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Characterization of interactors of the cellular prion protein

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1. Introduction

1.1 Historical background

Prion diseases or transmissible spongiform encephalopathies (TSEs) are rare, infectious and fatal neurodegenerative disorders that include Creutzfeldt-Jakob disease in humans, scrapie in sheep, and bovine spongiform encephalopathy in cattle. Some of these disorders have been know for over 100 years, but scientific progress in understanding these diseases started only 25 years ago with help of modern molecular biology and biochemistry. In 1982 the prion protein was isolated as the disease causing agent representing a unique pathogen. For the first time a transmissible agent consisted of nothing than a protein without genetic information encoded by nucleic acids. This was in contrast to all other known pathogens like viruses, bacteria or fungi.

Sporadic forms of dementia with spongiform degeneration of the brain were regarded as seldom events and did not arouse much interest even in the medical world. This changed dramatically as in 1986 a disease with such symptoms affected cattle. In this epidemic more than 180,000 cases of bovine spongiform encephalopathy (BSE) - or also called mad cow disease - affected the U.K. In other European countries also numerous cases were confirmed suggesting a huge threat to public health through dietary exposure. In 1996 an unsettling link between BSE and a novel form of the human Creutzfeldt-Jakob disease was found (Will *et al.*, 1996;Collinge and Rossor, 1996).

For many years the nature of the agent in these disorders was discussed. In 1732 the naturally occurring scrapie disease affecting sheep and goat was first described. In 1936 it was shown that inoculation between sheep (and goat) could transmit scrapie after prolonged incubation.

The character of transmission of the disease led to the assumption that the agent would be a virus. In 1954 Sigurdson termed it as a “slow virus”, referring to the incubation time as long as 20 years. A few years later Hadlow made mention of the similarities between scrapie and kuru. Kuru is a disease which is endemic among the Fore tribe of Papua New Guinea. Scrapie and kuru share common features as coordinative difficulties of patients and spongiform neurodegeneration of the brain. Consequently, Gajdusek and co-workers inoculated chimpanzees with kuru brain homogenates and confirmed the transmissibility of kuru in 1966 (Gajdusek, 1967). Indeed, the pathology of the central nervous system (CNS) was comparable to Creutzfeldt-Jakob disease (CJD), also a rare human neurodegenerative disease first

described in 1920. According to the before mentioned experiments the transmission of CJD to chimpanzees after intracerebral inoculation was reported in 1969 (Gibbs, Jr. *et al.*, 1969). In contrast to the expectations on the agent of TSEs, no indication on viruses or immunological responses was found. The agent also showed no response to nucleic acid inactivating treatments, e.g. UV-inactivation on ionizing radiation. Surprisingly the scrapie agent also showed weak response to procedures hydrolyzing or modifying proteins (e.g. treatment with urea or NaOH) (Alper *et al.*, 1967). Concluding to these findings Griffith suggested in 1967 that the transmissible agent might be a single protein (Griffith, 1967). In 1982 Prusiner and co-workers isolated a protease resistant glycoprotein which was the main component of the infectious fraction of infected brain homogenate. This isolated protein formed amyloid deposits in form of filamentous structures called scrapie-associated-fibrils (SAFs) or also called prion rods (Prusiner *et al.*, 1981;Prusiner, 1982;Hilmert and Diringer, 1984;Lehmann and Harris, 1996). Prusiner hence proposed the term “prion” for proteinaceous infectious particle, as this prion should lack nucleic acid. The isolated proteinase-K (PK)-resistent prion protein from infected Syrian hamster (Sha) brains was of 27-30 kDa (PrP27-30) designated as PrP^{Sc} – indicating the infectious character of scrapie - and was derived from 33-35 kDa protein.

After the N-terminal sequence of PrP^{Sc} had been determined by Edman degradation, molecular cloning studies of the PrP gene (prnp) were possible (Prusiner *et al.*, 1984). The mRNAs of scrapie infected and uninfected tissues were identical and antibodies raised in mice against scrapie infected hamster PrP also cross-reacted with uninfected tissues. This led to the isolation of a non-infectious isoform of the prion protein denoted PrP^{C} (for cellular prion protein) (Oesch *et al.*, 1985;Meyer *et al.*, 1986). These two isoforms – PrP^{Sc} and PrP^{C} – share the same amino acid sequence, but have different biochemical properties (see Tab.1).

Characteristics	PrP^C	PrP^{Sc}
Infectivity	No	Yes
Secondary structure	Mainly α-helical	Mainly β-sheet
Half life time	2-6 hours	16-24 hours and longer
PK digestion	Sensitive	Partially resistant
Ultracentrifugation in detergents	Soluble	Insoluble
Molecular weight (diglycosylated form) - PK → +PK	33-35 kDa → degraded	33-35 kDa → 27-30 kDa

Table 1 **| Comparison of the cellular and the scrapie isoforms of the prion protein.**

1.2 Animal prion diseases

As mentioned above, the oldest know TSE is scrapie in sheep and goats first described by McGowan (McGowan, 1922). In this disease the transmission from dam to unborn or newborns and other animals of the flock as well could be shown (Brotherston *et al.*, 1968;Dickinson *et al.*, 1974). Besides scrapie there are other prion diseases in animals, e.g. the transmissible mink encephalopathy (TME) (Hartsough and Burger, 1965;Burger and Hartsough, 1965), the chronic wasting disease (CWD) in elk and deer (Williams and Young, 1980), the bovine spongiform encephalopathy in cattle (Wells *et al.*, 1987) and the feline spongiform encephalopathy (FSE) in cats and big cats (Wyatt *et al.*, 1991).

Disease	Host	Cause
Scrapie	Sheep, Goat	Vertical and horizontal infection in genetically susceptible animals Oral transmission Sporadic
TME	Mink	Infection with contaminated meat from sheep and cattle
CWD	Deer, Elk	Unclear, possibly similar to scrapie
BSE	Cattle	Infection with prion- contaminated food Sporadic (?)
FSE	Cats	Infection with contaminated bovine tissue and food

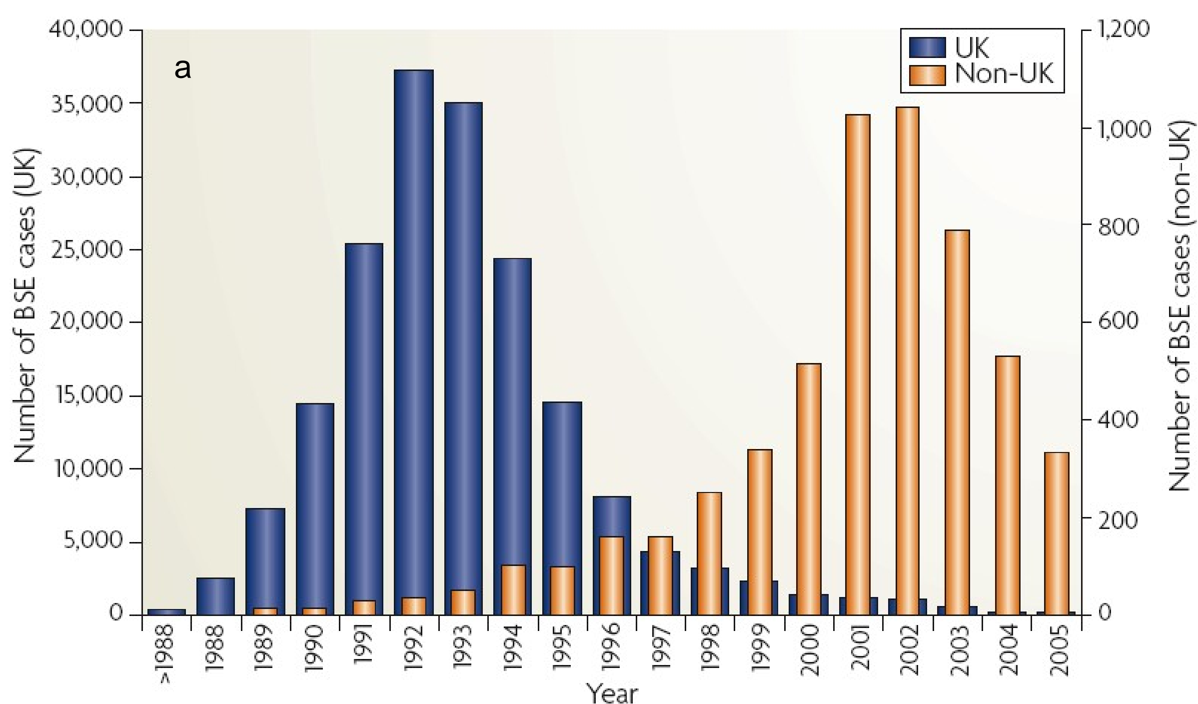
Table 2 | **Examples for TSEs in animals and their causes.**

FSE from cats and TME from minks are the sole know prion diseases in carnivores so far. Cause of these diseases is probably the feed with prion infected slaughter waste from cattle and sheep.

Scrapie in sheep is regarded as the source of the BSE epidemic 1986 in the U.K. Infected animals reacted at first hypersensitive for sensory stimuli like noise and light and later on developed progressive movement disorders (Wilesmith *et al.*, 1988). The transmission from scrapie from sheep to cattle resulted from feeding with meat and bone meal. In this way the species barrier was overcome. During the 70´s in the U.K. there was a new way of fat extraction method for meat and bone meal introduced. Part of this new method was the use of temperatures below 100°C and abandonment of NaOH. These conditions were not harsh enough to inactivate the scrapie agent which now was feed to cattle in high amounts. Perished cattle were then processed to meat and bone meal leading to an accumulation of prion proteins in the nutrition cycle for cattle. Another possible scenario would be that cattle afflicted by sporadic BSE were processed for meat and bone meal spreading the disease without any species barrier. More than 180,000 confirmed cases were reported in the U.K. But estimations range up to one million sick or infected animals (Anderson *et al.*, 1996). In

the year 1988 a ban for meat and bone meal was established leading to a drastic decrease of the epidemic. In the 90´s different European countries reported cases of BSE, e.g. Switzerland, Portugal, Ireland. The outbreak of an epidemic in Germany in the year 2001 caused by imported animal food from the U.K. led to a systematic European BSE testing of cattle older than 30 months (EU) or 24 months (Germany) with BSE tests. More than 400 BSE cases have been reported for Germany until today. But since 2005 the number of yearly reported BSE cases has declining to four cases for 2007.

CWD is exclusively appearing in North America and Canada affecting wild life animals as well as breeded ones (Spraker *et al.*, 1997). The routes of transmission are still unclear. CWD is supposed to be a sporadic disease which then spreads in the population. The characterization of the CWD agent led to the assumption that it has an entity of its own.



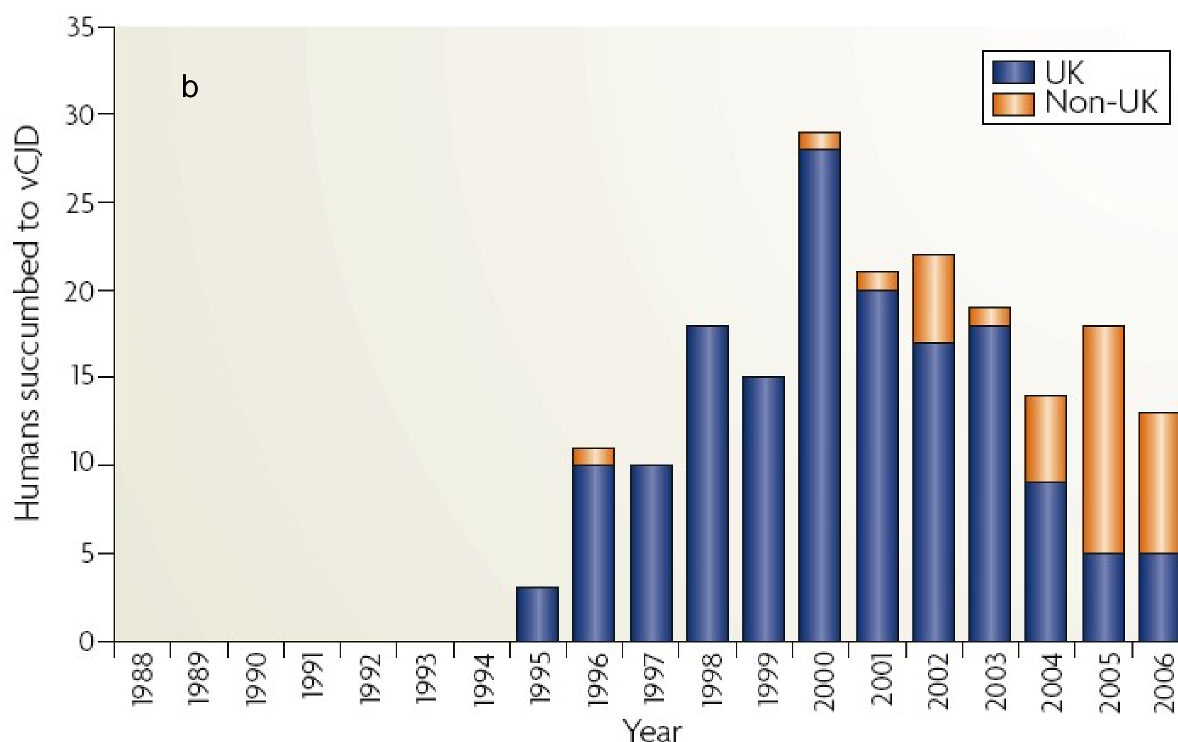


Figure 1 **| Incidence of BSE and vCJD cases reported worldwide. a** | Reported bovine spongiform encephalopathy (BSE) cases in the United Kingdom (UK; blue) and in countries excluding the UK (orange). **b** | Reported cases of variant Creutzfeldt–Jakob disease (vCJD) in the UK (blue) and in countries outside the UK (orange). From (Aguzzi *et al.*, 2007).

1.3 Human prion diseases

According to the etiology, human prion diseases can be divided into three categories – sporadic, acquired and inherited. About 80 – 90 % of all CJD cases are idiopathic or sporadic (sCJD) with a frequency of approx. 0.3 – 1.3 cases per million per year. As cause of the disease a somatic mutation of the prion gene (*Prnp*) or a spontaneous conformation change of the prion protein (PrP) is discussed, leading to a kind of chain reaction converting normal PrP^{C} into infectious PrP^{Sc}. In most cases the disease appears in the age of 45 to 75 and leads to death in approx. three to six months. Depression, insomnia, weight loss, exhaustion, head ache, ataxia, progressive amblyopia and pain with fast progressing dementia until the loss of speech are typical symptoms.

About 10 % of all CJD cases are genetically inherieted (fCJDs = familial CJD). These variants are commonly inherited autosomal-dominant. Like sCJD this disease strikes elderly people and is fatal. Studies revealed that in one in 2.5 million residents a family with inherited prion disease can be found. Over 20 different pathogenic *Prnp* mutations could be identified

until today. They can be divided into two groups: (i) point mutations resulting in an amino acid substitution or in two cases resulting in a generation of a stop codon, leading to a truncated prion protein. (ii) Insertions encoding additional copies of octapeptide repeats - up to nine. Usually five octapeptide repeats are present in the normal PrP. These mutation are believed to destabilize the prion protein resulting in spontaneous conformational changes (Owen *et al.*, 1989;Ghetti *et al.*, 1996).

Further genetically inherited prion diseases are Gerstmann-Sträussler-Scheinker disease (GSS) and the fatal familial insomnia (FFI). GSS is another autosomal dominant disorder with a prolonged clinical course than in CJD. GSS is presenting with chronic cerebellar ataxia and later occurring dementia. The course of disease takes about five to six years (with a range of 2 to 17 years, with onset at approx. 45 years of age (can range from 25 to 59 years). The most common GSS associated mutation in *PRNP* is P102L (Hsiao *et al.*, 1989). But there are different mutations for the codons 105, 117, 145, 198 and 217 (Goldgaber *et al.*, 1989). In 1981 GSS could be transmitted to monkeys and was sorted to TSEs since then (Masters *et al.*, 1981).

The most recent known prion disease is the FFI, which was first described in 1986 (Lugaresi *et al.*, 1986). The cause of disease is the mutation D178N in combination with methionine at codon 129. The age of the patients ranges from 20 to 60 years and the clinical course 8 to 30 months. For the most part a disorder in the circadian rhythm (insomnia) accompanied by progressing neurological deficits can be diagnosed. An experimental transmission of FFI was performed in 1995 (Tateishi *et al.*, 1995).

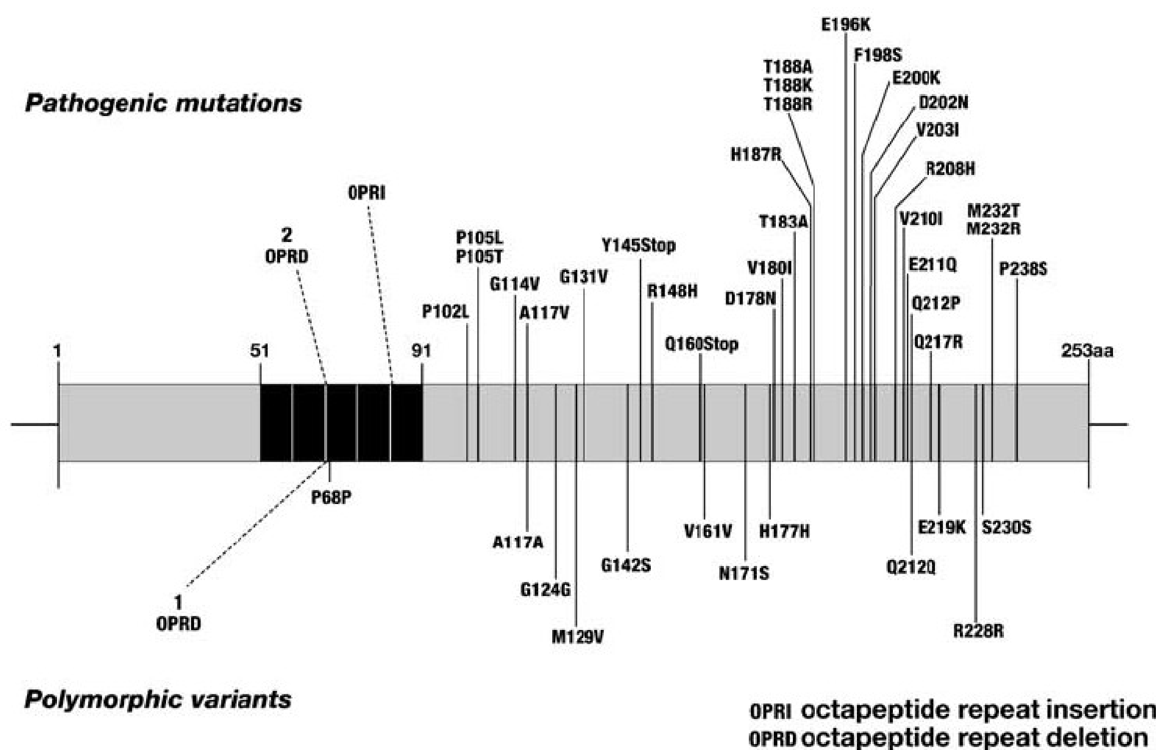


Figure 2 | **Mutations causing inherited prion diseases and polymorphisms in humans.** The grey bar depicts the human sequence of the prion protein (prnp), with the five octapeptide repeats as black boxes. The lines above indicate codon mutations that cause prion diseases. Below the lines are polymorphisms. Some are known to influence the susceptibility as well as the phenotype of prion diseases (Mead, 2006).

Also polymorphisms of the prion protein gene were detected. Some of them, but not all, seem to influence the susceptibility and/or the phenotype of the resulting prion disease. Probably the most important polymorphism is the codon for amino acid 129, which for humans can either code for methionine or valine. 51 % of the Caucasian population is heterozygous methionine/valine at codon 129, 37 % are homozygous for methionine and about 12 % are homozygous for valine. This codon has a key function in the susceptibility for prion diseases – most individuals with TSEs are homozygous (Palmer *et al.*, 1991;Collinge *et al.*, 1991). Some protection by heterozygosity at codon 129 can be observed also in familial prion diseases (Baker *et al.*, 1991). It is not possible to transmit TSEs like other infectious diseases by familial or sexual contact.

The so called acquired prion diseases – Kuru, iatrogenic CJD (iCJD), variant CJD (vCJD) – are transmitted similar to experimental inoculations. For the understanding of prion diseases in general Kuru from the Fore tribe in Papua New Guinea was of particular interest. First described in 1957, Kuru is transmitted through cannibalism (Gajdusek and ZIGAS, 1957;ZIGAS and Gajdusek, 1957). The kuru epidemic in the 20´s century was confined to

Papua New Guinea solely and there almost completely to the Fore tribe. Ritual cannibalism was practiced on the dead tribe members. Mainly women and children took part in these rituals consuming brain and inner organs of the dead, were the highest concentrations of prions are present. The origin of kuru is probably a dead tribe member which fell ill on sporadic CJD and later on was consumed by his relatives. Comparable to BSE the prions were enriched in following cycles of consumption. Cannibalism was banned in the mid 50´s in Papua New Guinea and the epidemic was stopped. The incubation time is varying between 4.5 years to over 40 years with a peak at 12 years. The clinic is showing fast progressive ataxia because of the mainly infected cerebellum. In the beginning no dementia is apparent in contrast to CJD. The course of disease ranges from three months to three years.

Iatrogenic CJD (iCJD) is a very rare disease and caused by medical casualties. The first reported case is from 1968, when a patient was infected using not effectively sterilized intracerebral electrodes. Later it was found out that not all surgical instruments can be adequate sterilized with regard to prions (Dickinson and Taylor, 1978). This led to the use of disposal surgical instruments in neurosurgery. The most common iCJD infections take place during the administration of human cadaver growth hormone or gonadotropin and dura mater or corneal grafting. More than 300 cases have been reported so far. The incubation time at intracerebral infection is about 19 to 46 months – for peripheral infections about 12 years. Not long ago even cases of patients infected with vCJD via blood transfusion have been reported (Llewelyn *et al.*, 2004;Peden *et al.*, 2004).

Due to the concern that BSE could be transmitted to humans 1990 a CJD surveillance program was started in the U.K. Several other European countries followed in 1993. In 1995 indeed the first three cases of a new variant of CJD (vCJD) were reported. Until today more than 200 confirmed cases have been reported. The clinical picture of vCJD strongly differs from all other human prion diseases. The most striking difference is the young age of patients – starting at 16 years up to 53 years. In contrast to sCJD the clinical course is slightly prolonged (9 to 35 months). After a longer phase of psychiatric disturbances the rest of the clinical presentation and course resembles sCJD with initial depression and behavioral changes (Zeidler *et al.*, 1997;Hill *et al.*, 1999). Further cerebellar syndromes and ataxia accompanied by dementia usually appear later on in the clinical course. The most distinctive neuropathological characteristic is the accumulation of amyloid plaques (“florid plaques”) in cerebral and cerebellar cortex similar to kuru-like spongiform vacuoles (Will *et al.*, 1996). Also unique is that PrP^{Sc} can be detected in the tonsil and other lymphoreticular tissues.

Today there is a strong indication that there is a direct link between BSA and vCJD. Collinge and co-workers could show by molecular strain typing that the glycoform ratios of PK digested PrP^{Sc} vCJD were identical with those seen in BSE (Collinge *et al.*, 1996). Inbred mice inoculated with homogenates of brains of vCJD patients showed the same BSE strain characteristic in incubation period and lesion profile (Bruce *et al.*, 1997;Hill *et al.*, 1997). Also macaque monkeys exhibited plaques similar to those found in vCJD when they developed neurological disease after the inoculation with bovine prions (Lasmezas *et al.*, 1996). Until 1989 when bovine offal in food for human consumption was banned in the U.K., homogenates of pooled bovine brains had been used as binders in food like hamburgers and sausages. In this way it was possible for the consumer to ingest food with extremely high titers of prions.

It is difficult to speculate about the total magnitude of a possible vCJD epidemic by extrapolating the current cases. Reports estimating the number of individuals who will develop vCJD in the next years vary in the range of hundreds to many thousands (Donnelly *et al.*, 2002;Ghani *et al.*, 2002).

1.4 Therapeutic and prophylactic approaches

Great efforts have been made in the recent years to find therapeutics or prophylactic drugs for prion diseases. The nature of the disease – inflicting sever damage to the brain including massive neuronal loss starting immediately in an early phase of the clinical course – makes it hard to believe that there is a proper pharmacological treatment possible that reverses these damages. Instead one different starting point might be the search for drugs prolonging the incubation time and/or slowing down the pace of the clinical course. This could be achieved by drugs either inhibiting the accumulation of PrP^{Sc} or the conversion of PrP^{C} in its pathogenic conformation.

Different substances have been reported to show an effect in animal models or cell culture systems, e.g. siRNA, peptide aptamers, Congo Red, polyene antibiotics, anthracycline derivatives, sulphated polyanions, porphyrins, branched polyamines, Suramin, STI571 and β-sheet breaker peptides. The conversion of PrP^{C} in its pathogenic conformation can be prevented by the inhibition of PrP^{C} synthesis, e.g. in $PrP^{0/0}$ mice (Bueler *et al.*, 1993;Weissmann *et al.*, 2001) or through RNAi (Tilly *et al.*, 2003). Or PrP^{C} is deflected from its place of conversion, e.g. with Suramin which leads to accumulation of PrP^{C} in the trans-Golgi-network. The aggregates are then directed to the lysosomes and degraded. PrP^{C} does

not reach the cell surface (Gilch *et al.*, 2001). STI571 however increases the lysosomal degradation of PrP^{Sc} and does not change the physiological properties of PrP^{C} (Ertmer *et al.*, 2004). In one proposed conversion model PrP^{C} is in equilibrium with an intermediate conformation PrP*. The intentional stabilization of the PrP^{C} conformation also inhibits the conversion (Cohen *et al.*, 1994;Prusiner, 1998;Clarke *et al.*, 2001). Chemical chaperones (Tatzelt *et al.*, 1996) and Congo Red (Head and Ironside, 2000) can function in this manner. The direct contact of the isoforms PrP*/PrP^{C} with PrP^{Sc} is important for the conversion. This can be prevented by anti-PrP aptamers, β-sheet breaker peptides and anti-PrP antibodies (Soto *et al.*, 2000;Head and Ironside, 2000;Enari *et al.*, 2001;Heppner *et al.*, 2001;Peretz *et al.*, 2001;Sigurdsson *et al.*, 2002;Proske *et al.*, 2002;Gilch *et al.*, 2003;White *et al.*, 2003). Soluble PrP dimers have been reported to restrain the binding of postulated co-factors to PrP*/PrP^{C} thereby inhibiting the production of PrP^{Sc} (Meier *et al.*, 2003). Very few substances are targeting PrP^{Sc} directly. Branched polyamines are a group of chemicals proposed to activate cellular clearance of PrP^{Sc} which leads to faster degradation (Supattapone *et al.*, 1999;Winklhofer and Tatzelt, 2000;Supattapone *et al.*, 2001).

