Aus dem Adolf-Butenandt-Institut für Lehrstuhl Stoffwechselbiochemie der Ludwig-Maximilians Universität München

Vorstand: Prof. Dr. rer. nat. Dr. h.c. Christian Haass

Functional characterization of Shadoo, a PrP-like protein with neuroprotective activity

Dissertation

Zum Erwerb des Doktorgrades der Naturwissenschaften (Dr. rer. nat.)

an der Medizinischen Fakultät

der Ludwig-Maximilians-Universität München

vorgelegt von

Vignesh Sakthivelu
aus
Coimbatore, Indien
2011

Gedruckt mit Genehmigung der Medizinischen Fakultät

der Ludwig-Maximilians-Universität München

Betreuer	:	Prof. Dr. rer. nat. Jörg Tatzelt
Zweitgutachter	:	Priv. Doz. Dr. rer. nat. Monika Bradl
Dekan	:	Prof. Dr. med. Dr.h.c. Maximilian Reiser, FACR,FRCR
Mündliche Prüfung am	:	30.05.2012

Acknowledgement

This doctoral thesis is a significant part of the research study that I did as a PhD student in the group of Prof. Dr. Jörg Tatzelt. Right from the beginning until the completion of the study, I had the good opportunity to work with many people whose contribution in different ways to the study have helped in making my PhD thesis very special. I would like to sincerely thank all these people who have made a noteworthy impact on my life.

Firstly, I would like to convey my immense gratefulness to my advisor Prof. Dr. Jörg Tatzelt, for giving me an interesting and important research topic, for his critical supervision, guidance and also for all his personal care and affection during my entire stay.

I am deeply indebted to PD. Dr. Konstanze F. Winklhofer for her valuable support and major discussions and suggestions during the entire PhD timeline.

Many heartfelt wishes are also in the offing to Dr. Ralf Seidel from the Max Planck Institute of Molecular Physiology, Dortmund, Germany for his enormous help in preparing the rabbit polyclonal Sho antibody.

I earnestly thank Prof. Dr. Christian Haass for agreeing to be a member of my PhD thesis committee and also for his helpful discussions during my research.

Frankly, much of my work has been possible due to the company and help of former and present members of the prion and Parkin group: Geli, Margit, Anita, Mareike, Lena and Julia. Special thanks to Veronika for her technical assistance in the lab; Sabine and Annette for their outstanding lab management. Heartfelt thanks to Uli, Natalie, Daniela, Viktoria and Sina for not just being good lab mates but also for being good friends and giving me the push and energy in all my high and low times; Anna, Alexandra, Kathrin, Elisa, Carolin, Maria Patra, Maria Funke and the people in Haass department are also fondly thanked.

My special thanks to Krishnaveni, Senthil Murugan and Katyayni for their love, special care and a critical help with my thesis.

My friends Santmoor, Sabari, Anu, Vidhya Shankar, Guru, Jeeva, Nagendran, Karthikeyan, Vigneshwaran, Ramesh, Vaideeswaran, Maha, Sudhakar, Bharati, Vaibhaoa, Shanaya, Kavin, Avinash, Paresh, Arun, Dilip, Sathish and Raghu deserve each and every word of goodness for their kind hospitality and never making me feel alone during my entire stay in Munich.

Words fail me, but the reason why I have reached this stage in life is because of the constant belief, unconditional love and wonderful prayers of my beloved parents Sakthivelu, Santhi and my brother Rajkumar that have helped me in all walks of my life.

Last but not the least, I am highly thankful to the DAAD (German Academic Exchange Service, Germany) for awarding me a fellowship that helped me to successfully complete my PhD in Germany.

Contents

Introduction

Prion diseases

Formation of aberrant protein conformers plays a crucial role in several neurodegenerative diseases like Alzheimer's disease, Parkinson's disease and prion diseases. Though altered conformations of proteins and neuronal cell death are characteristic features of all neurodegenerative diseases, prion diseases are unique among all the other diseases in that an infectious particle is generated, which is devoid of nucleic acids. Prion diseases (also called "transmissible spongiform encephalopathies" (TSEs) are a group of neurological disorders that include Creutzfeldt-Jakob disease (CJD) and Kuru in humans, bovine spongiform encephalopathy (BSE) in cattle and scrapie in sheep and goats. In prion diseases of humans and mammals, the host encoded cellular prion protein (PrP^C) is converted into a detergent-insoluble and partially proteinase K-resistant isoform, designated scrapie prion protein (PrP^{Sc}), which is the main component of infectious prions. Apart from the unconventional nature of the causative agent, prion diseases are peculiar in terms of their etiology; no other disease entity comprises sporadic, genetic and infectious variants.

Transmissible spongiform encephalopathies in animals

Scrapie

Although there is a discussion that scrapie, the first known TSE was present before the beginning of the 18th century in northern Europe and Austro-Hungary, the exact origin of the disease is not clear. The earliest recorded history about scrapie goes back to 1755 with a discussion at the British parliament about the spread of a new fatal disease in sheep because it affected the quality of wool, which was a highly commercial product in England at that time. Scrapie was documented for the first time in Germany in 1759. Clinical symptoms in sheep included difficulty in walking, rubbing their back against posts or trees, severe seizures and finally death. The only way to stop the spread of the disease was to isolate the infected sheep from the healthy stock and kill them.

Von denen mancherley Kranckheiten des Schaafviehes, und was vor Curen damit vorgenommen werden.

Der Trab ist auch eine Kranckheit der Schaafe, und ist anstekend.

Es bekommen auch manche Schaafe den Trab, welches eine Kranckheit ist, die daran zu erkennen, wenn sich das Stücke, das solchen bekommt, niederleget, und beisset mit dem Maule an den Füssen und um die Beine, und reiben sich mit dem Creuze an denen Stangen, verlieren das Gedeyen, fressen auch nicht recht, und verlahmen endlich; sie schleppen sich lange, verzehren sich nach und nach, und zuletzt müssen sie sterben. Welches Vieh diese Staupe bekommt, wird nicht besser. Daher denn das allerbeste ist, daß ein Schäfer, welcher ein Stücke von dem Trabe befallen, gewahr wird, es balde wegschafft, und vors Herrschafftliche Gesinde schlachtet. Es muß ein Schäfer ein solches Stücke Vieh also gleich von dem gesunden Vieh absondern, denn es stecket an, und kan vielen Schaden unter der Heerde verursachen.

Figure 1: First documentation of scrapie in Germany. First recorded proof of scrapie was described by Johann George Leopoldt in 1750, about the characteristic features and treatments that were used against scrapie (Source: Johann George Leopoldt (1750) Nuetzliche und auf die Erfahrung gegruendete Einleitung zu der Land-Wirtschaft, Part 5, Chapter 12 p.348. Sorau).

There was no focused research done on scrapie until the disease was successfully transmitted to healthy sheep by inoculating them with the brain and spinal cord extracts of infected animals (Cuille & Chelle, 1938). The infective nature of scrapie was further strongly confirmed once immunized sheep became sick after injecting a vaccine prepared from the brain, spinal cord and spleen of scrapie infected animals (Gordon, 1946). Successful transmission of scrapie to laboratory mice intensified scrapie research with the aim to identify and to characterize the biochemical properties of the infectious agent (Chandler, 1961). Initially, a virus was believed to be the causative agent because of the long incubation period. But scrapie agent's resistance to heat, ultraviolet light and formaldehyde, which are known to destroy viral particles, suggested that a virus was obviously not the infectious particle. Healthy sheep injected with brain homogenate from scrapie infected sheep developed clinical symptoms after five months, whereas goats injected with the sheep scrapie brain homogenate developed the clinical symptoms after 23 months. The incubation time was shortened to eight months when the brain homogenate from scrapie infected goat was injected in to a healthy goat. These were the first evidences describing a phenomenon denoted species barrier. Even though the scrapie transmission to the laboratory animals was experimentally confirmed but so far there is no proof available for the sheep or goat scrapie transmission into humans.

Bovine spongiform encephalopathy

BSE affects cattle and is commonly referred to as "mad cow disease". The first case of BSE was confirmed in the year 1986 in Great Britain. After this, the number of new cases increased significantly within the next few years in all parts of Great Britain, with a maximum of 36,680 cases identified in 1992. The clinical symptoms observed in BSE affected animals are difficulty in standing, lack of muscle coordination with trouble to walk and loss of weight. Affected cattle die within a few weeks or a few months after the onset of clinical symptoms. The source of BSE is still unknown, though it is believed that contaminated meat-and-bone meal (MBM) prepared from scrapie infected sheep might be responsible for the spread of the disease. New BSE cases in Great Britain are declining after the subsequent ban of MBM by the British government.

In the beginning of the 20th century the infectivity and disease transmission to heterologous animals was clearly demonstrated along with prolonged incubation period. In 1996, a new type of prion disease in humans called variant Creutzfeldt-Jakob disease (vCJD) was reported in United Kingdom (UK). The current idea is that vCJD is due to a transmission of BSE to humans, possibly through the consumption of BSE contaminated food stuffs. So far, 170 cases of vCJD have been recorded in Great Britain. In addition, a few vCJD cases have been reported outside the UK, but none so far in Germany.

Table 1. Number of BSE reported cases worldwide until the end of 2009.

Country	> - 2006	2007	2008	2009	Total
Austria	5	1	0	0	6
Belgium	133	0	0	0	133
Canada	10	3	4	1	18
Czech republic	26	2	0	2	30
Denmark	15	0	0	1	16
Finland	1	0	0	0	1
France	985	9	8	9	1011
Germany	411	4	2	2	419
Greece	1	0	0	0	1
Ireland	1593	25	23	9	1650
Israel	1	0	0	0	1
Italy	141	2	1	1	145
Japan	31	3	1	1	36
Sweden	1	0	0	0	1
Hungary	0	1	0	0	1
Netherlands	82	2	1	0	85
Poland	50	9	5	4	68
Portugal	1034	14	18	8	1074
USA	3	0	0	0	3
Spain	681	39	25	18	763
Switzerland	463	0	0	0	463
United Kingdom	184,481	65	42	10	184,598
Total	**190,148**	**179**	**130**	**66**	**190,523**

Chronic wasting disease

In the early 1960s, a peculiar disease called clinical wasting syndrome was observed in North American deer including mule deer (*Odocoileus Hemionus*), white-tailed deer (*Odocoileus Virginianus*) and the Rocky Mountain elk (*Cervus Canadensis*). The histopathology of the brain tissue from the diseased animals showed a spongy appearance, thus the disease was recognized as a form of transmissible encephalopathy and renamed as chronic wasting disease (CWD) (Williams & Young, 1992). Research on CWD was intensified after a possible link between BSE and vCJD was uncovered. CWD shares certain pathophysiological features with scrapie and BSE. Both farming and free ranging animals can be experimentally infected, with incubation periods ranging from 15-36 months. Clinical symptoms include behavioral abnormalities, difficulty in walking, excessive salivation, increased drinking, urination and finally death. The disease can be transmitted via animal to animal contact, saliva, feces and lateral transmission. Even though CWD transmission was reported to animals like sheep, goat, deer and cattle under experimental conditions, there is no reported evidence of transmission of CWD to humans (Belay et al, 2004; Hamir et al, 2005; Hamir et al, 2006; Hamir et al, 2007; Williams & Miller, 2002).

Prion diseases also occur in other animals such as Transmissible mink encephalopathy (TME) in mink (Hartsoug.Gr & Burger, 1965), Exotic ungulate encephalopathy (EUE) in zoo animals (Kirkwood et al, 1990) and Feline spongiform encephalopathy (FSE) in house and wild cats (Leggett et al, 1990).

Transmissible spongiform encephalopathies in humans

Prion diseases are not only restricted to animals but are also observed in humans. Human prion diseases can be classified into three different etiologic groups; sporadic, inherited and transmissible. The year, place of first occurrence and the etiology of the prion disease in humans are summarized in table 2.

Table 2. Overview of the human prion diseases.

Name of the prion disease	Place of first occurrence	Reference	Etiology
Sporadic Creutzfeldt-Jakob Disease (sCJD)	1920, Germany	Creutzfeldt,1920 Jacob, 1921	Unknown
Familial Creutzfeldt-Jakob Disease (fCJK)	1924, Germany	Kirschbaum 1924	Mutation in PrP gene
Gerstmann-Straussler-Scheinker Syndrome (GSS)	1928/1936, Austria	Gerstmann et al. 1928, 1936	Mutation in PrP gene
Iatrogenic Creutzfeldt-Jakob Disease (iCJK)	1974, USA	Duffy et al., 1974	Infection by medical treatment
Fatal familial insomnia (FFI)	1986, Italy	Lugaresi et al., 1986	Mutation in PrP gene
New variant Creutzfeldt-Jakob disease (nvCJD)	1996, Great Britain	Will et al., 1996	Infection

In humans, Kuru is the major acquired prion disease which was first noticed in the Fore tribes of Papua New Guinea in the late 1950s. Kuru means "to shake" in local Fore language. It is the first human form of prion disease which was experimentally confirmed to be infectious and transmissible. Ritual cannibalism practiced by the Fore tribes was touted to cause the transmission of the disease among the group, in which females and children were more severely affected than males. The disease was completely eradicated after the end of cannibalism. Gajdusek suggested that the disease progression occurs in three stages, in which the first stage includes trembling, deterioration and slurring of speech (Gajdusek, 1973). The secondary stage (also called as "sedentary stage") was characterized by severe ataxia, shock like muscle jerks and depression while the terminal stage was linked to the inability of the patient to sit without an external support, defective muscle coordination and difficulty in eating (Gajdusek, 1973).

Analyzing the postmortem brain of kuru patients, Igor Klatzo noted that Kuru is very similar to that of another human prion disease called Creutzfeldt-Jakob disease (CJD) (Klatzo et al, 1959). Kuru was successfully transmitted to chimpanzees by intracerebral injection of brain homogenate from Kuru infected individuals. Later, the nature of infectivity of CJD was confirmed in similar experiments (Gajdusek et al, 1966; Gibbs et al, 1968).

Sporadic human prion diseases occur spontaneously with no prior family history and mutations in the prion protein gene (*PRNP*). CJD was first described by two German neuropathologists Hans Gerhard Creutzfeldt and Alfons Jakob independently (Creutzfeldt, 1920; Creutzfeldt, 1921; Jakob, 1921). CJD is classified into four major types, sporadic CJD (sCJD), iatrogenic CJD (iCJD), variant CJD (vCJD) and familial CJD (fCJD). The most common form of CJD is sCJD, and accounts for approximately 85% of all CJD cases (Johnson, 2005; Prince et al, 2006). So far, the mechanisms which trigger sCJD have not been identified. However, sporadic somatic cell mutations in *PRNP* gene, spontaneous refolding of prion protein or unidentified infection have been proposed to be the cause of sCJD but none of these have been experimentally proven (Aguzzi et al, 2008).

fCJD is an inherited prion disease due to mutations in the *PRNP* and accounts for about 15% of all reported cases. The age of onset is around 45 years and a patient may live for several years after the onset of the disease. Mutations include insertions, deletions or substitutions and are located especially in the structured C-terminal domain of PrP (Figure 2).

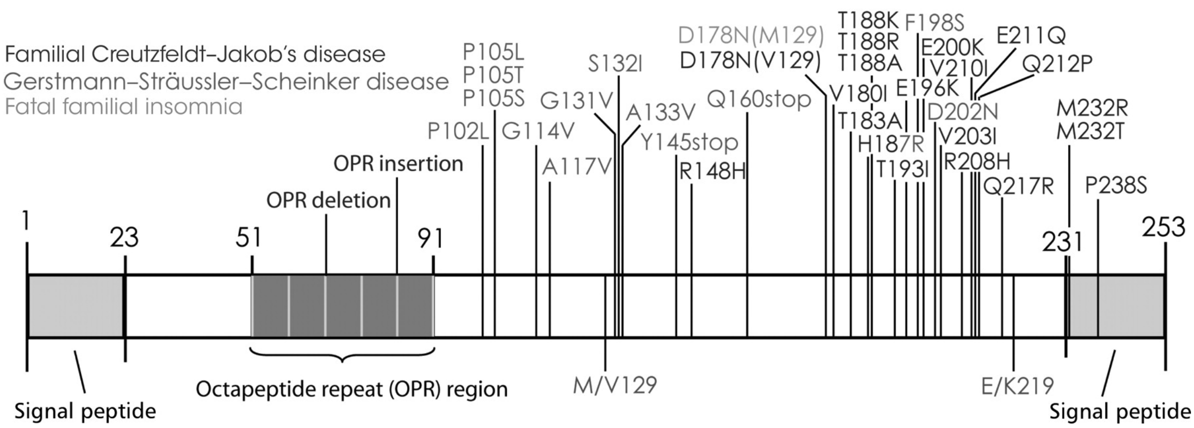

Figure 2: The human prion protein gene (*PRNP*) with all definite and suspected pathogenic mutations currently identified. The entire prion protein consists of residues 23-230, with 1-22 a signal peptide for targeting to the endoplasmic reticulum and 231-253 a signal peptide for GPI-anchor attachment. M/V and E/K219 are common polymorphisms that can influence the onset and phenotype of the disease (Adopted from Mead, 2006).

Gerstmann–Sträussler–Scheinker syndrome (GSS) is a rare genetic autosomal dominant prion disease first described by Josef Gerstmann, an Austrian neuropathologist, along with Ernst Sträussler and Ilya Scheinker (Gerstmann et al, 1935). Mice infected with intracerebral injection of GSS brain homogenate developed the disease symptoms comparable to the clinical symptoms of GSS, providing experimental evidence for the infectious nature of inherited prion diseases (Tateishi & Kitamoto, 1995).

Fatal familial insomnia (FFI) also belongs to the group of inherited prion diseases with a mutation in the *PRNP*. So far, there are 40 families found to possess the mutated gene worldwide. A disease with similar symptoms was described in patients with no PrP mutations called sporadic fatal insomnia (SFI).

The third group of the human prion disease is the infectious form that includes iCJD and the new variant Creutzfeldt-Jakob disease (nvCJD). Infectious forms account for less than 1% of all cases. Contaminated human growth hormones isolated from CJD infected individuals and contamination of surgical instruments from CJD infected tissues as a result of medical procedure accounted for the iatrogenic forms (Bernoulli et al, 1977; Davanipour et al, 1984; Duffy et al, 1974; Kondo & Kuroiwa, 1982). vCJD develops probably due to the intake of BSE contaminated food products and in contrast to the classical form has a very long incubation period (Aguzzi & Weissmann, 1996; Bruce et al, 1997; Collinge et al, 1996; Hill et al, 1997). So far, 280 vCJD cases have been confirmed worldwide since the first cases reported in 1996. The number of cases may rise in the future considering the unusually prolonged incubation periods of this disease. There is evidence that vCJD in contrast to sCJD, can be transmitted through blood products (Aguzzi & Glatzel, 2004; Llewelyn et al, 2004; Peden et al, 2004; Wroe et al, 2006). This indicates that prion contaminated blood products can significantly increase the risk of prion disease in humans. As a consequence people who lived in the UK between 1980 and 1996 were not allowed to donate blood in countries outside the UK.

Clinical signs and neuropathology of human prion diseases

The main characteristic features of all human prion diseases are prolonged incubation periods with complex etiology and differences in their disease duration, onset of clinical manifestation and neuropathology. Onset of the symptoms in sCJD is approximately around the age of 60 years. In fCJD and GSS, the symptoms are observed at an average age of 45-50 years, whereas in vCJD clinical signs are detected in relatively younger patients with an average age of 29 years. Kuru occurs at a wide range of ages between 4-60 years which is possibly associated with the concentration and the exposure time of the infectious particle. Prion diseases not only differ in their incubation periods but also in the duration between the onset of clinical signs and death. The average duration between the onset of the clinical symptoms and death in sCJD is only 2-3 months while in vCJD the average is 14 months and that of Kuru is 12 months. GSS shows an exceptionally long duration of the disease with an average of 5 years (Collinge, 2001; Johnson & Gibbs, 1998).

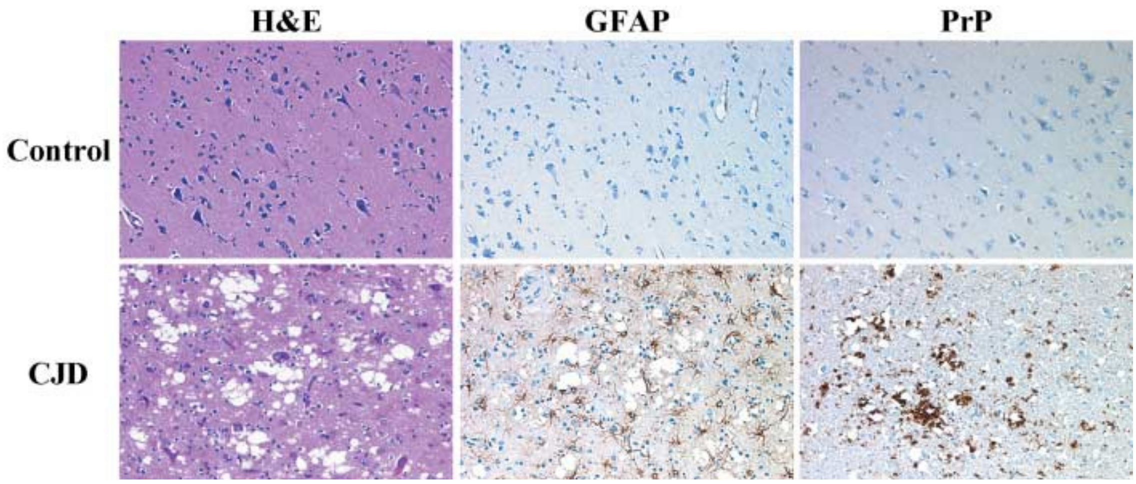

Figure 3: Neuropathological features of transmissible spongiform encephalopathies. Characteristic histological and immunohistochemical features between the brain samples of a control (upper row) and from Creutzfeldt–Jakob disease (CJD; lower row) patients. Brain samples were stained with hematoxylin and eosin (H&E), immunohistochemical staining with anti-GFAP antibody (GFAP) and an anti-prion antibody (PrP). Brain spongiform and neuronal cell death are observed by H&E staining. Astrogliosis and prion deposits are demonstrable using GFAP and PrP immunostains of CJD brain samples (Adopted from Aguzzi et al, 2001).

Clinical symptoms also differ among the human forms of prion diseases. Kuru is primarily characterized by tremor, swallowing difficulty, ataxia, and muscle in-coordination. CJD is primarily characterized by dementia, lack of muscle coordination and behavioral changes. The symptoms of FFI are phobias, paranoia, inability to sleep followed by the loss of weight and disruption of the autonomic nervous system. In common, cerebellar ataxia, slurred speech or visual impairment and unsteadiness with difficulty in walking are observed at the beginning whereas severe ataxia and dementia are observed in later stages of the disease (Wadsworth & Collinge, 2007).

In prion disease, neuropathological changes which include brain vacuolation, astrogliosis and accumulation of PrP amyloid deposits of various structure and size are observed (Figure 3). Kuru amyloid plaques were named after they were found in the brain of Kuru patients and are homogeneous deposits of protein aggregates (Klatzo et al, 1959). In addition, multicentric plaques were observed in postmortem brain of GSS patients and characteristic spiked-ball plaques were observed in vCJD patients (Brown, 1992); (Will et al, 1996).

Prion protein

Nature of the infectious agent

Although scrapie is known for more than 250 years and other prion diseases are known for several decades now, the cause of the infectious agent responsible for TSEs is still a mystery. Experiments to understand the biophysical and chemical properties are limited by difficult, time consuming and expensive bioassays. All the assays were performed in sheep and goats until successive transmission of scrapie to laboratory mice was discovered (Chandler, 1961). Scrapie was suggested to be caused by a slow virus, since healthy animals developed clinical signs after the intracerebral inoculation of brain homogenate contaminated with scrapie agent. However, the scrapie agent was found to be highly resistant towards formalin treatment that efficiently inactivates viral particles (Gordon, 1946). The observation that animals developed the disease after the inoculation of formalin treated scrapie brain suspension, laid a strong base for the speculation of a slow non-viral infection (Sigurdsson, 1954). The scrapie agent was also resistant to ultraviolet radiation, which causes damage to nucleic acids, suggesting that the infectious material was largely composed of protein rather than DNA/RNA (Alper et al, 1967; Griffith, 1967). In control experiments, however, scrapie infectivity was diminished upon treatment with proteinase K, diethyl pyrocarbonate, SDS, guanidinium thiocyanate, phenol and urea, suggesting that scrapie agent is composed of proteins required for infectivity (Prusiner et al, 1981). The term “Prion” (meaning proteinaceous infectious particles) was coined by Stanley B Prusiner in order to differentiate scrapie infectious particles from viruses or viroids (Prusiner, 1982). In 1982, Prusiner and colleagues reported a protein that co-purified with scrapie infectivity (Bolton, 1982).The protease resistant protein obtained from proteinase-K-treated Syrian hamster (SHa) brain suspension had a molecular weight of 27-30kD and was designated as scrapie prion protein 27-30 (PrP27-30), and was glycosylated (Bolton et al, 1985; Prusiner et al, 1983).

The subsequent determination of the amino acid sequence at the N-terminus of PrP27-30 allowed molecular cloning of the prion protein (PrP) gene. Interestingly, PrP is expressed by the host and no significant alteration in PrP mRNA level was found between healthy and infected animals (Oesch et al, 1985). The disease associated protease resistant form of PrP was designated as scrapie prion protein (PrP^{Sc}) while the normal protease sensitive cellular prion protein was designated as PrP^{C}. PrP^{Sc} is found in all forms of prion diseases and is absent in other neurodegenerative disease such as Alzheimer’s disease, Parkinson’s disease and amyotrophic lateral sclerosis (Bockman et al, 1985; Bockman et al, 1987; Brown et al, 1986; Manuelidis, 1985; Manuelidis et al, 1985). The expression level of glial fibrillary acidic protein (GFAP) is elevated in prion infected mice in parallel with PrP^{Sc} accumulation. However, mice devoid of GFAP did not show any alteration in disease progression (Gomi et al, 1995; Manuelidis et al, 1987; Tatzelt et al, 1996).

Many attempts to disprove the prion hypothesis and to demonstrate that viral particles are the pathogen have failed; however, the definite molecular composition of the infectious agent is still unknown. Host encoded 25-mer polynucleotides were co-purified with the infectious particles, which were later identified as non-essential components of the infectious units (Safar et al, 2005). Purified disease associated infectious molecules are not only composed of PrP^{Sc} but also contain significant amounts of lipids and carbohydrates (Appel et al, 1999; Dumpitak et al, 2005; Klein et al, 1998). Recent findings described that infectious prion particles can be generated from bacterially expressed recombinant prion protein (rPrP) which also cause prion diseases in mice (Kim et al, 2010; Legname et al, 2004; Wang et al, 2010). This result strongly supports the protein only hypothesis.

Conformational transition from PrP^C to PrP^{Sc}

Although PrP^C and PrP^{Sc} have the same amino acid (aa) sequence (primary structure) and posttranslational modification, PrP^{Sc} differs from PrP^C by its biochemical and biophysical properties such as solubility and secondary structure. This difference in secondary structure indicates that PrP^{Sc} must be an altered conformer of PrP^C. The conformational transition of PrP^C to PrP^{Sc} is believed to take place at the cell surface or in endosomes (Borchelt et al, 1992; Caughey & Raymond, 1991). The exact mechanism for the conformational change is not known but several theories have been proposed. The heterodimer model assumes that PrP^{Sc} interacts with PrP^C, thereby catalyzing its conversion to PrP^{Sc} (Figure 4) (Cohen et al, 1994).

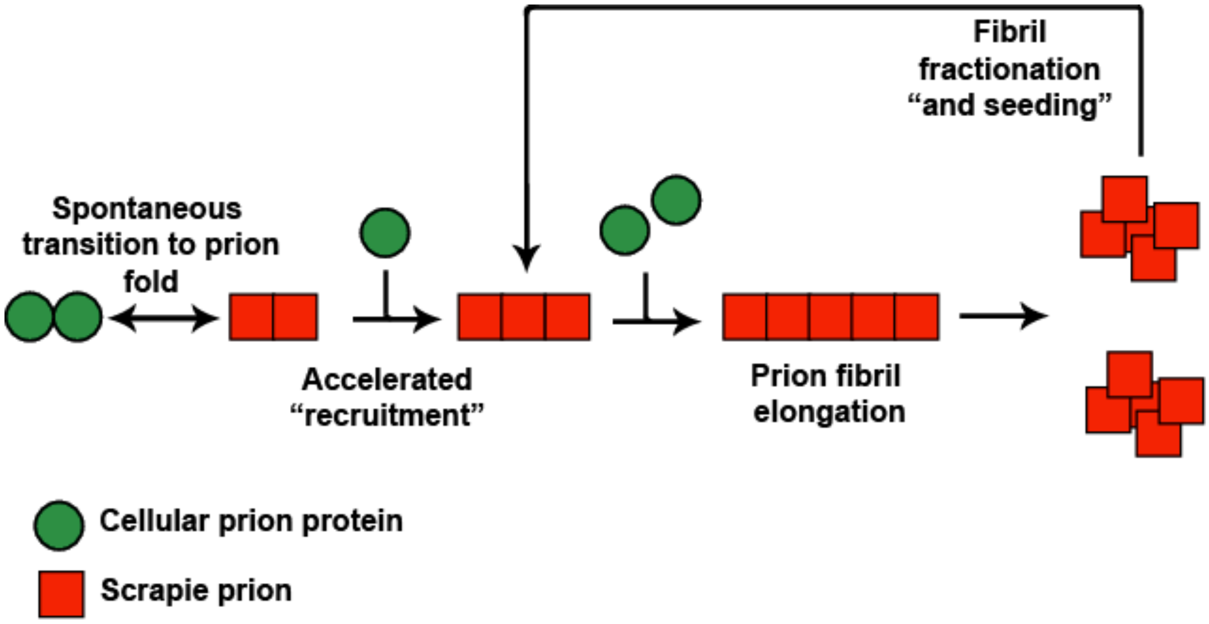

Figure 4: Heterodimer model for prion replication. Infectious prions are formed through autocatalytic process by direct interaction between PrP^C-PrP^{Sc}. Multiple PrP^{Sc} molecules are formed, stabilized and form elongated prion fibrils. Later fibrils are broken in to smaller unit that act as a seed for further PrP^{Sc} conversion (Adopted from Shorter & Lindquist, 2005).

In contrast, the nucleation dependent polymerization mechanism postulates that the nuclear core composed of PrP^{Sc} molecules act as a seed and catalyze the formation of PrP^{Sc}. This process continues until the formation of larger aggregates which act as a reservoir for PrP^{Sc} seeds (Jarrett & Lansbury, 1993). PrP^{Sc} replication *in vivo* takes place from some months to several years depending on the expression of PrP^C by the host. Interestingly, proteinase-K resistant and infectious PrP^{Sc} can be produced through an *in vitro* method called protein misfolding cyclic amplification (PMCA) (Soto et al, 2002). Propagation of PrP^{Sc} was achieved by the addition of PrP^{Sc} seeds to the hamster brain homogenate containing PrP^C in a test tube. PrP^{Sc} generated by PMCA method is infectious (Bieschke et al, 2004; Kim et al, 2010; Wang et al, 2010; Weber et al, 2007). Expression of PrP^C by the host is required for the replication of infectious prion (Bueler et al, 1992). Interestingly, the N-terminal domain (aa 23-90) and the C-terminal glycosylphosphatidylinositol (GPI) anchor are dispensable for the generation of infectious prions (Chesebro et al, 2005b; Fischer et al, 1996). Nucleic acids and lipids have been shown to be involved in conversion and propagation of prions from bacterially expressed recombinant PrP (Wang et al, 2010). But a different study revealed that infectious prions can be generated from recombinant PrP without any cofactors by PMCA technique (Kim et al, 2010).

Prion protein gene

In humans, chromosome number 20 possesses the PrP (*PRNP)* gene while in mice it is located on chromosome number 2. The PrP gene in humans and mice has 3 exons with the complete open reading frame (ORF) formed within the 3rd exon. The human prion protein is composed of 253 amino acids. The mouse and human prion protein genes were cloned in 1986 (Basler et al, 1986; Kretzschmar et al, 1986; Locht et al, 1986; Oesch et al, 1985; Sparkes et al, 1986). PrP is expressed ubiquitously in the embryonic stage (Kretzschmar et al, 1986), whereas in adults high level of expression is found in central nervous system (CNS) and to a lesser extent in spleen lymphocytes (Bendheim et al, 1992; Bueler et al, 1992). Lower PrP expression levels are also observed in muscle and lymphoid tissues (Bendheim et al, 1992).

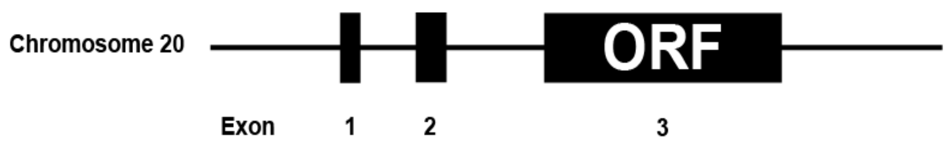

Figure 5: Schematic diagram of human PrP gene. *PRNP* gene located on chromosome number 20 and it has three exons with the entire ORF lying in the 3rd exon.

Biogenesis and structure of PrP

PrP biogenesis starts with the translocation of the nascent PrP amino acid chain into the lumen of the endoplasmic reticulum (ER). In the ER lumen, a series of posttranslational modifications takes place such as cleaving of the N-terminal signal sequence (aa 1-23), addition of glycans (aa 181 and 197), addition of GPI anchor to the C-terminal end after cleaving the GPI signaling sequence (aa 231-253) and formation of disulfide bond between aa 170 and 214. During trafficking through the secretory pathway the core glycans are processed into complex structure. Finally, mature PrP is transported to the outer leaflet of the plasma membrane. At the cell surface, PrP is present in three forms; unglycosylated, monoglycosylated and diglycosylated (Prusiner, 1989; Weissmann, 1994).

Structural studies with recombinantly expressed PrP (rPrP) revealed a large flexible disordered N-terminal region, containing an octa-repeat region, and a structured C-terminal domain (aa 126-226). This autonomously folding domain contains three α-helical regions (aa 144-154, aa 175-193 and aa 200-219) and a short two-stranded β-sheet (aa 128-131 and aa 161-164) (Donne et al, 1997; Riek et al, 1996; Riek et al, 1997). The C-terminal domain is characterized by extensive co- and posttranslational modifications, including two N-linked glycans with complex structure, a disulfide bridge and a C-terminal GPI anchor (rev. in (Tatzelt & Winklhofer, 2004). Interestingly rPrP from different species including frog, turtle and chicken show identical structural features with mammalian PrP, indicating that the physiological function of PrP is evolutionally conserved (Calzolai et al, 2005)

PrP^{Sc} is different from PrP^{C} with respect to its biochemical properties such as resistance to proteolytic digestion, formation of fibrillar structures and

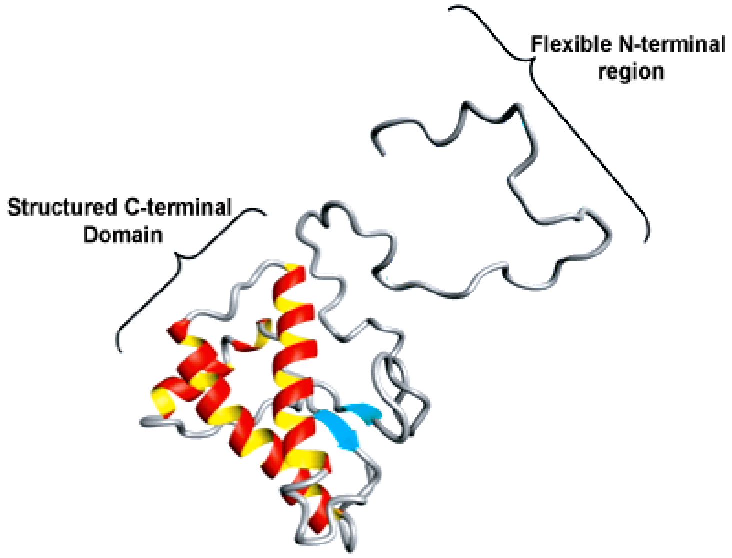

Figure 6: Three dimensional structure of recombinant Syrian hamster prion protein. Highly structured C-terminal domain showing with three α-helices (red and yellow) and two anti-parallel β-strands (blue) along with flexible N-terminal region (gray) (Adopted from Burns et al, 2003).

fluorescence birefringence in the presence of Congo red (Prusiner et al, 1983). Nuclear magnetic resonance (NMR) or X-ray crystallographic data on the structure of PrP^{Sc} are not available because of the insoluble character of PrP^{Sc}. Experiments with circular dichorism (CD) and infrared spectroscopes provided the first clue about the structure of PrP^{Sc} (Pan et al, 1993). PrP^{C} is mainly composed of α-helices (42%) with some β-sheets (3%), whereas PrP^{Sc} contains less α-helical (30%) domains and high β-sheet (43%) structure (Sakaguchi, 2007). A study using negative stain electron microscopy with 2D crystalline like arrays prepared from purified scrapie material suggested that PrP^{Sc} may be composed of parallel β-helix rather than β-sheet since both β-sheet and β-helix cannot be differentiated by spectroscopic methods (Wille et al, 2002). Structural data from brain derived PrP^{Sc} by mass spectrometry analysis of hydrogen-deuterium exchange also suggesting the presence of β-helix at the C-terminal region PrP^{Sc} (Smirnovas et al, 2011).

Function of prion protein

As mentioned above, PrP^C is expressed in all tetrapods and birds and the structural properties are highly conserved. Therefore, it is plausible to assume that the physiological function of PrP^C is also conserved. During the last three decades, a number of studies have been carried out to uncover the functional properties of PrP^C. However, the function of PrP^C is still enigmatic. Approaches to unmask the cellular function of PrP^C by several groups are discussed in detail below.

Studies from PrP knockout mice

Mice with a targeted disruption in the PrP gene (*Prnp*) do not show any distinct phenotype (Bueler et al, 1992). However, PrP^C expression is indispensable for the replication of scrapie prions; mice devoid of PrP^C are resistant to prion diseases and do not propagate infectious prions (Bueler et al, 1993; Bueler et al, 1992; Rambold et al, 2008a). Subsequent studies on PrP knockout mice exhibited slight phenotypic alterations, such as changes in circadian cycle rhythms (Tobler et al, 1996), olfaction (Le Pichon et al, 2009), abnormalities in neuronal excitability (Collinge et al, 1994), altered neurite outgrowth (Santuccione et al, 2005) and deficiency in proliferation of hematopoietic stem cells and neural precursor cells (Steele et al, 2006; Zhang et al, 2006). Upon using a stroke model, it became more clear that PrP knockout mice are highly sensitive to ischemic insult, hypoxia and seizures (McLennan et al, 2004; Mitteregger et al, 2007; Shyu et al, 2005; Spudich et al, 2005; Weise et al, 2006). Based on the above results, many functional roles have been attributed to PrP^C such as changes in synaptic transmission and neuronal excitability, protection against oxidative stress and a role in cell proliferation, differentiation and adhesion (rev. in (Linden et al, 2008; Nicolas et al, 2009).

Since PrP null mice do not show any significant phenotype, PrP^C does not seem to be an essential protein, at least under optimal conditions in a laboratory. However, alternatively compensatory mechanisms might have been activated in $PrP^{o/o}$ mice that overcame the loss of PrP^C. To identify if prion knockout mice develop any compensating mechanism during embryogenesis to balance the lack of PrP^C, Mallucci et al, generated a PrP conditional knockout mice, in which PrP expression is turned off during postnatal stage. However, the conditional knockout mice did not have any phenotype either (Mallucci et al, 2002). Thus, although $PrP^{0/0}$ mice have been available for more than 2 decades the definite physiological function of PrP^C is still enigmatic.

Stress-protective functions of PrP^C

The first line of evidence indicating a stress-protective function of PrP^C arose from experiments with hippocampal neurons isolated from the PrP knockout mice (Amitsuka et al, 1999). Later, it was found that PrP knockout mice are sensitive to ischemic brain damage, kainate induced seizure and to oxidative stress (Rangel et al, 2007). PrP^C knockout mice subjected to an ischemic brain injury show larger infarct volume with an increased activity of caspase-3 and expression of PrP^C rescues the brain injury from the ischemic insults and improves the neurological performance (Mitteregger et al, 2007; Spudich et al, 2005). In addition, up regulation of PrP^C mRNA and high immune reactivity of PrP^C is observed during an ischemic condition in humans and rodents (McLennan et al, 2004). In a cell culture model, recently established in our group, expression of cellular PrP^C protects human neuroblastoma (SH-SY5Y) cells from stress-induced apoptosis (Rambold et al, 2008a). Based on these findings, it appears that PrP^C may be involved in stress-protective and cell survival signaling pathways (Resenberger et al, 2011).

PrP protection against PrPΔHD

In an experiment to analyze PrP^C regions responsible for the conversion of PrP^{Sc}, it emerged that the deletion of the internal hydrophobic domain (HD) (aa 112-128) resulted in the generation of a neurotoxic mutant PrP, denoted as PrP ΔHD or PrPΔCR (Baumann et al, 2007; Li et al, 2007a; Shmerling et al, 1998). Interestingly removal of 20 amino acids within the HD is enough to cause formation of neurotoxic molecule (Li et al, 2007a). Transgenic mice expressing PrPΔHD develop severe ataxia and neurodegeneration in the cerebellum and die 100 days after birth. Surprisingly, this neurodegenerative phenotype was completely abolished by the co-expression of a single copy of PrP^C (Baumann et al, 2007; Li et al, 2007a; Shmerling et al, 1998). PrPΔHD-induced apoptotic cell death and protective function of wtPrP upon co-expression have been demonstrated also in cultured cells (Rambold et al, 2008b). In a different cell culture model expression of PrPΔ105-125 was shown to induce cation-permeable channels or membrane pore dependent current which was inhibited by over expression of PrP or by the addition of glycosaminoglycan (Solomon et al, 2010). Biochemical properties of PrPΔHD mutants indicate that the neurotoxic function resulted from the alteration in the normal function of PrP (Ballif et al, 2007; Christensen & Harris, 2009).

Role of PrP in copper binding and oxidative stress

Several *in vitro* and *in vivo* studies indicate that the histidine residues located within the octa-repeat region of the N-terminus of PrP^C are associated with Cu^{2+} binding activity (Brown et al, 1997b; Stockel et al, 1998; Viles et al, 1999). Reduced levels of Cu^{2+} were observed in the subcellular and synaptosomal fractions prepared from brain of PrP knockout mice (Brown et al, 1997a). However, alterations in brain Cu^{2+} level in $PrP^{0/0}$ mice were challenged by Waggoner *et al.,* (Waggoner et al, 2000). Further studies showed that binding of Cu^{2+} to PrP induces the formation of a misfolded PrP conformer distinct from PrP^{Sc} and that it stimulates endocytic trafficking of PrP (Pauly & Harris, 1998; Perera & Hooper, 2001; Quaglio et al, 2001). Since PrP^{C} is largely localized in the presynaptic membrane, so PrP^{C} might have an influence on synaptic Cu^{2+} homeostasis. In conclusion, the findings summarized above could suggest that PrP^{C} might be involved in modulating the Cu^{2+} dependent intracellular signaling cascade directly or indirectly in the presynaptic cleft.

For many years, oxidative stress has been linked to neuronal cell death in neurodegenerative diseases. An increase in oxidative stress biomarkers was described in $PrP^{0/0}$ mice, indicating that PrP^{C} might be involved in the suppression of oxidative stress (Wong et al, 2001). Decreased super oxide dismutase-1 (SOD-1) activity was identified in neuronal cells from PrP null mice (Brown et al, 1997b). SOD-1 requires cofactors such as Cu^{2+} and Zn^{2+} for its cellular function and hence the impaired Cu^{2+} levels in PrP null neurons could be responsible for the decreased SOD-1 activity that sensitize the cells to increased oxidative stress. PrP itself possess a SOD-like enzymatic activity that is abolished in mutants lacking the octa-repeat region, which is involved in Cu^{2+} binding (Brown et al, 1999).