Since 1796, when Edward Jenner applied the first vaccination against smallpox, vaccination is the first choice of prophylaxis. But PrP^{C} is expressed on almost every cell type and the resulting self-tolerance seems to circumvent any active immunization. However via an immunization it was possible to generate auto-antibodies (Gilch *et al.*, 2003;Sigurdsson *et al.*, 2003) capable of curing prion-infected cells (Gilch *et al.*, 2003). With the help of passive immunization in mice it was possible to extend the incubation time considerable and the PrP^{Sc} content in spleen and brain was clearly reduced (White *et al.*, 2003). The immunization with dimeric PrP induced a specific T cell response in wild-type mice (Kaiser-Schulz *et al.*, 2007). None of these generated auto-antibodies seem to have any drastic side effects. The application for humans is more complicated, because the time point of infection is normally not known and the vaccination should take place not long after it. So vaccination may only be introduced in course of post-exposition prophylaxis.

1.5 PrP gene structure

The PrP gene is highly conserved in evolution (Schatzl *et al.*, 1995;Wopfner *et al.*, 1999) and is mapped on the human chromosome 20, which is homologous to the region of mouse chromosome 2 (Robakis *et al.*, 1986;Sparkes *et al.*, 1986) – a strong hint for an important function. For all known PrP genes the open reading frame (ORF) resides in a single exon (Hsiao *et al.*, 1989;Gabriel *et al.*, 1992;Schatzl *et al.*, 1995) encoding ca. 250 amino acids. The promotor sequence has no TATA-box and is similar to house-keeping genes. A possible binding site for transcription factors SP1 and AP1 are given, but the regulation has to be explored further (McKnight and Tjian, 1986;Westaway *et al.*, 1987). The transcribed mRNA is 2.1 – 2.5 kb of size.

Figure 3 **| Structure of the murine PrP gene.** The gene contains three exons. Exon 3 encodes the complete ORF and the 3' untranslated region (3' UTR).

Some mice strains showed a prolonged incubation time by the inoculation with prion-infected brain homogenate. The murine PrP gene exists in two alleles PrP-A and PrP-B. These two alleles differ in two amino acid positions, which is the cause for the different incubation times (Carlson *et al.*, 1994). The PrP-B gene furthermore has an additional 7 kb insertion in his long intron (Westaway *et al.*, 1994). On the complementary strand there is a long antisense reading frame, which is even conserved between species (Rother *et al.*, 1997). Only the PrP genes of a few rodents, minks (Kretzschmar *et al.*, 1992) and of the most birds have a stop codon on this strand, but no mRNA was found for this antisense ORFs.

1.6 Structural properties of the prion protein

Various reports support the concept that prions consist in parts or completely of an abnormal isoform of the cellular prion protein, named PrP^{Sc}. The idea is that the pathogenic isoform PrP^{Sc} acts as a template promoting a posttranslational change in the conformation of PrP^{C} (Borchelt *et al.*, 1990;Caughey *et al.*, 1990;Prusiner, 1998).

After translation the mouse PrP has 254 amino acids, where the first 22 function as signal peptide for the entrance of the polypeptide chain into the endoplasmic reticulum (Oesch *et al.*, 1985). Another C-terminal signal peptide is cleaved, resulting in an attachment of a glycosyl-phosphatidyl-inositol (GPI) anchor at Ser231. Further posttranslational modifications follow – up to two N-linked carbohydrate chains (glycosylations) at asparagines at codon 180 and 196 (Bolton *et al.*, 1985;Manuelidis *et al.*, 1985). This leads to the typical three banding pattern in SDS PAGE, for non-, mono- and diglycosylated PrP. Finally, a single disulphide bond between cysteine residues 178 and 213 stabilizes the conformation of the protein (Hope *et al.*, 1986). After the cleavage of both signal peptides the mature prion protein comprises 209 amino acids.

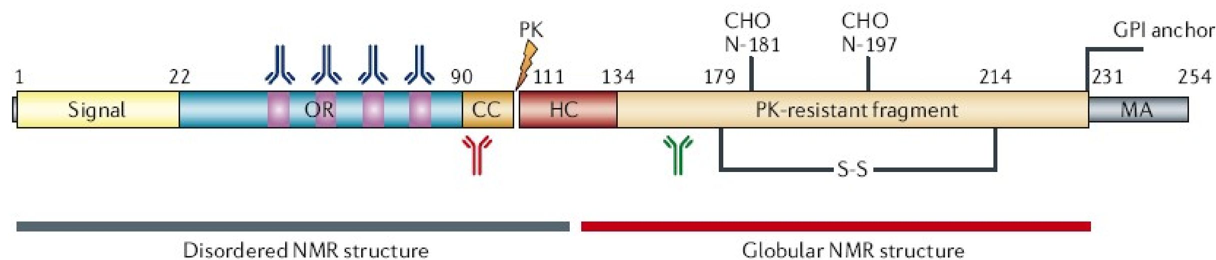


Figure 4 **| Primary structure of human PrP^{C} before and after maturation.** The translated PrP is cleaved of from the N- and C-terminal (MA) signal peptides and the glycosyl-phosphatidyl-inositol (GPI) anchor is attached to the C-terminal end. PrP molecules can be N-glycosylated twice (CHO) and a disulfide bond (S-S) is built, resulting in a final PrP protein with 209 amino acids. The estimated cutting site of proteinase K (PK) within PrP^{Sc} is indicated by the lightening symbol. CC is the charged cluster and HC stands for hydrophobic core. From (Aguzzi and Heikenwalder, 2006).

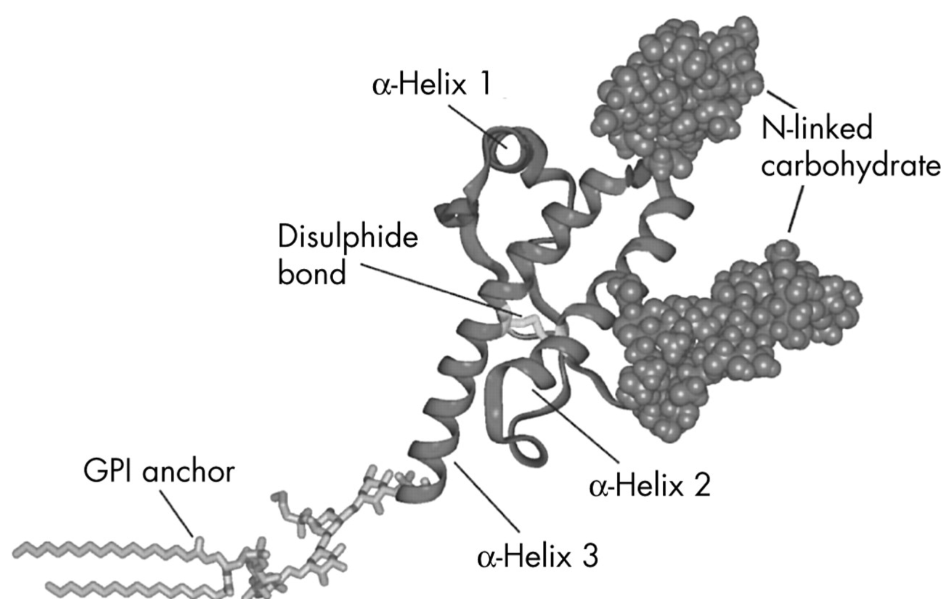


Figure 5 | **Structure of murine PrP^C at the cell surface.** Structure of PrP^C attached via GPI anchor to the outer leaflet of the plasma membrane. GPI anchor, the three helices, disulfide bond and the carbohydrates are indicated. From (Collinge, 2005).

The Golgi compartment is the next stop for properly folded PrP^C, which then after passing the secretory pathway is attached to the outer leaflet of the plasma membrane via its GPI anchor (Borchelt *et al.*, 1990;Taraboulos *et al.*, 1990a;Caughey *et al.*, 1991).

The internalization of PrP is assumed to occur through caveolae-like domains or in rafts, specialized plasma membrane regions rich in cholesterol and glycosphingolipids (Taraboulos *et al.*, 1995). Once in the endosomes, PrP can either be recycled to the cell surface again (Vey *et al.*, 1996) or during this recycling be directed to autosomes for degradation in multi-vesicular bodies (MVBs). These multivesicular bodies can either fuse with lysosomes for degradation or fuse with the plasma membrane, resulting in the release of exosomes, vesicles capable of fusing with nearby cell membranes (van Niel *et al.*, 2006). The localization of PrP^C on the cell surface is essential for the conversion process into PrP^Sc (Borchelt *et al.*, 1990;Taraboulos *et al.*, 1990b;Caughey and Raymond, 1991;Caughey, 1991). In cell culture models the treatment of prion-infected cells with Suramin induced the aggregation of PrP^C in the trans-golgi-network with a simultaneous decrease of cell surface PrP^C, resulting in decrease of PrP^Sc quantity within a few days (Gilch *et al.*, 2001). The actual belief is that the conversion takes place directly at the plasma membrane or in caveolae-like domains, rafts or in early compartments of the endocytotic pathway (Borchelt *et al.*, 1992;Naslavsky *et al.*, 1997;Nunziante *et al.*, 2003). Following conversion PrP^Sc is directed to the lysosomes and gets slowly degraded by the cell.

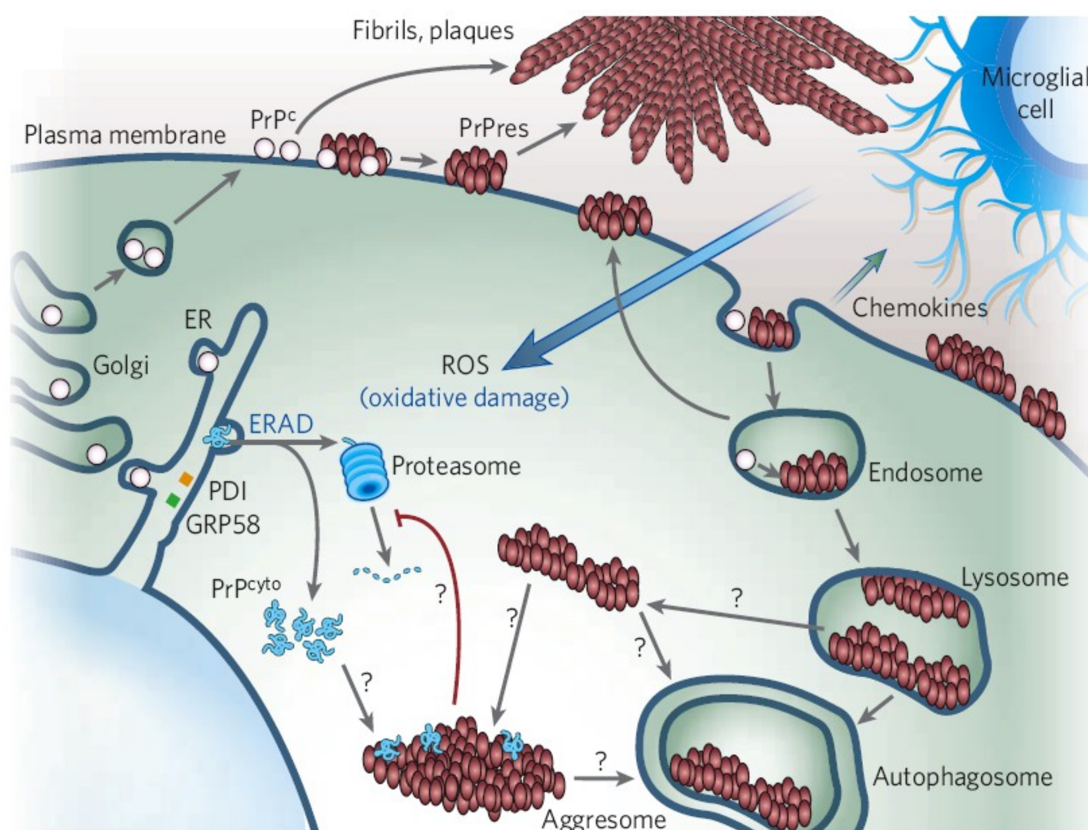


Figure 6 **| Subcellular trafficking of prion protein.** Novel synthesized PrP^C (white spheres) is transported along the secretory pathway through the endoplasmic reticulum (ER) and the Golgi. PrP^C on the cell surface is localized in cholesterol-rich domains (rafts or caveolae) by its GPI anchor. PrP^C is subjected to endocytosis and recycles to the cell surface or is directed to final degradation in lysosomes. Conversion into PrP^{Sc} (red ovals) occurs at the cell surface in rafts or in compartments along the endocytic pathway. Due to its partially protease resistance PrP^{Sc} is not efficiently degraded in lysosomes and therefore accumulates. Misfolded PrP (in blue) can be retrograde translocated into the cytosol (PrP^{cyto}). There it is either degraded by the proteasome or induces neurotoxicity by accumulating. From (Caughey and Baron, 2006)

1.7 The conformation of PrP^{C} and PrP^{Sc}

In 1993 the secondary structure of purified PrP^{C} and PrP^{Sc} were compared by optical spectroscopy and found different (Pan *et al.*, 1993). This was in contrast to the dogma, that the amino acid sequence defines the biological active conformation (Anfinsen, 1973). It was possible for the prion protein to adopt two different conformations. It could be shown, that PrP^{C} contains about 42 % α-helix and 3 % β-sheets, whereas PrP^{Sc} is compromising about 30 % α-helix and 45 % β-sheets. This was done with the help of Fourier-transform infrared spectroscopy (FTIR), mass spectrometry and circular dichroism (CD) studies (Pan *et al.*, 1993;Stahl *et al.*, 1993;Gasset *et al.*, 1993;Pergami *et al.*, 1996). So far is has been impossible to produce crystals of PrP to perform crystallographic analysis. Data about the three dimensional structure was gained by the analysis of purified and refolded mouse PrP (aa 121-231) expressed in *E. coli*. This PrP was analyzed by nuclear magnetic resonance (NMR) and mainly confirmed previous computer modelling (Cohen *et al.*, 1994;Riek *et al.*, 1996). It consisted of two stranded antiparallel β-sheet and three α-helices (four were predicted by computer modelling). Except the N-terminal part – until aa 120, which showed not defined structure. Full length PrP (aa 23-231) was characterized later (Riek *et al.*, 1997). Octarepeats are eight residue long peptides, rich in glycine, proline and histidine. Most know PrPs contain a region formed by the repetition of five consecutive octarepeats, complying the amino acid 50 to 90 in mouse. Nevertheless a defined globular conformation might be achieved *in vivo* by binding of metal ions, e.g. Cu^{2+} (Hornshaw *et al.*, 1995a). Also a poly-L-proline type II – helix structure has been reported for PrP in vivo (Gill *et al.*, 2000).

The structure of aggregated PrP^{Sc} was explored further with computer modelling and 2D-structure comparisons. PrP^{Sc} structure is dominantly shaped by β-helices. In this model the flexible part (residues 89 to 175) has the conformation of a left-handed β-helical sheet. Three of these molecules form a trimer in shape of a disc. These discs can form fibrilliar structures – so called “prion rods”. In these trimers the β-helices are in the middle, while the C-terminal α-helices are positioned at the outside (Govaerts *et al.*, 2004).

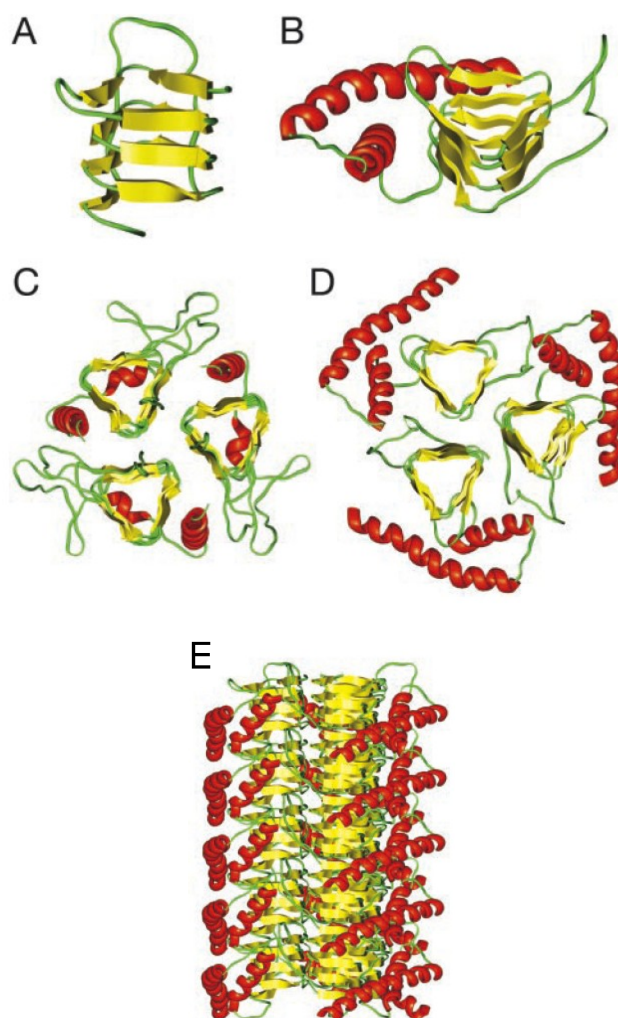
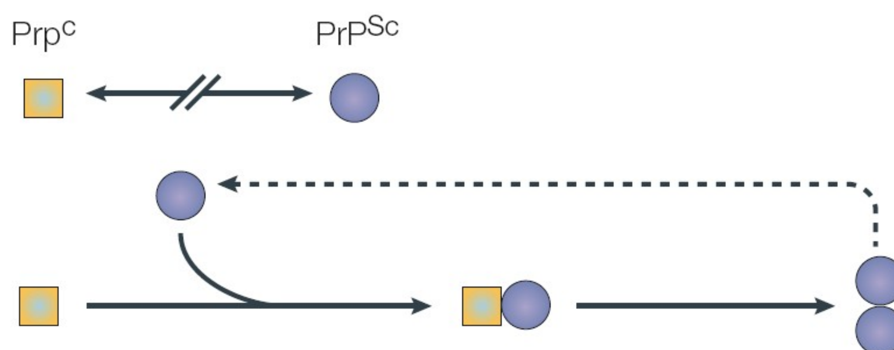


Figure 7 **| Suggested structure of PrP^{Sc} and a model for "prion rods". a** | N-terminal part of PrP^{Sc} (aa 89-143) is forming a left-handed β-helical sheet. **b** | Model of a PrP^{Sc} monomer. Via NMR defined α-helical structure (aa 177-227) was combined with the β-helical model from **a**. **c** | Trimeric model of PrP^{Sc} prepared through computer and structural analysis. **d** | Two dishes of trimeric PrP^{Sc} attach via polar interaction of the upper coil of the β-helix of the lower dish with the lower coil of the β-helix of the upper dish. This way enough space for the α-helices and the glycosyl groups is build. **e** | Model of more PrP^{Sc} trimers attaching as a "prion rod". For enhanced clarity the glycosyl groups were erased. From (Govaerts *et al.*, 2004).

1.8 Mechanisms of prion conversion

The prion-only hypothesis states that host PrP^{C} is converted into PrP^{Sc} by direct interaction with scrapie protein. A relative small part of PrP^{C} is thought to be involved in the process of intra molecular refolding into a pathogenic conformation. One idea is that the region between residues 90 to 112 is refolded, followed by the conversion of the two short β-sheet structures and the first α-helix into a large β-sheet formation. Only the remaining other two α-helices and the disulphide bonds need to be preserved for PrP^{Sc} to be infectious (Hornemann *et al.*, 1997;Prusiner, 1998;Wille *et al.*, 2002). In other words, the predominantly β-sheet conformation of PrP^{Sc} is transmitted to the native α-helical structure of PrP^{C}. There are currently two different models proposed providing possible explanations for this mechanism.
There is the heterodimer hypothesis proposed by Prusiner (Prusiner *et al.*, 1990;Cohen *et al.*, 1994) – also called “refolding model” – where PrP^{C} is partially unfolded into a transitional state called PrP*. This PrP* then gets refolded under the influence of PrP^{Sc} – while this contact is called heterodimer – which results in the formation of a homodimer of PrP^{Sc} molecules. While building the heterodimer, there might be a molecular chaperone enhancing the process. Newly generated PrP^{Sc} is now able to convert PrP^{C} in an autocatalytic process. This conversion step has an very high energy barrier (Cohen *et al.*, 1994) and spontaneous forms of the disease only appear at an advanced age of patients, indicating that age dependent decrease in efficiency of cellular quality control also might play a role (Wickner *et al.*, 1999). The energy barrier might be lowered by specific mutations, promoting β-sheet conformation, would be an explanation for hereditary forms of the prion disease.
Gajdusek and his group first defined the nucleation dependent polymerization model – also called seeding model – in 1990 (Brown *et al.*, 1990). Later on it was further developed by Landsbury together with Byron Caughey (Come *et al.*, 1993;Caughey *et al.*, 1995).

According to their theory the cellular prion protein can deposit on a polymerization seed consisting of a PrP^{Sc} oligomer. PrP^{C} thereby adopts the conformation of PrP^{Sc} in the seed leading to growing aggregates of PrP^{Sc} – no unfolded transition state is needed in this model. The newly formed aggregates disintegrate randomly and thereby form new seeds. Elegantly this model also explains the existence of different prion strains. The PrP^{Sc} seed determines the structure of the polymeric aggregates, meaning that the PrP monomer does not to have a special intrinsic conformation. These two models are applicable to prion diseases in general, since the presence of a pathogenic mutation most likely favors the spontaneous formation of either PrP^{Sc} nuclei or PrP*.



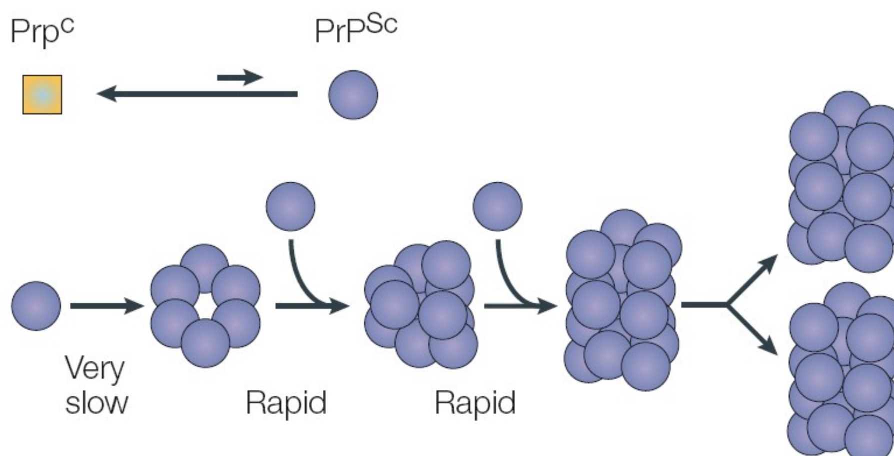


Figure 8 | **Two models for prion conversion and replication. a** | Refolding model (=heterodimer model). **b** | Seeding model (=polymerization model). From (Weissmann C., 2004).

1.9 Prion strains and the species barrier

NMR studies on recombinant hamster- (Donne *et al.*, 1997), human- (Zahn *et al.*, 2000) and bovine-PrP (Lopez *et al.*, 2000) showed, that there is no principle difference in mammalian prion proteins, except small length differences of the α-helices. The experimental transmission of prion diseases into other species showed an increase in incubation time. Passaging the disease in the same species shortened the incubation time. This phenomenon was called "species barrier" in the 60´s (Pattison and Jones, 1968). The concept of species barrier was confirmed through many experiments in transgenic mice. Cause of the species barrier seems to be primarily the amino acid sequence homology of the prion proteins of donor and recipient (Scott *et al.*, 1989;Scott *et al.*, 1993;Schatzl *et al.*, 1995;Prusiner and Scott, 1997). It appears that the species barrier is less pronounced when two species are more closely related, e.g. sheep and cattle. When prion proteins differ much, e.g. in Syrian hamster and mouse, the species barrier is higher. Experiments with chimeric prion proteins in transgenic mice confirmed this (Scott *et al.*, 1989;Scott *et al.*, 1993;Telling *et al.*, 1994;Telling *et al.*, 1995). But still it is not clear which regions or PrP are responsible for the species barrier. Analyses of primate and rodent PrP suggest that amino acid 112 has an important role (Schatzl *et al.*, 1995). Studies on transgenic animals suggest that amino acids at the C-terminus are important (amino acids 168,172, 215, 219), matching the site for binding the postulated co-factor for conversion, factor "X" (Telling *et al.*, 1994;Telling *et al.*, 1995;Kaneko *et al.*, 1997a). The existence of various prion strains could be shown through the purification of PrP^{Sc} isolates from different natural occurring prion diseases in humans and animals. These strains differ in biological and biochemical features, e.g. causing different incubation times and neuropathological patterns by experimental inoculation.

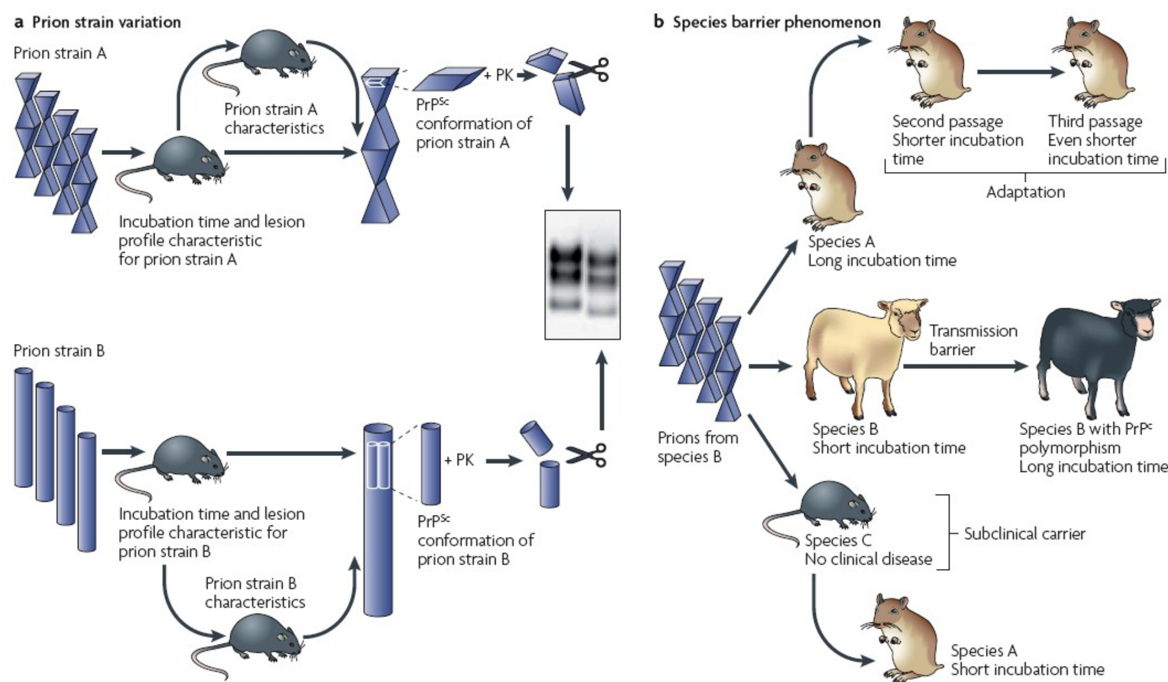


Figure 9 | **Models for prion strain variation and species barrier. a** | Two prion strains (A and B) cause different incubation times and lesion profiles by inoculation in genetically identical hosts. These features persist through many serial passages in new hosts. **b** | Prions from one species are often less infectious to other species. After serial passages in the same host the incubation time constantly decreases. From (Aguzzi *et al.*, 2007).