Neurotoxic signaling through PrP^{C}

Prion propagation and neurotoxicity are the two central events in prion disease and the expression of PrP^{C} is essential for both. Brandner and colleagues were the first to show an important role of PrP^{C} as a mediator of PrP^{Sc}-induced neurotoxicity in prion disease. They grafted PrP^{C} over expressing neural tissue into the brain of $PrP^{0/0}$ mice. After the intracerebral inoculation with scrapie prions the grafted PrP^C expressing brain tissue propagated PrP^{Sc} and developed clinical characteristic features of prion disease, but the neighboring tissue devoid of PrP^C stayed healthy although PrP^{Sc} spread from graft to the host brain (Brandner et al, 1996). The role of PrP^C as a mediator of PrP^{Sc}-induced neurotoxicity was further supported by other transgenic mouse models, scrapie-infected mice expressing non-neuronal PrP^C did not develop clinical symptoms, although they accumulate PrP^{Sc} in addition with astrogliosis (Mallucci et al, 2003). Similarly, transgenic mice expressing anchorless PrP and infected with PrP^{Sc} do not develop clinical disease though they propagate infectious prions (Chesebro et al, 2005b).

Cell culture experiments from our group support the idea that the expression of PrP^C is required to transmit neurotoxic signals linked to PrP^{Sc}. Furthermore, the intrinsically disordered N-terminal domain and GPI anchor are required for this activity (Rambold et al, 2008b; Resenberger et al, 2011). The *in vivo* and *in vitro* studies described above support the scenario that PrP^{Sc} mediates its toxic effects through an interaction with PrP^C. This PrP^C/PrP^{Sc} complex could possibly modulate PrP^C dependant signaling pathways (Resenberger et al, 2011).

Putative co-receptors for PrP

PrP^C attachment via GPI moiety to the detergent resistant microdomains (DRMs) of plasma membrane would suggests that PrP^C might be involved in signal transduction, since DMRs are widely recognized as membrane signaling platforms (Allen et al, 2007; Haigh et al, 2009). Antibody mediated PrP cross-linking activates Fyn tyrosine kinase and as a consequence phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) in a caveolin-1 dependent manner (Mouillet-Richard et al, 2000; Schneider et al, 2003; Toni et al, 2006).

Establishment of synaptic like structure in cultured primary hippocampal neurons was observed after the addition of rPrP, however, this effect was blocked by protein kinase C and SRC kinase inhibitors (Kanaani et al, 2005). Binding of Cu^{2+} to PrP^{C} activates phosphatidylinositol 3-kinase (PI3K), thereby triggering the neuroprotective signals (Vassallo et al, 2005). A recent study shows that increased levels of phosphorylated mitogen-activated protein kinases (MAPKs) are involved in neuro protection against PrP^{Sc} induced toxicity (Uppington & Brown, 2008).

In order to transmit intracellular signals, PrP^{C} would require a co-receptor since it does not have any direct contact to the cytosol. Several biological molecules are proposed to interact with PrP and are discussed in detail below. So far, 37/67 kDa laminin receptor (Gauczynski et al, 2001; Rieger et al, 1997), an unknown 66 kDa membrane protein (Martins et al, 1997) and the stress-inducible transmembrane protein 1 (STI1) are proposed as interacting partners of PrP^{C} (Zanata et al, 2002). It has been shown that amino acid residues 230-245 from STI1 interact with the hydrophobic region (aa113-128) of PrP through which PrP transduces the neuroprotective signals (Zanata et al, 2002). A recent study reported that the recruitment of PrP^{C}-STI1complex at the cell surface induces the neuroprotection and neuritogenesis, with increased protein synthesis via PI3K-mTOR signaling and this neuroprotective translational stimulation is abolished in scrapie infected cells (Roffe et al, 2010).

Using yeast two-hybrid technology, Rieger and colleagues demonstrated that a 37 kDa laminin receptor precursor (LRP) interacts with PrP^{C}, thereby acting as a cellular receptor or co-receptor for PrP^{C} (Rieger et al, 1997). The 37 kDa LRP/67 kDa LR and PrP^{C} are co-localized at the cell surface of neuronal and non-neuronal cells (Gauczynski et al, 2001). PrP^{C} has two different sites for LRP/LR binding; the direct binding domain at C-terminal region (aa 144-179) and the indirect binding domain at N-terminal region (aa 53-93). Similar to that, LRP/LR aa regions 161-179 involved in direct and indirect interaction with PrP^C via amino acids 180-285.

Additional PrP interacting proteins

Proteins associated with intracellular vesicles or caveolae-like domains such as synapsin, growth factor receptor-bound protein 2 (Grb-2), prion interactor 1 (Pint1), p75, caveolin and casein kinase 2 (CK2) were also described to form complexes with PrP^C (Della-Bianca et al, 2001; Meggio et al, 2000; Mouillet-Richard et al, 2000; Spielhaupter & Schatzl, 2001). Interestingly, PrP^C associated with caveolin or CK2 induces intracellular signaling through Fyn kinase or phosphotransferase activity of CK2α respectively (Meggio et al, 2000; Mouillet-Richard et al, 2000). Abnormally folded PrP Q217R, a mutant associated with GSS, was shown to bind to Bip (heat shock 70 kDa protein 5(HSP70). As a consequence PrP Q217R was retained in the endoplasmic reticulum. Further, Bip-PrPQ217R enhanced the proteasomal degradation of abnormally folded mutant PrP, thus preventing the formation of protein aggregates, suggesting that Bip might play a significant quality control role in PrP biogenesis (Jin et al, 2000). GFAP and Bcl2 were also found to interact with PrP (Kurschner & Morgan, 1995; Oesch et al, 1990). Cytosolic prion protein co-aggregates with Bcl2 and thereby triggers apoptotic cell death. This toxicity is abolished by the co-expression of cytosolic heat shock proteins (Rambold et al, 2006).

The function of glutamate receptors was also proposed to be modulated by PrP^C, but evidences are inconsistent. Electrophysiological findings suggest that PrP^C binds to the NR2D subunit of the N-Methyl-D-aspartic acid receptor (NMDAR) complex and there by suppresses the NMDAR complex activity

(Khosravani et al, 2008). Recent study shows PrP^C-laminin γ1 complexes along with group I metabotropic glutamate receptors (mGluR1/5) and initiates signaling cascade for neurite outgrowth (Beraldo et al, 2011). More biochemical evidences are needed to establish the functional link between NMDAR and PrP^C. Apart from these, PrP^C is proposed to be a cellular receptor for amyloid-β (Aβ) oligomers. Binding of Aβ oligomers with PrP^C initiates synaptic dysfunction and altered long-term potentiation (LTP) in hippocampal neurons (Lauren et al, 2009). This was challenged by Kessels *et al.,* in a study, where Aβ oligomers induced synaptic dysfunction, loss of dendritic spines and altered LTP were irrespective of PrP^C expression (Kessels et al, 2010). At the same time, independent studies by different groups supported the hypothesis that PrP^C might be a receptor for Aβ oligomer (Barry et al, 2011; Caetano et al, 2011; Chung et al, 2010; Freir et al, 2011; Resenberger et al, 2011). Recently, our group identified that PrP^C not only can mediate toxic signals induced by Aβ oligomers, but also can transmit deadly signals by different β-sheet rich oligomeric conformers (Resenberger et al, 2011).

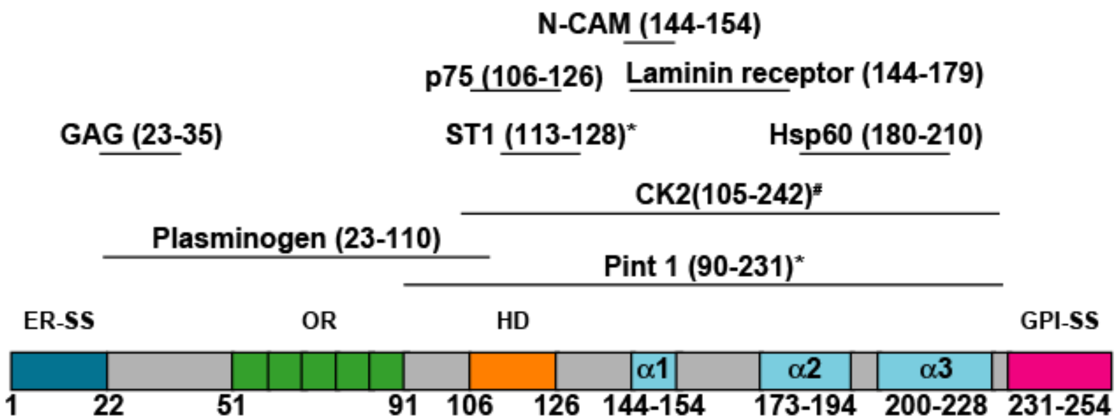

Figure 7: Schematic representations of the binding domains of PrP^C ligands on human PrP^C molecule. Human PrP^C molecule contains a ER-SS: endoplasmic reticulum signal sequence, OR: octa-repeat region, HD: hydrophobic domain, α: α-helical region, GPI-SS: glycosylphosphatidylinositol anchor signal sequence. Lines are indicating the binding site for each PrP^C binding molecule, which is also represented by amino acid numbers in parentheses (Adopted from Lee et al, 2003).

Different domains of PrP^C are engaged in binding with a variety of proteins favoring the macromolecular complex theory. Even though several proteins are considered to be associated with PrP^C at the cell surface, in cytosol, in endocytic compartments and in the secretory pathway, the role of PrP binding protein and the complete signaling cascades are still elusive. Identifying the cellular receptor for PrP^C and its function would have a beneficial role in designing therapeutic strategies in prion disease.

Table 3. Proposed cellular receptors for PrP and its subcellular binding sites.

PrP^C interacting molecules	**Subcellular binding sites**
Bcl-2	unknown (Kurschner and Morgan, 1995)
Hsp60	unknown (Edenhofer *et al.*, 1996)
Nrf2	unknown (Yehiely *et al.*, 1997)
Aplp1	cell surface (Yehiely *et al.*, 1997)
Caveolin-1	caveolae raft (Mouillet-Richard *et al.*, 2000)
Laminin	cell surface (Graner *et al.*, 2000)
CK2	caveolae raft (Meggio *et al.*, 2000)
N-CAM	caveolae-like domain (Schmitt-Ulms *et al.*, 2001)
Synapsin 1b	intracellular vesicles (Spielhaupter and Schätzl, 2001)
p75	caveolae raft (Della-Bianca *et al.*, 2001)
Grb2	intracellular vesicles (Spielhaupter and Schätzl, 2001)
Laminin receptor	cell surface (Gauczynski *et al.*, 2001b)
Pint 1	unknown (Spielhaupter and Schätzl, 2001)
STI 1	cell surface (Zanata *et al.*, 2002)
GAG	cell surface (Pan *et al.*, 2002)
NRAGE	cytosol (Bragason and Palsdottir, 2005)
NR2D(NMDAR)	cell surface (Khosravani et al, 2008)

Shadoo

Sprn gene and its polymorphisms

In search for homologs of PrP^C, a new gene termed *Sprn* (“shadow” of the prion protein) that is highly conserved from fish to mammals was identified. Expression of *Sprn* gene in all mammals suggests that Shadoo (Sho) might have an important physiological function. A *SPRN* pseudogene was described in humans and primates and may have arisen due to the segmental duplication (Harrison et al, 2010; Premzl et al, 2004). A human *SPRN* pseudogene has an overlap with the non-coding exon of SYCE1 gene that is involved in meiosis in mammals (Harrison et al, 2010). Chromosomal rearrangements in fish have produced multiple paralogs of *Sprn* gene and at least 2 *Sprn* gene copies are present in fish genome (Harrison et al, 2010; Premzl et al, 2004; Premzl et al, 2003; Strumbo et al, 2001; Strumbo et al, 2006).

Figure 8: Schematic diagram of human *SPRN* gene. *SPRN* gene has two exons and the entire ORF of human *SPRN* gene is located within the second exon. E1, exon 1; E2, exon 2; ORF, open reading frame.

A study in humans revealed allelic variations in *SPRN* gene. A common A to G change at the 11th position downstream from the start codon, amino acid change T7M within the N-terminal signal peptide and silent polymorphism at codon 61 were identified in human *SPRN* gene (Beck et al, 2008; Daude et al, 2009a; Daude et al, 2009b). Many polymorphisms have been identified in sheep. A common silent polymorphism at Y112Y and allelic variation at V71A were observed. Surprisingly, several allelic variations within the internal HD were also identified (Daude et al, 2009). The mouse genome was also analyzed, but genetic variations in mouse *Sprn* gene have not been identified as yet.

Table 4. Polymorphisms in *SPRN* gene (Adopted from Daude et al, 2009).

Position	-11	T7M	G61G
Genotype (%)	A/A 37.6	T/T 45.2	C/C 49.4
	G/G 15.1	C/T 39.8	C/T 43
	A/G 47.3	C/C 15.1	T/T 7.5
Predominant allele	A = 61.7%	T = 65.1%	C = 71.0%

Expression of *Sprn* gene

Sho expression in mice and sheep is restricted to the CNS especially to the hippocampus, cerebellum and to a lesser extent to the cerebral cortex, thalamus and medulla (Lampo et al, 2010; Watts et al, 2007). The immunohistochemistry and *in situ* hybridization analysis suggested that Sho might be primarily distributed at synapses (Lampo et al, 2010) and it may have an overlapping expression with PrP in certain regions of the brain (Watts et al, 2007). Prompted by these experimental data, expression patterns of *Sprn* mRNA and protein level were examined in prion infected mice. In one study it was shown Sho protein level was drastically reduced upon prion infection, whereas mRNA level was unaltered or a little elevated (Lloyd et al, 2009; Watts et al, 2007). Sho degradation in parallel with prion infection might be coupled with a proteostatic effect (Westaway et al, 2011) and during disease progression Sho might be degraded by cellular compartments such as proteaosome or lysosome. A different study illustrated that Sho reduction in prion diseased mice is not a general feature and might depend on the prion strain (Miyazawa & Manuelidis, 2010). Furthermore, transgenic mice over expressing Sho infected with scrapie prions developed the clinical disease similar to that of wild type mice. Thus, it is not likely that Sho expression has an effect on the pathogenesis of prion disease (Wang et al, 2011).

Structure of Sho

Sho is a neuronal glycoprotein, having positively charged N-terminal BR/RG repeats, a central HD and a glycosylation site at the C-terminal region and is attached to the plasma membrane through a GPI anchor. Notably, there is significant sequence homology of the HD of PrP and Sho. So far, no experimental evidence is available to state that the N-terminal region of Sho binds to copper. However, it contains a RGG box that is associated with RNA binding. CD spectroscopic analysis of recombinant mouse Sho (mSho) suggests that it might be completely unstructured (Watts et al, 2007).

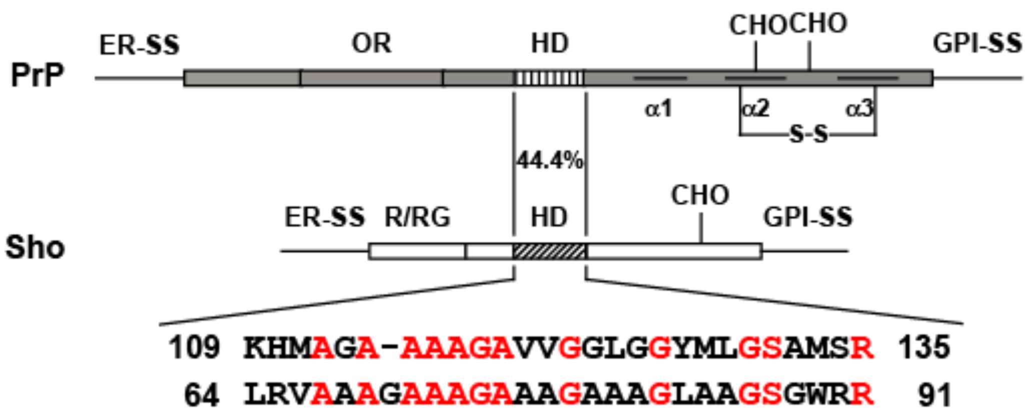

Figure 9: Schematic presentation of the similarities in hydrophibic domains between PrP and Sho. ER-SS: endoplasmic reticulum signal sequence, R/RG: arginine and glycine rich basic repeats, OR: octa-repeat region, HD: hydrophobic domain, α: α-helical region, CHO: N-linked glycosylation acceptor site, S-S: disulfide bridge, GPI-SS: glycosylphosphatidylinositol anchor signal sequence. Sequence alignment of the hydrophobic domains between PrP and Sho and conserved amino acids are marked in red.

Biological function of Sho

The conserved features such as unstructured N-terminal domain, internal HD and a C-terminal GPI anchor between PrP and Sho prompted the hypothesis that both proteins are functionally related (Premzl et al, 2003). Interestingly, Sho can protect cerebellar granule neuronal (CGN) cells from PrPΔHD induced neurotoxicity (Watts et al, 2007). Blocking the expression of Sho in a PrP null background leads to a lethal phenotype in mouse embryos suggesting that Sho might be required for early embryogenesis (Young et al, 2009). *SPRN* null allele and single nucleotide polymorphisms (SNPs) were identified in CJD patients in the UK, supporting a possible role of *SPRN* genetic variants in prion diseases (Beck et al, 2008). The conserved RGG boxes in Sho might be associated with binding of RNA molecules (Corley & Gready, 2008). RGG box containing proteins are shown to be involved in RNA processing and in some proteins RGG boxes mediate the interaction with its binding partners (Lukasiewicz et al, 2007). Similar to PrP, localization of GPI-anchored Sho in lipid rafts might indicate a role in neural cell signaling.

Aim of the study

As described above, PrP^C is ubiquitously expressed and structural similarities in PrP between different species suggest that its function might be evolutionarily conserved (Calzolai et al, 2005; Wopfner et al, 1999). Despite numerous studies, the physiological function of PrP is largely unknown. However, different studies in transgenic animals and cultured cells are now supporting the idea that PrP^C can protect neuronal cells against stress-induced cell death (rev in (Westergard et al, 2007). From one class of PrP mutants (PrPΔHD) it emerged that PrP^C can acquire a neurotoxic potential by deleting the internal HD (Baumann et al, 2007; Li et al, 2007b; Shmerling et al, 1998). Interestingly, expression of PrP^C can completely prevent the neurotoxic activity of PrPΔHD suggesting that PrP^C and PrPΔHD can induce neurotrophic or neurotoxic signaling via similar signaling pathway (Li et al, 2007b; Rambold et al, 2008b).

A genomic analysis indicated the presence of a PrP-related gene (*SPRN*) that encodes Sho (Premzl et al, 2003). Sho is expressed in the CNS. The sequence homology between Sho and PrP is found within the internal HD, however, certain features such as, a N-terminal repeat region and a C-terminal GPI anchor are also conserved and provoked the hypothesis that Sho and PrP are functionally related (Premzl et al, 2003). Moreover similarly to PrP, Sho can rescue neurons from PrPΔHD-induced neurotoxicity (Watts et al, 2007). From the above studies, it is reasonable to assume that PrP and Sho might transmit their neuroprotective signals by activating similar intracellular signaling cascade.

Hence, the aim of the present study was:

- To analyze the biogenesis of human Sho in SH-SY5Y cells, in particular; ER import, glycosylation patterns, maturation, dimerization and cellular localization.
- To provide insight into the stress-protective activity of Sho. In particular, we aimed to identify domains of Sho that are required for its stress-protective activity. To explore the stress-protective activity of Sho two different stress paradigms are employed in this study, which includes exposition of SH-SY5Y cells to the excitotoxin glutamate and the expression of neurotoxic PrP mutant PrPΔHD.
- To test for the possibility of a conserved function of the N-termini of Sho and PrP.
- To elucidate the role of Sho in PrP^{Sc}-induced apoptosis.

Results

Sho is highly conserved from fish to mammals and it was predicted to be glycosylated and anchored onto the plasma membrane via a GPI moiety (Premzl et al, 2003). Although Sho has no overall sequence homology with mammalian PrP, some characteristic features are conserved, such as the internal HD, N-linked glycosylation and a GPI anchor at the C-terminal. Similar to mammalian PrP, zebra fish Sho (zeSho) and mSho were found to be complex glycosylated and targeted to the plasma membrane via the GPI anchor (Miesbauer et al, 2006; Watts et al, 2007). As an initial step, this study aimed to analyze wild type human Sho and the impact of different domains on maturation, trafficking and stress-protective activity.

Generation of antibodies against human Sho

Sho antibodies are not commercially available. Production of antibodies against Sho will be useful to detect endogenous Sho level in cells and tissues and for further functional characterization of Sho in prion diseases. For the generation of the antibodies, the human Sho gene was cloned into the pET-19b vector using the restricting enzymes NdeI and XbaI. Further, Sho gene was transformed and expressed in *E.coli-BL-21* strain. Expression levels of recombinant Sho (rSho) in *E.coli* were high, but the protein was exclusively in the insoluble fraction. For immunization, inclusion bodies were purified and solubilized in guanidine hydrochloride (GndHCl). With this solution two rabbits were immunized (Eurogentec, Belgium). After 90 days, serum samples were collected from the immunized rabbits.

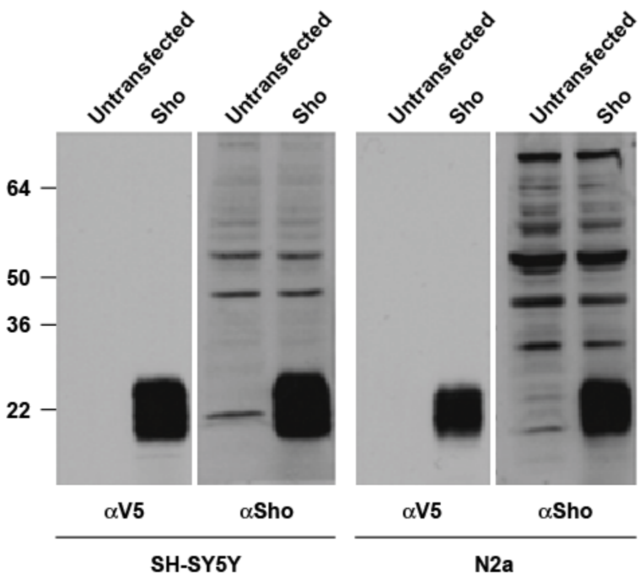

Figure 10: Specificity of anti-human Sho antibody. Human Sho containing a C-terminal V5 tag was transfected in mouse neuroblastoma (N2a) or SH-SY5Y cell lines and the expression was tested by Western blotting using an anti-V5 antibody or the newly generated anti-Sho antibody (αSho).

To examine the activity of anti-Sho antibodies, mouse neuroblastoma (N2a) or human neuroblastoma (SH-SY5Y) cell lines were transiently transfected with Sho containing a C-terminal V5 tag. After 24 h, cells were washed with cold PBS, scraped off the plate, pelleted and lysed in cold detergent buffer and the proteins were analyzed by Western blotting using anti-V5 antibodies or serum isolated from rabbits immunized with rSho. As shown in figure 10, the newly generated polyclonal antibodies against rSho specifically recognized the over expressed human Sho.

Biochemical characterization of mammalian Sho

Cloning of human Sho and mutants thereof

Similar to mammalian PrP, human Sho has an N-terminal ER-SS (aa 1-24) which mediates ER import. Further, a single glycosylation site is found in the C-terminal domain (N111) and a glycosylphosphatidylinositol signaling sequence

(GPI-SS) at the C-terminus (aa 127-151) (Premzl et al, 2003). zeSho biogenesis was previously analyzed in our group. It starts with the translocation of nascent Sho polypeptide into the ER lumen, where it undergoes a series of posttranslational modifications such as glycosylation and GPI anchor attachment at the C-terminal (Miesbauer et al, 2006). Thereafter, the protein is complex glycosylated and transported to the outer surface of the plasma membrane (Miesbauer et al, 2006).

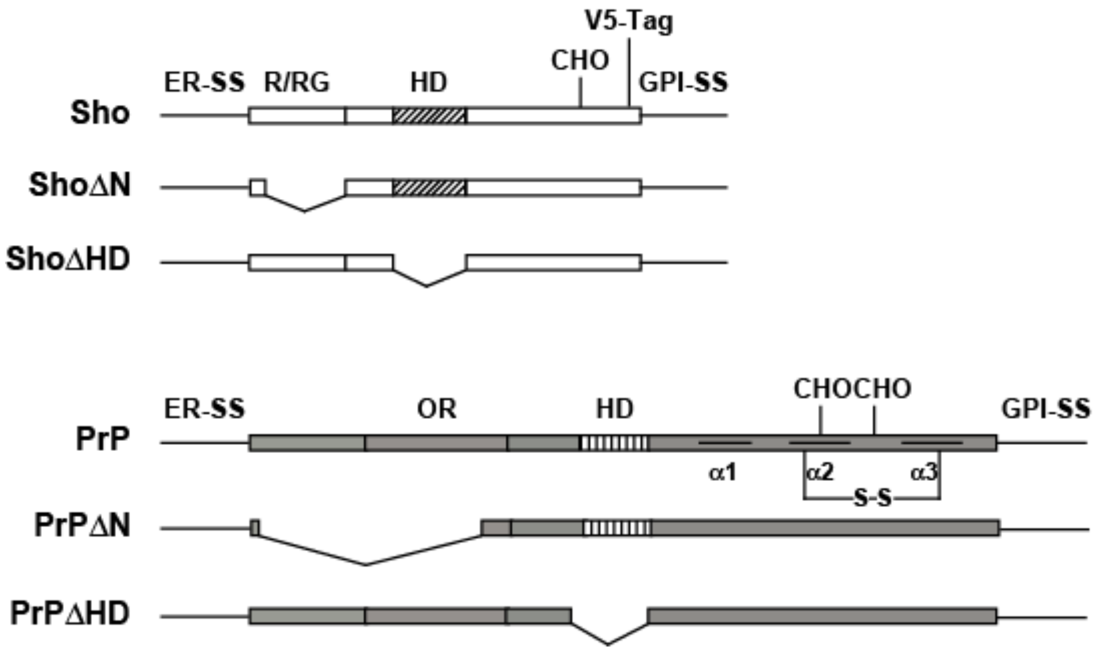

Figure 11: Schematic presentation of the PrP, Sho and their mutant constructs used in this study. ER-SS: endoplasmic reticulum signal sequence, R/RG: arginine and glycine rich basic repeats, OR: octa-repeat region, HD: hydrophobic domain, α: α-helical region, CHO: N-linked glycosylation acceptor site, S-S: disulfide bridge, GPI-SS: glycosylphosphatidylinositol anchor signaling sequence and V5-tag: GKPIPNPLLGLDST.

For biochemical and functional analysis of mammalian Sho, the human homologue was synthesized using ligation-based chemical gene synthesis using Sloning building block technology (Sloning, Puchheim) (Van den Brulle et al, 2008) and cloned into a mammalian expression vector pcDNA 3.1/Zeo(+). A V5 tag (5′ GGT AAA CCG ATA CCG AAC CCG CTC CTC GGT CTC GAT TCG

ACG 3′) or HA tag (5′ TAC CCA TAC GAT GTT CCA GAT TAC GCT 3′) was introduced between amino acids 124 and 125. Sho was used as a template to generate the subsequent deletions and mutants by standard polymerase chain reaction (PCR) method: ShoΔN (aa 30-56 deleted) and ShoΔHD (aa 68-89 deleted). The design of the Sho mutants was based on PrP mutants that have been characterized previously.

Wild type Sho and Sho mutants are complex glycosylated

Protein glycosylation maintains the folding, physiological structural and cellular localization, thereby enhancing the protein-protein interaction, solubility and increases the resistance against proteolysis (Shental-Bechor & Levy, 2008; Winklhofer et al, 2003a; Zhou et al, 2005). PrP mutant devoid of unstructured N-terminal domain (PrPΔN) shows altered neuroprotective activity but still could endorse propagation of infectious prions (Fischer et al, 1996; Mitteregger et al, 2007; Rambold et al, 2008b). Removal of intrinsic HD (aa 113-133 deletion) from PrP showed a gain of neurotoxic function which can be repressed by the expression of a single copy of PrP^C (Baumann et al, 2007; Li et al, 2007a; Rambold et al, 2008b; Shmerling et al, 1998). Importantly, these PrP mutants are complex glycosylated and are targeted to the outer leaflet of the plasma membrane through their GPI anchor (Winklhofer et al, 2003b).

As previously mentioned, earlier experiments showed that zeSho expressed in mammalian cells is complex glycosylated and anchored via a GPI moiety to the plasma membrane (Miesbauer et al, 2006). To examine the co and post-translational modifications of human Sho and the respective mutants indicated in

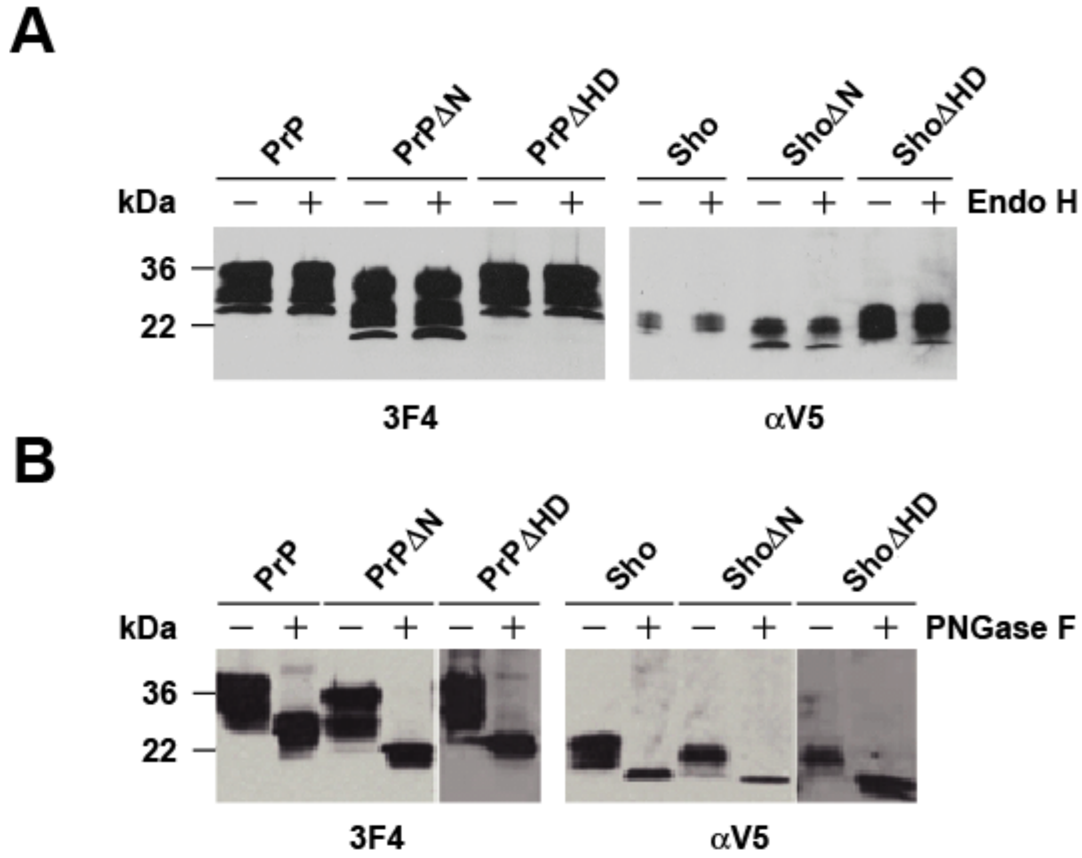

Figure 12: Deletion of the N-terminal or the hydrophobic domain does not interfere with biogenesis of Sho. Sho and its mutants are complex glycosylated. SH-SY5Y cells were transiently transfected with the constructs indicated in figure 10. Total cell lysates were treated with Endo H (A) or PNGase F (B) (+) or left untreated (-) and PrP or Sho proteins were detected by Western blotting.

figure 11 the constructs were expressed in SH-SY5Y cells. To monitor N-linked glycosylation, cell lysates were treated with Endoglycosidase H (Endo H), an enzyme that cleaves only high mannose structure or Peptide: *N*-glycosidase F (PNGase F), which can remove all N-linked glycans. An increase in the electrophoretic mobility of the proteins after PNGase F digestion (Figure 12B) indicated that all constructs are modified with N-linked glycans. Endo H treatment did not show any difference in the electrophoretic mobility of the proteins (Figure 12A) indicating that all the constructs are modified with N-linked glycans of complex structure.

Sho and the mutants are targeted to the outer leaflet of the plasma membrane via a GPI anchor

0.5% of the proteins located at eukaryotic cell membrane are GPI anchored (Eisenhaber et al, 2001). Experimental evidence for the cell surface localization of Sho was first shown for zeSho (Miesbauer et al, 2006) and later also for mSho (Watts et al, 2007). To analyze the cellular localization of the human homologues,

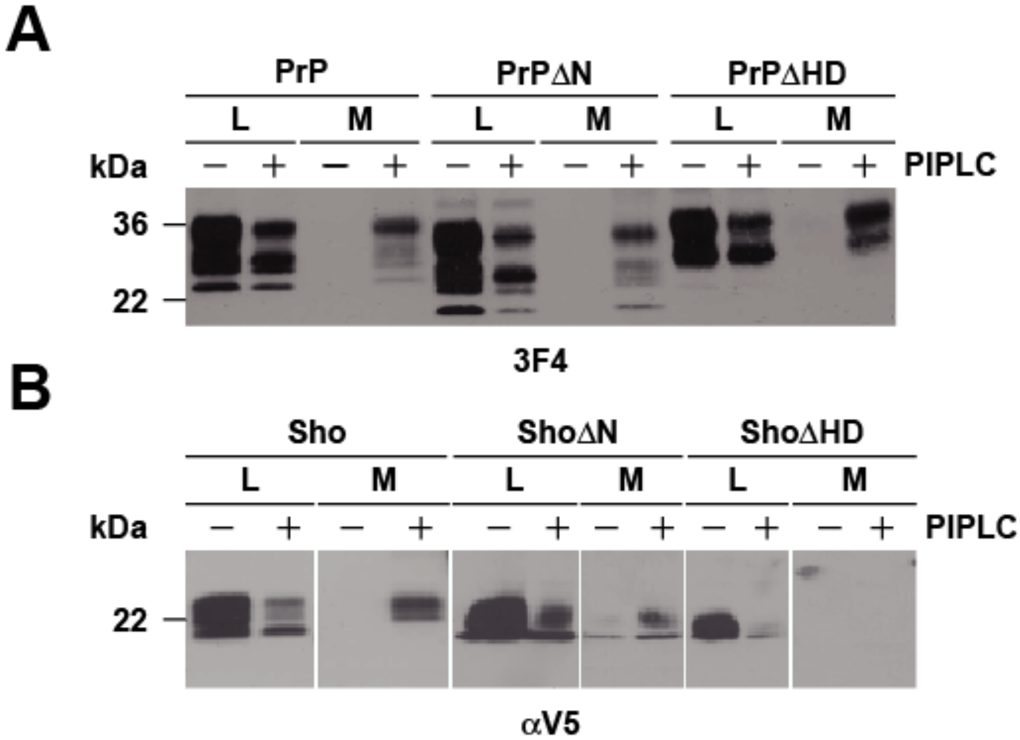

Figure 13: Sho and its mutants are tethered to the outer leaflet of the plasma membrane via a GPI anchor. Sho and the different mutants contain a C-terminal GPI anchor. Transiently transfected cells were incubated at 4°C for 3 h with PIPLC to release GPI-anchored proteins from the cell surface (PIPLC +) or mock-treated (PIPLC -). PrP (A) or Sho (B) present in the cell lysates (L) or the cell culture supernatant (M) were analyzed by Western blotting.

Sho and PrP constructs were expressed in SH-SY5Y cells and live cells were treated with PIPLC, an enzyme that liberates GPI-anchored proteins from the cell membrane. Indeed, PrP and PrP mutants were found in the cell culture medium (M) concomitantly with the disappearance of the respective proteins in the cell lysates (L) (Figure 13A). Similarly, levels of Sho and the different mutants were diminished in the cell lysates upon treatment with PIPLC. In parallel, Sho and ShoΔN levels were increased in the cell culture medium (M) of PIPLC treated cells; however, significant amounts of ShoΔHD were not detected in the supernatant of PIPLC treated cells, using neither anti-V5 antibody nor newly generated rabbit polyclonal anti-Sho antisera (αSho). However, the levels of ShoΔHD were significantly decreased in PIPLC treated cells lysates (L) (Figure 13B). So far, we have not been able to identify the molecular mechanism responsible for this peculiar phenomenon.

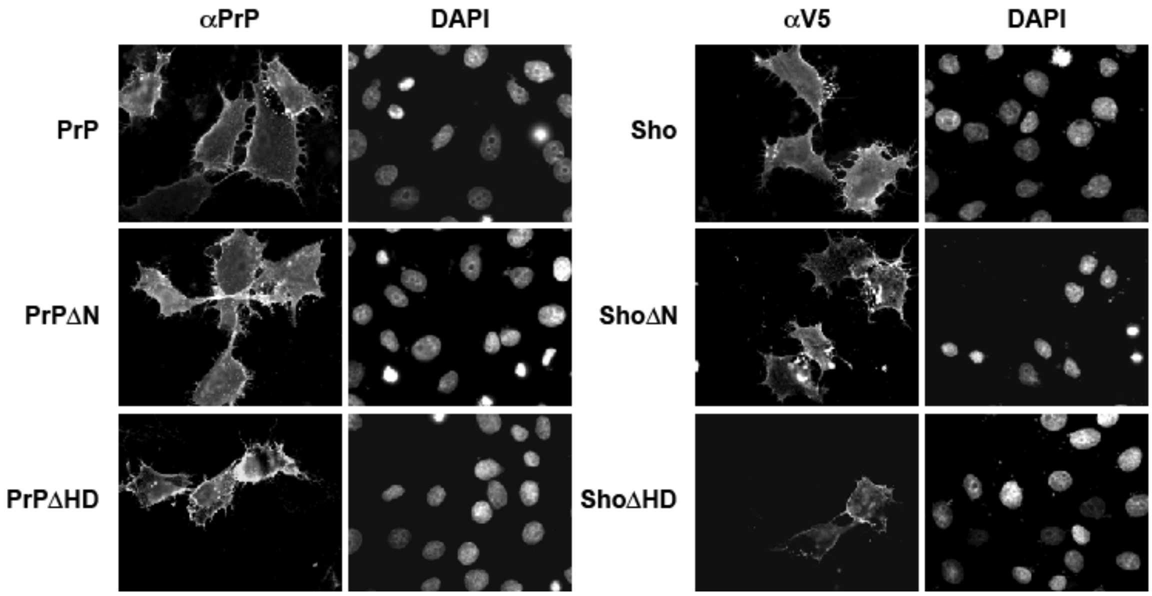

Figure 14: Sho and the different mutants are present at the outside of the plasma membrane. SH-SY5Y cells grown on cover slips were transiently transfected and localization of the constructs indicated was analyzed by indirect immunofluorescence of non-permeabilized cells. Nuclei were stained with DAPI.

To provide further evidence for the cellular localization of the Sho constructs, an indirect immunofluorescence analysis was performed. Transiently transfected SH-SY5Y cells were fixed with 3.7 % paraformaldehyde treatment and anti-V5 or anti-PrP antibodies were incubated with the fixed cells. The antibodies bind only to the proteins expressed on the cell surface since they cannot penetrate the cell membrane. All constructs including ShoΔHD were detected on the outer surface of the plasma membrane (Figure 14).

Collectively, these findings revealed that full length Sho, as well as its mutants, lacking N-terminal or internal HD are complex glycosylated and attached to the outer leaflet of the cell membrane through a GPI anchor.

Sho attenuates glutamate induced excitotoxic stress

Excessive stimulation of neuronal cells by neurotransmitters such as glutamate can damage the neuronal cells through a pathological process called excitotoxicity. As previously mentioned, altered LTP and increased neuronal excitability have been observed in PrP knockout mice (Collinge et al, 1994; Curtis et al, 2003; Maglio et al, 2004; Mallucci et al, 2002). A recent study suggests that PrP knockout mice exhibit enhanced NMDAR dependent neuronal excitability (Khosravani et al, 2008). These results would indicate that PrP^C might be involved in attenuating the neuronal excitability by regulating the glutamate receptor's activity. This is the rationale behind the use of glutamate as a physiological stress agent to analyze the role of PrP and Sho in stress-induced toxicity.

SH-SY5Y cells transiently transfected with the constructs indicated in figure 11, were grown on cover slips. The cells were treated with 500 µM glutamate for 3 h, followed by paraformaldehyde fixation. Apoptotic cells were identified by indirect immunofluorescence assay using an antibody against activated caspase-3. In this context, it is important to note that SH-SY5Y cells are characterized by low levels of endogenous PrP^C (Figure 15, right panel, pcDNA, 3F4). Consistent with previous results, PrP^C was able to protect cells against excitotoxic cell death whereas the deletion of the intrinsically disordered

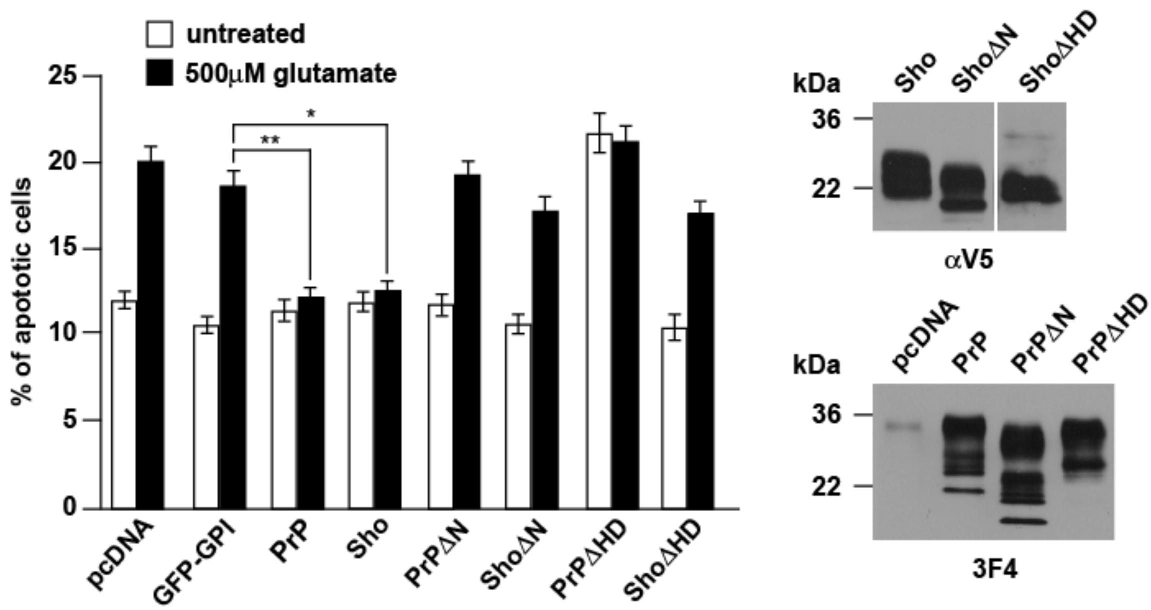

Figure 15: Sho protects against glutamate stress-induced apoptosis. SH-SY5Y cells expressing the constructs indicated were stressed with glutamate (500 µM) for 3 h at 37°C, fixed, permeabilized and activation of caspase-3 was analyzed by indirect immunofluorescence. To detect cells undergoing apoptosis, the number of activated caspase-3-positive cells out of at least 1100 transfected cells was determined in at least three independent experiments. Percentage of apoptotic cells among transfected cells is shown. Expression levels were analyzed by immunoblotting (right panel). *$P<0.05$, **$P<0.005$, ***<0.0005.

N-terminal domain (PrPΔN) leads to loss of protective activity (Figure 15) (Rambold et al, 2008b).

Similarly, expression of Sho had a stress-protective ability, which was abolished by the deletion of the N-terminal domain (Figure 15). These results indicate that the deletion of the N-terminal domain from Sho and PrP has similar outcome, i.e, the loss of a stress-protective activity. However, deletion of the hydrophobic region (HD) had different consequences. Earlier experiments from cell culture and transgenic mice expressing PrPΔHD showed that PrPΔHD obtained a toxic activity (Baumann et al, 2007; Li et al, 2007a; Rambold et al, 2008b; Shmerling et al, 1998). As illustrated in figure 15, PrPΔHD expression was toxic to SH-SY5Y cells and also it does not interfere with glutamate induced excitotoxicity. Surprisingly, ShoΔHD expression did not induce apoptotic cell death in SH-SY5Y cells, but it was also devoid of a stress-protective activity to interfere with glutamate-induced cell death.