The glycosylations profile of different prion strains show distinct differences in SDS-PAGE when analyzed as proteinase K digested isolates. The strains differ in fragment size as well as in the ratio of the three glycosylation forms (non-, mono-, diglycosylated). There were different human prion strains isolated, causing different disease characteristics (Collinge *et al.*, 1996;Parchi *et al.*, 1996). Passaging in another species does not change biological and biochemical properties of prion strains. This means there are different conformation of PrP^{Sc} existent, coding for different strains (Telling *et al.*, 1996). There have been three strains reported for humans taking sporadic and iatrogenic CJD together. A fourth strain was reported for vCJD cases (according to London terminology).

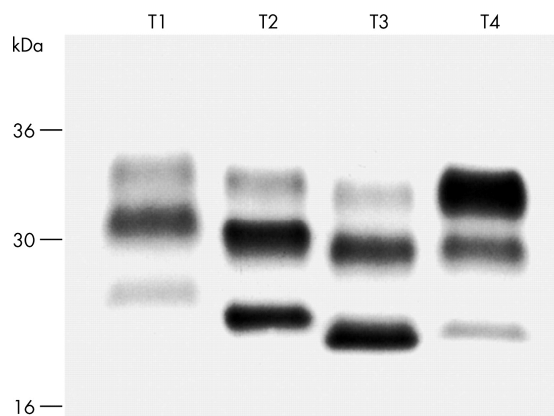


Figure 10 | **Molecular strain typing for human prions.** Western blot of proteinase K treated brain homogenates. Strains differ in molecular mass and glycoform ratios. Sporadic or iatrogenic CJD (T1-3) and vCJD (T4). From (Collinge, 2005).

1.10 Hypothetical functions of the cellular prion protein

The prion protein has been identified in mammals, birds, marsupials, amphibians and fish (Harris *et al.*, 1993;Windl *et al.*, 1995;Wopfner *et al.*, 1999;Strumbo *et al.*, 2001;Oidtmann *et al.*, 2003). The highest concentrations of PrP^C can be found in neurons, especially at synaptic ends, where the protein travels through axonal transport (Kretzschmar *et al.*, 1986;Borchelt *et al.*, 1994;Fournier *et al.*, 1995). Most PrP^C is concentrated in the pre-synaptic region (Herms *et al.*, 1999). Nonetheless its expressed in most tissues, particularly in cells of the immune system (Dodelet and Cashman, 1998). The definite function of PrP^C could not be resolved yet. $PrP^{0/0}$ showed no noteworthy phenotype (Bueler *et al.*, 1992). These mice were in contrast to wild type mice totally resistent against an infection with PrP^{Sc} and did not got sick after inoculation with prion-infected brain homogenate (Bueler *et al.*, 1993). Some minor neurological abnormalities were found later – in circadian rhythm and sleep, reduction of slow after-hyperpolarizations evoked by trains of action potentials (Collinge *et al.*, 1994;Colling *et al.*, 1996;Tobler *et al.*, 1996). This effect could possibly explained by abnormal homeostasis of intracellular calcium (Colling *et al.*, 1996).

Some data support the idea that PrP is part of copper metabolism, because PrP is able to bind copper ions (Hornshaw *et al.*, 1995b;Brown *et al.*, 1997a;Pauly and Harris, 1998) due to its N-terminal octarepeats (Stockel *et al.*, 1998). The amount of copper in the cell membrane is reduced in $PrP^{0/0}$ mice compared to wild type mice (in liver about 50 %, in brain about 90 %)

(Brown *et al.*, 1997a) . In these mice an increased sensibility to oxidative stress in neurons can be found (Brown *et al.*, 1997b;Pauly and Harris, 1998), with a decreased concentration of copper ions at the synaptic membranes and a reduced copper/zinc superoxide-dismutase (SOD) activity. Hence a SOD activity or a transporter function for the uptake of metal ions – from the synaptic gap – was discussed for PrP (Brown *et al.*, 1997a;Brown *et al.*, 1999;Wong *et al.*, 2000). These ideas could not be approved by experiments *in vivo* with transgenic mice. So the participation in SOD activity of PrP^C is questionable (Hutter *et al.*, 2003).

1.11 Interactors of the prion protein

Another possibility would be that PrP^C functions as a receptor for an unknown ligand. Via Yeast-Two-Hybrids screenings a couple of extra- and intracellular interactors could be found. In total, over 20 different interactors have been identified. For a complete overview sight Table 3. Some of the prominent interactors are e.g. Laminin receptor precursor (Rieger *et al.*, 1997;Gauczynski *et al.*, 2001), Caveolin-1 (Harmey *et al.*, 1995), HSP 60 (Edenhofer *et al.*, 1996), Bcl-2 (Kurschner and Morgan, 1995), Synapsin Ib and Grb2 (Spielhaupter and Schatzl, 2001). Some of these interactors play a role in cellular signal transduction, proposing a function of PrP^C in signal transduction pathways.

In the last years different groups found growing evidence that PrP^C is part of cellular signal transduction. Signal transduction cascades start mostly at the cell surface by binding of ligands to specific receptors, which then forward the signal into cytoplasm or the nucleus. Receptors of this kind mainly gather in caveolae or rafts, specialized compartments on the outer leaflet of the plasma membrane. These compartments comprise high concentrations of cholesterol and glycosphingolipids. Also the GPI anchored prion protein localizes in rafts (Taraboulos *et al.*, 1995). The missing contact to cytoplasmic proteins does not necessarily prevents signal transduction since other GPI anchored proteins playing roles in signal transduction are present in rafts, too. E.g. in neuronal cell line 1C11, crosslinking of PrP^C with antibodies lead to dephosphorylation and activation of the intracellular Fyn-kinase. This effect is Caveolin-1 dependent, which is also localized in caveolae and rafts (Mouillet-Richard *et al.*, 2000). In a $PrP^{0/0}$ fibroblast cell line a regulation of some genes, important for proliferation and differentiation, could be reported (Satoh *et al.*, 2000). Another evidence for the role of PrP^C in survival and differentiation of neuronal cells could be seen, when it was reported that PrP^C activated signal transduction pathways important for neurite outgrowth and survival of neuronal cells (Chen *et al.*, 2003). Also it was reported previously that PrP^C can

directly interact with proteins involved in signal transduction, e.g. the adaptor protein Grb2 and Synapsin Ib, for which the interaction could be shown *in vitro* and in cell culture (Spielhaupter and Schatzl, 2001).

PrP^C-interacting protein	Sub-cellular localization	Method of discovery	Binding epitope on PrP^C	Reference(s)
GFAP	Cytoplasmic (cytoskeleton)	Ligand blots	Unknown	(Oesch *et al.*, 1990)
Bcl-2	The cytoplasmic face of organelles	Yeast 2-hybrid	Residues 72–254	(Kurschner and Morgan, 1995)
Grb2	Cytoplasmic	Yeast 2-hybrid	N-terminal and C-terminal binding sites	(Spielhaupter and Schatzl, 2001)
Synapsin-1b	Synapse	Yeast 2-hybrid	N-terminal and C-terminal binding sites	(Spielhaupter and Schatzl, 2001)
Pint1	Unknown	Yeast 2-hybrid	Residues 90–231	(Spielhaupter and Schatzl, 2001)
Glycosaminoglycans (i.e. heparin)	Cell membrane, extracellular matrix	Heparin-agarose pull-down on PI-PLC-treated cells	Residues 23–52, 53–93, and 110–128	(Caughey *et al.*, 1994;Harmey *et al.*, 1995)
Caveolin	Caveolae (cell membrane)	Co-purification in N2a cells	Unknown	(Harmey *et al.*, 1995)
Dystroglycan	Cell membrane (transmembrane)	Co-immunoprecipitation	Unknown	(Keshet *et al.*, 2000)
Synaptophysin	Synaptic vesicles (transmembrane)	Co-immunoprecipitation	Unknown	(Keshet *et al.*, 2000)
Neuronal nitric oxide synthase (nNOS)	Peripheral membrane protein	Co-immunoprecipitation	Unknown	(Keshet *et al.*, 2000)
ApoE	Secreted	Co-immunoprecipitation/pull-downs with recombinant proteins	Residues 23–90	(Gao *et al.*, 2006)
Plasminogen	Secreted	Immobilized serum proteins probed with PrP^C- or PrP^{Sc}-containing material	Binds to both PrP^C and PrP^{Sc} ($PrP^{27–30}$)	(Fischer *et al.*, 2000;Ellis *et al.*, 2002)
ER Chaperones (calnexin, calreticulin, protein disulfide isomerase, BiP, grp94)	Endoplasmic reticulum	Two sets of immunoprecipitations on radio-labeled cells	Unknown	(Capellari *et al.*, 1999)
Hsp60	Mitochondria	Yeast 2-hybrid	Residues 180–210	(Edenhofer *et al.*, 1996)

NRAGE	Cytoplasm and peripheral membrane protein	Yeast 2-hybrid	Residues 122–231	(Bragason and Palsdottir, 2005)
TREK-1	Cell membrane (transmembrane)	Bacterial 2-hybrid	Residues 128–230	(Azzalin *et al.*, 2006)
Rdj2	Cytoplasmic face of membranes	*In vitro* pull-downs with GST fusions	Unknown	(Beck *et al.*, 2006)
Tubulin	Microtubules	Chemical crosslinking	Unknown	(Nieznanski *et al.*, 2005)
αB-crystallin	Cytoplasm	Yeast 2-hybrid	Unknown	(Sun *et al.*, 2005)
ZAP-70	Cytoplasm	Co-immunoprecipitation	Unknown	(Mattei *et al.*, 2004)
Fyn	Cytoplasmic face of membranes	Co-immunoprecipitation	Unknown	(Mattei *et al.*, 2004)
Casein kinase 2 α/α′ subunits	Cytoplasmic	Far-Western blots and plasmon resonance using recombinant proteins	Residues 105–242	(Meggio *et al.*, 2000)
Nrf2	Cytoplasm/nucleus	Alkaline phosphatase-PrP^C fusion used to screen a mouse brain cDNA library	Unknown	(Yehiely *et al.*, 1997)
APLP1	Cell membrane	Alkaline phosphatase-PrP^C fusion used to screen a mouse brain cDNA library	Unknown	(Yehiely *et al.*, 1997)
RNA aptamers	N/A	GST-PrP^C fusion and library of RNA sequences	N-terminus (residues 23–52)	(Weiss *et al.*, 1997)
Laminin	Secreted	Recombinant GST-PrP^C and laminin co-purification	Unknown	(Graner *et al.*, 2000)
37-kDa/67-kDa laminin receptor	Cell membrane	Yeast 2-hybrid	Residues 144–179 (direct) and residues 53–93 (heparan sulfate proteoglycan-dependent)	(Rieger *et al.*, 1997;Gauczynski *et al.*, 2001)
Stress-inducible protein 1	Cytoplasm	Complementary hydropathy	Residues 113–128	(Zanata *et al.*, 2002)
N-CAM	Cell membrane	Mild formaldehyde crosslinking in N2a cells	Residues 141–176	(Schmitt-Ulms *et al.*, 2001)
'Protein X' (hypothetical protein)	Unknown	Inferred from prion inoculations in transgenic mice expressing chimeric human/mouse PrP^C.	Discontinuous epitope comprising residues 167/171 and 214/218	(Telling *et al.*, 1995;Kaneko *et al.*, 1997b)

Table 3 | **Reported interactors of PrP^C or PrP^{Sc}.** From (Watts *et al.*, 2006).

1.12 Aim of this work

Although PrP^C is evolutionary highly conserved and crucial for neurotoxicity in TSEs the physiological function is not yet properly elucidated. The aim of this study was to shed light on the physiological function of PrP by characterizing novel interactors. Four novel interactors were picked and in a first step, the direct interaction should be tested by co-immuno-precipitations in mammalian cell culture system. Subsequently, the subcellular localization should be tested immunofluorescence experiments. By over-expressing the interactors in cell culture, physiological alterations of PrP should be investigated, including changes in the raft localization of PrP^C. The surface expression of PrP^C should be elucidated via FACS analysis and by the use of radioactive metabolic labelling, the turn-over of PrP^C should be determined. Surface-biotinylation assays were performed to monitor the re-internalization of PrP^C, while cytotoxic effects in consequence of the over-expression were explored by MTT-assay. Finally, PrP^{Sc} levels were studied in scrapie-infected cells regarding possible interference due to the interactors.

2. Material and Methods

2.1 Material

2.1.1 Chemicals

2-Propanol	Roth, Karlsruhe
β-Mercaptoethanol	Sigma, Muenchen
Pure acetic acid	Roth, Karlsruhe
Agarose	Invitrogen, Karlsruhe
Ammoniumperoxodisulfate (APS)	Roth, Karlsruhe
Adenosintriphosphate	Sigma, Muenchen
Bacto Agar Becton	Dickinson, Heidelberg
Bacto Yeast Extract	Becton Dickinson, Heidelberg
Bacto Trypan	Becton Dickinson, Heidelberg
Boric acid	Roth, Karlsruhe
Dimethylsulfoxide	(DMSO) Sigma, Muenchen
Ethanol	Roth, Karlsruhe
Ethidiumbromid	Invitrogen, Karlsruhe
Ethylenediamine tetraacetic acid (EDTA)	Roth, Karlsruhe
Glycine	Roth, Karlsruhe
Methanol	Roth, Karlsruhe
Milk powder	VWR, Darmstadt
N-Lauroylsarcosin (Sarkosyl)	Roth, Karlsruhe
PBS	Gibco, Karlsruhe
Pefabloc SC	Roche, Mannheim
Proteinase K (PK)	VWR, Darmstadt
Protogel	Biozym, Hess. Oldendorf
(30 % (w/v), Acrylamid : Bisacrlyamid (37.5:1))	
Sodium chloride	Roth, Karlsruhe
Sodium dodecylsulfate (SDS)	Roth, Karlsruhe
Sodium deoxycholate (DOC)	Roth, Karlsruhe
Tris-Hydroxy-Methyl-Amino-Methan (Tris)	Roth, Karlsruhe

TEMED	Sigma, Muenchen
Triton X-100	Sigma, Muenchen
Tween-20	Roth, Karlsruhe

All other not itemized chemicals were ordered from Invitrogen (Karlsruhe), Roth (Karlsruhe), Sigma (Muenchen) and VWR (Darmstadt).

2.1.2 Antibiotics

Ampicillin	Sigma, Muenchen
Kanamycin	Sigma, Muenchen

2.1.3 Radioactive compounds

(^{35}S)-Met/Cys (Promix; 1000 Ci/mmol)	Amersham, Freiburg

2.1.4 Restriktionendonucleases und DNA-modifying enzymes

Restriktionendonucleases und DNA-modifying enzymes were ordered at New England Biolabs (Frankfurt), Roche (Mannheim) and Invitrogen (Karlsruhe).

2.1.5 Antibodies

The following antibodies were used for detection of PrP in immunoblot, immunoprecipitations or FACS analysis:

Name	Specificity	Origin	Company / Source
3F4	3F4 epitope (residues 109-112 in human PrP)	Mouse monoclonal	Signet Pathology System, USA
A7	Prion Protein	Rabbit polyclonal	(Proske *et al*. 2002).
4H11	Prion Protein	Mouse monoclonal	generated in collaboration with Dr. Kremmer (GSF, Muenchen)

The following antibodies were used for detection of protein-tags in immunoblot, immunoprecipitations or FACS analysis:

Name	Specificity	Origin	Company
HA-probe (F-7)	internal region of the influenza hemaglutinin (HA) protein	Mouse monoclonal	Santa Cruz Biotechnology, Heidelberg, Germany
c-Myc	epitope mapping within the C-terminus of c-Myc of human origin	Rabbit polyclonal	Santa Cruz Biotechnology, Heidelberg, Germany
Nrage (H-300)	Nrage	Rabbit polyclonal	Santa Cruz Biotechnology, Heidelberg, Germany

The following antibodies were used as secondary antibodies for the detection of PrP and tagged Proteins:

Name	Specificity	Origin	Company
Peroxidase - labeled IgG	Anti-mouse	Sheep	Amersham Pharmacia, Freiburg
Peroxidase - labeled IgG	Anti-rabbit	Donkey	Amersham Pharmacia, Freiburg
Peroxidase - labeled IgG	Anti-goat	Donkey	Santa Cruz Biotechnology, Heidelberg, Germany
Fluorescein – Isothyocianate (FITC)- linked IgG	Anti-mouse	Donkey	Dianova, Hamburg
Cy3 linked IgG	Anti-mouse	Donkey	Dianova, Hamburg

2.1.6 Cell lines

FCS was inactivated for 30 min at 56°C for the usage in cell culture media.

The mouse neuroblastoma cell lines N2a (ATCC CCL 131) and ScN2a have been described ((Butler *et al.*, 1988) and (Schatzl *et al.*, 1997)). Hpl3-4 cells were described previously (Kuwahara *et al.*, 1999). Hpl3F4 cells were made by stably introducing 3F4-tagged PrP into Hpl 3-4 cells (Maas *et al.*, 2007).

Cell line	Origin	Cultivation
N2a	neuroblastoma cell line from spontaneous tumor in A/J mice ($PrP^{a/a}$) (Klebe und Ruddle, 1969)	Opti-Mem Glutamax (Gibco, Karlsruhe) added 55 ml fetal calf serum (PAA, Marburg), 5 ml Penicillin/Streptomycin (10000 IU/ml; 10000 UG/ml) (Gibco, Karlsruhe)
ScN2a	neuroblastoma cell line from mice persistent infected with mouse adapted scrapie strain RML (Rocky-Mountain-Laboratories), stably transfected with 3F4-PrP (Scott *et. al.*, 1992)	as prescribed above for N2a cells
Hpl 3-4	hippocampus cell line, $PrP^{0/0}$, $Dpl^{0/0}$ (Kuwahara *et al.*, 1999)	DMEM Glutamax (Gibco, Karlsruhe) added 55 ml fetal calf serum (PAA, Marburg), 5 ml Penicillin/Streptomycin (10000 IU/ml; 10000 UG/ml) (Gibco, Karlsruhe)
Hpl 3F4	cells were made by stably introducing 3F4-tagged PrP into Hpl 3-4 cells (Maas *et al.*, 2007)	as prescribed above for Hpl 3-4 cells
ScHpl 3F4	cells were made by infection with brain homogenate of strain 22L (Maas *et. al.*, 2007)	as prescribed above for Hpl 3-4 cells

2.1.7 Bacteria

XL1-Blue (Stratagene, Amsterdam): bacteria strain for cloning DNA.

Luri-Bertani-Medium (LB)	10 g/l Bacto Typton 5 g/l Bacto Yeast Extract 10 g/l NaCl
LB-Agar-dishes	15 g Bacto Agar/1 l LB-Medium
LB-Agar-dishes + antibiotics	15 g Bacto Agar/1 l LB-Medium + 50 mg Antibiotic/1 l LB-Medium

2.1.8 Plasmids

The following vectors with noted inserts were used in this work:

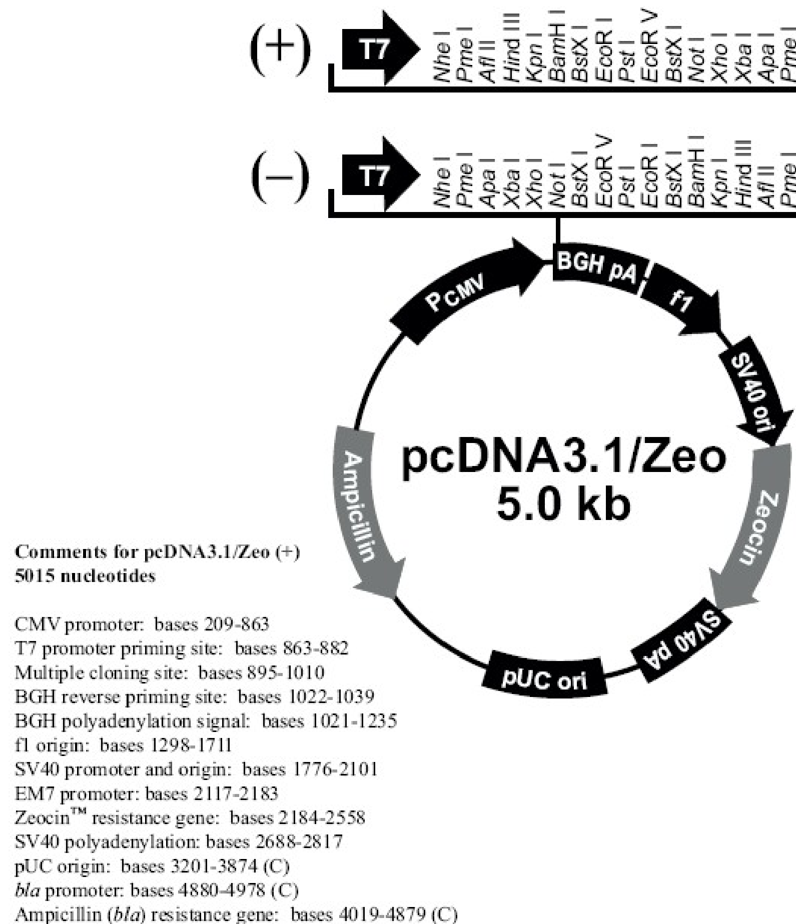


Figure 11 | **Vector map of the plasmid pcDNA3.1/Zeo(+).**
Myc-tagged full-length *mSlit 1* was cloned into the EcoRV site of the vector. According to (Kidd *et al.*, 1999).

Furthermore for the expression of *cytoPrP*, PrP23-230 carrying a epitope tag for monoclonal antibody 3F4 was cloned into this vector (Krammer *et al.*, 2008).

This vector was also used for the expression of ^{Ctm}*PrP*, carrying a epitope tag for monoclonal antibody 3F4 (Stewart and Harris, 2001).

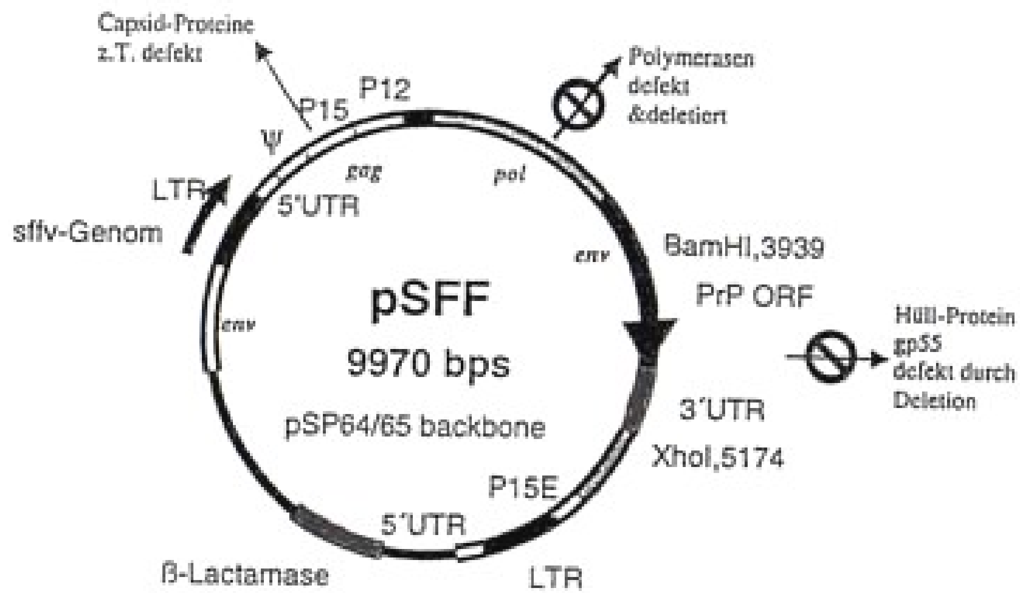


Figure 12 | **Vector map of the plasmid psFF.** According to (Tumas *et al.*, 1996). Full length mITSN-1L was cloned into the BamH1 site of the vector (data presented in this work).

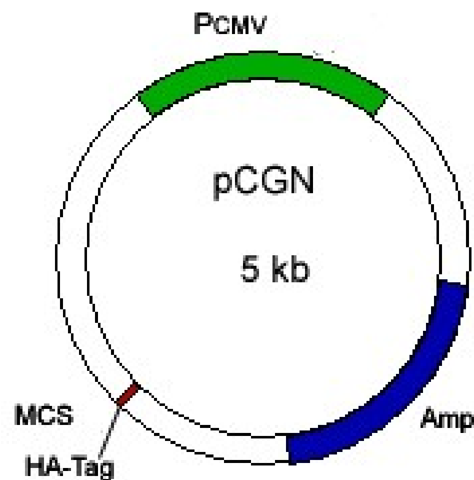


Figure 13 | **Vector map of the plasmid pCGN-HA.**
According to (Hussain *et al.*, 1999). Full length mITSN-1L was cloned into the BamH1 site of the vector.

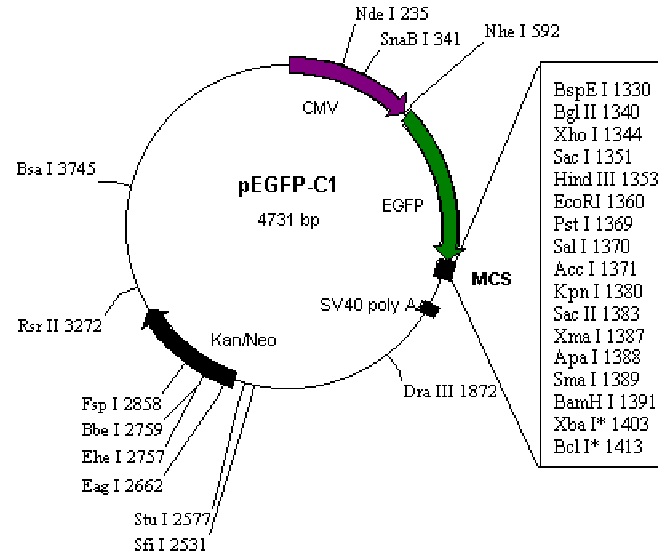


Figure 14 | **Vector map of the plasmid pEGFP.**
Full length myc-tagged NRAGE was cloned into the XhoI/BamH1 site of the vector. According to (Salehi *et al.*, 2000).