Sho protect cells against PrPΔHD-induced toxicity

Transgenic mice expressing PrPΔ105-125 in a PrP null background exhibit neurodegenerative phenotype such as cerebellar atrophy, tremor, granule neuronal loss and astrogliosis. This phenotype is eliminated upon expression of full length PrP (Li et al, 2007a). Moreover, mSho has been shown to protect neurons from PrPΔHD induced neurotoxicity as well (Watts et al, 2007). Therefore, we have decided to use the expression of PrPΔHD as a second model for neurotoxic insult in order to identify Sho domains required for its activity to protect neurons against PrPΔHD induced toxicity. Sho and its mutants were transiently co-transfected with PrPΔHD in SH-SY5Y cells. As a control, mock transfection of pcDNA or expression of GFP-GPI constructs was used. After 24 h, transfected cells were fixed with 3.7% paraformaldehyde solution and stained with anti-active caspase-3 antibody in order to identify apoptotic cells. PrP mutants corresponding to Sho constructs were analyzed in parallel.

As illustrated earlier, PrPΔHD expression mediated neurotoxicity was suppressed by co-expression of full length PrP (Figure 16). Consistent with previous results employing cerebellar granule neurons (CGN) (Watts et al, 2007), expression of Sho was able to inhibit the toxicity induced by the expression of PrPΔHD; whereas control cells (pcDNA and GFP-GPI) do not protect the cells from PrPΔHD induced apoptosis (Figure 16). Notably, co-expression of PrPΔN or

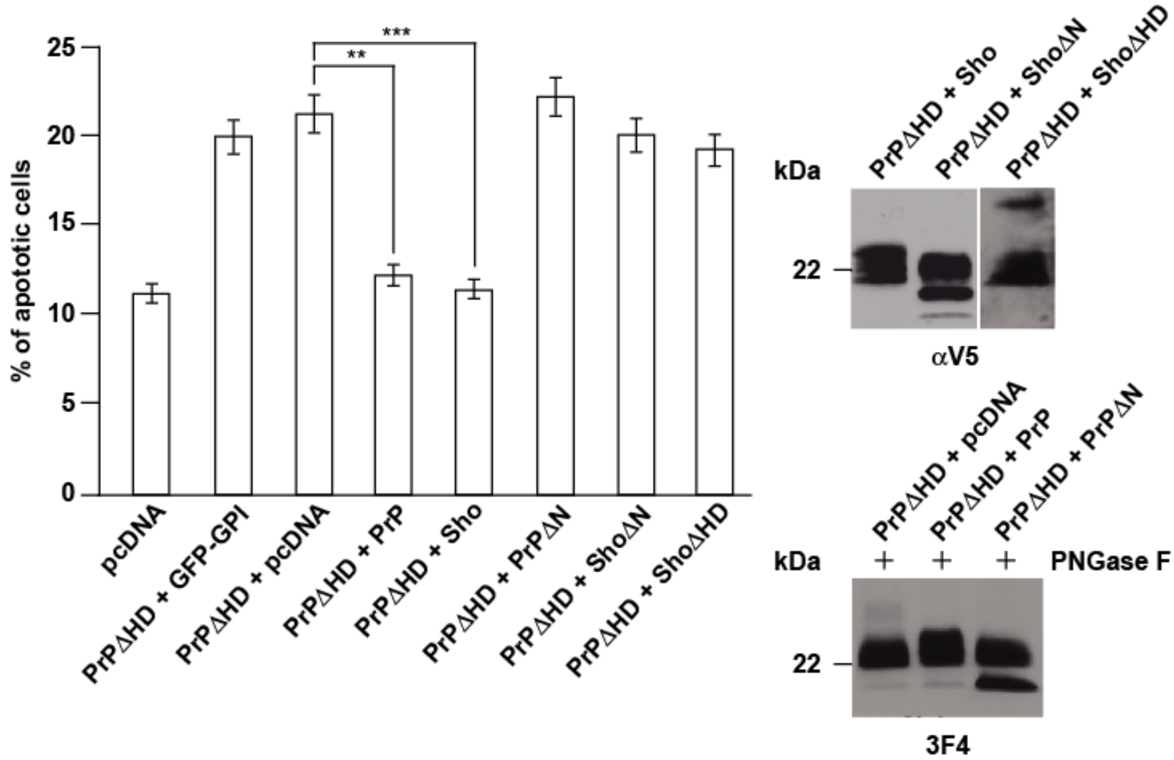

Figure 16: Expression of Sho interferes with toxic effects of PrPΔHD. SH-SY5Y cells were transiently co-transfected with PrPΔHD and the constructs are indicated in the figure 11. Apoptotic cell death was determined as described under figure 15. Expression levels were analyzed by immunoblotting (right panel). To specifically detect PrPΔN cell lysates were treated with PNGase F prior to the Western blot analysis (3F4). $^*P<0.05$, $^{**}P<0.005$, $^{***}<0.0005$.

Sho ΔN did not prevent apoptosis in cells expressing PrPΔHD. Similarly, ShoΔHD does not interfere with toxic effects of PrPΔHD expression (Figure16).

The hydrophobic domain mediates homodimerization of Sho

Oligomerization is frequently linked to the physiological activity of proteins and regulation of enzymes and receptors. Dimer formation of GPI-anchored proteins cannot only direct their recruitment to the lipid rafts but also promote their interaction with their receptors (Cunningham et al, 2003; Simons & Toomre, 2000). Previous studies have demonstrated that PrP form dimers at the cell surface and the HD mediate dimerization of PrP^C and are part of the dimer interface

(Priola et al, 1995a) (Meyer et al, 2000b; Rambold et al, 2008b). Internal HD of Sho shares a high sequence homology to the HD of PrP (44.4%; Figure 9). Thus, it is possible that HD of Sho might also exhibit similar dimer forming activity. To analyze the dimer forming ability of Sho, ShoS87C (serine at aa 87 replaced with cysteine) was cloned in pcDNA 3.1/ Zeo (+) vector using PCR method (Figure 17). The strategy behind this approach is that when two cysteine residues are close together, a stable disulfide bond is formed between them under the physiological condition. A similar method was successfully employed to analyze the domains involved in dimer forming ability of amyloid precursor protein (APP) (Munter et al, 2007)

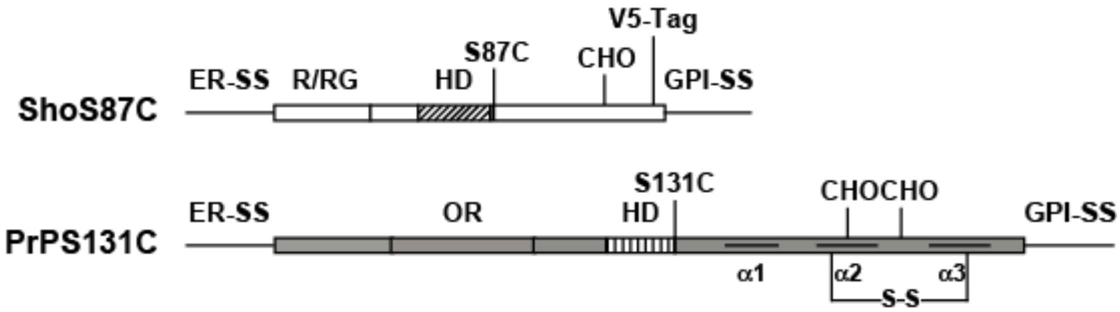

Figure 17: Schematic presentation of the Sho and PrP cysteine mutants used. For the generation of ShoS87C serine at aa 87 replaced with cysteine, whereas for PrPS131C generation serine at aa 131 replaced with cysteine.

To stabilize a potential Sho dimer, serine 87 was replaced by cysteine. If the internal HD is involved in Sho homodimerization, newly introduced cysteine molecule could form an intermolecular disulfide bond which is stable under non-reducing conditions. This dimer formation can be observed using Western blot by preparing the protein lysates with the sample buffer without reducing agents such as 2-mercaptoethanol (ß-ME*)* or *dithiothreitol* (DTT). This strategy was successfully used before to show dimerization of PrP^C (Rambold et al, 2008b). SH-SY5Y cells were transiently transfected with ShoS87C and in parallel PrPS131C was transfected into cells, and was used as control. Cell lysates were prepared in the presence of Laemmli sample buffer with or without ß-ME or DTT and proteins were analyzed by Western blotting. Corroborating the earlier results, PrP dimers were found in the cell lysates prepared from PrPS131C under non-reducing

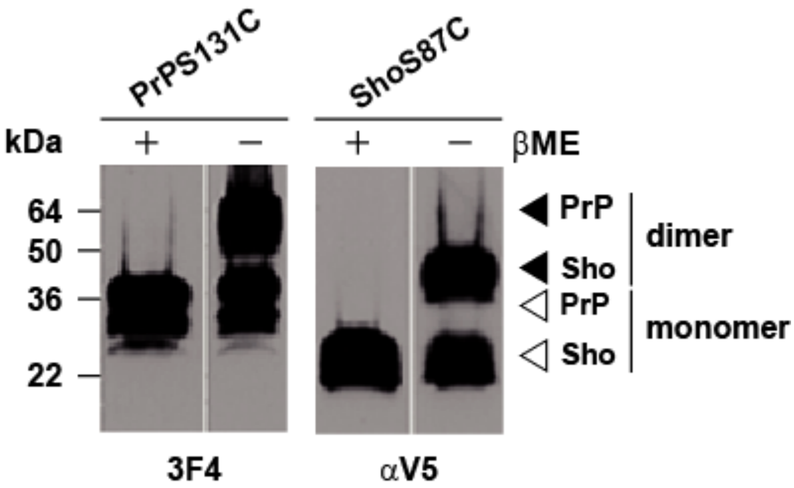

Figure 18: The hydrophobic domain is part of the dimer interface. SH-SY5Y cells were transiently transfected with PrPS131C or ShoS87C and cell lysates were analyzed under reducing (+ βME) or non-reducing (- βME) conditions. Proteins were detected by immunoblotting.

conditions (Figure 18). Similarly, higher molecular weight species appeared with the molecular mass similar to Sho dimer indicating the formation of Sho homodimer (Figure 18). In the presence of reducing agents the migration pattern of ShoS87C was identical to that of wild type Sho, indicating that the introduced cysteine residues induced the formation of intermolecular disulfide bond.

Furthermore, the possible involvement of the N-terminal domain on homo dimer formation of Sho was studied. To this end amino acid residues 30-56 were deleted from the ShoS87C (ShoΔN,S87C) mutant (Figure 19A). SH-SY5Y cells were transiently transfected with ShoΔN,S87C and characterized by Western blotting. As shown in figure 19B, ShoΔN,S87C formed dimers similarly to ShoS87C.

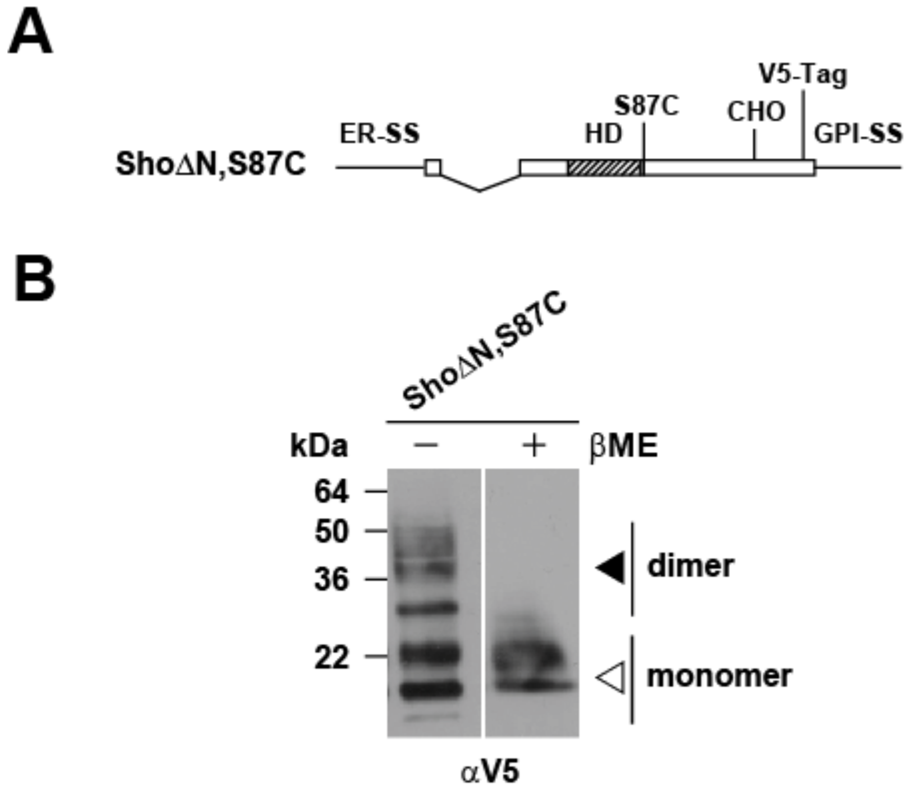

Figure 19: The N-terminal domain is dispensable for dimer formation. Cells transiently expressing ShoΔN,S87C were lysed and the protein sample was analyzed by Western blotting under either reducing (+βME) or non-reducing condition (-βME).

To analyze N-linked glycosylation, lysates from ShoS87C and PrPS131C expressing cells were incubated with Endo H, or PNGase F. The increased electrophoretic mobility of the proteins after PNGase F digestion (Figure 20A; lower panel) indicates that cysteine mutants of Sho and PrP were glycosylated. Endo H treatment (Figure 20A, upper panel) did not yield any difference in the electrophoretic mobility of the proteins revealed that the mutants were complex glycosylated.

To analyze the cellular localization, live SH-SY5Y cells transfected with Sho and PrP cysteine mutants were treated with PIPLC. Indeed, both of the mutants were found in the cell culture medium after PIPLC treatment (Figure 20B). This analysis revealed that biogenesis and post-translational modifications of ShoS87C and PrPS131C were similar to that of wild type PrP or wild type Sho:

ShoS87C and PrPS131C were complex glycosylated, tethered to the outer leaflet of the plasma membrane via a GPI anchor.

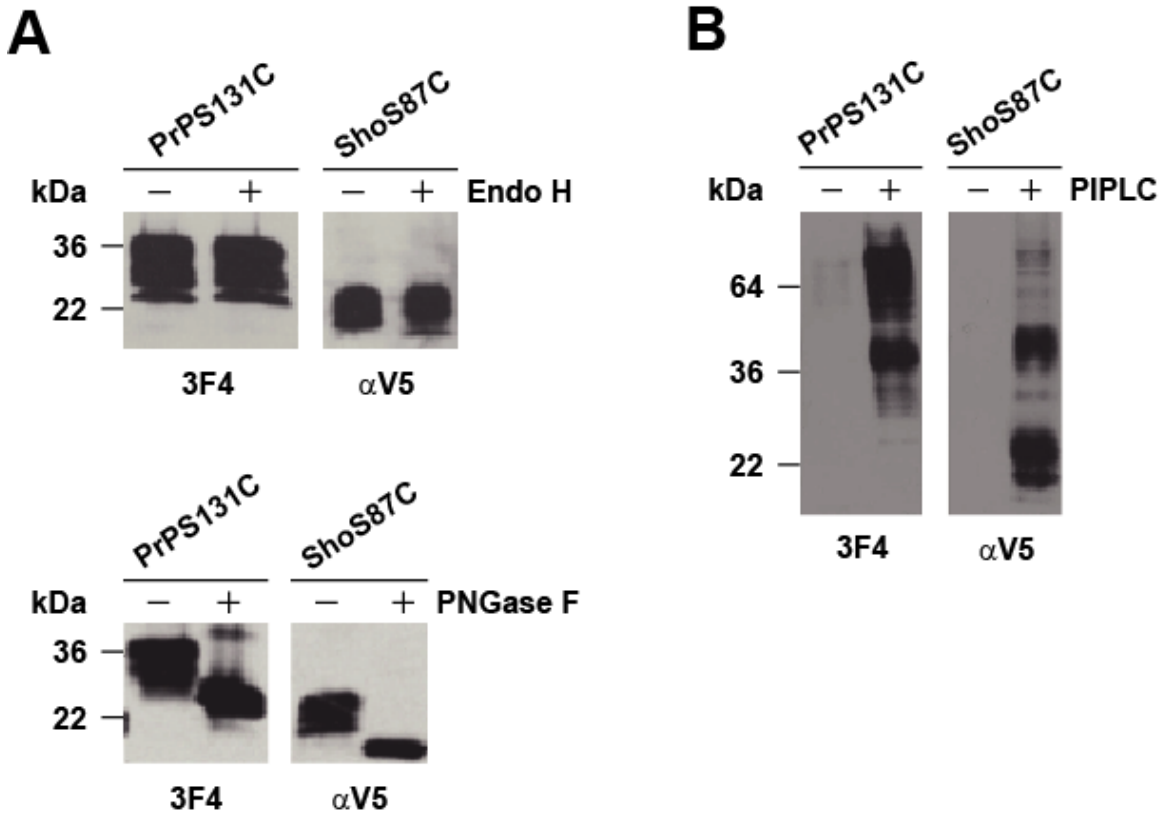

Figure 20: Cysteine mutants are complex glycosylated attached to the outer leaflet of the plasma membrane via a GPI anchor. (A) SH-SY5Y cells expressing the constructs indicated were lysed and lysates were treated with Endo H (+), or PNGase F (+), or left untreated (-) prior to a Western blot analysis under reducing conditions. (B) Transiently transfected cells were incubated at 4°C for 3 h with PIPLC to release GPI-anchored proteins from the cell surface (PIPLC +) or mock-treated (PIPLC -). PrP or Sho cysteine mutants present in the cell culture supernatant was analyzed by Western blotting under non-reducing conditions.

Sho and PrP homodimers are formed within the cell

Dimerization has been well described for many membrane and transmembrane proteins and can be linked to cell adhesion, migration, proliferation and various cellular signaling processes. Sometime dimerization occurs within the lumen of ER, for example, some G-protein coupled receptors (GPCRs) are known to dimerize within the lumen of ER and then transported to the plasma membrane

(Overton & Blumer, 2000; Overton & Blumer, 2002), while other GPCRs dimerize at the cell surface upon agonist stimulation (Tateyama et al, 2004). Various GPCRs are known to be glycosylated and the functional effects of glycosylation differ from one receptor to another receptor (Wheatley & Hawtin, 1999). β1-adrenergic receptor deglycosylation affects its dimerization and recruitment to the plasma membrane (He et al, 2002). Glycosylation was found to be indispensable for folding and trafficking of vasoactive intestinal peptide (VIP)-1 receptor, thyroid-stimulating hormone (TSH) receptor and follicle stimulating hormone (FSH) receptor (Couvineau et al, 1996; Davis et al, 1995; Russo et al, 1991).

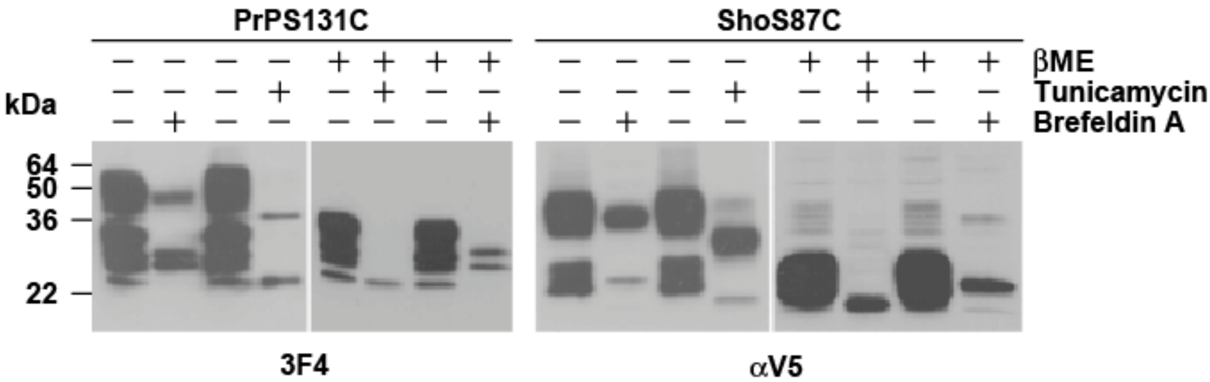

Figure 21: Sho and PrP homodimers are formed within the secretory pathway independent of N-linked glycosylation. Transiently transfected SH-SY5Y cells were grown overnight in the presence of tunicamycin or brefeldin A. Protein extracts were prepared and analyzed under reducing (+βME) or non-reducing condition (-βME) by immunoblotting.

We then went on to analyze the role of N-linked glycosylation in the homodimer formation of Sho and whether the homodimer formation occurs within the cell or at the cell surface. Transiently transfected SH-SY5Y cells expressing either PrPS131C or ShoS87C were cultivated overnight in the presence of tunicamycin or brefeldin A and dimer formation was analyzed by Western blotting as described in the figure 21. Tunicamycin efficiently inhibits the N-linked glycosylation proteins and brefeldin A effectively blocks protein transport from ER to golgi complex. Homodimers of Sho or PrP could be detected under both conditions (Figure 21) indicating that dimer formation apparently occurs in the secretory pathway and is independent of N-linked glycosylation.

No evidence for the *trans* dimers at the cell surface

Trans or cis dimers of proteins are often formed at the cell surface depending upon the specific cellular signaling pathways being activated. *Cis* dimer of glial-cell-line-derived neurotrophic factor receptor α1 (GFRα1) was considered to interact with glial cell-derived neurotrophic factor (GDNF) molecules (Bespalov & Saarma, 2007). This study addresses the possibility that dimer formation occurs in *trans* at the plasma membrane between Sho molecules located on adjacent cells.

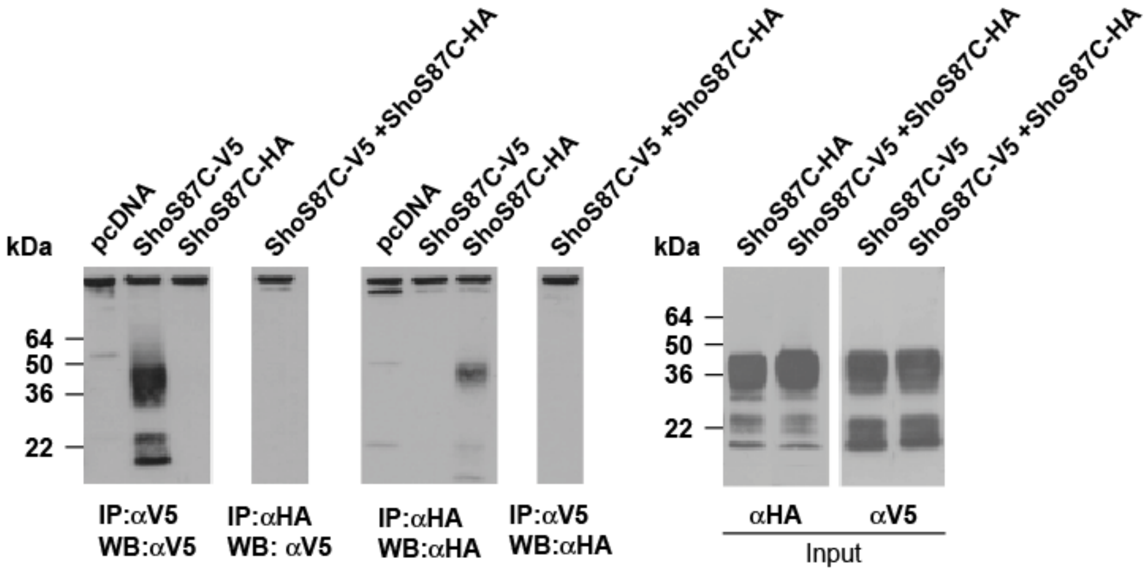

Figure 22: No evidence for the formation of Sho *trans*-dimers. Separately transfected cells expressing either ShoS87C-HA or ShoS87C-V5 were mixed and co-cultivated for 24 h. Cells were lysed and Sho was immunoprecipitated under non-reducing conditions with an anti-HA or anti-V5 antibody. The immunopellet was analyzed by Western blotting using the anti-HA or anti-V5 antibody. Western blot analysis of the input is shown in the right panel.

For this purpose, a ShoS87C construct with a C-terminal HA instead of the V5 tag tag was generated. Separately transfected SH-SY5Y cells expressing either ShoS87C-V5 or ShoS87C-HA were mixed and co-cultivated for additional 24 h.

The density was chosen to allow cell-cell contact. Cell lysates were prepared and incubated with anti-V5 or anti-HA antibodies overnight at 4^{o}C. The protein antibody complexes were precipitated using protein A/G agarose beads and Western blot was developed using anti-V5 or anti-HA antibodies respectively. As shown in figure 22 the co-immunoprecipitation analysis did not indicate the formation of *trans*-dimers. However, we cannot exclude the possibility that Sho *trans*-dimers can form *in vivo* under certain conditions.

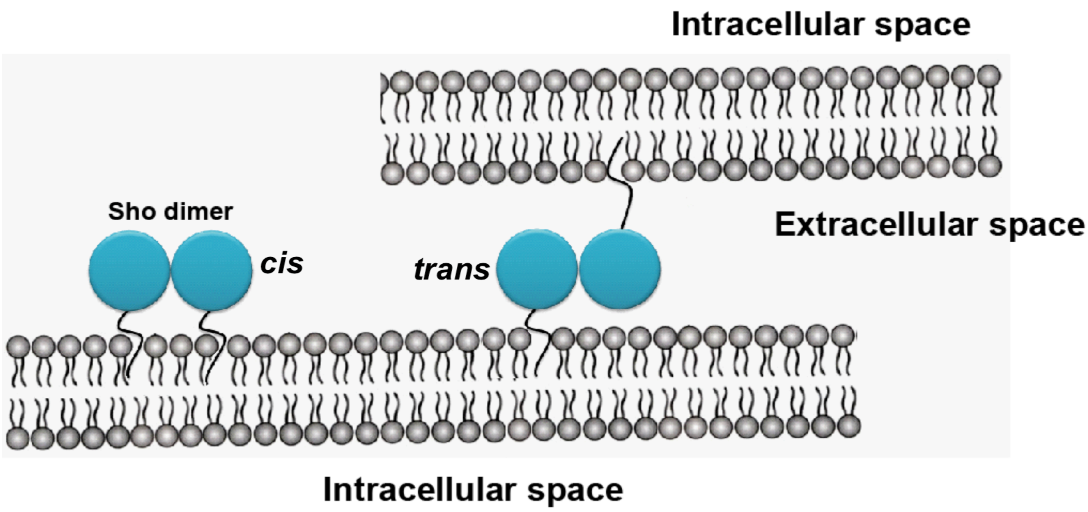

Figure 23: Possible forms of Sho dimers at the cell surface. Schematic diagram illustrates Sho homodimer in *cis* and *trans* forms.

Interaction between Sho and PrP

By various methods, such as co-immunoprecipitation, yeast two hybridization systems and cross-linking experiments, various studies have described multiple proteins that can interact with PrP at the cell surface or inside the cell. As previously mentioned, the HDs of PrP and Sho mediate their homodimerization. Thus, it is possible that PrP and Sho might also form PrP/Sho heterodimers via the HDs. Such an interaction of Sho with PrP was described in a study using yeast two hybridization system and the interaction was mediated through the internal HD (Jiayu et al, 2009).

This study could suggest that Sho might interact with PrP under physiological conditions via its HD. HD induced homodimerization of Sho and PrP was illustrated in figure 18. To analyze the possibility of a mixed PrP/Sho heterodimer, transiently transfected SH-SY5Y cells expressing both PrPS131C and ShoS87C were lysed in ice cold detergent buffer (0.5% Triton X-100, 0.5% sodium deoxycholate in PBS) and cell lysates were incubated with the anti-V5 antibody overnight at 4^{o}C. Sho immuno complex was precipitated with protein A/G agarose

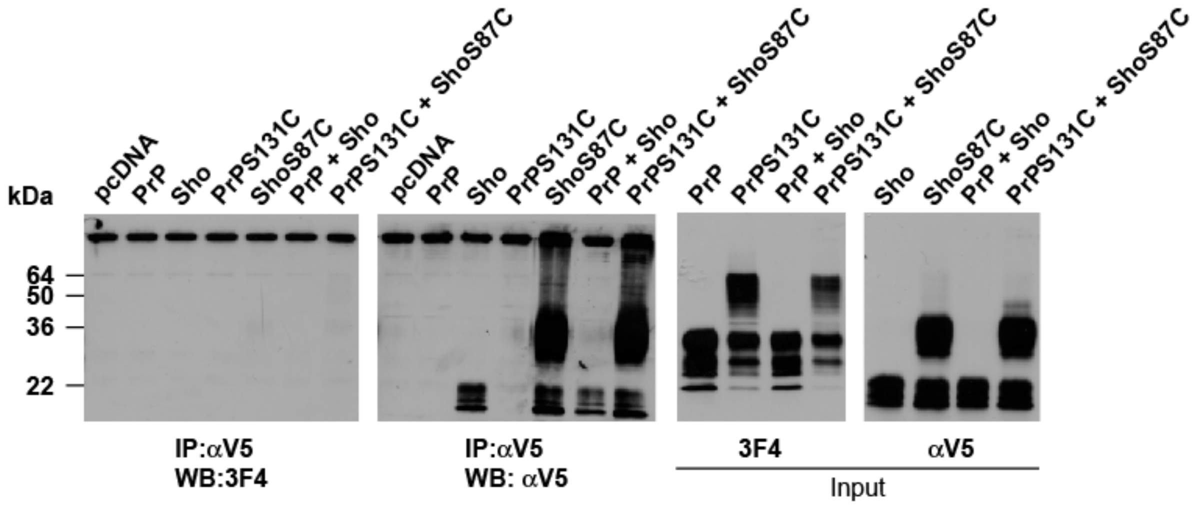

Figure 24: No evidence for the formation of a mixed PrP/Sho dimer. Transiently transfected cells co-expressing PrPS131C and ShoS87C-V5 were lysed and Sho was immunoprecipitated under non-reducing conditions using the anti-V5 antibody. The immunopellet was then analyzed by Western blotting using the anti-PrP antibody 3F4 or the anti-V5 antibody. Sho and PrP present in the lysates prior to the immunoprecipitation were analyzed by immunoblotting (right panel, input).

beads and the immunopellet was then analyzed by Western blotting using a anti-PrP antibody. Sho homodimers were efficiently detected by developing a control Western blot using anti-V5 antibody, however, using this method we were not able to show the formation of mixed PrP/Sho dimmers (Figure 24).

The N-terminal domain of Sho can restore stress-protective activity of PrPΔN

The functional characterization of Sho mutants presented above revealed a critical role of the N-terminal domain for the stress-protective activity of Sho.

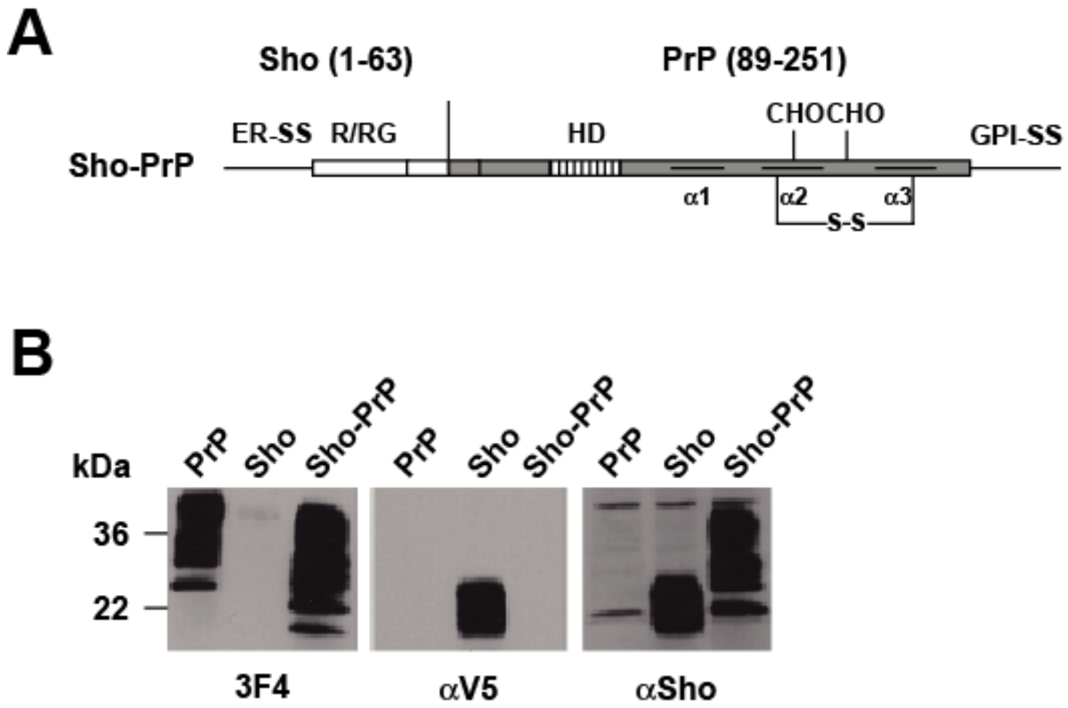

Figure 25: The chimeric protein N-Sho/PrP-C (Sho-PrP) was expressed in human neuroblastoma cells. (A) Schematic presentation of the chimeric protein Sho-PrP. (B) Sho-PrP is efficiently expressed in SH-SY5Y cells. Cell lysate from transiently transfected cells were analyzed by Western blotting using 3F4, anti-V5 and αSho antibodies.

Similarly, PrPΔN lacks a stress-protective activity in cell culture and animal models (Figure 15 and 16) (Mitteregger et al, 2007; Rambold et al, 2008b). To test for the intriguing possibility of a conserved function of the N-terminal domains of PrP^C and Sho, the N-terminal domain of Sho (aa 1-63) was fused to PrPΔN (aa 89-251) (Figure 25A). The fusion N-Sho/PrP-C (Sho-PrP) gene was inserted into a mammalian expression vector pcDNA3.1/Zeo (+) using the EcoRI and BamHI restriction enzymes for further biochemical and functional characterizations.

N-Sho/PrP-C is complex glycosylated and GPI-anchored

To analyze N-linked glycosylation, lysates from Sho-PrP expressing cells were incubated with Endo H or PNGase F. The increased electrophoretic mobility of the proteins after PNGase F digestion (Figure 26B) indicates that Sho-PrP was complex glycosylated, since Endo H treatment (Figure 26A) did not yield any difference in the electrophoretic mobility of the proteins.

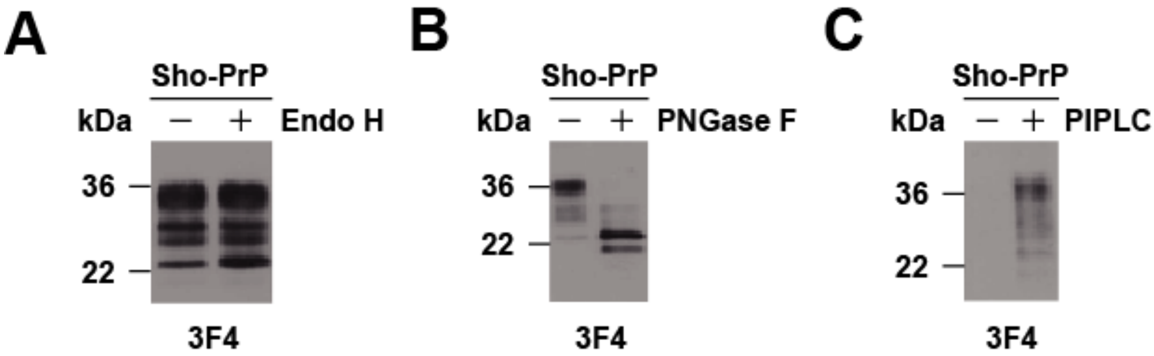

Figure 26: Post-translational modifications of N-Sho/PrP-C. Cell lysate from transiently transfected cells were subjected to the EndoH treatment (A) or incubated with PNGase (B) prior to the Western blot analysis. (C) Intact SH-SY5Y cells were treated with PIPLC for 3 h, the medium was collected and analyzed by Western blotting using the 3F4 antibody.

To analyze the cellular localization, live SH-SY5Y cells transfected with Sho-PrP gene were treated with PIPLC. Indeed, Sho-PrP was found in the cell culture medium after PIPLC treatment (Figure 26C). This analysis revealed that biogenesis and post-translational modifications of Sho-PrP were similar to that of wild type PrP or wild type Sho: the chimera was complex glycosylated and tethered to the outer leaflet of the plasma membrane via a GPI anchor.

Sho-PrP has a stress-protective activity

Next, the stress-protective activity of Sho-PrP was compared to that of PrPΔN. As described earlier, two different toxic conditions were used. 1.Exposure

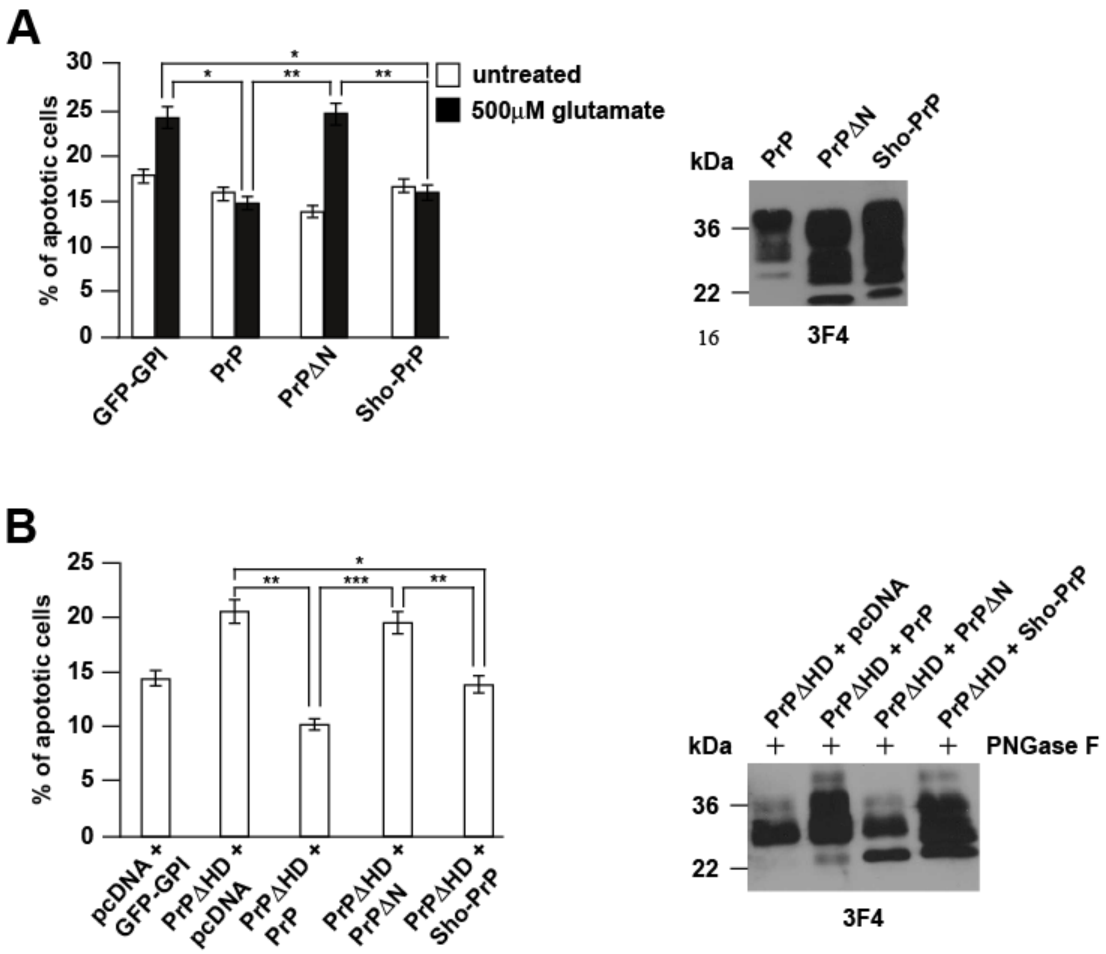

Figure 27: The N-terminal domain of Sho can functionally replace that of PrP. (A) Sho-PrP protects against stress-induced apoptosis. SH-SY5Y cells expressing the constructs indicated were stressed with glutamate (500 μM) for 3 h at 37°C. Apoptotic cell death was determined as described under figure 15. Protein expression levels were analyzed by immunoblotting (right panel). Expression of Sho-PrP interferes with toxic effects of PrPΔHD. SH-SY5Y cells were transiently transfected with PrPΔHD or PrPΔHD and the constructs indicated. Cells undergoing apoptosis were analyzed as described under figure 15. Percentage of apoptotic cells among transfected cells is shown. Expression levels were analyzed by immunoblotting (right panel). *P<0.05, **P<0.005, ***<0.0005.

to the excitotoxin glutamate 2. Expression of the neurotoxic PrP mutant PrPΔHD. SH-SY5Y cells were transfected with Sho-PrP and treated with 500 μM glutamate or co-transfected with PrPΔHD. The cells were then fixed with 3.7% formaldehyde solution and stained with anti-active caspase-3 antibody in order to identify apoptotic cells. In contrast to control transfected cells (GFP-GPI), Sho-PrP expressing cells were significantly protected against cell death induced by the exposure to glutamate (Figure 27A) or the expression of PrPΔHD (Figure 27B). In summary, these experiments revealed that the N-terminal domain of Sho can restore the stress-protective capacity of PrPΔN.

A possible role of Sho in PrP^{Sc}-induced toxicity

Studies in transgenic mice and cultured cells revealed that neuronal expression of GPI-anchored PrP is required to mediate prion-induced toxicity (Brandner et al, 1996; Chesebro et al, 2005a; Mallucci et al, 2002; Rambold et al, 2008b). Similar to PrP, Sho also exhibited protective activity against stress-induced apoptosis in cultured cells (Figure 15 and 16). These findings indicate that Sho and PrP^{C} could have overlapping signaling activities and that Sho might also to be able to mediate prion-mediated toxicity.

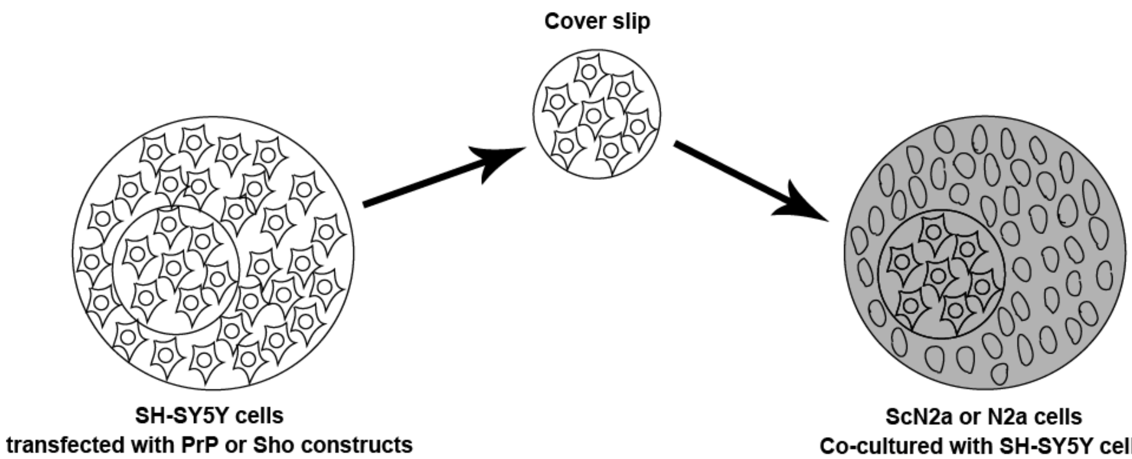

Figure 28: A schematic representation of co-cultivation assay. SH-SY5Y cells grown on cover slip were transiently transfected with PrP or Sho constructs. 3 h later the SH-SY5Y cells were washed and the cover slip was transformed in to a cell culture dish containing ScN2a or N2a cells and then co-cultivated for 16-18 h (Adopted from Rambold et al, 2008b).