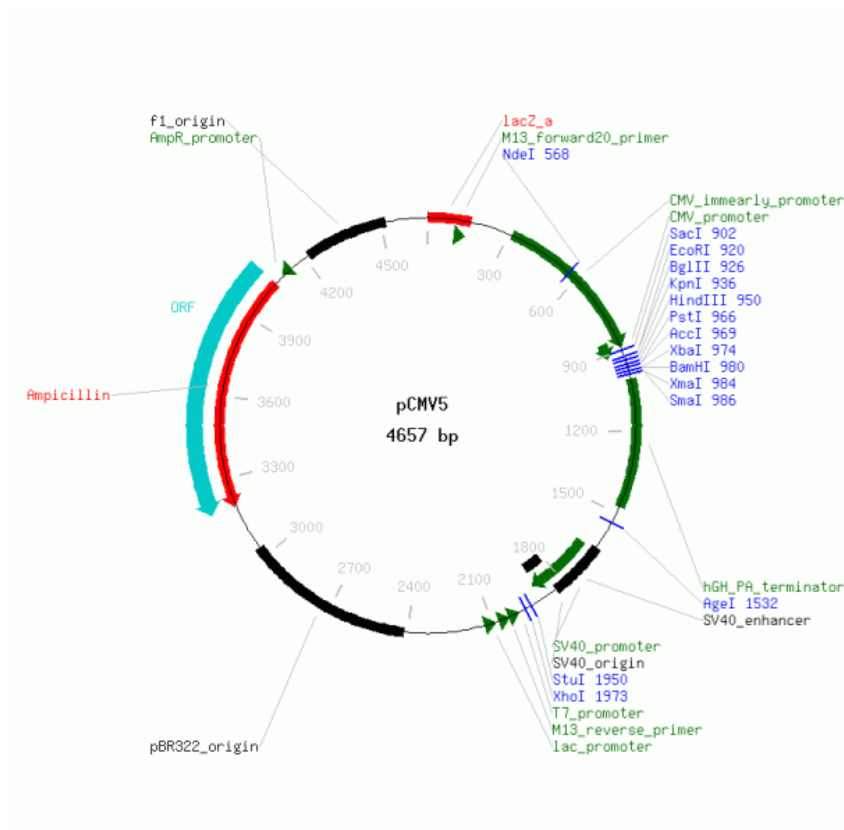


Figure 15 | **Vector map of the plasmid pCMV5.** Full length mSynXIII was cloned into the MCS of the vector. According to (von Poser and Sudhof, 2001).

2.2 Molecular biological methods

All procedures were executed at 4°C – exceptions are annotated in the protocols. All used buffers, media and solutions were made with twice distillated water and autoclaved or filtered sterile before application.

2.2.1 Quantification of nucleic acid concentrations

Concentrations of nucleic acids in solution can be determined by photometric measurement. At this the absorption of ultraviolet light at the wavelength of λ = 260 nm is measured. Conversion in molar concentration is done by different factors according to the kind of nucleic acid measured:

ds DNA: 1 OD_{260nm} = 50 µg/ml
ss DNA/RNA: 1 OD_{260nm} = 40 µg/ml
oligos: 1 OD_{260nm} = 20 µg/ml

This measurement is only exact for absorptions between 0.1 and 0.7. For higher absorptions it is useful to dilute the sample and measure again. To check the purity of the sample the absorption at λ = 260 nm is measured to and the ratio of OD_{260nm}/OD_{280nm} is calculated. A value of about 1.8 is sufficient.

2.2.2 Enzymatic digestion of DNA

Restriction enzymes were used for DNA digestion applying the manufacturer´s recommended buffers in a 20 or 40 µl final volume. 1-3 µg DNA were usually digested with at least 10 units of the required enzyme, either for 2 h or over night at 37° C.
Restriction endonucleases and the 10 x reaction buffer were obtained from New England Biolabs, USA.

2.2.3 Isolation and elution of DNA fragments from agarose gel

After electrophoresis separation, agarose gel fragments were excised from the gel with a scalpel and eluted to 20-50 µl in water using GFX^{TM} Purification Kit (Amersham Pharmacia, Freiburg, Germany) according to manufacturer ´ s procedures.

2.2.4 DNA dephosphorylation

Treatment of digested DNA with alkaline phosphatase (calf intestinal phosphatase, CIP, NEB) which catalyses the removal of 5´ phosphate groups was carried out in order to prevent self-re-circularisation of the plasmide vector. The reaction was set by adding 0.1 units CIP/ pmol DNA and 1 X CIP buffer to the digested DNA. The reaction mixture was incubated at 37° C for 1 h.

2.2.5 Agarose gel electrophoresis

Agarose	Gibco/ BRL Life Lab., Paisley, Scotland
TAE- buffer	40 mM Tris-Acetate 1 mM EDTA, pH 8.0
5x loading buffer	50 % Glycerol in TAE- buffer 0.05 % Bromphenol blue
100 base pair DNA ladder	Gibco/ BRL Life Lab., Paisley, Scotland

Agarose gels (0.5 to 2 % depending on the length of the DNA fragment) were prepared by dissolving agarose in TAE-buffer by boiling the buffer in a microwave. After the solution had cooled to approx. 60° C, 1.5 µl ethidium bromide were added and the mixture was transferred into the appropriate gel chamber for solidification. The gel was then covered with TAE buffer and DNA samples to which the sample buffer had been added as well as 1.5 µl of the DNA ladder marker were loaded onto the gel. The gel was run under constant voltage (110 V) in order to separate DNA fragments. After the electrophoresis, the ethidium-bromide-stained

DNA was visualized under UV-light and photographed with an “eagle eye” processor (Peq Lab, Germany).

2.2.6 DNA ligation

Vector and DNA fragments were ligated with a molecular ratio of 1:3 using either the T4-ligase (Gibco/ BRL Life Lab., Paisley, Scotland) or the DNA Quick Ligation Kit (Roche Diagnostic, Mannheim). For ligation with T4-ligase, the 10 x ligation buffer and 1 unit of the ligase were used in a total volume of 20 µl and the reaction mixture was incubated over night at 16°C. Ligation with the Quick Ligation Kit was carried out according to manufacturer´s procedures and the reaction was incubated at room temperature for 8-9 min.

2.2.7 Cloning into the pSFF vector

The handling of the pSFF vector is rather difficult due to the size of the vector (9.9 kbp). So a specialized protocol had to be used for cloning ITSN-1L into the pSFF vector.
5 µg of vector were cut with 2 µl BamH1 according to manufacturer´s procedures (NEB). The restricted DNA was precipitated with Sodiumacetate. Therefore 5 µl Sodiumacetate (3 M, pH 5.2) and 150 µl EtOH (96 %) were added to the complete 50 µl restriction mix and incubated for 2 h at -20°C. The complete mix was centrifuged for 30 min at 14000 rpm and 4°C. The resulting pellet was redissolved in 30 µl $H_2O_{bidest.}$.
9 µg of BamH1-cutted ITSN-1L and 1 µl pSFF vector were used for ligation with T4 ligase according to manufacturer´s procedures (Roche) and subsequently analyzed by agarose gel electrophoresis. The ligated plasmid of the expected size was cutted out (see 2.2.3) and the insert was sequenced.

2.2.8 Polymerase chain reaction (PCR)

Oligonucleotides	Metabion (Germany)
Deoxynucleotide 1 mM each dATP, dTTP, dGTP, dCTP	(Amersham Pharmacia, Freiburg, Germany)
DNA polymerase Taq Gold	(Roche Diagnostic, Mannheim),
Pfu Turbo	(Stratagene, The Netherlands)
10x PCR reaction buffer	Roche Diagnostic, Mannheim; Stratagene, The Netherlands

Polymerase chain reaction procedure was used to amplify specific DNA sequences in vitro. A 50 µl reaction was set as follows:

DNA template	50-100 ng
5´ primer	2 µl (100 nM)
3´ primer	2 µl (100 nM)
dNTP-mix	50 µM
Polymerase	10 units
10 x polymerase reaction buffer	10 % volume
H_2O dest.	ad 50 µl

Reaction mix was set in 0.5 ml reaction tubes and was carried out in a PE 9600 Thermocycler (Perkin Elmer). The temperature for hybridization of oligonucleotides with single strand parental DNA (Annealing) was generally set at 55° C. For more specific reactions the temperature was adjusted according to the following formula:

T_A (°C) = 60+ [(G+ C) x 41/ Nt] – (600/ Nt)

T_A represents the annealing temperature, C+G the amount of guanine and cytosine in the primary sequence of the priming nucleotide and Nt the length (number of nucleotides) of the primers).

Amplification parameters:

95° C	5-10 min initial denaturation (once)
35 cycles with:	
95° C	1 min amplification
55° C (or required T)	1 min annealing temperature
72° C	1.5 min elongation
72° C	7 min final elongation (once)

Amplified DNA fragments were loaded on agarose gels for electrophoresis and purification.

2.2.9 Preparation of competent bacteria

SOC medium	2 % Trypton
	0.5 % Bacto Yeast Extract
	10 mM NaCl
	2.5 mM KCl
	10 mM $MgCl_2$
	10 mM $MgSO_4$
	20 mM Glucose

200 ml LB medium were inoculated with one colony from an LB agar plate and transferred to a shaking incubator at 37°C until an OD_{600nm} of 0.6 - 0.7 was reached. The bacterial culture was placed on ice for 15 min. After centrifugation at 3000 g for 15 min at 4°C the pellet was first suspended in 50 ml ice cold 100 mM $MgCl_2$, placed on ice again for 30 min and then, after a new centrifugation step suspended in 50 ml cold 100 mM $CaCl_2$ and placed on ice for 30 min Bacteria were pelleted again and incubated overnight in 5 ml $CaCl_2$ at 4°C. 0.5 ml glycerol and 2.5 ml $CaCl_2$ were added and 100 µl aliquots were stored at –80°C.

2.2.10 Heat shock transformation

Heat shock competent E. coli (100 µl aliquot) were thawed on ice and up to 1 µg plasmid DNA or ligation mixture (max. 10 µl) were added and incubated on ice for 30 min. Bacterial cells were then warmed to 42°C for 90 sec and immediately chilled on ice for 2 min. 400 µl

SOC medium was added and the bacteria were transferred to a shaking incubator set at 37°C to allow the bacteria to recover and to express the antibiotic resistance. After incubation for 30 min, the cultures were plated onto an LB agar plate with the appropriate selective antibiotic and incubated at 37°C overnight.

2.2.11 DNA extraction and purification

Solution I	10 mM EDTA, 400 µg/ ml RNase I 100 mM Tris-HCl, pH 7.5
Solution II	1 M NaOH 5.3 % dodecylsulfate sodium salt (SDS)
Solution III	Buffered solution containing acetate

For isolation of plasmid DNA from bacterial culture, the GFX Micro Plasmid Prep Kit (Amersham Pharmacia, Freiburg, Germany) was used according to manufacturer´s directions. 1.5 ml of an overnight bacterial cell culture was pelleted in a microcentrifuge tube and suspended in an isotonic solution containing RNA (Solution I). Cells were then lysed by alkali treatment, chromosomal DNA and proteins were denatured with addition of solution II. The pH of the solution was neutralized with an acetate solution (Solution III) and cell debris, chromosomal DNA and proteins were precipitated. After centrifugation, the supernatant cell lysate was transferred to a GFX glass fiber matrix column to bind plasmid DNA. The matrix bound DNA was washed with an ethanolic buffer to remove salts and other residual contamination. The DNA was eluted from the matrix in 100 µl sterile bidest. water. The total yield of DNA was between 1 and 5 µg.

Large scale preparation of plasmid DNA from bacteria (Maxipreparation)

Resuspension buffer (P1)	50 mM Tris/HCl, pH 8.0 10 mM EDTA 100 µg/ ml RNase A

Lysis buffer (P2)	2 mM NaOH, 1% SDS (w/v)
Neutralization buffer	3.0 M potassium acetate, pH 5.5
Equilibration buffer (QBT)	750 mM NaCl 50 mM MOPS 15 % (v/ v) EtOH 0.15 % (w/ v) Triton X-100
Wash buffer (QC)	1 M NaCl 50 mM MOPS 15 % (v/v) EtOH pH 7
Elution Buffer (QF)	50 mM Tris/ HCl, pH 8.5 1.25 M NaCl 15 % (v/ v) EtOH

For purification of up to 500 µg plasmid DNA from bacterial culture, 2 ml of an overnight clone culture were diluted into 100 ml selective LB medium and were grown overnight at 37°C. After pelletting the cells at 4000 g and 4° C for 15 min, the commercially available Quiagen Maxi Purification Kit was applied according to manufacturer's direction. The protocol is based on an alkaline lysis procedure, followed by binding of plasmid DNA to an anion exchange resin columns under low salt condition. The DNA was eluted, concentrated and desalted by isopropanol precipitation. The pellet was dissolved in sterile H_2O dest.

2.2.12 Determination of cell density

The number of cells in the growing culture was monitored by measurement of the optical density at a wavelength of $\lambda = 600$ nm in 1.5 ml plastic cuvettes, culture medium was used as a control.

2.2.13 Storage of bacteria

For storage of bacteria, 1 ml aliquots from a liquid culture ($OD_{600nm} \approx 0.8$) were added to 400 µl glycerol and preserved in 1.5 ml storage tubes equipped with screw caps and stored at -80°C.

2.3 Working with mammalian cells

2.3.1 Culture media

DMEM, Dulbecco´s modified Eagle medium	
(Gibco/ BRL Life Lab., Paisley, Scotland)	
supplemented with	2 mM L-Glutamine
	100 IU/ml Penicillin
	0.1 mg/ ml Streptomycin
	10 % (v/v) Fetal Calf Serum (FCS)
	(inactivated at 56°C for 30 min)
Opti-Mem	100 IU/ml Penicillin
(Gibco/ BRL Life Lab., Paisley, Scotland)	0.1 mg/ ml Streptomycin
	10 % (v/v) Fetal Calf Serum (FCS)
	(PAA, Marburg)
	(inactivated at 56°C for 30 min)
Trypsin-EDTA	
(Gibco/ BRL Life Lab., Paisley, Scotland)	0.25 % Trypsin, 1 mM EDTA
Phosphate buffered saline (PBS)	
(Gibco/ BRL Life Lab., Paisley, Scotland)	

2.3.2 Culture of mammalian cells

Cells were cultivated on appropriate culture dishes (NUNC) and kept in an incubator in a 5 % CO_2, 90 % humidity atmosphere at 37°C. Medium was changed every two days. When 80 % confluency was reached, cells were washed with PBS, briefly treated with 1 ml Trypsin-EDTA in order to remove cells from the dish. Cells were then suspended in culture medium and transferred to a new culture dish in a 1:10-1:20 volume ratio, unless otherwise specified.

2.3.3 Storage of eukaryotic cells

Dimethylsulfoxide (DMSO) Sigma, Munich

For storage of mammalian cells, 75-80 % confluent adherent cultures were subjected to mild treatment with trypsin and suspended in culture medium with addition of 20 % total FCS and 10 % DMSO. 1 ml aliquots of the suspension were transferred to 1.5 ml cryotubes (NUNC) and frozen to –80°C for 24 h. Tubes were then transferred to liquid nitrogen for long-term storage.

2.3.4 Thawing of cells

Thawing was achieved by incubating frozen cells at 37°C for 10-15 min. Cells were resuspended in 10 ml culture medium with 10 % FCS and centrifuged for 10 min at 1000 g in order to remove toxic DMSO. The supernatant was removed and the pellet resuspended in 10 medium and plated on culture dish.

2.4 Cell biological methods

2.4.1 Cell surface biotinylation

Sulfo-biotin-X-NHS (Pierce)	250 µ/ml
Lysis buffer	50 mM Tris-HCl pH 7.5
	150 mM NaCl
	0.5 mM EDTA
	1 mM NaF
	0.5 % (v/v) Triton-X 100
	1 % (v/v) Tween-20

The interaction of ITSN-1L and PrP^C located on the cell surface was assessed by surface biotinylation followed by co-immunoprecipitation. Hpl 3F4 cells were plated on 6 cm Petri dishes and transiently transfected with ITSN-1L.
After 72 h, cells were placed on ice, rinsed twice with cold PBS and incubated on ice for 25 minutes with 1 ml PBS containing 250 µg/ml membrane-impermeable sulfo-biotin-X-NHS (Pierce). Cells were washed again three times with cold PBS to remove unbound biotin and culture medium before incubation at 37°C for 45 min in normal culture medium. Cells were washed twice with PBS, harvested with 1 ml lysis buffer on ice for 10 minutes and cellular debris was removed by centrifugation at 14,000 rpm for 5 min. The proteins were harvested and prepared for immunoblotting or immunoprecipitation followed by immunoblotting. Blots were developed with horseradish peroxidase-conjugated Streptavidin and visualized using the enhanced chemoluminescence blotting kit from GE Healthcare (ECL plus).

2.4.2 MTT-Assay

The MTT-Assay is a non-radioactive colorimetric test, which is based on the reduction of yellow tetrazolium salt 3-[4.5-Dimethylthiazol-2-yl]-2.5-Diphenyl Tetrazolium Bromide (MTT) to purple formazan crystals via cellular mitochondrial dehydrogenases. These dehydrogenases cleave the tetrazolium ring in MTT-tetrazolium salts resulting in MTT-formazan. These water insoluble formazan crystals are dissolved in isopropanol. The absorption of this formazan solution can be measured at a wavelength of $\lambda = 550 – 600$ nm.

Cleavage of the tetrazolium ring is direct proportional to the cell count and displays the viability of the cells. For the MTT-Assay 10000 cells were seeded in a 96-well plate in 100 µl medium. On the following day cells were transiently transfected with constructs as mentioned in the relating results. Per construct 10 wells were treated in parallel and mock-treated cells additionally treated with Staurosporin (inhibitor of protein kinases) were used as positive control. As negative control mock-treated cells were also measured. 72 h later 20 µl of MTT solution were added to each well. After 3 h of incubation at 37°C and 0.5 % CO_2 medium was removed and 150 µl 2-Propanol/0.03 M HCl was added to the cells. After an additional incubation of 2 h (37°C, 0.5 % CO_2) the absorption of the resulting solution was measured with a Sunrise ELISA-Reader (Tecan, Maennedorf, Schweiz) at λ = 570 nm. The resulting absorptions were statistically evaluated with MS Office Excel.
Stock solution: MTT (3-[4.5-Dimethylthiazol-2-yl]-2.5-Diphenyl Tetrazolium Bromide)
5 mg/ml in PBS
Cells treated with Staurosporin (1 µM in DMSO) were used as positive control. Staurosporin is an inhibitor of ATP-dependent proteases, simulating cell death.

2.4.3 Metabolic labelling and radio-immunoprecipitation

Cys/ Meth-free medium	RPMI without cysteine or methionine (Gibco/ BRL Life Lab., Paisley, Scotland) with 1% FCS
$[^{35}S]$- Cysteine and methionine Promix	L-$[^{35}S]$- in vitro Cell labelling mix 1000 Ci/mmol (Amersham Pharmacia, Freiburg)
Chase medium	complete culture medium with 10 % FCS
Lysis buffer	100 mM NaCl 10 mM Tris-HCl, pH 7.5 10 mM EDTA 0.5 % Triton X-100 0.5 % DOC
RIPA buffer-SDS	0.5 % Triton X-100

	0.5 % DOC 1% SDS in PBS
Protein A- sepharose	50 % suspension in RIPA buffer-SDS (Amersham Pharmacia, Freiburg)
Pefabloc protease inhibitor	1 % stock solution (Roche Diagnostic, Mannheim)

For immunoprecipitation transiently transfected (ITSN-1L, Slit 1) N2a cells (6 cm Petri dishes) with 80-90 % confluency were used. Cells were washed twice with phosphate-buffered saline (PBS) and placed into an incubator at 37°C for 1 h in the presence of 1 ml RPMI medium without methionine/cysteine containing 1% FCS to promote metabolism of endogenous methionine and cysteine. Subsequent radioactive labelling of proteins was carried out by adding 400 µCi/ml $[^{35}S]$-Met/Cys to the medium for 1 h at 37°C (pulse). After incubation, cells were washed twice in cold PBS and harvested with 1 ml lysis buffer on ice for 10 min or further incubated at 37 °C for different lengths of time in 5 ml complete culture medium to allow transport to the cell surface (chase). After appropriate chase times, cells were washed with PBS and directly harvested. 1 ml lysis buffer was added to the dishes for 10 min on ice. After lysis, cell lysates were transferred to 1.5 ml tubes and debris was removed by centrifugation for 40 sec at 18000 g. After addition of 1% N-lauryl sarcosine, postnuclear lysates were boiled at 95°C for 10 min. Samples were placed on ice and 20 µl Pefabloc protease inhibitor were added; lysates were then incubated with antibody 4H11, as indicated, overnight at 4 °C (dilution 1:100). The protein-antibody complexes were precipitated by addition of 100 µl protein A-Sepharose beads for 90 min at 4°C. The beads were then centrifuged at 18000 g for 1 min and washed in RIPA buffer supplemented with 1 % SDS at 4°C. The washing procedure was repeated five times. All samples were analyzed by 12.5 % SDS-PAGE after elution with 4x sample buffer and boiling for 10 min at 95°C. SDS-polyacrylamide gels containing proteins radiolabelled with ^{35}S-labelled amino acids were subsequently fixed in blotting buffer for 15 min before placing on Whatman paper and fitting on a gel for 2 h at 80°C. Dried gel was then placed in a light-tight X-ray holder and covered with a sheet of X-ray film (Kodak Biomax MR). The film holder was stored at –80°C for about 2 weeks and then developed with an automatic film processor.

2.4.4 Transient transfection of mammalian cells

Transfection with Fugene 6 (Roche Diagnostic, Mannheim) using N2a cells

Fugene 6 transfection reagent is a blend of lipids and other components supplied in 80 % ethanol. Standard transfection was performed by dissolving 2 µg DNA in serum-free medium. Transfection reagent was then added and the mix was incubated at room temperature for 15 min before adding to the cells.

Transfection with Lipofectamine 2000 (Invitrogen, Carlsbad, USA) using Hpl 3-4 cells

Medium and Lipofectamine 2000 were warmed to room temperature.

Solution A	500 µl Opti-Mem w/o FCS/AB 10 µl Lipofectamine 2000 5 min incubating at RT after mixing
Solution B	500 µl Opti-Mem w/o FCS/AB 4 µg DNA 5 min incubating at RT after mixing

Solution A and B were mixed and incubated for 20 min at room temperature. Cell culture medium (DMEM, 10 % FCD, Pen/Strep) was changed (2 ml per 6 cm Petri dish). 400 µl mixed Solution A+B was added per 6 cm dish and was dispensed by panning.

Transfection using Lipofectamine 2000 (for siRNA)

The siRNA was dissolved in the provided amount of water resulting in a concentration of 10 µM/µl. 4 µl siRNA per transfection of one 6 cm dish was used. Solution A and B were mixed and incubated for 20 min at room temperature.

Solution A	500 µl Opti-Mem w/o FCS/AB 10 µl Lipofectamine 2000 5 min at RT incubating after mixing
Solution B	500 µl Opti-Mem w/o FCS/AB 4 µl siRNA 5 min at RT incubating after mixing

50 % confluent cells were washed twice with PBS. 4 ml medium without FCS and Antibiotics were added to the cells. 1ml of mixed solution A+B was applied drop wise on the cells.

2.4.5 Generating viral particles for transduction

The cells of the two package cell lines (PA 317, ψ 2 – culture medium DMEM) were mixed 1:1 in a 6-well dish. A total of 300,000 cells were plated. The cells were transiently transfected with pSFF-ITSN1 via Lipofectamine 2000 according to 2.4.4. When cells reached a confluency of ca. 90 %, they were trypsinised and split into the next 6-well. After three passages in 6-well plates the cells were expanded to 6 cm dish and from there after reaching 90 % confluency onto 10 cm dishes. The cell culture medium was harvested, centrifuged for 10 min at 1000 rpm and from the supernatant were aliquots of 1 ml immediately stored at -80°C.

2.4.6 Transduction of mammalian cells

250,000 cells were plated on a 6 cm dish and were grown for 24 h in an incubator. Afterwards 4 µg/ml Polybrene were added to the cell culture medium and incubated for at least 2 h. 1 ml of retroviral particles were thawed in a 37°C waterbath as fast as possible and applied tropwise directly onto the cells. Cells were grown for additional 24 h, followed by extensive

washing with PBS, before medium change. The expression of the gene of interest was examined by SDS-PAGE.

2.4.7 Preparation of Protein A-Sepharose

1.5 g of Sepharose were added to 50 ml H_2O and incubated for 15 min at room temperature. Sepharose was washed twice in H_2O (1000 g, 10 min) before it was redissolved in 15 ml H_2O. Aliquots of 1 ml were stored at 4°C. Before protein a-sepharose can be used in immunprecipitation it was equilibrated twice in the according buffer and resuspended in 1 volume.

2.4.8 Co-Immunoprecipitation

Co-IP lysis buffer	50 mM Tris-HCl pH 7.5
	150 mM NaCl
	0.5 mM EDTA
	1 mM NaF
	0.5 % (v/v)Triton-X 100
	1 % (v/v) Tween-20
Pefabloc SC stock solution	1 % (w/v) in H_2O
Protein-A-Sepharose (storage)	50 % (w/v) in H_2O (Amersham, Freiburg)
Protein-A-Sepharose (usage)	50 % (w/v) in appropriate buffer

All procedures were done on ice. Cells were washed twice in ice cold PBS and lysed in 1 ml lysis buffer for 10 min on ice. After removal of insoluble debris by centrifugation at 14000 rpm for 5 min at 4°C, antibody (dilution 1:100) and 0.5 mM of Pefabloc were added and the sample was incubated for 3 h at 4°C. Protein-A Sepharose was added for an additional 1.5 h of incubation at 4°C. The beads then were washed five times with ice cold PBS, and the precipitated proteins were eluted by incubation at 95 °C for 5 min in 60 µl of 4 x sample buffer and used in immunoblotting. An aliquot (20 µl) of each sample was analyzed on 12.5 % SDS-PAGE for PrP and 7.5 % SDS-PAGE for the detection of ITSN-1L and Slit 1.

2.4.9 Preparation of postnuclear lysates

Lysis buffer	100 mM NaCl
	10 mM Tris-HCl, pH 7.5
	10 mM EDTA
	0.5 % Triton X-100
	0.5 % DOC

Adherent cells were washed once with PBS and were subsequently covered with 1 ml lysis buffer at room temperature for 10 min. Total lysates were transferred to 1.5 ml reaction tubes for separation of cell debris by centrifugation for 40 sec at 14.000 rpm. 20 µl of the 1 % Pefabloc stock solution were added to the supernatants before precipitation with 10 vol. methanol overnight at -20°C or -80°C for 2 h. Samples were then centrifuged at 3500 rpm for 30 min at 4°C and the resulting pellet was resuspended in 50-100 µl TNE and 4x SDS-sample buffer was added. Samples were heated at 95°C for 10 min and placed on ice.

2.4.10 Protein preparation from cell culture medium

Hpl3-4 cells were transiently transfected with a plasmid encoding myc-tagged Slit 1. 24 h post transfection cells were grown for an additional 24 h in medium without FCS. The medium was taken and cellular debris was removed by centrifugation at 14000 rpm for 5 min. 5 volumes of methanol were added to the medium and incubated for 24 h at -25°C. Precipitated proteins were centrifuged for 25 minutes at 3500 rpm and the pellets were redissolved in TNE buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA) with 4 x SDS-sample buffer. After boiling for 10 minutes, an aliquot was analyzed by SDS-PAGE.

2.5 Biochemical methods

2.5.5 Proteinase K (PK) digestion

Proteinase K (PK)	1 mg /ml stock solution (Merck, Darmstadt)

Aliquots of post-nuclear lysates were incubated for 30 min at 37°C with 20 µg/ml PK; the digestion was stopped by addition of protease inhibitor Pefabloc. Samples were precipitated with ethanol and analyzed in immunoblot assay.

2.5.6 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

4 x Resolving solution	1.5 M Tris 0.4 % SDS pH ad 8.8
4 x Stacking solution	0.5 M Tris 0.4 % SDS pH ad 6.8
Ammoniumpersulfate (APS)	10% (w/ v) stock solution
N,N,N,N-tetramethylethylendiamin (TEMED)	Pharmacia/ LKB, Upplasa, Sweden
Resolving gel (12.5 % acrylamide)	25.9 ml 30 % (w/ v) Acrylamide/ Bisacrylamide (37.5:1) Protogel (National Diagnostics, Atlanta, USA) 15.4 ml resolving solution 20.3 ml aqua dest. 90 µl TEMED

	192 µl 10% (w/v) Ammoniumpersulfate (APS) in H_2O
Stacking gel	2.8 ml 30 % (w v) Acrylamide/Bisacrylamide (37.5:1)
(5 % acrylamide)	4.2 ml stacking solution
	9.9 ml aqua dest.
	30 µl TEMED
	168 µl 10% (w/v) APS
10 x SDS electrophoresis buffer	250 mM Tris
	2.5 M Glycine
	1 % SDS

Electrophoresis separation of proteins was carried out on discontinuous, denaturing SDS gels containing 12.5 % or 7.5 % acrylamide (Laemmli, 1970). The gels consist of two parts, the upper stacking gel and the lower resolving gel. Due to the pH-Value and ion concentrations proteins get more focused in a distinct band running through the stacking gel. After that proteins get separated by size in the resolving gel. Once polymerization was completed, the glass plates containing stacking and resolving gels were mounted in an electrophoresis chamber (Midi Protein Gelkammer, Peqlab) and covered with electrophoresis buffer. Samples were loaded on the gel (approx. 15-25 µl) together with a protein standard (Rainbow-Protein-Size marker (Amersham, Freiburg)). Constant ampere - 25 mA for the time in stacking gel and 45 mA for the time in resolving gel - was applied for mass separation until the proteins had reached the bottom of the resolving gel.

2.5.7 Western blot

Blotting buffer	20 % Methanol 3 g Tris 14.5 g Glycine
10 x TBST	0.5 % Tween 20 100 mM NaCl 10 mM Tris-HCl pH 7.4
Skim milk buffer 5 %	(2.5 g) skim milk powder (Merk, Darmstadt) in TBST buffer (ad 50 ml)
PVDF membrane	Amersham Pharmacia Freiburg, Germany
Semi-Dry transblot machine	Bio-Rad, Richmond, USA
Detection kit ECL ^{Plus} Western Blot Detection kit	Amersham Pharmacia, Freiburg, Germany
X-ray films	Kodak Biomax XS

After electrophoretic mass separation, proteins were electro-transferred from polyacrylamide gels onto a Hybond-P:PVDF membrane using a semi-dry-method. The membrane was activated with methanol and then equilibrated with dest. water. Gel was placed in contact with the membrane. They were then sandwiched together between 6 layers of Whatman paper which had been soaked in Blotting Buffer. A Semi-Dry Transblot electrophoresis apparatus (Biorad) was used for the transfer. The gel was blotted for 2 h at 0.8 mA/cm^2 gel. The membrane was then incubated in 5% skim milk buffer (or gelatin for goat antibodies) for 30 min at room temperature for saturation of unspecific binding sites and then in TBST with the specific primary antibody and shaken overnight at 4° C. The membrane was washed 5 times (5 min each) with TBST and the secondary antibody (peroxidase conjugated) was subsequently added to TBST at a dilution of 1:7500 for 30 min. The membrane was washed

again 3 times with TBST, finally with dest. water. The antibody-protein complex was visualized with an enhanced chemoluminescence ECL-plus kit by incubating the membrane for 3 min in the ECL-solution (solution 1 : solution 2 = 1:40), drying it with Whatman paper and exposing it to X-ray films.

2.5.8 Flotation assay for detergent-resistant microdomains (DRM)

Lysis buffer	150 mM NaCl
	25 mM Tris–HCl pH 7.5
	5 mM EDTA
	1 % Triton-X 100
TNE	150 mM NaCl
	25 mM Tris–HCl pH 7.5
	5 mM EDTA

For the isolation of detergent-resistant microdomains (DRM) or rafts, 3×10^7 cells were solubilized in 400 µl lysis buffer and incubated on ice in the cold room for 30 min. The cell lysate was mixed with Nycodenz 70% in TNE to a final concentration for Nycodenz of 35% and loaded into an ultracentrifuge tube. This fraction was overlaid by a discontinuous Nycodenz gradient formed by 200 µl fractions of Nycodenz solutions with concentrations of 25, 22.5, 20, 18, 15, 12, and 8%. After ultracentrifugation (200,000 g, 4 h, 4°C, Beckmann TLS55 rotor), 200 µl fractions were collected from the top to the bottom of the gradient and precipitated with 5 volumes of methanol. After centrifugation for 30 min at 3500 rpm, the pellets were resuspended in TNE, and an aliquot of each fraction was analyzed in immunoblot.

2.5.9 Confocal laser scanning microscopy

Cells were plated on glass cover slips (Marienfeld, Germany) in a concentration that they were about 70 % confluent after 72 h. 24 h after plating they were transiently transfected via lipofection as described above and let grown for additional 24 h. After that cells were washed three times with PBS and fixed in Histofix (Roth) for 30 min at room temperature. Cells were washed three times with PBS after fixation and were subjected to NH_4Cl (50 mM in 20 mM glycine), Triton X-100 (0,1 %), and Gelatin (0,2 %) treatment for 10 min each at room temperature, each time followed by three washing steps with PBS. The first antibodies were added 1:100 in PBS and incubated for 45 min at room temperature. After three times washing with PBS cells were incubated with Cy2- or Cy3-conjugated secondary antibodies diluted 1:100 in PBS and incubated for 45 min in the dark. This was followed by three washings steps with PBS. The cover slips were mounted on microscope slides, dried for 60 min at room temperature and kept at -25°C in the dark. Confocal laser scanning microscopy was done using a Zeiss LSM510 Confocal System (Zeiss, Goettingen, Germany).

2.5.10 Fluorescence Activated Cell Sorting (FACS) analysis

FACS buffer	2.5 % fetal calf serum (FCS) 0.05 % Sodium-Acide in PBS
EDTA	1 mM in PBS

For detection of PrP surface expression in transfected cells, 5 ml EDTA were applied to detach cells from culture dishes. 1 ml cells was harvested and centrifuged at 1200 rpm for 2 min, redissolved in 500 µl FACS buffer and incubated on ice for 5 min. Cells were incubated with primary antibodies (dilution 1:100 in FACS buffer) for 45 minutes on ice and washed afterwards three times with FACS buffer. Secondary antibodies (Cy2-labelled, Dianova) were incubated for 45 minutes on ice and in the dark. After washing 20 µl 7-AAD (Becton and Dickinson) was added for staining of dead cells and incubated additional 15 min on ice and in the dark. Flow cytometry was performed in a FACS EP CS XL (Coulter). As a negative

control, cells incubated with the secondary antibody alone were used and the resulting signals were subtracted from the values obtained using both antisera. Forward and side scatters were collected as linear signals and all fluorescent emissions on a logarithmic scale. A gate based on FSC and SSC was set to separate living and dead cells and ten thousand events were acquired inside the gate using EXPO 32 ADC (Coulter) software.

3. Results

The N-terminal portion of PrP has been implicated in subcellular trafficking and cellular quality control mechanisms (Nunziante *et al.,* 2003; Gilch *et al.,* 2004). To search for N-terminal interactors of PrP^C a yeast two-hybrid system (Y2H), screening a murine, neuronal cDNA library with the N-terminal part of PrP (residues 23 – 100) as bait was performed (Schätzl, unpublished data).

Several novel interactors of the prion protein were identified. Intersectin 1 (ITSN-1L), Slit 1, Nrage and Synaptotagmin 13 (SynXIII) were chosen to work on, as they were the most interesting candidates.

3.1 Co-immunoprecipitation experiments with candidate interactors from Y2H screen

In a first step the interaction of candidate interactors from the Y2H screen with PrP had to be confirmed by a method unrelated to the original Y2H screening. So, co-immuno-precipitations were performed. The cell lines N2a, CHO, Hpl3-4, Hpl3F4 were transiently transfected with ITSN-1L (HA tag), Slit 1 (myc tag), Nrage (Flag tag) and SynXIII (myc tag). The Hpl3-4 cell line is a PrP knock-out cell line carried along just as a control for transfection efficiency and acting also as a control for the immunoprecipitation. Cell lysates were subjected to co-immunoprecipitation using the tag antibodies. This means, in these experiments the interactors where used as the "bait" to pull out PrP, because the applied antibody for Co-immunoprecipitaion was directed against the interactors. Using the Y2H candidate interactors ITSN-1L and Slit 1 as bait PrP^C could be detected via immunoblot (Fig. 17, lanes 1, 2). Nrage and SynXIII showed no interaction with PrP in co-immunoprecipitations (Fig. 16, C, D and Fig. 17, lanes 3, 4). Neither PrP nor the candidate interactors showed an intrinsic affinity to Protein-A Sepharose (Fig. 16, A lane 5, B lane 10, C lane 15, D lane 20 and Fig. 17 lane 5). Furthermore, PrP could not be precipitated by the anti-tag antibodies alone (Fig. 17 lane 6). It was also tested to use PrP as bait (the antibody in the Co-immuoprecipitation was directed against PrP) and co-precipitate the candidate interactors – this way around also ITSN-1L and Slit 1 were also the sole confirmed interactors (Fig. 16 A lane 4, B lane 9).

For the following characterizations only ITSN-1L and Slit 1 were used.

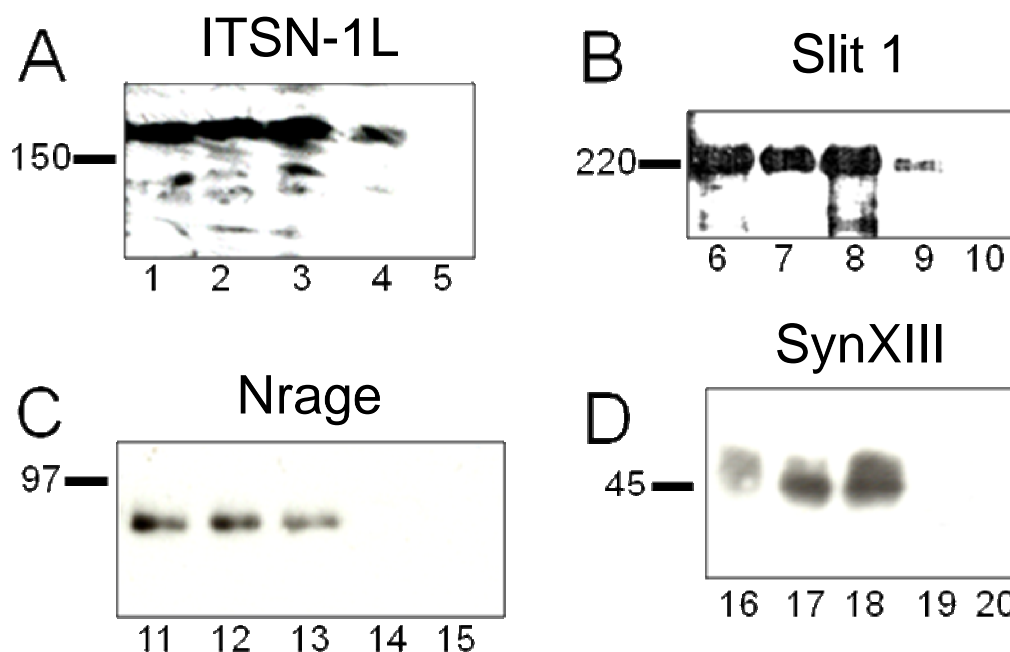


Figure 16 | **Transfections and co-immunoprecipitations of candidate interactors. a** | Cells transfected with ITSN-1L. Cell lysates of: (1) N2a, (2) CHO, (3) Hpl3-4. (4) Co-immunoprecipitation in Hpl3F4 cells with PrP as bait. (5) Immunoprecipitation with Protein-A sepharose only as control. **b** | Cells transfected with Slit 1. Cell lysates of: (6) N2a, (7) CHO, (8) Hpl3-4. (9) Co-immunoprecipitation in Hpl3F4 cells with PrP as bait. (10) Immunoprecipitation with Protein-A sepharose only as control. **c** | Cells transfected with Nrage. Cell lysates of: (11) N2a, (12) CHO, (13) Hpl3-4. (14) Co-immunoprecipitation in Hpl3F4 cells with PrP as bait. (15) Immunoprecipitation with Protein-A sepharose only as control. **d** | Cells transfected with SynXIII. Cell lysates of: (16) N2a, (17) CHO, (18) Hpl3-4. (19) Co-immunoprecipitation in Hpl3F4 cells with PrP as bait. (20) Immunoprecipitation with Protein-A sepharose only as control. The western blots were analyzed each with the adequate anti-tag antibodies.

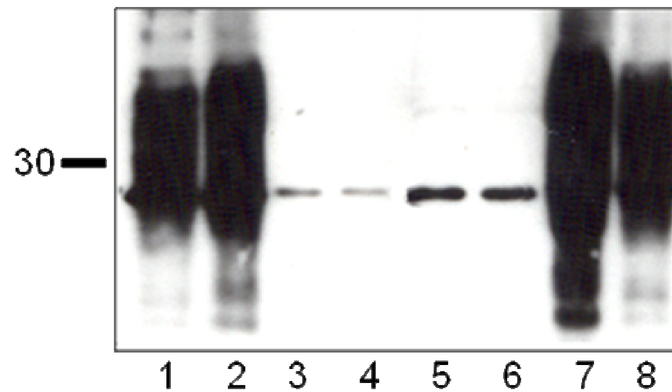


Figure 17 | **Co-immunoprecipitations of candidate interactors.** N2a cells were transfected with ITSN-1L (1), Slit 1 (2), Nrage (3) and SynXIII (4). In this experiment the candidate interactors were used as baits. (5) Affinity test of PrP to Protein-A sepharose. (6) Immunoprecipitation with anti-HA antibody for PrP in untransfected N2a cells. (7) + (8) Immunoprecipitations with anti-PrP antibodies A7 and 4H11 as positive controls. The western blot was developed with mAb 4H11. The unspecific signals in (3), (4), (5) and (6) result from the light chain cross-reacting with 4H11.

3.2 Control experiments for the specificity of co-immunoprecipitations

The next experiments were performed to rule out the possibility that the interaction of PrP with ITSN-1L and Slit 1 happens after lysis in the lysis buffer, indicating physiological unspecific interactions.

For this, lysates of cells that separately expressed PrP and lysates of cells that separately expressed ITSN-1L or Slit 1 were mixed after lysis. No PrP was detectable in immunoblot after co-precipitation with HA-probe (anti ITSN-1L) or anti-c-myc (anti Slit 1) (Fig. 18 A lanes 2, 4). This means no interaction of PrP with ITSN-1L or Slit 1 occurred after lysis in the buffer, giving a proof that shown interactions are not a Co-imunoprecipiation artefact. Figure 18 A lanes 1 and 5 are cell lysates as control. Figure 18 A lanes 3 and 6 are immunoprecipitations with 4H11 as control for precipitations. As a positive control PrP was co-precipitated with Grb2, an already described interactor of PrP (Spielhaupter and Schatzl, 2001) (Fig. 18 B lane 3). Grb2 is expressed ubiquitously in cells. Figure 18 B lane 2 is a control if Grb2 binds unspecific to Protein-A Sepharose, which is not the case.

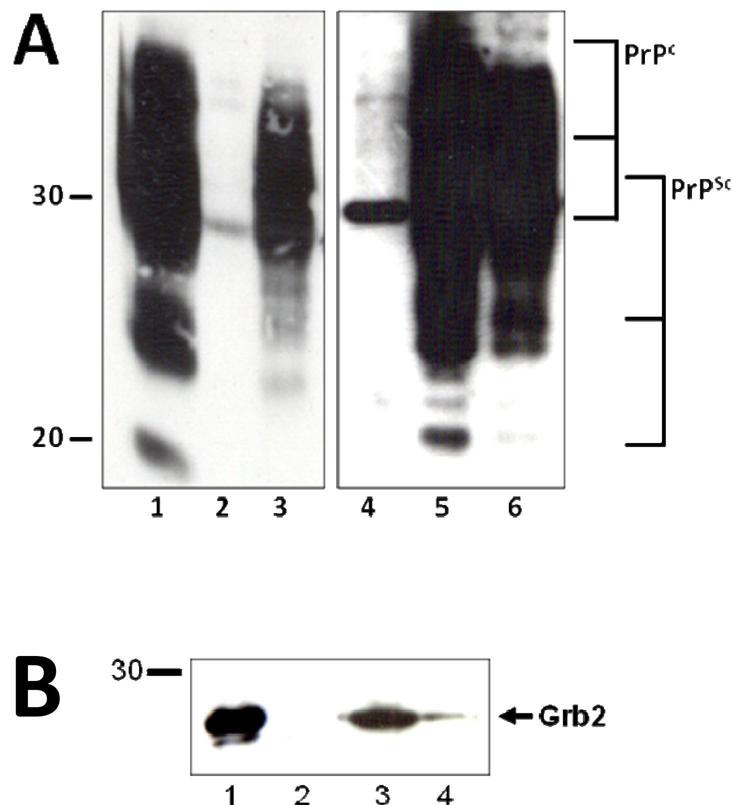


Figure 18 | **Control co-immunoprecipitations with mixed lysates. a |** Western blots developed with 4H11. Lysates were mixed after lysis. No interaction of PrP with ITSN-1L (lane 2) or Slit 1 (lane 4) can be detected. Lanes 1 and 5 are crude cell lysates. Lanes 3 and 6 are immunoprecipitations with 4H11 as control. **b |** Western blot analyzed with anti-Grb2. (1) Co-IP using PrP as a bait in Hpl3F4 cells, as a positive control. (2) Control for unspecific binding of Grb2 to Protein-A sepharose. (3) IP using anti-Grb2 antibody. (4) ITSN-1L-transfected Hpl3-4 cells. Co-IP with HA-probe (anti-ITSN-1L).

Using HA-Probe as antibody for co-immunoprecipitation also Grb2 could be precipitated with ITSN-1L (Fig. 18 B lane 4) in Hpl3-4 cells not expressing PrP.

3.3 Further characterization of the interaction between PrP and Intersectin 1

3.3.1 Intersectin 1 co-precipitates with cytosolic PrP mutants

The interaction between ITSN-1L and PrP^C could be shown before. The amount of PrP^C interacting with ITSN-1L is low due to the fact that ITSN-1L is a cytosolic protein (Hussain *et al.*, 1999) whereas PrP^C is mostly located at the cell surface. In this experiment PrP^C mutants directly expressed in the cytosol, called cytoPrP and ^{Ctm}PrP, were used for co-immunoprecipitation with ITSN-1L. It was reported that mice expressing a PrP mutant (cytoPrP) which lacks the N-terminal ER targeting signal sequence showed strong ataxia caused by cerebellar degeneration and gliosis (Ma *et al.*, 2002). Furthermore, cell culture models revealed that binding of cytoPrP to Bcl-2, an anti-apoptotic protein (Danial and Korsmeyer, 2004), triggered apoptosis (Rambold *et al.*, 2006). ^{Ctm}PrP, however, is one of two possible transmembrane topologies of PrP while being imported into the ER (Yost *et al.*, 1990). Mice expressing increased amounts of ^{Ctm}PrP due to a triple mutation (AV3) in the transmembrane domain of PrP showed progressive neurodegeneration (Stewart *et al.*, 2005). Neither cytoPrP nor ^{Ctm}PrP showed any cytotoxic effects when transiently expressed in an cell culture model (see 3.8).

To investigate if ITSN-1L also binds to these PrP mutants Hpl 3-4 cells were transiently transfected with plasmids encoding cytoPrP or ^{Ctm}PrP and ITSN-1L. The lysates were subjected to immunoprecipitation with anti-HA mAb and analyzed by immunoblotting using the anti-PrP mAb 4H11 for detection of the co-precipitated PrP mutants (Fig. 19). The first lane in each blot (Fig. 19 lanes 1, 3) represents a cell lysate of transfected cells analyzed by anti-PrP mAb 4H11 as a transfection control. CytoPrP as well as ^{Ctm}PrP could be co-precipitated with ITSN-1L (Fig. 19 lanes 2, 4) resulting in a banding patter comparable to the transfection control.

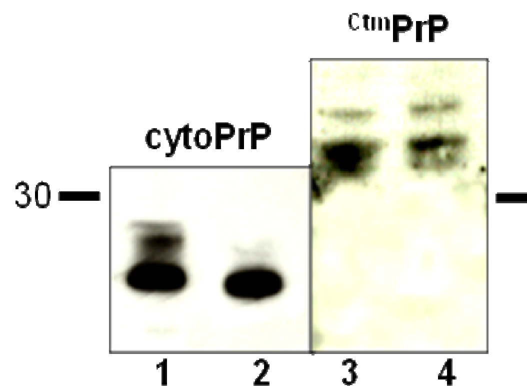


Figure 19 | **Co-immunoprecipitations with cytoPrP and ^{Ctm}PrP.** Western blot analyzed with 4H11. (1) Positive control. Cell lysate of Hpl3-4 cells transfected with cytoPrP. (2) Co-IP using ITSN-1L as a bait in Hpl3-4 cells transfected with cytoPrP. (3) Positive control. Cell lysate of Hpl3-4 cells transfected with ^{Ctm}PrP. (4) IP using anti-Grb2 antibody. (4) Co-IP using ITSN-1L as a bait in Hpl3-4 cells transfected with ^{Ctm}PrP.

3.3.2 Surface PrP co-precipitates with Intersectin 1

One previous experiment (see 3.1) suggested that ITSN-1L binds PrP that once was on the cell surface, because clearly the three banding pattern of PrP for non-, mono- and diglycosylated could be seen. To further examine the interaction between the cytosolic protein ITSN-1L and PrP it was examined if surface-located PrP could interact with ITSN-1L as well. Surface proteins (including PrP) were biotinylated in Hpl3F4 cells transiently transfected with plasmids encoding ITSN-1L (Fig. 20). Figure 20 lane 1 was a control for the specificity of the anti-HA mAb. The cell lysates were all subjected to immunoprecipitation with anti-HA mAb - except Figure 20 lane 3 which was subjected to anti-PrP mAb 4H11 as a positive control. The co-immunoprecipitations were analyzed by immunoblotting. The detection of the biotinylated surface PrP was done by Streptavidin. Figure 20 lane 2 shows a signal for biotinylated PrP comparable to the control Figure 20 lane 3. This indicates that once surface-located PrP interacts with ITSN-1L.

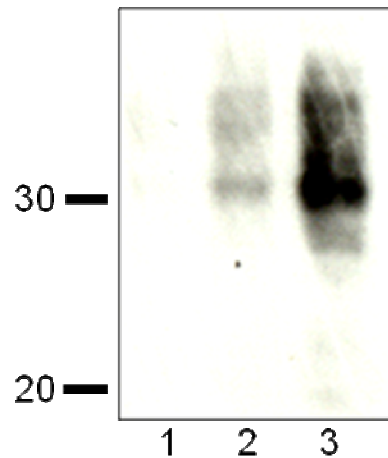


Figure 20 | **Biotinylated surface PrP co-immunoprecipitates with ITSN-1L.** Immunoprecipitations of biotinylated surface PrP in Hpl3F4 cells. The cell lysates were all subjected to immunoprecipitation with anti-HA mAb (except (3) which was subjected to anti-PrP mAb 4H11 as a positive control) and analyzed by immunoblotting with Streptavidin.

3.3.3 Intersectin 1 colocalizes with cytoPrP

As shown in Figure 19 ITSN-1L interacts with cytoPrP. To confirm this "biochemical proof" in an unrelated experiment, indirect immunofluorescence and confocal laser scanning microscopy was used. For this Hpl3-4 cells were transiently transfected with a plasmids encoding HA- ITSN-1L and cytoPrP followed by indirect immunofluorescences using the anti-PrP mAb 4H11 (α-PrP) with anti-mouse Cy2-labeled secondary antibody and anti-HA mAb (α-ITSN1) with anti-rabbit Cy3-labeled secondary antibody (Fig. 21). In the merged picture the green cytoPrP and the red ITSN-1L signals resulted in a yellow staining, showing that the two proteins co-localize.