A novel co-cultivation assay previously established in our group (Rambold et al, 2008b) was used to analyze the possible role of Sho in scrapie-induced

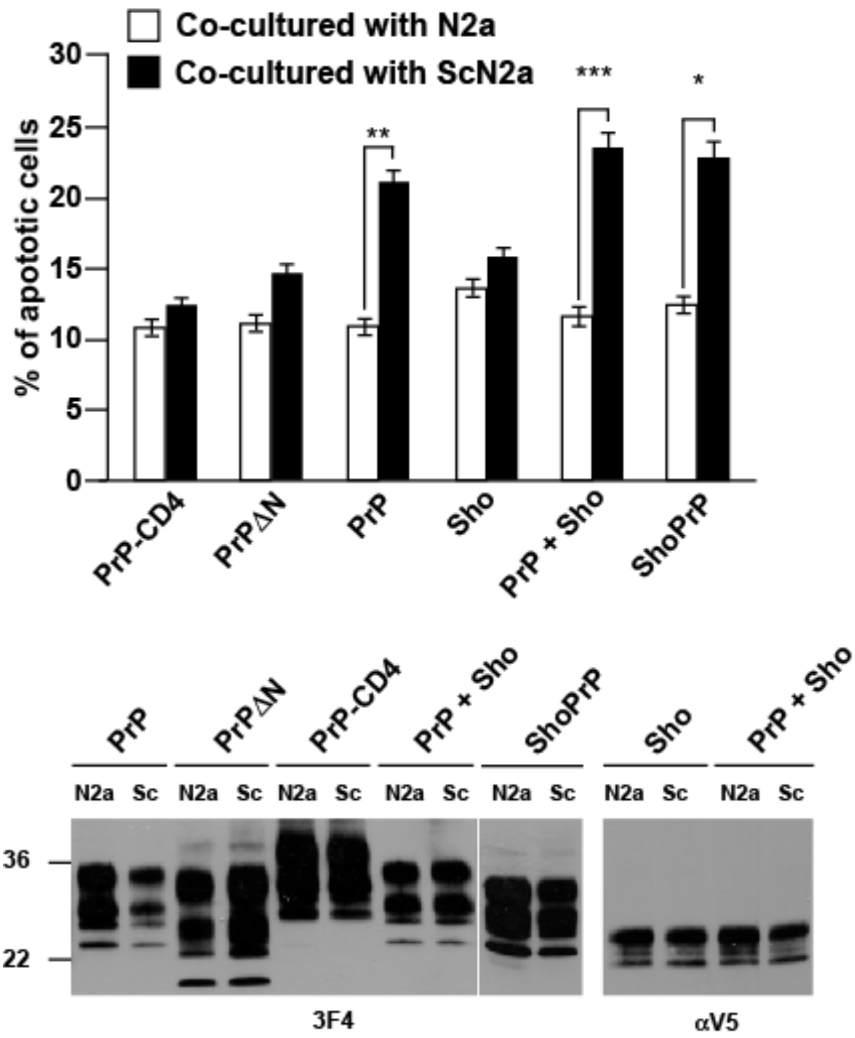

Figure 29: Sho does not protect against scrapie prions-induced apoptosis. SH-SY5Y cells were transiently transfected with the Sho only or PrP constructs as indicated and co-cultivated with N2a or ScN2a cells. To detect the apoptotic cells fixed cells were stained against anti-active caspase-3 antibody and the apoptotic cells were counted as explained in figure 15. Expression of transfected constructs in the SH-SY5Y cells co-cultivated with N2a or ScN2a cells was analysed by immunoblotting using 3F4 or anti-V5 antibodies (lower panel). $P<0.05$, $^{**}P<0.005$, $^{***}<0.0005$.

cytotoxicity. In this method, the uninfected cells grown on a coverslip were co-cultured along with N2a or ScN2a cells. No cell death was observed in SH-SY5Y cells expressing low level PrP^{C}, whereas cells overexpressing PrP undergo apoptosis in the presence of PrP^{Sc}. Interestingly overexpression of PrP-CD4 is not able to induce apoptosis. PrP-CD4 is a mutant PrP, a heterologous C-terminal transmembrane domain instead of a GPI anchor. This mutant is located at the plasma membrane but not in lipid rafts and has no stress-protective activity (Rambold et al, 2008b; Winklhofer et al, 2003c). Since ScN2a cells were reported to release PrP^{Sc} molecules consistently in the cell culture medium via exosomes (Fevrier et al, 2004; Vella et al, 2007), this approach could be a valid tool to identify the role of Sho in prion infection.

SH-SY5Y cells grown on cover slips were transiently transfected with PrP and Sho constructs. 4 h later, the transfected cells on cover slips were extensively washed with DMEM without FCS and placed in to cell culture dishes either with N2a or ScN2a. After 18 h the SH-SY5Y cells were fixed and stained with anti-active caspase-3 antibody and the apoptotic cells were analyzed as mentioned in figure 15. Corroborating previous finding (Rambold et al, 2008b), SH-SY5Y cells expressing PrP-CD4 did not undergo apoptosis when co-cultivated with N2a or ScN2a cells (Figure 29; PrP-CD4). However, significant increase in apoptotic cell death was observed, when SH-SY5Y cells expressing GPI-anchored PrP^{C} were co-cultivated with ScN2a (Figure 29; PrP). Expression of wild type Sho did not decrease viability of SH-SY5Y cells co-cultured with ScN2a cells (Figure 29; Sho).

Further-on, two more questions need to be addressed more elaborately. Firstly, can Sho-PrP also transmit a toxic signal similar to PrP^{C}? Expression of PrPΔN does not sensitize SH-SY5Y cells to PrP^{Sc}-induced cell death (Figure 29; PrPΔN). Similarly PrPΔN does not protect the cells against stress-induced cell death. But the fusion protein Sho-PrP restores stress-protective signaling. When Sho-PrP expressing SH-SY5Y cells were co-cultivated with ScN2a cells, indeed, Sho-PrP expression sensitized the SH-SY5Y cells to PrP^{Sc}-induced apoptosis indicating that the chimeric protein can efficiently transmit its toxic signals identical to PrP^{C} (Figure 29; Sho-PrP).

Secondly, does Sho protect cells against PrP^{Sc}-induced toxicity? Sho has been shown to be down regulated in prion infected mice brain (Watts et al, 2007). Moreover, the hypothesis from the cell culture experiment is that Sho protected neurons from death and the loss of Sho could be implicated in neuronal cell death in prion disease. We now have the best cell culture model (Co-cultivation assay) to test this hypothesis. However, co-expression of Sho with PrP does not interfere with PrP^{Sc}-induced apoptosis in SH-SY5Y cells (Figure 29; PrP + Sho). Interestingly, a recent study shows that transgenic overexpression of Sho does not prolong scrapie disease in mice (Wang et al, 2011).

PrPΔHD toxic signaling is blocked by NMDA receptor antagonist

The experiments in transgenic mice and cultured cells showed that the expression of PrPΔHD is neurotoxic. Similar to PrP^{C}, PrPΔHD is localized to the plasma membrane via GPI moiety and has no direct contact to cytosol. Therefore, PrPΔHD requires a transmembrane protein in order to transmit its neurotoxic signal. Moreover, wtPrP attenuates PrPΔHD-induced neurotoxicity, glutamate and NMDA mediated excitotoxicity. This gives us a clue that PrP and PrPΔHD might use the same receptor for their intracellular signaling. Previous results from our group indicated that memantine blocks PrP^{Sc}-induced toxicity (Resenberger et al, 2011). Memantine is an antagonist of glutamatergic NMDA receptors. Hence, we cautiously wanted to analyze whether memantine has similar effects on PrPΔHD-induced toxicity. PrPΔHD was expressed in SH-SY5Y cells with or without the

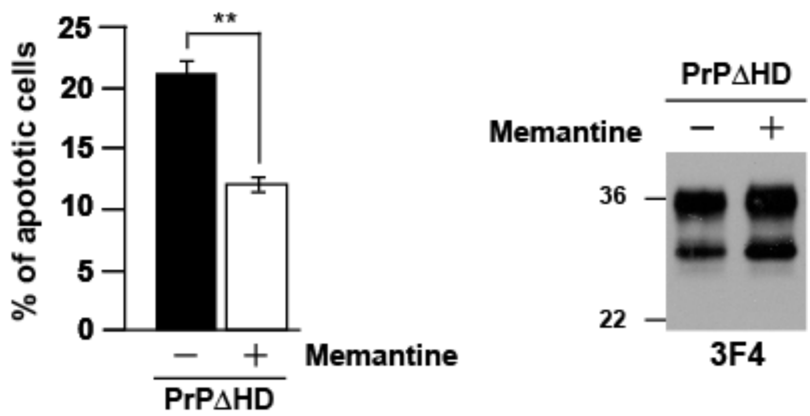

Figure 30: Memantine rescues SH-SY5Y cells from PrPΔHD induced apoptosis. SH-SY5Y cells were transiently transfected with PrPΔHD and cultured overnight in the presence of memantine. Apoptotic cells were identified by indirect immunofluorescence using the anti-active caspase-3 antibody as explained in figure 15. Protein expression was analyzed by immunoblotting using the 3F4 antibody (right panel). * $P<0.05$, ** $P<0.005$, *** <0.0005.

presence of memantine. Indeed, upon the treatment with memantine the apoptotic cell death in PrPΔHD expressing cells was significantly reduced (Figure 30).

Discussion

The prominent role of PrP in prion diseases is well characterized and so far no other molecule has been found to be connected to these diseases. Less than a decade ago, in a search for PrP-related proteins using comparative genomics, Premzl et al., identified a new PrP-related gene called Shadoo (Sho; Shadow of prion protein) (Premzl et al, 2003). Evolutionary origin of PrP and Sho are not known. However, a study suggests that the genes of PrP might have co-evolved from a common ancestral gene (Premzl et al, 2004). Sho is expressed mainly in CNS and is highly conserved from fish to man (Premzl et al, 2003). Although there is no overall sequence homology, PrP and Sho have a conserved internal HD and both are anchored on the plasma membrane via a GPI moiety. This study provides new insights into biogenesis of human Sho and its physiological activity, specifically its ability to protect cells against stress-induced apoptosis.

Biogenesis of human Sho

Sho is complex glycosylated and attached to the plasma membrane.

Previously our group analyzed the biogenesis of zeSho in mammalian cells and provided the first experimental evidence that Sho is complex glycosylated and targeted to the outer leaflet of the plasma membrane (Miesbauer et al, 2006), a finding corroborated for mSho later (Watts et al, 2007). In this study, the biogenesis of human Sho was analyzed in detail. PNGase F and Endo H, enzymes that remove the N-linked glycans from proteins were employed to study the glycosylation pattern of Sho. Corroborating previous findings for zeSho and mSho, human Sho expressed in SH-SY5Y cells is sensitive to PNGase F but resistant to Endo H, indicating that Sho is complex glycosylated. In addition, a possible role of N-terminal and HD in Sho biogenesis was also addressed. Similar to wtSho, mutants devoid of N-terminal and HD treated with PNGase F also show difference in their migration pattern in comparison with untreated protein samples indicating that these mutants are also modified with complex glycans.

As described above, zeSho and mSho are attached to the plasma membrane via GPI moiety. So the next question addressed was whether the human homologue is also anchored to the plasma membrane via a GPI moiety. To test this, Sho expressing SH-SY5Y cells were treated with PIPLC. As expected, Sho was found in the cell culture medium after the PIPLC treatment. Sho mutant devoid of the N-terminal domain was also released in to the cell culture medium after the PIPLC treatment, whereas, ShoΔHD was undetectable using either anti-V5 antibody or αSho. But reduced level of ShoΔHD was observed in the cell lysates treated with PIPLC. So the expression of ShoΔHD was further analyzed by non-permeable fluorescence microscopy technique. Indeed, similar to wtSho, expression of ShoΔHD at cell surface was confirmed by fluorescence microscopy. Collectively, these results confirm that Sho and its deletion mutants are modified with complex glycans, targeted to the plasma membrane through a GPI moiety. Moreover, deletion of the N-terminal or the HD does not have any detectable impact on Sho biogenesis.

Sho forms homodimers

Many GPI anchored proteins have been shown to form dimers. Dimer formation is not only linked to the physiological function but also to cellular trafficking and targeting to lipid rafts (Mayor & Riezman, 2004; Paladino et al, 2004; Simons & Toomre, 2000). For example, dimerization was demonstrated for CD59, a GPI anchored complement regulatory protein (Hatanaka et al, 1998), GFRα, urokinase-type plasminogen activator receptor (uPAR/CD87) and CD55 (Airaksinen & Saarma, 2002; Hatanaka et al, 1998). Formation of PrP homodimers have been reported previously and that the internal HD is required for dimerization and is a part of the dimer interface (Meyer et al, 2000a; Priola et al, 1995b; Rambold et al, 2008b). PrP homodimerization was experimentally shown by introducing a cysteine residue within the HD of PrP (PrPS131C) (Rambold et al, 2008b). A similar approach was previously used to analyze the dimerization of human epidermal growth factor receptor 2 (Erb-2/Her2) and the amyloid precursor protein (APP) (Cao et al, 1992; Munter et al, 2007). Since the HD of Sho is highly homologues to the HD of PrP, it is reasonable to assume that Sho might also be able to form homodimers via its HD.

To address this possibility, a modification was introduced into Sho: the serine residue at 87^{th} position was replaced by a cysteine residue (ShoS87C). If Sho forms a dimer under physiological conditions, the cysteine residues come closer and form a disulfide bond. Under non-reducing conditions the dimers can be detected by a shift in protein migration on SDS/PAGE. Western blot analysis from cultured mammalian cells expressing ShoS87C showed an additional slow migrating band in comparison to wtSho under non-reducing conditions. The slower migrating band disappears in the presence of reducing agents such as β-ME or DDT indicating that Sho forms homodimers and the homodimer is stabilized by an intermolecular disulfide bond via the introduced cysteine residues. PNGase F and PIPLC treatment of ShoS87C revealed that the biogenesis is not altered by the cysteine modification i.e. similar to wtSho, ShoS87C is also complex glycosylated and attached to the plasma membrane through a GPI moiety.

The next question we addressed was whether the N-terminal domain of Sho had an impact on homodimerization. To test this, ShoΔN,S87C (aa 30-56 deleted in ShoS87C) was expressed in cultured mammalian cells and the protein samples was analyzed by Western blotting under non-reducing conditions. Indeed, similar to ShoS87C, ShoΔN,S87C also forms dimers. These results indicate that the N-terminal domain is not required for Sho homodimerization.

Many membrane proteins form dimers within the cell and are transported to the outer membrane. For example presence of immature GPCR dimers within the ER lumen suggests that dimerization might be an integral part of GPCR maturation and an initial step in the production of functional GPCRs (Milligan, 2004). Sometimes substrate binding can also induce dimerization of the target proteins at the cell surface. For example brain derived neurotrophic factor (BDNF) binding to TrkB tyrosine kinase receptor induces receptor dimerization (Blum & Konnerth, 2005; Scharfman & McNamara, 2010).

After we obtained experimental evidence for Sho and PrP homodimerization, we further wanted to know if Sho and PrP homodimerization take place within the cell or at the plasma membrane. To test this, Sho and PrP were expressed in mammalian cells in the presence of brefeldin A, a lactone antibiotic that blocks the protein transport from ER to golgi. Western blot analysis revealed that the Sho and PrP homodimers could be detected after the treatment with brefeldin A. This result indicated that Sho and PrP homodimers are formed within the secretory pathway. Further, the effect of glycosylation on homodimerization was investigated. Mammalian cells expressing ShoS87C and PrPS131C were treated with tunicamycin and homodimer formation was analyzed by Western blotting. Indeed, Sho and PrP homodimers can be detected under this condition. Thus, glycosylation is not required for Sho or PrP homodimer formation.

Taken together, Sho and PrP homodimer formation occurs within the secretory pathway and does not require N-linked glycosylation. Since dimerization is a general basic characteristic feature of several membrane receptors, it could be possible that homodimerization of Sho and PrP might be linked with cellular processes such as maturation, cellular trafficking, binding to their receptors, regulation of intracellular signals and endocytosis.

Signal transduction can be initiated between two different cells through protein-protein interaction at the outer surface of the plasma membrane. For example, a homodimer of N-Cadherin, a cell adhesion molecule interacts with another N-Cadherin homodimer of a neighboring cell to form functional *cis-trans* homotetramer (Kim et al, 2005). *Cis* homodimers of junctional adhesion molecule 1 (JAM 1) can bind to other *cis* homodimer of JAM 1 of the adjacent cell and regulate the paracellular permeability and leukocyte transmigration (Kostrewa et al, 2001). Thus, the question arose whether Sho can form *trans* homodimers at the cell surface. To address this experimentally, two different tags (V5 and HA tags) were introduced into ShoS87C. Cells expressing Sho constructs with two different tags were mixed together and grown until the establishment of cell to cell contacts. Further, the cells were lysed and the possible formation of a *trans* homodimer was analyzed by co-immunoprecipitation analysis. Using co-immunoprecipitation analysis, Sho *cis* homodimer could be readily detected but not *trans* homodimers or *cis-trans* homotetramers. However, we cannot rule out the possibility that under certain circumstances Sho *trans* dimers might be formed *in vivo* in response to a specific stimulus.

No formation of PrP/Sho mixed dimer

As described earlier, Sho and PrP contain a highly homologues HD. Since, both Sho and PrP can form homodimers via their HD, it is tempting to speculate that Sho and PrP may form heterodimers and HD might be a part of the dimer interface. Using yeast two-hybrid system, it was demonstrated that Sho can interact with PrP, which is mediated by the HD (Jiayu et al, 2009). Further, collision induced dissociation (CID) spectral studies suggested that PrP can be co-purified with Sho or vice versa (Watts et al, 2009). To analyze a possible interaction, PrP along with Sho or PrPS131C with ShoS87C were co-transfected, protein samples were co-immunoprecipitated and analyzed by Western blotting. If Sho and PrP formed a mixed dimer under physiological conditions, introduced cysteine molecules might form a disulfide bridge and different migration patterns for Sho/PrP heterodimer might be seen on Western blot. However, using this approach we were not able to show a PrP/Sho interaction.

The stress-protective activity of Sho

Since Sho and PrP share certain structural features, it seems plausible that the two proteins have also similar physiological functions. Indeed in 2007, Joel Watts and colleagues described a PrP-like neuroprotective activity of Sho. Similar to wtPrP, Sho can protect CGN cells against PrPΔHD-induced toxicity (Watts et al, 2007). In our study we investigated whether this protective activity of Sho is limited to PrPΔHD-induced apoptosis or can also protect cells against other physiological stress agents. To test this, glutamate was employed as a model for excitotoxic stress. Glutamate is considered to be the main mediator of excitotoxicity in the CNS by changing the LTP. Moreover several studies suggested that PrP can ameliorate altered LTP and excitotoxicity induced neuronal cell death (Collinge et al, 2004; Khosravani et al, 2008; Rambold et al, 2008b; Whittington et al, 1995).

Mammalian cells expressing PrP or Sho were stressed with acute concentration of glutamate and apoptotic cell death was then measured by staining cells for active caspase-3. Indeed, similar to PrP, Sho can protect SH-SY5Y cells from glutamate stress-induced apoptosis. Further, to map the domains involved in this stress-protective activity of Sho, the deletion mutant clones coding for Sho protein without the N-terminal or HD were prepared. Stress-protective activity of Sho mutants was analyzed by apoptotic cell death as readout. Similar to PrPΔN mutant, N-terminally truncated version of Sho lost its stress-protective activity and lack of HD also makes Sho functionally inefficient. These results indicate that the stress-protective activity of Sho and PrP depend on similar domains i.e, N-terminal domain and internal HD. The impaired stress-protective activity of Sho mutants is not due to improper cellular trafficking, since both the mutants are complex glycosylated and attached to the plasma membrane via a GPI moiety.

With the above results, it is possible to assume that the N-terminal region and the HD are required for the stress-protective function of Sho and PrP. But it is still puzzling as to how the N-terminal and HD are involved in stress-protective signaling? It has been suggested that the intrinsically disordered domains of protein are implicated in protein-protein interaction (Tompa et al, 2009). The N-terminal domain of PrP is intrinsically disordered and by CD spectroscopic analysis of rSho it has been suggested that whole protein might be intrinsically disordered (Watts et al, 2007). Therefore, it could be reasonable to assume that the N-terminal domain of Sho and PrP may interact with their unidentified co-receptors involving intracellular signaling cascade.

Deleting HD in Sho does not lead to neurotoxic species

Experiments in transgenic mice revealed the unexpected finding that by deleting the intrinsic HD, PrP can gain a neurotoxic potential (Baumann et al, 2007; Li et al, 2007a; Shmerling et al, 1998). Interestingly, the neurotoxic potential of PrPΔHD is independent of replication of infectious prion (rev.in (Winklhofer et al, 2008). Co-expression of a single copy of wtPrP can completely abolish the neurotoxic phenotype although the cellular mechanism involved in PrPΔHD mediated toxicity is unknown (Shmerling et al, 1998). Similar to wtPrP, PrPΔHD is also complex glycosylated and targeted to the plasma membrane through a GPI anchor (Winklhofer et al, 2003c). An interesting finding is that similar to PrP^C, co-transfection of Sho can counteract PrPΔHD induced toxicity in CGN cells indicating that Sho has PrP-like activity in terms of neutralizing the neurotoxicity induced by PrPΔHD (Watts et al, 2007).

As previously mentioned, sequence similarity between Sho and PrP lies within the HDs. So the next question addressed was, does the removal of the HD from Sho generate neurotoxic species similar to that of PrPΔHD? To test this, ShoΔHD expressing mammalian cells were fixed and stained for active caspase-3 and the apoptosis level was measured. Using a cell culture assay developed in our lab, we could reproduce PrPΔHD-induced toxicity. Mammalian cells expressing PrPΔHD undergo apoptosis and this phenotype is rescued upon co-expression of wtPrP or wtSho. Interestingly, expression of ShoΔHD does not induce apoptosis in cultured mammalian cells indicating that removal of the intrinsic HD of Sho does not result in toxic species at least under the experimental conditions tested. At the same time, mammalian cells undergo apoptosis when ShoΔHD and PrPΔHD are co-expressed indicating that ShoΔHD does not interfere with PrPΔHD-induced apoptosis. Although Sho and PrP contain identical HD, acquiring a neurotoxic conformer might be exclusive for PrP.

The N-terminal domain of Sho can functionally replace that of PrP

Sho and PrP can protect cells against different toxic insults and their mutants devoid of N-terminal domain (ShoΔN and PrPΔN) are impaired in their stress-protective activity. The next question we addressed was to whether the fusion of the N-terminal domain of Sho with PrPΔN can restore its stress-protective activity. To analyze this, cultured mammalian cells expressing the chimeric protein Sho-PrP (N-terminus (aa 1-63) of Sho fused to PrPΔN (aa 89-251) were stressed with glutamate or co-expressed along with PrPΔHD and apoptotic cell death was measured using activated caspase-3 staining. Indeed, similar to wtPrP, Sho-PrP protects cultured cells against glutamate or PrPΔHD-induced apoptosis.