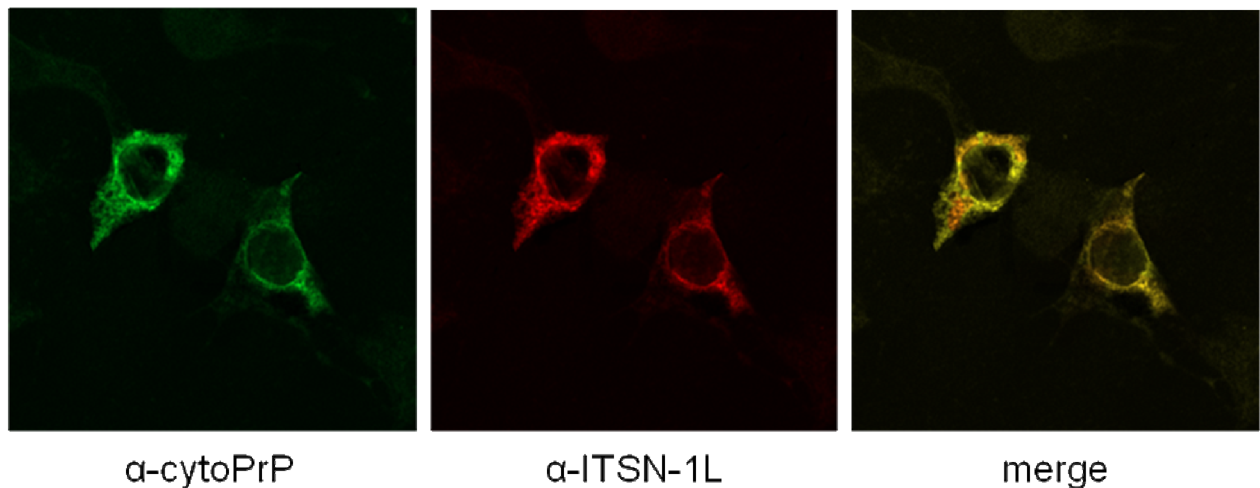


Figure 21 | **Intersectin 1 colocalizes with cytoPrP.** Hpl3-4 cells were transiently transfected with a plasmids encoding ITSN-1L and cytoPrP followed by indirect immunofluorescences using the anti-PrP mAb 4H11 (α-PrP) and anti-HA mAb (α-ITSN-1L).

3.3.4 Intersectin 1 colocalizes with Flotillin 1 in exosomes

As previously reported PrP^{C}, PrP^{Sc} and prion-infectivity can be found in exosomes of brain homogenate and in cultured cells (Baron *et al.*, 2006;Vella *et al.*, 2007). Previous experiments above showed that ITSN-1L can be co-immunoprecipitated with non-, mono- and diglycosylated PrP^{C} as well as with surface biotinylated PrP^{C}. This is indicating that PrP during its recycling comes in contact with ITSN-1L. Hypothetically, a contingent of PrP is trafficking through endosomes into MVBs and gets secreted via exosomes. When endosomes fuse into MVBs the membrane orientation is reversed. During this process PrP can come into contact with intracellular ITNS-1L. Afterwards they could be released together in exosomes.
Testing this hypothesis we used Hpl3F4 cells transiently transfected with a plasmid encoding ITSN-1L, followed by indirect immunofluorescence staining using the anti-Flotillin 1 Ab with anti-goat Cy3-labeled secondary antibody and anti-HA mAb (α-ITSN-1L) with anti-rabbit Cy2-labeled secondary antibody (Fig. 22). In the merge picture there is a colocalization of Flotillin 1 and ITSN-1L indicating that both proteins are present in late endosomes/exosomes.

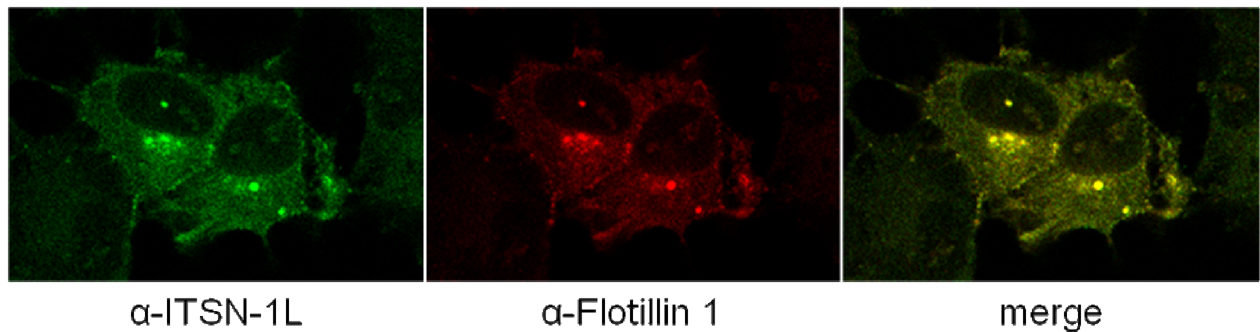


Figure 22 | **Intersectin 1 colocalizes with Flotillin 1.** Hpl3-4 cells were transiently transfected with a plasmid encoding ITSN-1L followed by indirect immunofluorescences using the anti-Flotillin 1 Ab and anti-HA mAb (α-ITSN-1L).

3.4 Further characterizations of the interaction between PrP and Slit 1

3.4.1 Slit 1 colocalizes with cellular PrP on the cell surface

Slit 1 interacts with PrP^C as shown by co-immunoprecipitation. For verification of this result the subcellular interaction site of Slit 1 and PrP had to be examined. Hpl3F4 cells were transiently transfected with a plasmid encoding Slit 1 followed by indirect immunofluorescence assay using the anti-PrP mAb 4H11 (α-PrP) with anti-mouse Cy2-labeled secondary antibody and anti-myc mAb (α-Slit 1) with anti-rabbit Cy3-labeled secondary antibody. In the merged picture (Fig. 23) the red PrP-staining and the green Slit 1 signal result in a yellow staining, when both proteins co-localize. Notably, PrP and Slit 1 colocalize exclusively on the cell surface.

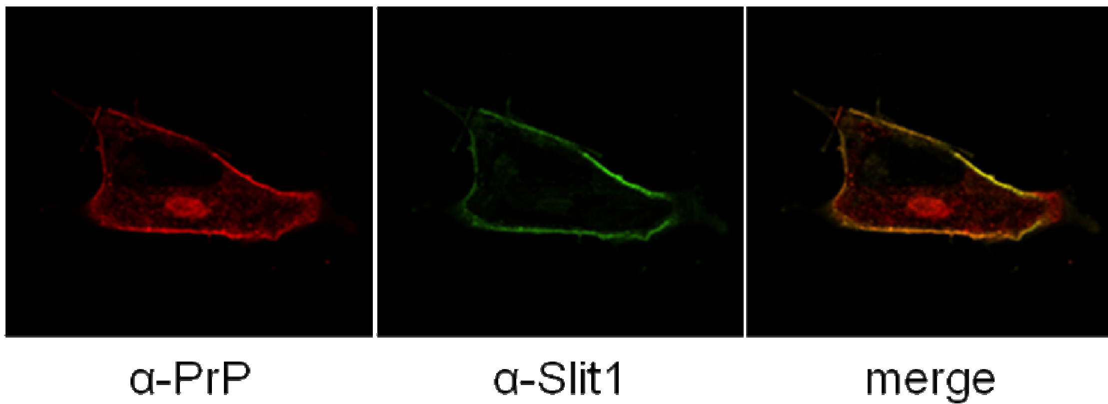


Figure 23 | **Slit 1 colocalizes with PrP^C on the cell surface.** Hpl3F4 cells were transiently transfected with a plasmid encoding Slit 1 and analyzed by indirect immunofluorescence using the anti-PrP mAb 4H11 (α-PrP) and anti-myc mAb (α-Slit 1).

3.4.2 Interaction with Slit 1 changes the raft-localization of cellular PrP

Most GPI-anchored proteins are enriched in cholesterol- and sphingolipid-rich lipid rafts within the plasma membrane, which is also true for PrP^C. Lipid rafts can be isolated by a flotation assay of cell lysates prepared in cold detergents. To test if the interaction with Slit 1 changes the distribution of PrP^C on the cell surface, Hpl3F4 cells were transiently transfected with plasmids encoding myc-tagged Slit 1. Cold detergent lysates of these cells were applied to a flotation assay. From top to bottom of the gradient, an aliquot of each fraction was collected and subjected to immunoblot analysis (Fig. 24). In the mock-treated control cells (Fig. 25, -Slit 1 Transfection) most PrP^C was found in fractions 3 and 4 with low amounts in fractions 5 and 6. In transfected cells (Fig. 24, +Slit 1 Transfection), the majority of PrP was found in fractions 1 and 2. The distribution of Slit 1 (Fig. 26) in these fractions is matching the distribution of PrP (Fig. 24), indicating the interaction of PrP and Slit 1.

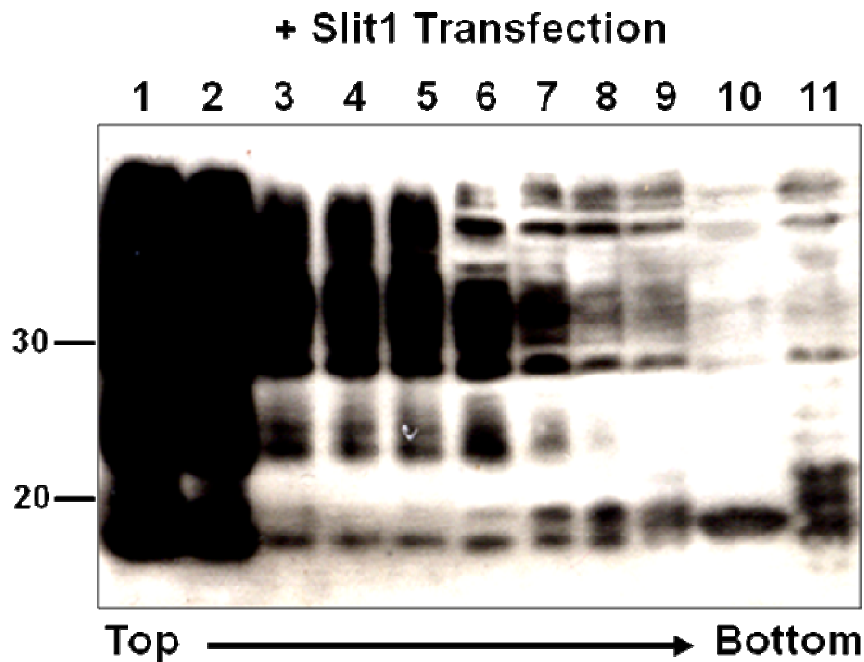


Figure 24 | **PrP changes its raft localisation in cells expressing Slit 1.** Hpl3F4 cells transfected with Slit 1 were subjected to flotation assay. Fractions were analyzed for the distribution of PrP by immunoblotting using the anti-PrP mAb 4H11.

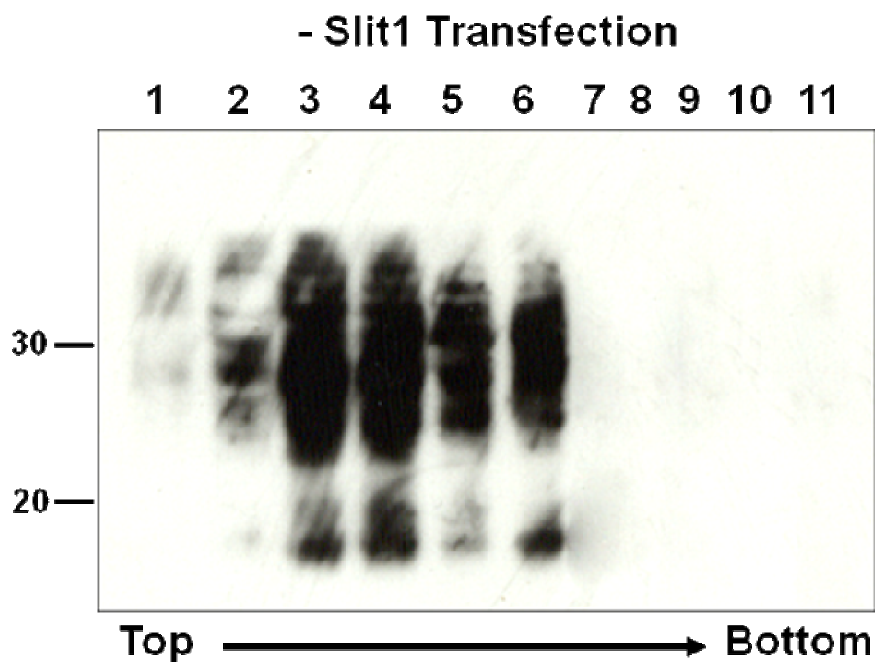


Figure 25 | **PrP changes its raft localisation in cells expressing Slit 1.** Untransfected Hpl3F4 cells were subjected to flotation assay. Fractions were analyzed for the distribution of PrP by immunoblotting using the anti-PrP mAb 4H11.

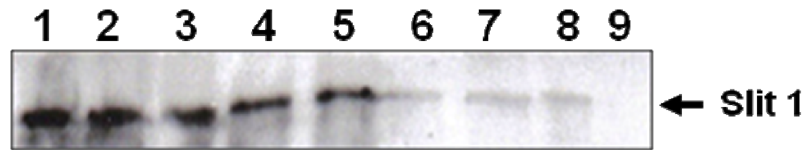


Figure 26 | **Distribution of Slit 1 in flotation assay.** Hpl3F4 cells transfected with Slit 1 were subjected to flotation assay. Fractions analyzed for the distribution of Slit 1 by immunoblotting using the anti-myc mAb.

3.4.3 Secreted Slit 1 binds to surface PrP^C

In this experiment Hpl3-4 and Hpl3F4 cells were transiently transfected with myc-tagged Slit 1. After 48 h the cell culture medium was collected und examined for content of soluble Slit 1 by immunoblot. In cells expressing PrP significant less soluble Slit 1 is available in cell culture medium (Fig. 27 A lanes 1, 2, 3) than in PrP knock-out cells (Fig. 27 A lanes 4, 5, 6). Three independent experiments are statistically analyzed in Figure 27 C. The amount of free Slit 1 in the cell culture medium is reduced by 60 % compared to cells not expressing PrP. Using the Student´s t-test the result was tested as significant. In another experiment cell culture medium of Hpl3-4 cells transiently transfected with Slit 1 was applied to Hpl3F4 cells not expressing myc-tagged Slit 1 (Fig. 27 B). This medium contained secreted Slit 1 (Fig. 27 B lane 1). After eight hours Hpl3F4 cells were lysed and subjected to immunoprecipitation with anti-PrP mAb 4H11. Immunoprecipitations were analyzed by immunoblot with anti-myc mAb. In Figure 27 B lane 2 a signal for Slit 1 can be seen, indicating that Slit 1 from the cell culture medium was co-precipitated with PrP^C.

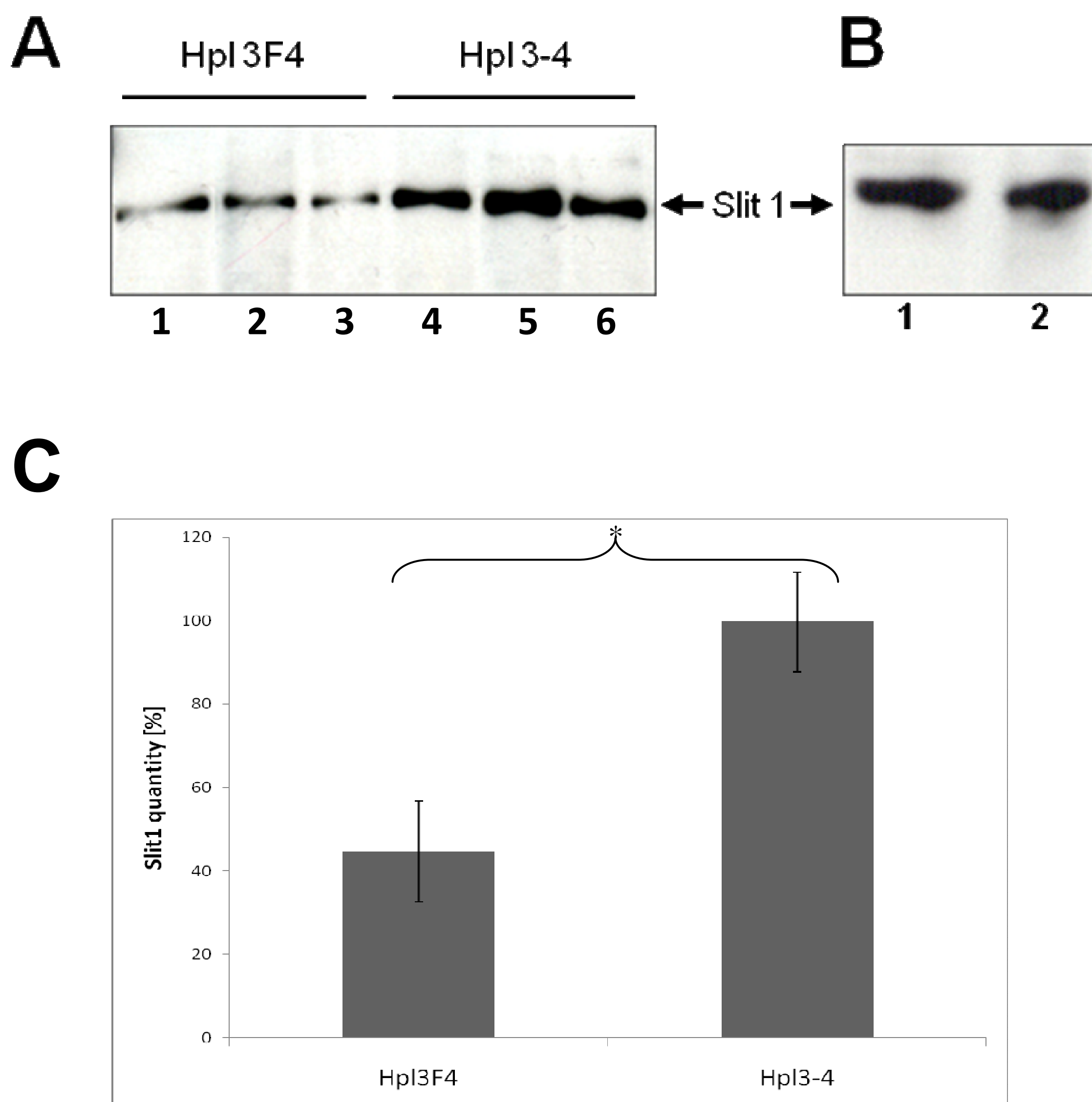


Figure 27 | **Slit 1 binds PrP on the cell surface. a** | Hpl3-4 and Hpl3F4 cells were transfected with Slit 1. After 48 h the cell culture medium was collected and analyzed by immunoblot with three experiments in parallel (n = 3). **b** | Cell culture medium of Hpl3-4 cells (Lane 1) transiently transfected with Slit 1 was applied to Hpl3F4 cells. Hpl3F4 cells were subjected to immunoprecipitation with anti-PrP mAb 4H11 and analyzed by immunoblotting using the anti-myc mAb for detection of Slit 1 (Lane 2). **c** | Statistical analysis of **a.** * indicates that using Student´s t-test the result was calculated as significant for three independent experiments ($t_{exp.} > t(0,98; 2)$).

3.5 PrP^{Sc} levels upon over-expression of Intersectin 1 and Slit 1

If PrP and ITSN-1L or Slit 1 bind in a physiological environment there is possibly an effect on the conversion of PrP^{C} into PrP^{Sc}. To investigate this ScN2a cells were transiently transfected with either ITSN-1L or Slit 1. After three and four days cells were harvested, cell lysates split up and one part PK digested and one not. All lysates were analyzed via immunoblot for levels of PrP^{Sc}. The over-expression of ITSN-1L and Slit 1 as well did not lead to a decrease or increase of PrP^{Sc} levels in ScN2a cells (Fig 28).

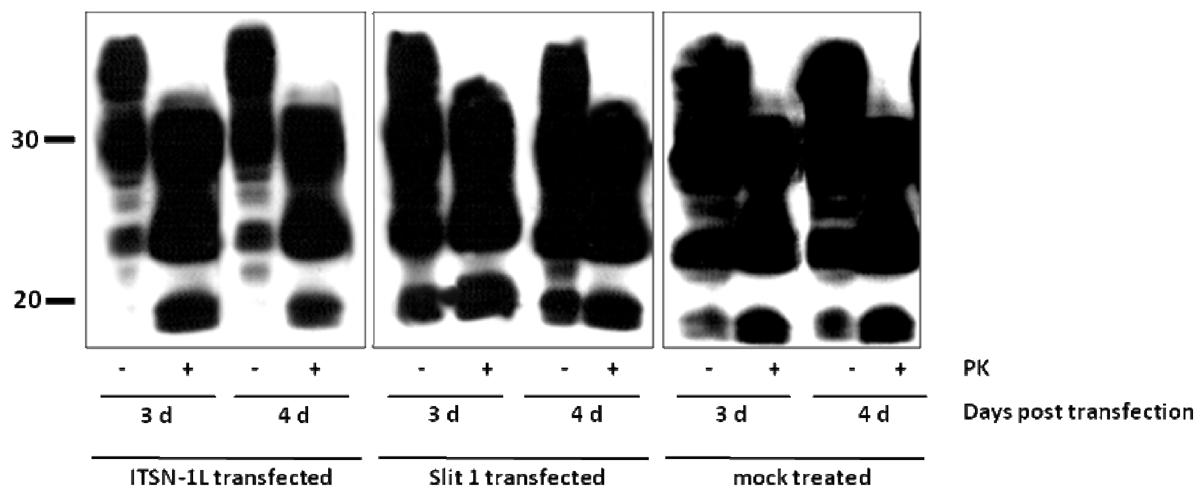


Figure 28 | **PrP^{Sc} levels in ITSN-1L and Slit 1 transfected ScN2a cells.** ScN2a cells were transfected either with ITSN-1L or Slit 1. After three and four days cells were harvested and analyzed by immunoblot for PrP^{Sc} levels. The western blot was developed with 4H11.

3.6 Half-life of PrP^{C} in Intersectin 1 or Slit 1 transfected cells

To further examine the physiological environment PrP and ITSN-1L or Slit 1 interact within, the kinetic of PrP degradation was characterized in a pulse-chase experiment. The half-life of PrP in ITSN-1L transfected, Slit 1 transfected and mock-treated N2a cells were compared.
N2a cells were metabolically labelled with [^{35}S]-methionine/cysteine for one hour and either directly harvested or chased for noted intervals of time (Fig. 29) in [^{35}S]-free culture medium before lysis. PrP^{C} in these lysates was immunoprecipitated with mAb anti-PrP antibody 4H11. For densitometric analysis PrP was diglycosylated with PNGase F and analyzed by SDS-

PAGE (Fig. 29). The resulting PrP bands were evaluated by densitometric analysis and quantified as fractions of the bands observed immediately after pulse (Fig. 30). This analysis revealed a half-life of PrP of 2.2 h (Fig. 30 A). This turnover was comparable with previous reports (Nunziante *et al.*, 2003). The turnover rates for ITSN-1L ($t_{1/2}$ = 2.4) and Slit 1 ($t_{1/2}$ = 2.2) transfected cells were virtually unaffected (Fig. 30 B and C).

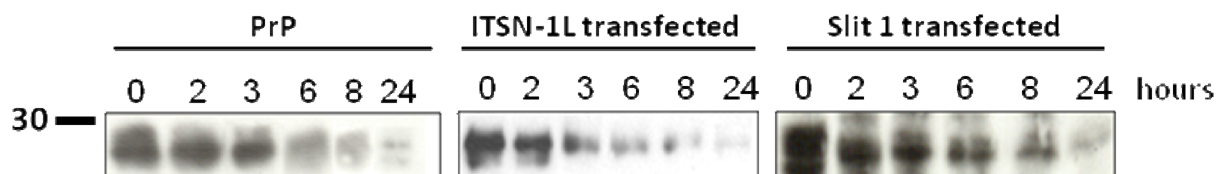


Figure 29 | **PrP turnover in ITSN-1L and Slit 1 transfected N2a cells.** N2a cells were transfected either with ITSN-1L or Slit 1 and metabolically labelled with [^{35}S]-methionine/cysteine. After different time points lysates were analyzed for PrP level via SDS-PAGE. The autoradiograms indicate that degradation kinetics of PrP in ITSN-1L and Slit 1 transfected cells are not changed.

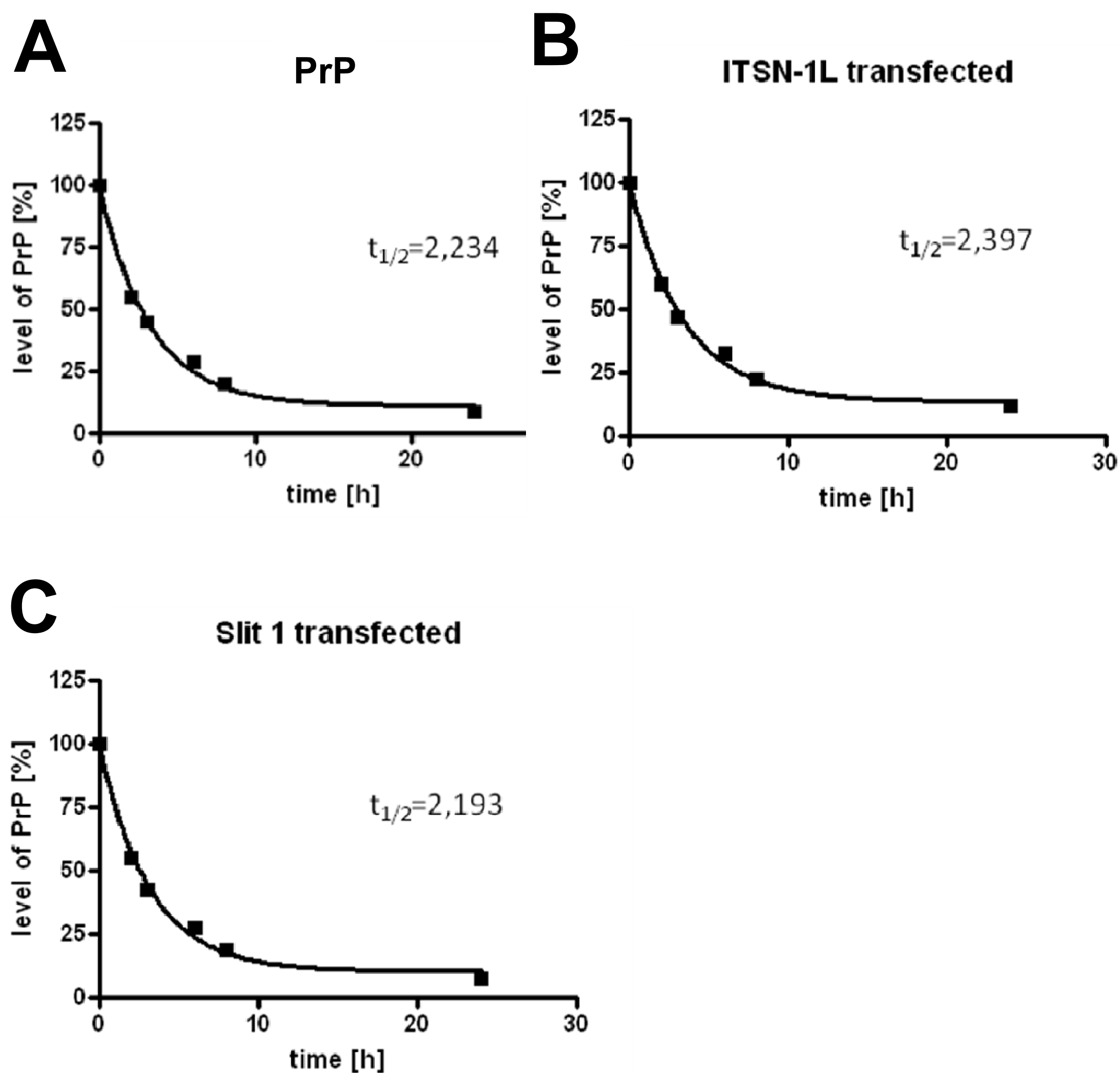


Figure 30 | **Evaluation of autoradiograms from the experiments as described in (Fig 29).** The amounts of PrP are expressed as percentage of total PrP precipitated directly after labelling and are plotted as a function of the chase time points. Data points were fitted to an exponential decay curve using non-linear regression analysis.

3.7 Surface FACS analysis of expression of PrP in Intersectin 1 and Slit 1 over-expressing cells

The interaction of ITSN-1L and Slit 1 with PrP could influence the level of PrP on the cell surface. To examine this N2a cells were transiently transfected with either ITSN-1L or Slit 1. Slit 1 is a secreted protein and can be found on the cell surface (Fig. 31 A), binding PrP (see Fig. 23). When Slit 1 is expressed the amount of cell surface PrP is unaffected (Fig. 31 C). Via FACS analysis no ITSN-1L can be found on the cell surface (Fig. 31 B) and the levels of surface PrP are unchanged by the expression of ITSN-1L (Fig. 31 D).

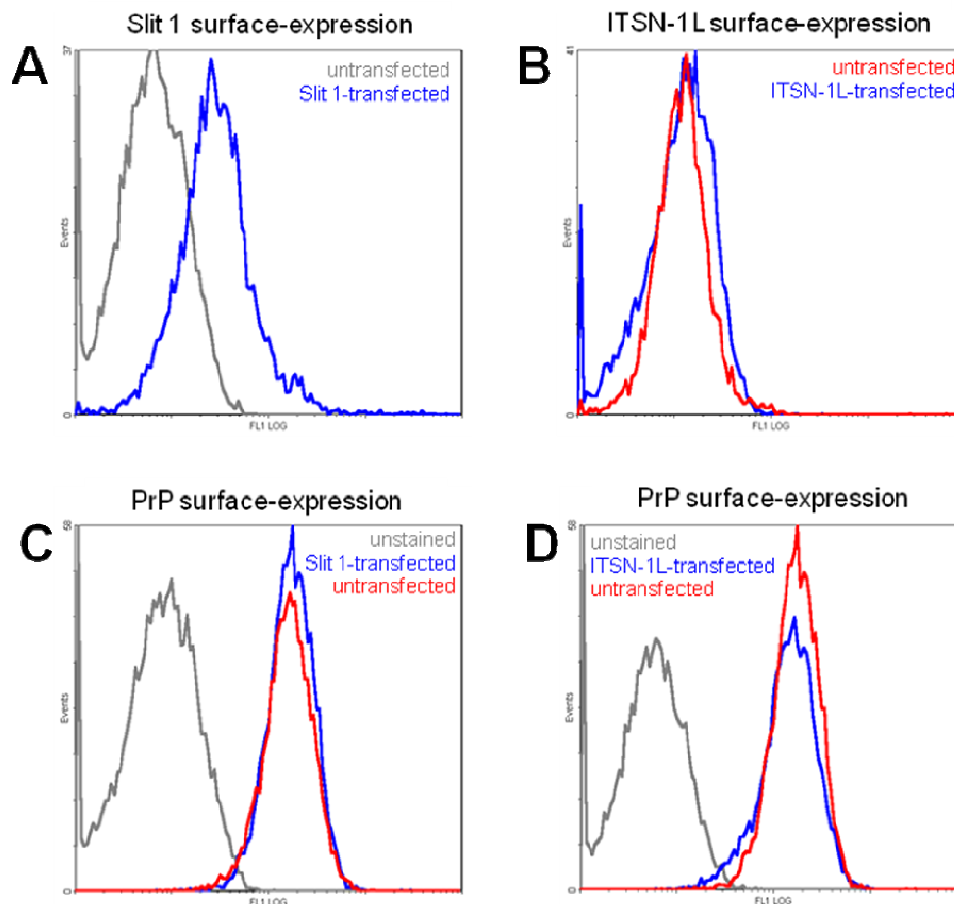


Figure 31 | **Surface expression of PrP, ITSN-1L and Slit 1 in N2a cells. a** | Cells were transfected with Slit 1, which can be found on the cell surface. **b** | Cells transfected with ITSN-1L do not show ITSN-1L on the cell surface. **c** | The over-expression of Slit 1 does not influence the PrP levels on cell surface. **d** | The over-expression of ITSN-1L does not influence PrP levels on cells.

3.8 Test for cytotoxicity of cytoPrP and ^{Ctm}PrP, Slit 1 and Intersectin 1 transiently expressed in PrP$^{0/0}$ and cells expressing PrP

As mentioned above (see 3.3.1), cytoPrP and ^{Ctm}PrP showed no cytotoxic effects when expressed in cells with PrP or PrP knock-out cells. To quantify these phenotypic findings, MTT-assays were performed. Additional the cytotoxicity of ITSN-1L and Slit 1 was tested. Hpl3F4 and Hpl3-4 cells were transiently transfected with cytoPrP, ^{Ctm}PrP, ITSN-1L and Slit 1. After 24 h, 48 h and 72 h the cell vitality was measured with MTT-assay. The MTT-assay is based on the reduction of yellow tetrazolium salt (MTT) to purple formazan crystals via cellular mitochondrial dehydrogenases. The amount of build formazan crystals can be measured at 0 wavelength of 570 nm. It is crucial therefore to start with exact cell counts. To get statistical profound results eight 96-wells were used in parallel and the whole experimental setting was repeated twice. In parallel cells were mock-treated (transfection with empty vector) and treated with Staurosporin, as positive control. For the analysis the viability of the mock-treated cells is set to 100 % for 1 d post treatment (1 d p.t.).

Hpl3-4 cells transfected with ITSN-1L and Slit 1 showed no significant difference to mock-treated (transfected with empty vector) cells regarding viability (Fig. 32, 34). Also the presence of PrP in Hpl3F4 cells did not alter the results in a significant manner (Fig. 33, 35). In contrast to published data also the expression of cytoPrP and ^{Ctm}PrP did not change the cell viability in Hpl3-4 cells as well as in Hpl3F4 cells (Fig 36, 37).

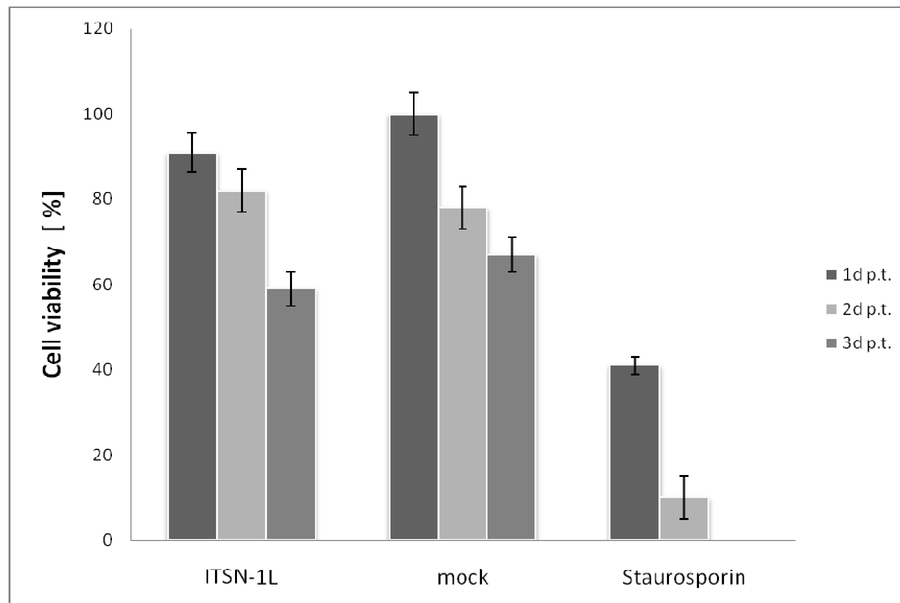


Figure 32 | **MTT-assay with ITSN-1L transfected Hpl3-4 cells.** No significant differences between ITSN-1L transfected and mock-treated cells. Cell viability was tested after 24 h, 48 h and 72 h post treatment (p.t.).

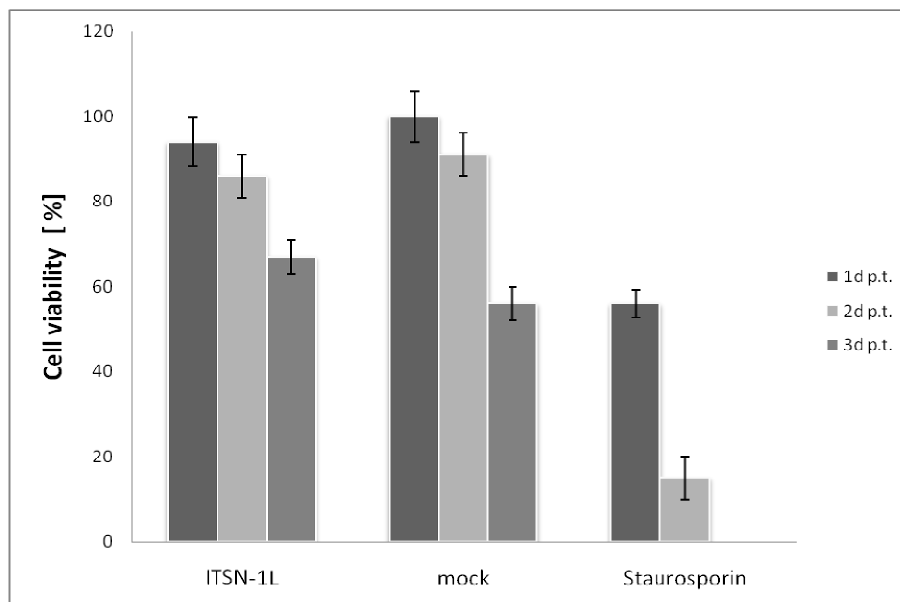


Figure 33 | **MTT-assay with ITSN-1L transfected Hpl3F4 cells.** No significant differences between ITSN-1L transfected and mock-treated cells. Cell viability was tested after 24 h, 48 h and 72 h post treatment (p.t.).

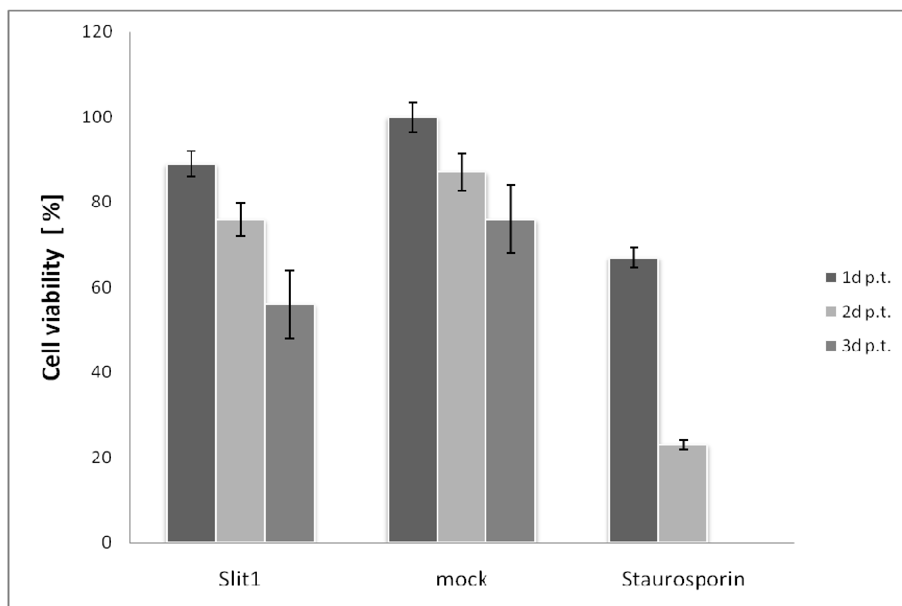


Figure 34 | **MTT-assay with Slit 1 transfected Hpl3-4 cells.** No significant differences between Slit 1 transfected and mock-treated cells. Cell viability was tested after 24 h, 48 h and 72 h post treatment (p.t.).

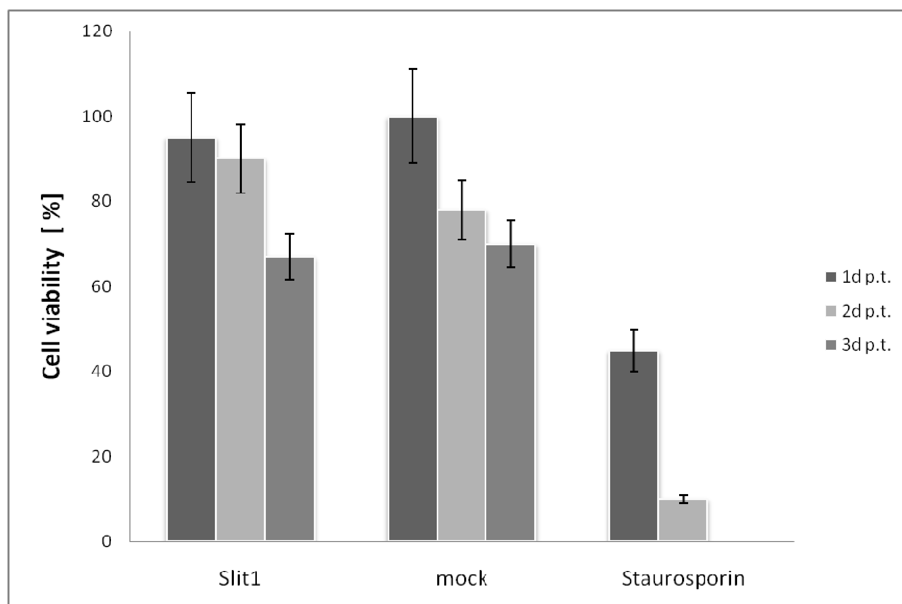


Figure 35 | **MTT-assay with Slit 1 transfected Hpl3F4 cells.** No significant differences between Slit 1 transfected and mock-treated cells. Cell viability was tested after 24 h, 48 h and 72 h post treatment (p.t.).

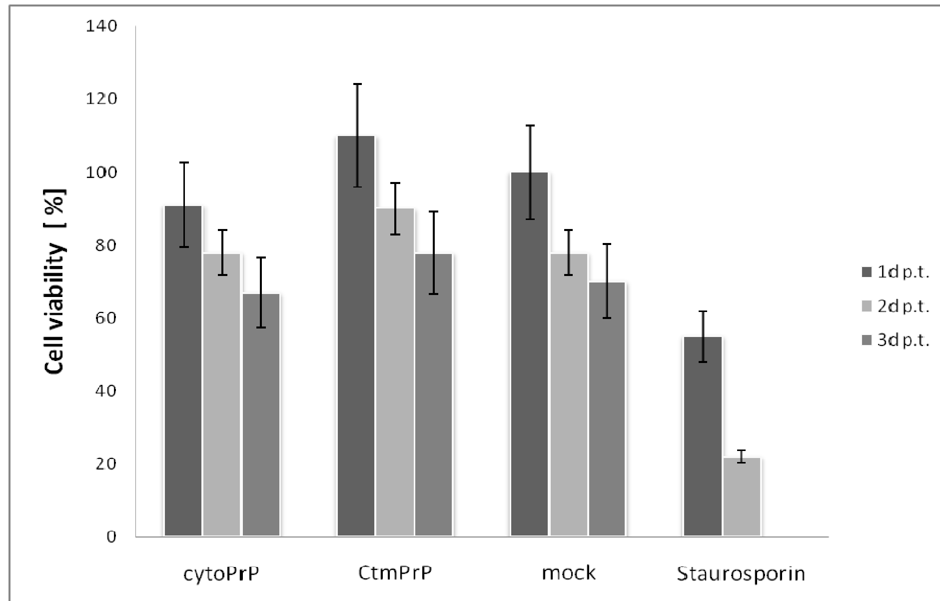


Figure 36 | **MTT-assay with cytoPrP and ^{Ctm}PrP transfected Hpl3-4 cells.** No significant differences between cytoPrP and ^{Ctm}PrP transfected and mock-treated cells. Cell viability was tested after 24 h, 48 h and 72 h post treatment (p.t.).

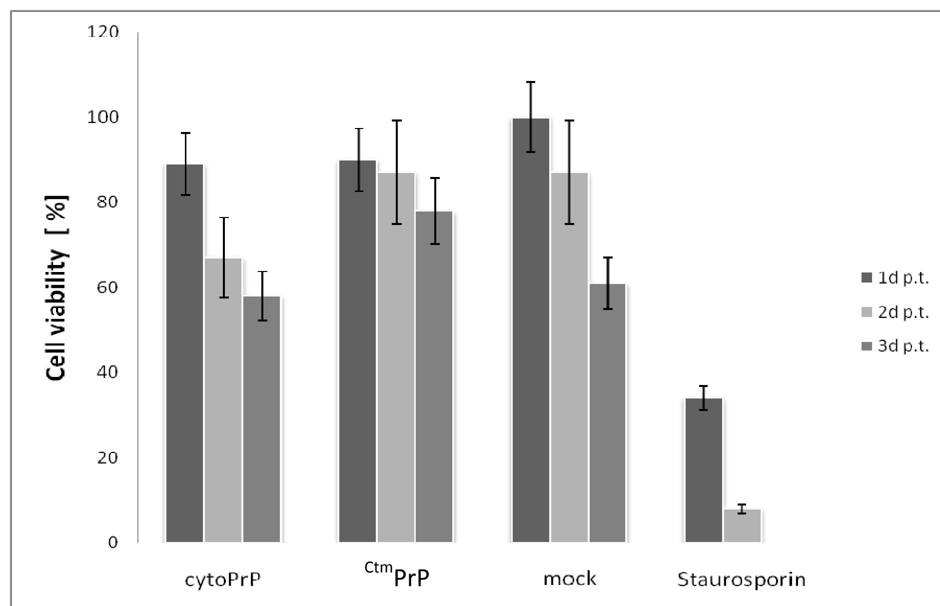


Figure 37 | **MTT-assay with cytoPrP and ^{Ctm}PrP transfected Hpl3F4 cells.** No significant differences between cytoPrP and ^{Ctm}PrP transfected and mock-treated cells. Cell viability was tested after 24 h, 48 h and 72 h post treatment (p.t.).

3.9 Retroviral transduction with pSFF-ITSN-1L

The endogenous expression of Intersectin 1 in the available neuronal cell lines is too low to be detected by immunoblot or immunofluorescence. Throughout this work ITSN-1L was transiently transfected for all experiments for this reason. This is rather impracticable, because transfection efficiency is variable, depending on the passage number of cells and the confluency at transfection. The stable expression of HA-tagged ITSN-1L in neuronal cell lines would be a welcome tool for future experiments, especially when additional transfections are needed in one experiment, multiplying the variability of results. Hence the retroviral transduction was chosen for stable expression of ITSN-1L. HA-tagged ITSN-1L was therefore cloned into the pSFF vector. Subsequently, this vector was transfected into package cell lines PA 317/ψ 2 for the production of replication-deficient retroviral particles. These particles can be harvested in the cell culture supernatant and be used to transduce other cell lines. With these retroviral particles N2a and Hpl3-4 cells were transduced, expressing HA-tagged ITSN-1L (Fig. 38).

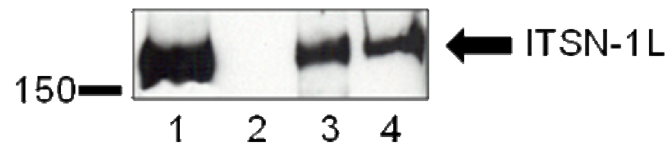


Figure 38 | **N2a and Hpl3-4 cells expression transduced ITSN-1L.** Cells expressing HA-tagged ITSN-1L introduced by retroviral transduction with vector pSFF-ITSN-1L. (1) Transient transfection of N2a cells with ITSN-1L as positive control. (2) Transfection with empty vector as negative control. Transduction with HA-tagged ITSN-1L in N2a cells (3) and Hpl3-4 (4) cells.

4. Discussion

The definite function of PrP^C is still elusive. To gain further insights, one way is to look at proteins PrP^C interacts during its trafficking through the cell. Many different proteins have been proposed (for review see (Caughey and Baron, 2006;Westergard *et al.*, 2007)), but still no link to the function of PrP^C could be made. Of particular interest where interactors binding to the N-terminus of PrP^C. It has been shown that the N-terminus of PrP is able to bind copper ions due to its octarepeats, supporting the idea that PrP is part of the copper metabolism (Hornshaw *et al.*, 1995b;Brown *et al.*, 1997a;Pauly and Harris, 1998;Stockel *et al.*, 1998). Deletions within the N-terminus of murine PrP^C caused a significant reduction of internalization of PrP in neuroblastoma cells (Nunziante *et al.*, 2003). These date indicate that the N-terminus of PrP could be a targeting element and be important for transport to the plasma membrane as well as the modulation of endocytosis. And at the same time the N-terminal region of PrP is highly conserved in evolution (Wopfner *et al.*, 1999) indicating an important, yet not properly understood function.

First of all the yeast two-hybrid system was applied to identify novel protein interactors of PrP^C. In this approach murine PrP^C (amino acids 23–100) was used as bait to screen a mouse brain cDNA expression library. Using this highly homologous system, several novel interactors where identified (Schätzl *et. al.*, unpublished data). The aim of this work was to verify and explore the results in a physiological context. Co-immunoprecipitations were performed, which confirmed the Y2H data for Intersectin 1 and Slit 1. With these two interactors further characterization were performed.

4.1 Finding interactors of the prion protein

Since the physiological function of PrP^C is still elusive the interaction with other proteins can provide new insights, not only for the function, but also for the conversion of PrP^C into PrP^{Sc}. Probably there is also a chance finding “Factor X” or “Protein X” (Telling *et al.*, 1994;Telling *et al.*, 1995;Kaneko *et al.*, 1997a) among the interactors. Further experiments have to show, which interactors play a main role in this complex disease. Interactors may then become a target for therapeutic approaches, e.g. through antibodies or specially directed compounds, since no striking progress could be made, until now, in searching effective and applicable anti-prion drugs.

Through the years many different methods have been applied to find novel interactors of the prion protein. Overlay-assays were performed with radioactive labelled PrP and scrapie-infected brain homogenate, whereas the proteins were separated by SDS-PAGE (Oesch *et al.*, 1990). These so called ligand blots were optimized by using PrP as a fusion protein with alkaline phosphatase. Instead of brain homogenate a cDNA library generated by phage display-system was used (Yehiely *et al.*, 1997). The Y2H assay has been applied by different groups (Edenhofer *et al.*, 1996;Rieger *et al.*, 1997;Spielhaupter and Schatzl, 2001;Bragason and Palsdottir, 2005). Also co-immunoprecipitations were performed in different ways (Keshet *et al.*, 2000;Mattei *et al.*, 2004;Gao *et al.*, 2006) and cross-linking was used (Schmitt-Ulms *et al.*, 2001;Nieznanski *et al.*, 2005). Another idea was to use affinity chromatography followed by mass spectrometry (Petrakis and Sklaviadis, 2006).

4.2 Various topological isoforms of PrP^{C} interact with Intersectin 1

ITSN-1 is a cytosolic, multi-domain scaffold protein. The long isoform ITSN-1L is expressed exclusively in neurons (Guipponi *et al.*, 1998;Hussain *et al.*, 1999;Ma *et al.*, 2003). There are three ways for PrP to interact with ITSN-1L in the cytosol. There are two different transmembrane topologies, ^{Ctm}PrP and ^{Ntm}PrP, which can occur when PrP enters the ER (Yost *et al.*, 1990). GSS patients with an additional A117V mutation show increased levels of ^{Ctm}PrP, probably leading to progressive neurodegeneration (Hegde *et al.*, 1998;Stewart *et al.*, 2005). A small proportion of normal PrP can occur in the cytosol due to inefficiency of its ER signal sequence (Rane *et al.*, 2004). Here, cytoPrP, ^{Ctm}PrP, PrP^{C} could be immunoprecipitated together with ITSN-1L. There is indication that cytoPrP can produce cytotoxic effects which seems to be dependent of the compartment in which PrP misfolding occurs. The binding of cytoPrP to Bcl-2 probably inhibits its anti-apoptotic function causing cytotoxicity (Rambold *et al.*, 2006). In this context the binding of ITSN-1L to cytoPrP can be of importance for this effect. ITSN-1L could be rescuing the toxic phenotype by preventing the interaction of cytoPrP and Bcl-2. It was demonstrated in recent studies that Intersectin 1 has an anti-apoptotic function in endothelia cells. The knock down of Intersectin 1 by RNAi initiated apoptosis through the activation of proapoptotic Bcl-2 family members (Predescu *et al.*, 2007).

4.3 Intersectin 1 may interact with PrP^{Sc} in late endosomes/exosomes during its recycling

However, normally PrP^{C} is present on the outer leaflet of the plasma membrane. After a few minutes PrP leaves the raft environment and recycles via clathrin coated pits between cell surface and recycling endosomes (Morris *et al.*, 2006). During this recycling, endosomes can be directed to fuse into multivesicular bodies (MVBs). These MVBs can either fuse with lysosomes for degradation or fuse with the plasma membrane for exosome release (van Niel *et al.*, 2006). During the event of fusion into MVBs the membrane orientation of the endosomes is inverted, enabling the contact of cytosolic proteins with proteins of the outer leaflet of the plasma membrane, which could be the case for ITSN-1L and PrP. It could be shown by others that exosomes contain PrP^{C} as well as PrP^{Sc} (Fevrier *et al.*, 2004). Bioassays in mice and cell culture demonstrated, that these exosomes can effectively transport infectivity (Baron *et al.*, 2006). A standard marker protein for exosomes is Flotillin-1. This lipid raft marker is also present in endosomes (Kokubo *et al.*, 2000;Salzer and Prohaska, 2001). By using immunofluorescence assays in this work, it could be shown that ITSN-1L and PrP reside in late endosomes/exosomes together, making it the most practicable interaction site for PrP and ITSN-1L. All exosomes have an endocytotic origin. ITSN-1 is known to be part of the endocytotic machinery. ITSN-1 directly binds other proteins of the endocytic machinery and expression of ITSN-1 inhibited the endocytosis of transferrin receptor (Okamoto *et al.*, 1999;Hussain *et al.*, 1999;Sengar *et al.*, 1999;Adams *et al.*, 2000;Predescu *et al.*, 2003;Marie *et al.*, 2004).

Data presented in this work showed that surface biotinylated PrP can be immunoprecipitated with ITSN-1L (3.3.2). This is a strong indication that PrP and ITSN-1L binds after the endocytosis of PrP. All these data taken together, PrP^{C}, PrP^{Sc} and ITSN-1L can be located together in endosomes. Exosomes were already proposed as possible conversion site for PrP^{C} into PrP^{Sc} (Arnold *et al.*, 1995). It may be that ITSN-1L plays a role in the conversion process. On the other hand ITSN-1L has an anti-apoptotic capacity (Predescu *et al.*, 2007). As reported in this work ITSN-1L can be co-immunoprecipitated along with PrP^{Sc}.

4.4 Intersectin 1 can also interact with Grb2

Using ITSN-1L as bait also Grb2 can be co-immunoprecipitated (3.2). Grb2 is an intracellular adaptor protein which main role seems to be linking extracellular signals with intracellular

signalling molecules (Koch *et al.*, 1991). Grb2 is consisting of two SH3 domains and one SH2 domain. The SH2 domain enables Grb2 to interact with tyrosine kinases, while the SH3 domain binds to proline-rich segments in proteins (Anderson *et al.*, 1990). ITSN-1L has multiple proline-rich motifs and also PrP has an appropriate motif at aa 101-104 in murine PrP. Grb2 is capable of binding PrP, but the interaction is abolished when one of these prolines is replaced by leucine (Spielhaupter and Schatzl, 2001;Lysek and Wuthrich, 2004). These mutations correspond to the Gerstmann-Sträussler-Scheinker (GSS) syndrome in human patients. Like Grb2 also ITSN-1L is involved in intracellular signalling. This could indicate a kind of super-complex with Grb2, PrP and ITSN-1L which enables and regulates the cross-linking of PrP controlling complex signal transduction cascades. E.g. it has been reported that the cross-linking of PrP leads to an activation of the tyrosine kinase Fyn (Mouillet-Richard *et al.*, 2000). The loss of interaction between Grb2 and PrP might be of importance for the pathology of GSS patients.

4.5 Intersectin and apoptosis

Recently it could be shown that the down-regulation of Intersectin 1 in cultured lung microvascular endothelial cells (EC) triggers apoptosis through the activation of Bcl-2 family members (Predescu *et al.*, 2007). Furthermore it was reported that Intersectin 1 can be a substrate of granzyme B, a protease able to activate apoptosis by caspase-dependent and caspase-independent pathways (Loeb *et al.*, 2006). The cleavage of Intersectin 1 could possibly promote apoptosis. In EC cells with depleted Intersectin 1 caveolae vesicle trafficking is impaired. This leads to nutrition reduction, which also promotes apoptosis (Predescu *et al.*, 2007).

As mentioned above Intersectin 1 can be precipitated with cytoPrP (see 3.3.1), which can induce apoptosis by coaggregation with Bcl-2 (Rambold *et al.*, 2006). Presumably the cytotoxic effect of cytoPrP is accomplished by the indirect activation of Bcl-2 family members, since the function of Intersectin 1 is impaired due to the binding to cytoPrP. There is another recent report showing that decreasing ITSN expression in both neuroblastoma cells and primary cortical neurons dramatically increased apoptosis (Das *et al.*, 2007), but the endocytotic pathway was not severely inhibited. Class II phosphoinositide 3'-kinase-C2beta (PI3K-C2beta) was found via Y2H screen as an ITSN binding protein. ITSN may regulate a PI3K-C2beta-AKT survival pathway.

4.6 Intersectin and other neuronal diseases

Lately there has been a report that mentioned Intersectin in context with the Huntington´s disease. Huntington´s disease is a rare inherited neurological disorder approximately affecting 1:10000 people of Western descent (0.01:10000 for Asian or African descent). Huntington's disease is caused by a trinucleotide repeat expansion in the Huntingtin (Htt) gene and is one of several polyglutamine (or PolyQ) diseases. This altered form of the Htt protein, mutant Huntingtin (mHtt), leads to neuronal cell death in defined areas of the brain. The more extra glutamines are inserted in the Htt gene the higher the likeliness of disease and the faster the neuronal degeneration progresses. It was reported that Intersectin increased aggregate formation by mutant Htt through activation of the c-Jun-NH2-terminal kinase (JNK)-MAPK pathway. Silencing ITSN or inhibiting JNK attenuated aggregate formation (Scappini *et al.*, 2007).

Another remarkable perspective is that the Intersectin gene is located on the human chromosome 21. Down syndrome (DS) patients have three chromosomes 21. This means every gene encoded on chromosome 21 is potentially over-expressed in these individuals. Interestingly, Alzheimer's disease (AD) usually affects DS individuals by their mid 40s. This may mean that genes located on chromosome 21 are involved in neurodegeneration of DS patients. Current studies suggest that endosomal disorders may cause the earliest pathology of AD. Consequently, proteins like Intersectin that are involved in endocytosis and vesicle trafficking and are over-expressed in DS could be novel candidates in the pathogenesis of AD (Keating *et al.*, 2006).

4.7 Slit 1 interacts with PrP on the cell surface

Slit 1, the other interactor presented in this work, belongs to a family of proteins that are secreted, highly conserved proteins involved in commissural axon guidance, mediating normal neuronal development (Rothberg *et al.*, 1988;Rothberg *et al.*, 1990;Kidd *et al.*, 1999;Brose *et al.*, 1999;Brose and Tessier-Lavigne, 2000). They also play an important role in the development of the vasculature and other organs (Hinck, 2004;Strickland *et al.*, 2006). Slit 1 acts as a signalling ligand for the guidance receptor Roundabout 1 (Robo 1) and is

expressed in the midline of the CNS in both invertebrates and vertebrates (Tessier-Lavigne and Goodman, 1996).

As presented in this work Slit 1 can directly bind to PrP on the cell surface. This was shown in co-immunoprecipitations and immunofluorescence experiments. Moreover, both proteins have a cell surface interaction partner in common. Heperan sulfate (HS) can bind PrP as well as Slit 1 and is important for prion pathology and the metabolism of the prion protein (Diaz-Nido *et al.*, 2002;Johnson *et al.*, 2004). For Slit 1 it was reported that signalling requires Slit/Robo to be co-expressed on the same cell with the HS proteoglycan syndecan (Steigemann *et al.*, 2004;Johnson *et al.*, 2004;Rhiner *et al.*, 2005).

4.8 Interaction with Slit 1 alters PrP raft localization

As the flotation assay shows, there is an interaction of PrP and Slit 1 on the surface. PrP changes its raft localization when expressed with Slit 1 in the same cell (see 3.4.2). Slit 1 is a very large protein possibly interacting with more than one protein at once. As mentioned above Slit 1 and PrP both bind to HS. Perhaps PrP and Slit 1 interact while binding a third partner.

It is also possible that PrP is involved in Slit - Robo signalling. In this case PrP could act as a competitor for Robo, meaning that PrP could be a regulator for Slit/Robo signalling. On the contrary PrP could be a kind of stabilizer for the Slit – Robo interaction in context of a bigger complex together with HS. These results shed new light on the possible physiological role PrP might play. Most other PrP interactors are located in the cytosol. As discussed earlier, only a little amount of PrP is normally present in the cytosol. Slit 1 represents a PrP interactor binding to wild type PrP in a conceivable situation.

4.9 Half-life of PrP^C in Intersectin 1 or Slit 1 transfected cells

It has been reported that the half-life of PrP^C/PrP^{Sc} can be influenced by drugs (Ertmer *et al.*, 2004;Nunziante *et al.*, 2005). Also the N-terminus of PrP^C seems to be of special importance for the turnover (Nunziante *et al.*, 2003). The interacting proteins that were identified in Y2H assay were supposed to interact with the N-terminus. Hence it was investigated if the half-life of PrP^C can be influenced by ITSN-1L or Slit 1. The half-life of PrP^C in ITSN-1L-transfected or Slit 1-transfected cells did not change due to the transfection (see 3.6). The interaction

should not impede PrP^C in his function. As recently shown the knock-down of Intersectin in neuroblastoma cells did not alter endocytosis, probably indicating that Intersectin in this cell lines is involved in other functions besides endocytosis (Das *et al.*, 2007).

4.10 FACS analysis of surface PrP in Intersectin 1 and Slit 1 over-expressing cells

Further characterizations of the interaction of PrP with ITSN-1L and Slit 1 were done by the analysis of the surface expression of PrP. Previous publications showed that the level of surface located PrP can be influenced by different drugs (Gilch *et al.*, 2001;Ertmer *et al.*, 2004), which also reduce the amount of produced PrP^{Sc}. To check whether the expression of ITSN-1L and Slit 1 has an effect on the amount of surface PrP, N2a cells were transfected with ITSN-1L and Slit 1. As shown in (3.7) the expression of ITSN-1L or Slit 1 did not alter the amount of surface-located PrP. This fits into the idea that ITSN-1 L interacts with PrP that recycles from the cell membrane (see 3.3.2). Slit 1 is thought to interact with PrP of neighboring cells *in trans*. It is therefore reasonable that Slit 1does not influence surface expression of PrP.

4.11 Interaction of PrP^C with Intersectin 1 or Slit 1 does not alter PrP^{Sc} levels

Although ITSN-1L and Slit 1 both interact with PrP^C the over-expression of each of them in prion infected cells does not alter the conversion of PrP^C in PrP^{Sc} regarding the amount of PrP^{Sc} (3.5). This is especially interesting because the interactions of ITSN-1L and Slit 1 with PrP take place in different cellular compartments – late endosomes/exosomes for ITSN-1L and the outer leaflet of the cell membrane for Slit 1. But both interactions do not interfere with the conversion of PrP^C in PrP^{Sc} in a transient transfection situation. Interesting for further research would be to analyze the conversion of PrP^C in PrP^{Sc} in cells permanently overexpressing ITSN-1L and Slit 1 or both of the proteins – e.g. with retroviral transfection.

4.12 Future aims

An interesting starting point for future research is the involvement of Intersectin 1 in apoptosis, with regarding the interaction with PrP. The quantity of conflicting data indicates the need for clarification. Therefore, the influence of different PrP mutants and PrP^{Sc} should

be considered, concerning the interaction with Intersectin 1. For this it is important to turn the attention to endogenous Intersectin 1. Very recently there have been some publications concerning this topic using neuroblastoma cells. With this new knowledge the most urgent questions can probably be answered. In this context the role of Intersectin 1 in endocytosis has to be addressed first. Endocytosis is the major overlap of prion and Intersectin research, since it is believed that conversion of PrP^{C} into PrP^{Sc} occurs along the endocytotic pathway.
The interaction of Slit 1 has effects on the physiology of PrP. Consequently, changes in the conversion of PrP^{C} to PrP^{Sc} should be tracked in detail. Expanding the experiments presented in this work, the *de novo* synthesis of PrP^{Sc} in cell culture system should be studied. Further, the components of rafts under the influence of Slit 1 over-expression should be further investigated. This may elucidate the mechanism, which is responsible for the change in raft localization of PrP. Yet another approach to characterize the interaction of Slit 1 and PrP further would be to analyze axon growth, influence be different Slit 1 levels or by using different PrP mutants, because it has been reported that the expression level of PrP^{C} is increased in differentiating cells. Differentiating cells show axon growth which probably can be influenced by Slit 1 expression. For these kinds of experiments primary neuronal cells should be used, because their axon growth may respond to Slit 1 gradients, recreating the situation in embryonic brain.
Another interesting research direction would be to search for interactors directly binding to amino acids 168, 172, 215 and 219 of the prion protein. These are the proposed binding sites for “Factor X”. Some already described interactors could possibly bind in this area of the prion protein: e.g. Bcl-2, Pint1, NRAGE, TREK-1, Casein kinase 2 α/α′ subunits, 37-kDa/67-kDa laminin receptor and N-CAM. It would be interesting to find out if one of these proteins has any influence on the conversion of PrP^{C}. Probably there are interesting starting points for therapeutic approaches.

5. Summary

English

Prion diseases are rare, infectious and fatal neurodegenerative disorders that include Creutzfeldt-Jakob disease in humans, scrapie in sheep, and bovine spongiform encephalopathy in cattle. The pivotal event is characterized by the conversion of PrP^C into its pathological isoform PrP^{Sc}, which is believed to be the infectious agent (Weissmann, 1995;Prusiner, 1998;Aguzzi and Heppner, 2000;Chiesa and Harris, 2001;Collinge, 2001;Priola and Vorberg, 2004;Aguzzi and Polymenidou, 2004). However growing evidence proposes that PrP^{Sc} itself is not neurotoxic, since e.g. mice lacking endogenous PrP are immune to the toxic effects of PrP^{Sc} (Bueler *et al.*, 1993;Brandner *et al.*, 1996).
Although PrP^C is evolutionary highly conserved and crucial for neurotoxicity in TSEs (transmissible spongiform encelophalopathies) the physiological function is not yet properly elucidated (Schatzl *et al.*, 1995;Brown *et al.*, 1997c;Wopfner *et al.*, 1999;Westergard *et al.*, 2007). Knock-out mice lacking PrP do not show apparent incapabilities (Bueler *et al.*, 1992), except effects on the neuronal level, such as altered long term potentiation and defects in GABA-ergic receptor-mediated synaptic inhibition (Collinge *et al.*, 1994;Whittington *et al.*, 1995). Recent results indicate that these might not be the only effects, showing that PrP knock-out mice displayed age-related defects in motor coordination and balance (Nazor *et al.*, 2007). PrP^C can bind Cu(II) ions and may have a superoxide dismutase function (Brown *et al.*, 1997a;Brown *et al.*, 1997c), so it could possibly have a protective function against oxidative stress. It was also proposed that PrP plays a role in signal transduction, activating the tyrosine kinase Fyn (Mouillet-Richard *et al.*, 2000). It was also shown, that the cellular prion protein plays a protective role against apoptosis (Kuwahara *et al.*, 1999).
One way to elucidate the physiological role of the cellular prion protein could be to investigate the interaction of PrP^C with other proteins, while it is trafficking through the cell.
It was reported that the N terminus is involved in trafficking of PrP^C. Deletions in the N terminal region of PrP lead to a significant reduction of internalisation and prolonged the half-life of PrP while delaying the transport through the secretory pathway (Nunziante *et al.*, 2003). Naturally neurons have a high PrP expression. Consequently a yeast two-hybrid screen was performed using a cDNA library derived from BALB/c mice with N terminal murine PrP (aa 23-100). Intersectin 1 (ITSN-1L), Slit 1, NRAGE and Synaptotagmin 13 were identified interacting with PrP^C. The physiological relevance was confirmed here by methodically

unrelated experiments, namely co-immunoprecipitations and co-localisations in murine neuronal cell lines. For further experiments ITSN-1L and Slit 1 were chosen, since their interaction with PrP could be shown in mammalian cells.

ITSN-1L is a protein involved in endocytosis, regulation of actin polymerization, and Ras/MAPK signalling (Tong *et al.*, 2000;Ma *et al.*, 2003). It could be shown in this work that ITSN-1L colocalizes with PrP in late endosomes/exosomes. Exosomes are coming more and more into focus of prion research, because of growing evidence that they could be transmitting and disseminate the prion disease (Vella *et al.*, 2007). In addition it could be reported that ITSN-1L also interacts with cytoPrP, ^{Ctm}PrP and Grb2, indicating a more complex role, possibly in regulation of apoptosis, besides the involvement in endocytosis. The over-expression of ITSN-1L in murine cell culture system did not alter the physiological state of PrP regarding, half-life and cell surface expression.

Slit 1 on the other hand is unique as an interactor, because it is one of few described interactors that are not located in the cytosol - as a secreted protein Slit 1 binds PrP on the cell surface. Slit 1 is involved in commissural axon guidance, mediating normal neuronal and organ development (Rothberg *et al.*, 1988;Rothberg *et al.*, 1990;Kidd *et al.*, 1999;Brose *et al.*, 1999;Brose and Tessier-Lavigne, 2000;Hinck, 2004;Autiero *et al.*, 2005;Strickland *et al.*, 2006). As shown in this work the interaction of Slit 1 has a physiological influence by changing PrP raft localisation, while overexpression Slit 1 in murine cell lines. This could denote a possible involvement of PrP in axon guidance, since PrP^C is highly expressed in the embryonic brain, when most of the axon growth occurs in animal development.

Deutsch

Prionenerkrankungen sind seltene, infektiöse und tödliche neurodegenerative Erkrankungen, die sowohl die Creutzfeldt-Jakob Krankheit, als auch Scrapie in Schafen und die Bovine Spongiforme Enzephalopathy in Rindern umfassen. Das entscheidende Ereignis ist die Konversion von PrP^{C} in die pathologische Isoform PrP^{Sc}, welches als infektiöses Agens angesehen wird (Weissmann, 1995;Prusiner, 1998;Aguzzi and Heppner, 2000;Chiesa and Harris, 2001;Collinge, 2001;Priola and Vorberg, 2004;Aguzzi and Polymenidou, 2004). Andererseits konnte gezeigt werden, dass PrP^{Sc} allein nicht neurotoxisch ist, da Mäuse, denen endogenes PrP fehlt, immun gegen die toxische Wirkung von PrP^{Sc} sind (Bueler et al., 1993;Brandner et al., 1996).
Obwohl PrP^{C} ein in der Evolution stark konserviertes Protein ist und es für die Neurotoxizität bei TSE-Erkrankungen (transmissible spongiform encelophalopathies) entscheidend ist, wurde seine physiologische Rolle noch nicht ausreichend geklärt (Schatzl et al., 1995;Brown et al., 1997c;Wopfner et al., 1999;Westergard et al., 2007). Knock-out Mäusen, denen PrP gänzlich fehlt, zeigen keine offensichtlichen Behinderungen (Bueler et al., 1992), ausgenommen auf neuronaler Ebene - wie die veränderte Langzeit-Potenzierung und Einschränkungen GABAerger rezeptorvermittelter, synaptischer Inhibition (Collinge et al., 1994;Whittington et al., 1995). Neueste Untersuchungen zeigen, dass dies vermutlich nicht die einzigen Effekte sind. PrP knock-out Mäuse zeigten altersabhängige Defekte in motorischer Koordination und Balance (Nazor et al., 2007). PrP^{C} kann Cu^{2+}-Ionen binden und könnte somit eine Superoxiddismutase-Funktion haben, die eine schützende Funktion gegen oxidativen Stress darstellen würde (Brown et al., 1997a;Brown et al., 1997c). Zudem wurde PrP, über die Aktivierung der Tyrosinkinase Fyn, eine Rolle in der Signaltransduktion zugeschrieben (Mouillet-Richard et al., 2000). Das zelluläre Prion Protein zeigte darüber hinaus auch eine Schutzfunktion gegen Apoptose (Kuwahara et al., 1999).
Über die physiologische Rolle des zellulären Prion Proteins könnte die Untersuchung der Interaktion von PrP^{C} mit anderen Proteinen während dessen intrazellulären Transports Aufschluss geben. Es wurde berichtet, dass der N-Terminus beim intrazellulären Transport involviert ist. Deletionen in der N-terminalen Region von PrP führten zu einer signifikanten Verringerung der Internalisation und verlängerten die Halbwertszeit von PrP, während gleichzeitig die Sekretion verzögert wurde (Nunziante et al., 2003).
Typischerweise zeigen Neuronen eine hohe PrP-Expression, deswegen wurde ein Yeast Two-Hybrid Assay mit einer aus BALB/c Mäusen mit n-terminalen, murinem PrP (Aminosäuren

23-100) gewonnenen cDNA Bibliothek durchgeführt. Intersectin 1 (ITSN-1L), Slit 1, NRAGE und Synaptotagmin 13 wurden als Interaktoren für PrP^C identifiziert. Die physiologische Relevanz wurde in methodisch unabhängigen Experimenten, wie der Co-immunopräzipitation und der Co-lokalisation in murinen Zelllinien, nachgewiesen. Für die weiterführenden Experimente wurden ITSN-1L und Slit 1 ausgewählt, da sie eine Interaktion mit PrP in Säugetier-Zelllinien zeigten.

ITSN-1L ist ein Protein, das an der Endozytose, der Regulation der Aktin-Polymerisierung und Ras/MAPK Signalwegen beteiligt ist (Tong et al., 2000;Ma et al., 2003). In dieser Arbeit konnte gezeigt werden, dass ITSN-1L mit PrP in späten Endosomen/Exosomen co-lokalisiert. Exosomen geraten zunehmend in das Interesse der Forschung, da sich die Hinweise häufen, dass Exosomen für die Übertragung und Ausbreitung der Prionenerkrankungen verantwortlich sind (Vella et al., 2007). Desweiteren konnte in dieser Arbeit gezeigt werden, dass ITSN-1L auch mit cytoPrP, CtmPrP und Grb2 interagiert. Dies deutet auf eine weit komplexere Rolle unter anderem in der Regulation der Apopotose hin. Die Überexpression von ITSN-1L in murinen Zelllinien hingegen veränderte die physiologische Situation von PrP hinsichtlich Halbwertszeit und Zelloberflächenexpression nicht.

Slit 1 unterscheidet sich von den anderen in dieser Arbeit untersuchten Interaktoren, da es sich nicht um ein cytosolisches Protein handelt, sondern von der Zelle sekretiert wird und an PrP auf der Zelloberfläche bindet. Slit 1 ist beteiligt an der Regulation der Richtung des Axonwachstums und steuert Neuronen- und Organentwicklung im Allgemeinen (Rothberg et al., 1988;Rothberg et al., 1990;Kidd et al., 1999;Brose et al., 1999;Brose and Tessier-Lavigne, 2000;Hinck, 2004;Autiero et al., 2005;Strickland et al., 2006). In dieser Arbeit konnte gezeigt werden, dass die Interaktion von Slit 1 mit PrP auf der Zelloberfläche einen direkten physiologischen Einfluß auf PrP nimmt. Bei der Überexpression von Slit 1 in murinen Zellen, veränderte sich die Raft-Lokalisation von PrP. Dies lässt eine Beteiligung von PrP in der Regulation der Richtung des Axonwachstums vermuten. PrP^C wird im Gehirn von Embryonen stark exprimiert, während zeitgleich der Großteil des Axonwachstums in der Entwicklung von Tieren stattfindet.

6. Abbreviations

A	adenosine
Aa	amino acid
Ala	alanin
APS	ammoniumpersulfate
Asp	aspartate
ATP	adenosintriphosphate
Bisacrylamide	N,N´-Methylenbisacrylamide
bp	base pair
BSE	bovine spongiform encephalopathy
C	cytidine
cDNA	complementary DNA
CJD	Creutzfeldt-Jakob disease
cpm	counts per minute
Ci	Curie
CNS	central nervous system
CWD	chronic wasting disease
Cys	cysteine
DMEM	Dulbecco´s Modified Eagle ´s Medium
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
dNTP	desoxynucleotide
ER	endoplasmic reticulum
EDTA	ethylenediamine tetraacetic acid
et al.	et altera (and others)
EtOH	ethanol
FCS	fetal calf serum
FFI	fatal familiar insomnia
FITC	Fluorescein - Isothyocianate
FSE	feline spongiform encephalopathy
G	guanosine
Glu	glutamate

GPI	glycosyl-phosphatidyl-inositol
GSS	Gerstmann-Sträußler-Scheinker disease
Gly	glycine
IC_{50}	concentration of a substance resulting in a 50 % inhibition
iCJD	iatrogenic CJD
in vitro	in cell culture / reaction vessel
in vivo	in living organism
kb	kilo base pair
kDa	kilo dalton
mAB	monoclonal antibody
mRNA	messenger RNA
MEM	Minimum Essential Medium
Met	methionine
OD	optical density
ORF	open reading frame
pAB	polyclonal antibody
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PrP	prion protein
PrP^{C}	cellular non-pathogenic form of the prion protein
PrP^{Sc}	pathogenic form of the prion protein
RT	room temperature
PBS	Phosphate Buffered Saline
Phe	phenylalanine
SDS	sodium dodecyl sulfate
PK	Proteinase K
PMSF	phenylmethylsulfonylfluorid
Pro	proline
PVDF	polyvinyldifluorid
RNA	ribonucleic acid
RNAi	RNA interference
RNase	ribonuclease
sCJD	spontanious CJD
SDS	sodium dodecylsuflate

Ser	serine
siRNA	small interfering RNA
SV40	Simian Virus 40
T	thymidine
TAE	tris-acetate-EDTA-buffer
TE	tris-EDTA-buffer
TEMED	N,N,N,N-tetramethylethylendiamin
TGN	trans-Golgi network
TME	transmissible mink encephalopathy
TSE	transmissible spongiform encephalopathy
Tyr	tyrosin
U	unit
U	uracil
V	volt
Val	valin
vCJD	variant CJD
v/v	volume per volume
w/v	weight per volume
wt	wild type

7. Reference List

Reference List

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8. Publications

Original articles:
Bruns C.P., Schätzl H.M. (2009). Novel interactors of the prion protein involved in signaling and axon growth. *(In preparation)*

Talks:
Bruns C.P., Schätzl H.M. (2005). Characterization of cellular interactors of the prion protein. *EU Prion Meeting*. (Crete, Greece)

Bruns C.P., Schätzl H.M. (2005). Characterization of cellular interactors of the prion protein. *Annual Symposium des Institutes für Virologie TUM*. (Garmisch, Germany)

Bruns C.P., Schätzl H.M. (2004). Characterization of cellular interactors of the prion protein. Identification and characterisation of cellular interactors of the prion protein regarding trafficking and quality control. *Annual Symposium des Institutes für Virologie TUM.* (Garmisch, Germany)

Poster presentations:

Bruns C.P., Schätzl H.M. (2007). Secreted Slit 1 interacts with the prion protein on the cell surface. *Third European Congress of Virology*. (Nürnberg, Germany)

Bruns C.P., Schätzl H.M. (2007). Characterization of cellular interactors of the prion protein. *Abschlusssymposium des Bayerischen Forschungsverbundes Prionen, For Prion*. (München, Germany)

Bruns C.P., Schätzl H.M. (2006). Characterization of cellular interactors of the prion protein. *European Network of Excellence (NoE), NeuroPrion, Prion 2006*. (Turino, Italy)

Bruns C.P., Schätzl H.M. (2006). Characterization of cellular interactors of the prion protein. *Jahrestagung 2006 der Gesellschaft für Virologie, Annual Meeting*. (München, Germany)

Bruns C.P., Schätzl H.M. (2005). Cellular interactors of the prion protein. *TSE-Forum, European Network of Excellence (NoE), Prion 2005*. (Düsseldorf, Germany)

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10. Curriculum vitae

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