There is no experimental evidence that either copper or any other metal co-factors bind to the N-terminal domain of Sho. There is no sequence homology between the N-termini of Sho and PrP and the only similarity seems to be that both domains are intrinsically disordered. Based on the hypothesis, that the intrinsically disordered domains can be involved in protein-protein interaction, it is reasonable to assume that N-terminal domain of Sho and PrP bind with same co-receptor to mediate stress-protective signaling.

Sho does not protect cells from PrP^{Sc}-induced apoptosis

Formation of PrP^{Sc} in the CNS of infected individuals is the crucial event in prion diseases. Apart from PrP, there is no solid evidence of any other protein to play a curtail role in prion disease. After the discovery of Sho, it has been hypothesized that it may have an important role in prion disease. Interestingly, it was reported that Sho is downregulated in prion infected mice. However, Sho mRNA level seems to be unchanged (Lloyd et al, 2009; Watts et al, 2007). Identifying the *SPRN* null allele in two patients affected with vCJD also supports the involvement of Sho in prion diseases (Beck et al, 2008).

To address a possible effect of Sho on PrP^{Sc}-induced toxicity, a novel method called co-cultivation assay, which is developed in our laboratory, was used. PrP and Sho were expressed in cultured mammalian cells and these cells were then co-cultivated with ScN2a cells, which are constantly secreting PrP^{Sc} in the medium. Corroborating previous findings, PrP^{C} expressing cells underwent apoptosis in the presence of PrP^{Sc}, whereas in contrast, Sho expressing cells were not affected by the presence of PrP^{Sc}. Further-more, we checked whether co-expression of Sho with PrP can block PrP^{Sc}-induced apoptosis. To test this, Sho was co-expressed with PrP and these cells were grown in the presence of PrP^{Sc}. Co-expression of Sho with PrP failed to protect cells against PrP^{C} dependent PrP^{Sc}-induced apoptosis. A recent study in transgenic mice shows that Sho overexpression does not alter scrapie pathogenesis (Wang et al, 2011). Moreover, down regulation of Sho in mice infected with different prion strains has been studied by Kohtaro Miyazawa and Laura Manuelidis and Sho reduction seems to be prion strain specific. For example Sho level seems to be unaffected in mice infected with Asian CJD strain whereas Kuru infected mice show a significant reduction in Sho level (Miyazawa & Manuelidis, 2010).

In summary, our co-cultivation assay results suggest that Sho can neither protect cells from PrP^{Sc}-induced apoptosis nor can it mediate PrP^{Sc}-induced toxicity. Together with recently published studies, it appears that Sho cannot modulate prion pathogenesis.

Summary

Shadoo (Sho) is the only known protein that has some similarities with the cellular prion protein (PrP^C). Both proteins are evolutionarily highly conserved glycoproteins that are mainly expressed in the brain. Both the proteins share common characteristic features such as an unstructured N-terminal domain, a homologous internal hydrophobic domain (HD) and a C-terminal glycosylphosphatidylinositol (GPI) anchor. Preliminary data also showed that PrP and Sho have apparently a similar stress-protective activity. In the present study, the biogenesis and physiological function of the human homologue Sho was studied. Compared with PrP show here some similarities and differences.

Similarities

- Both the proteins are complex-glycosylated and selectively transported by a GPI anchor to the outer plasma membrane. Both dimerize within the secretory pathway. The dimerization is mediated here by the hydrophobic domain.
- Both have a stress-protective effect, for which the N-terminal domain is required.
- PrP and Sho probably activate similar cellular pathways that protect cells from stress-induced apoptosis.

Differences

- Unlike PrPΔHD, deletion of the hydrophobic domain of Sho leads to the formation of no toxic conformers.
- Sho does not mediate toxicity induced by PrP^{Sc}.

Taken together, this study reinforces the view that PrP and Sho could mediate their neuroprotective potential by common cellular co-receptors. The formation and propagation of toxic conformers is specific for PrP. Although Sho is unlikely to contribute to prion pathogenesis, studying the physiological function of Sho could be a useful tool to clarify the physiological function of PrP. The discovery of the physiological role of PrP is important to understand the pathological role of PrP in prion diseases. This knowledge can help to develop new therapeutic strategies against prion diseases.

Zusammenfassung

Shadoo (Sho) ist das bisher einzig bekannte Protein, das gewisse Ähnlichkeiten mit dem zellulären Prion-Protein (PrP^C) aufweist. Beide Proteine sind evolutionär hochkonservierte Glycoproteine, die hauptsächlich im Gehirn exprimiert werden. Chakterische Merkmale beider Proteine sind eine unstrukturierte N-terminale Domäne, eine homologe interne hydrophobe Domäne und ein C-terminaler Glycosylphosphatidylinositol (GPI)-Anker. Erste Daten zeigten darüber hinaus, dass Sho und PrP offenbar eine ähnliche stress-protektive Aktivität besitzen. Im Rahmen der vorliegenden Studie wurde die Biogenese und physiologische Funktion des menschlichen Sho-Homologs untersucht. Verglichen mit PrP zeigen sich hier einige Ähnlichkeiten und Unterschiede.

Ähnlichkeiten

- Beide Proteine werden complex-glykosyliert und gezielt durch einen GPI-Anker an die äußere Plasmamembran transportiert. Beide dimerisieren innerhalb des sekretorischen Signalweges. Die Dimerisierung wird dabei durch die hydrophobe Domäne vermittelt.
- Beide besitzen eine stress-protektive Wirkung, wofür die N-terminale Domäne benötigt wird.
- PrP und Sho aktivieren vermutlich ähnliche zelluläre Signalwege, um Zellen vor stress-induzierter Apoptose zu schützen.

Unterschiede

- Im Gegensatz zu PrPΔHD führt eine Deletion der hydrophoben Domäne von Sho nicht zur Bildung von toxischen Konformeren.
- Sho vermittelt keine PrP^{Sc}-induzierte Toxizität.

Zusammengenommen festigt die vorliegende Studie die Ansicht, dass PrP und Sho ihr neuroprotektives Potenzial durch gemeinsame zelluläre Co-Rezeptoren vermittlen könnten. Die Bildung und Propagierung toxischer Konformere ist jedoch spezifisch für PrP. Obwhol Sho somit vermutlich nicht zur Prion-Pathogenese beiträgt, könnte die Studie der physiologischen Funktion von Sho ein nützliches Werkzeug sein, um die physiologische Funktion von PrP zu klären. Die Aufdeckung der physiologischen Rolle von PrP ist bedeutend, um die pathologische Rolle von PrP in Prion-Erkrankungern zu verstehen. Dieses Wissen kann dazu beitragen neue therapeutische Strategien gegen Prion-Erkrankungen zu entwicklen.

Methods

Molecular biology methods

Cloning and site directed mutation by polymerase chain reaction (PCR)

PCR method was employed for the selective amplification of DNA fragments using thermostable DNA-polymerase and primers as listed below (see primer list) (Saiki et al, 1988). To clone various Sho mutants, the cDNA of wtSho in pcDNA 3.1/Zeo (+) was used. To delete the entire domain or to substitute the single amino acid of Sho, a two step PCR strategy was used; first DNA fragments with oligonucleotides (primers) containing desired mutations with overlapping sequence homology were amplified. Further, these amplified fragments are then used in a second reaction as template and the hybridizing sequence homologies are used as internal primers. By appropriate selection of the internal primers both mutations and insertions or deletions are inserted into a gene fragment, while the external primers each contain an interface for a restriction endonuclease and amplification of the product used.

Reaction mixer for PCR:

ddH_2O	38.5 µl
forward primer 10 µM	1 µl
reverse primer 10 µM	1 µl
plasmid (1 µg/µl)	1 µl
Pfu-Buffer 10x with MgSO4	5 µl
dNTPs 10 mM	2.5 µl
Pfu-Polymerase (2.5 U/µl)	1 µl
final volume	50 µl

Table 3. Reaction mixture for PCR program.

To amplify the Sho cDNA the following PCR program was used:

Temperature	Time	Cycle
95^{o}C	5 min	1x
95^{o}C 50^{o}C 72^{o}C	50 sec 45 sec 2 min	30x
72^{o}C 10^{o}C	10 min ∞	1x

Table 4: PCR program for Sho amplification

Agarose gel electrophoresis

To separate linearized DNA fragments from supercoiled DNA or to analyze PCR products, 1-2% (w/v) agarose gels in 1x Tris/Borate/EDTA (TBE) buffer and 0.2 µg/ml ethidium bromide were used depending on the expected size of the fragment. A 1 kb size marker was used to define the size of the fragment. 6x loading dye was added to the DNA samples and gels were run at 80 V.

Isolation and purification of DNA fragments from agarose gel

DNA fragments were cut out of the agarose gel on a UV illuminator and purified with the Nucleo Spin Extract kit (Macherey-Nagel) according to the manufacturer's instructions.

Enzymatic modification of DNA fragments

Purified DNA fragments were digested with 10 U restriction enzyme and the respective reaction buffer according to the manufacturer's instructions either overnight for digestion close to the end of DNA fragments or 1 h at 37°C to digest circular DNA. DNA fragments were purified as described above.

Alkaline phosphatase treatment

To avoid self-ligation, the linearized vectors were dephosphorylated with shrimp alkaline phosphatase (SAP) before ligation. SAP and SAP reaction buffer was added to the digested vector according to the manufacturer’s instructions. The mixture was incubated at 37°C for 10 min and heat inactivated at 65°C for 10 min.

Ligation of cDNA fragments into vector DNA

To ligate the digested and purified DNA fragment into a respectively linearized plasmid, 100-200 ng of the plasmid was mixed with 1-2 µg DNA fragment, T4 ligase buffer and T4 ligase in a final volume of 20 µl. The mixture was incubated for 3 h at room temperature and heat inactivated for 10 min at 65°C. 7 µl were used for transformation of competent bacteria of the *E. coli* strain DH5α

Preparation of competent bacteria

A DNA molecule cannot usually pass through the bacterial cell membrane since it is a highly hydrophilic molecule. So, the bacterial cells might be competent to take up the plasmid DNA into the cells. This is done by making pores and destabilizing the cell wall using high concentration of divalent cations. Single colony of freshly grown *E.Coli* DH5α strain was dissolved in 2 ml of Luria broth (LB) medium and shaken for 16 h at 37°C. Then the mixture was poured into 250 ml LB medium and the cells were cultivated for 2-3 h until an OD 590 value of 0.4-0.6 was reached. Further, the culture was centrifuged at 3750 rpm for 5 min at 4°C and the bacterial pellet was resuspended in 100 ml of ice cold TFB1 buffer. The suspension was incubated on ice for 5 min and then centrifuged at 3750 rpm for 5 min at 4°C. The pellet was then resuspended in 10 ml of ice cold TFB2 buffer and incubated for 30-60 min on ice. Further, it was aliquoted up to 100 ml and competent cells were stored in liquid nitrogen or in -80°C until the use.

Transformation of competent bacteria

The transformation is used for receiving and amplifying the plasmid by *E.Coli* (Sambrook, 1989). 100 ml of competent bacterial cells were gently thawed on ice and mixed with ligated or 1 mg of plasmid DNA. After 30 min of incubation on ice, the suspension was incubated for 90 seconds at 42^{o}C (heat shock) and then kept on ice for 5 min. After adding 400 ml of LB medium without antibiotics the culture was shaken for 60-90 min at 37^{o}C and plated in different concentrations of antibiotic containing agar plates. Then, the plates were incubated at 37^{o}C for 16-20 h.

Plasmid DNA preparation from bacterial culture

For the preparation of plasmid DNA Qiagen-Mini/Maxi-Kit was used and followed according to the manufacturer's instructions.

Sequencing

The DNA sequencing was performed based on the Sanger's chain termination method (Sanger et al, 1977) by GATC company in Konstanz., Germany.

Cell biology methods

Cell culture

Cultivation of cells

Human neuroblastoma cells (SH-SY5Y) cells were cultured in Dulbecco's modified Eagle's medium (DMEM). The complete medium contained 10% heat inactivated fetal calf serum (FCS), 1% antibiotics solution (final concentration was 1U/ml of penicillin G, 1mg/ml of streptomycin) and 2 mM glutamine. The cell line was cultured as an adherent single monolayer in cell culture flasks at 37^{o}C with 5% CO_2.

Passaging

The passaging of the cell line was done on an average every 3-4 days. After aspiration of the cell culture medium the cells were rinsed with phosphate buffered saline (PBS -/-) and then incubated with trypsin (0.5 g/L) for several minutes. Further, the cells were scrutinized carefully, resuspended in prewarmed complete medium and divided with the desired seeding ratio into new cell culture flasks.

Plating the cells

For plating the cells, the existing quantity of cells was determined using a Neubauer cell counting chamber. SH-SY5Y cells were plated at a density of 5 x10^5 cells in 3.5 cm culture dishes. For immunofluorescence analysis, cells were thinly plated in order to detect individual cells more efficiently. In this case, 4x 10^5 SH-SY5Y cells were platted on sterile cover slips in a 3.5 cm cell culture dish.

Transfection

The cells were plated 24 h before transfection and then the cells were washed with medium without FCS. For transient transfection, the plasmid DNA was mixed with Lipofectamine and Plus (Invitrogen) in OptiMEM according to the manufacturer's instructions. After 3 h the transfection mixture was replaced with complete medium and the cells were incubated for 24 h at 37^{o}C with 5% CO_2 before proceeding to the experiments as indicated.

Harvesting the cells

Cells were harvested 24 h after the transfection. These cells were washed twice with PBS -/- and then scraped off with a cell scraper in PBS -/-. Then, cells were centrifuged for 3 min at 3000 rpm and the cells pellet was placed on ice before processing for the experiments as indicated.

Total cell lysate

The cell pellets were resuspended on ice cold in detergent buffer (0.1% Triton X-100 or 0.5% Triton X-100/sodium desoxycholate (DOC) in PBS-/- with protease inhibitors and incubated on ice for 10-20 min with harsh vortexing in between. The resulting total cell lysate was mixed with Laemmli sample buffer, boiled for 10 min at 95^{o}C and analyzed by the SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE

The proteins were separated using one-dimensional, discontinues SDS-PAGE (Laemmli, 1970). The concentration of stacking gel was 4% and for the separating gel depending on the size of the protein, an 8-14% polyacrylamide concentration was used. Electrophoresis was done at 150-250V in a Hoefer SE600 chamber.

Western blot analysis

For subsequent immunodetection, previously separated proteins by SDS-PAGE were transferred onto a nitrocellulose membrane (Towbin et al, 1979). The protein transfer was performed in transfer buffer at a constant current of 1000 mA for 2 h at 4 ° C.

Ponceau S staining

After blotting the membrane was incubated for 5 min in Ponceau S solution and rinsed in distilled H_2O to check for complete transfer of proteins from the gel to the nitrocellulose membrane. Before the immune reaction the membrane was decolorized by PBST.

Immunodetection of proteins

The immunodetection of proteins was performed using the Enhanced Chemiluminescence (ECL) system according to the manufacturer's instructions. First the nitrocellulose membrane was blocked for non-specific binding with 5% skimmed milk solution for 1 h at room temperature (RT) and followed by incubation with primary antibody or antiserum for 16 h at $4^{o}C$. The membrane was washed 3 times with PBST for 10 min and incubated with horseradish peroxidase (HRP) conjugated secondary anti-mouse or anti-rabbit antibody in PBST for 45-60 min at room temperature. Subsequently, the membrane was again washed 3 times with PBST and incubated with HPR-substrate, and then the blots were exposed to the X-ray film to visualize the signals.

Glycosylation analysis

For the detection of glycosylation of proteins various methods that were employed have been described below.

Treatment with tunicamycin

To analyze the protein core glycosylation, tunicamycin which blocks the synthesis of all N-linked glycoproteins (N-glycans) was used. Transiently transfected cells were incubated with 0.5 mg/ml of tunicamycin at $37^{o}C$ with 5% CO_2 and the cell lysates were analyzed by Western blotting.

Digestion with Endo H or PNGase F

To identify whether the N-linked glycosylation of proteins was present in the form of a high mannose structure or complex glycosylated, an enzymatic digestion of cell lysates with Endo H or PNGase F was performed. Endo H cleaves the glycoforms of high mannose structure (Maley et al, 1989; Robbins et al, 1984) whereas PNGase F digests the complex glycans (Plummer et al, 1984; Tarentino et al, 1985; Tarentino & Plummer, 1987). The cell lysates were mixed with denaturing buffer, boiled at 95^{o}C for 10 min and incubated on ice for 5 min. After the addition of reaction buffer and the enzyme, the sample was incubated for 1-3 h at 37^{o}C and mixed with Laemmli sample buffer. Further-on, the sample was resolved on the SDS-PAGE gel and analyzed by Western blotting.

Treatment with brefedin A

Transiently transfected SH-SY5Y cells were grown in the presence of 1μg/ml of brefeldin A, which blocks the protein transport from ER to golgi complex and triggers the retrograde protein transport Golgi complex to ER. After 24 h of transfection the cell lysates were prepared and analyzed by Western blotting.

Indirect immunofluorescence microscopy

Transiently transfected SH-SY5Y cells were grown on glass cover slips and fixed 24 h post transfection with 3.7% PFA for 20 min. Fixed cells were incubated with primary antibody for 45 min at 37°C in PBS containing 1% BSA. After extensive washing with cold PBS, incubation with the Cy3 conjugated secondary antibody followed at 37°C for 30 min. Cells were mounted onto glass slides and examined by fluorescence microscopy.

Co-immunoprecipitation

To analyze formation of a mixed PrP/Sho dimer SH-SY5Y cells were co-transfected with PrPS131C and ShoS87C. At 24 h post-transfection the cells were harvested and lysed in ice-cold detergent lysis buffer (0.5% Triton X-100, 0.5% sodium deoxycholate in PBS) supplemented with protease inhibitors. Precleared lysates were incubated with αV5 antibody overnight at 4°C. The immunocomplex was precipitated with protein A sepharose beads and analyzed by Western blotting. To analyze formation of Sho trans-dimers, separate dishes of SH-SY5Y cells were transfected with either ShoS87C-V5 or ShoS87C-HA. 3 h post-transfection cells were extensively washed, trypsinized, mixed together and seeded in one cell culture dish. 24 h later, the cells were harvested and analyzed as described above.

Co-cultivation assay

SH-SY5Y cells grown on coverslips were transiently transfected with PrP and/or Sho constructs using lipofectamine plus reagent. At 3 h after the transfection, the cover slips were transferred into cell culture dishes containing ScN2a or N2a cells. 16 h later, the apoptotic cell death in transiently transfected SH-SY5Y cells was analyzed.

Apoptosis assay

As described earlier (Rambold et al, 2006), SH-SY5Y cells were grown on cover slips. 24 h after transfection, the cells were incubated with glutamate (500 μM) for 3 h. The cells were then fixed and activated caspase-3 detected by indirect immunofluorescence using an anti-active caspase-3 antibody. To detect cells undergoing apoptosis, the number of activated caspase-3 positive cells out of at least 1100 transfected cells was determined using a Zeiss Axioscope 2 plus microscope (Carl Zeiss, Göttingen, Germany). Quantifications were based on triplicates of at least three independent experiments.

Statistical analysis

Data were expressed as means ± SE. All the experiments were performed in triplicates and repeated at least three times. Statistical analysis was carried out using student's t-test. P-values are as follows: * $P<0.05$, **$P<0.005$, ***<0.0005.

Materials

Biological materials

Bacterial strain

DH5α	Genotype: supE44, _lac169 (_80lacZ_M15) hsdR17, recA1, endA1, gyr96, thi-1, eLA1 Source: Hanahan, 1983

Vectors

pcDNA3.1/ZEO(+)	Invitrogen, Karlsruhe
pET-19b	Novagen, Darmstadt

Cell lines

SH-SY5Y cells	Human neuroblastoma cells (ATCC-Nr. HTB11)
N2a cells	Mouse neuroblastoma cells (ATCC-No. CCL - 131)

Antibodies

Anti-PrP 3F4 monoclonal	Signet Laboratories, Dedham, MA, USA
Anti-V5 monoclonal	Invitrogen, Karlsruhe
Anti-HA monoclonal	Covance, Münster
Anti-active caspase-3 polyclonal	Promega, Mannheim
Cy3 conjugated anti rabbit	Dianova, Hamburg
Cy3 conjugated anti mouse	Dianova, Hamburg
Anti-GFP monoclonal	Roche Diagnostics, Mannheim
Anti-Sho polyclonal (αSho)	(Sakthivelu et al, 2011)

Primer list

PrP_For HindIII_Forw	CCCAAGCTTATGGCGAACCTTGGCTAC
PrP_Zsho_rev-1	GCCCAGTCCGTAGCCGATCATGGCGCTCCCCAGCAT
PrP_Zsho_forw-2	ATGCTGGGGAGCGCCATGATCGGCTACGGACTGGGC
ZSho_Cter_XhoI_rev	CGCCTCGACTCAAGCCCACATAATAAC
ZSho_PrP_For_New-2	TCCCAGAGCAAAGGCTCAGCAGGGGCTGCGGCAGCT
ZSho_PrP_Rev-1	AGCTGCCGCAGCCCCTGCTGAGCCTTTGCTCTGGGA
HSho-F-1BamHI	CGCGGATCCGCCGCCACCATGAACTGGGCACCCGC
HSho-PrP-R-2-EcoRI	CCGGAATTCTCATCCCACGATCAGGAAGATGAGG

HuSho-PrP-New-reve-1	CTGATTATGGGTACCCCCTCCTTGCAGGGA GGAACCCGGGGCACCG
HuSho-PrP-New-For-2	CGGTGCCCCGGGTTCCTCCCTGCAAGGAGG GGGTACCCATAATCAG
7-HuSho-ΔHD_Rever-1	TCCCCGGGTCCCGCGGCCCTTCTAGCCACG CGCAGGGAGGAACC
7-HuSho-ΔHD_Foew-2	GGTTCCTCCCTGCGCGTGGCTAGAAGGGCC GCGGGACCCGGGGA
8-HuSho-C87-Rever-1	GGCCCTTCTCCAGCCGCAGCCCGCCGCCAG GCC
8-HuSho-C87-Forw-2	GGCCTGGCGGCGGGCTGCGGCTGGAGAAG GGGC
9-HuSho-ΔN-Reve-1	GAGGAACCCGGGGCACCGTATCCGCGGCC GCCCTTGGCTG
9-HuShoΔN-Forw-2	CAGCCAAGGGCGGCCGCGGATACGGTGCC CCGGGTTCCTC
5-HuSho_NdeI_Forw_ PET	GGGAATTCCATATGAACTGGGCACCCGCA ACGTGCTGGGCT
5HuSho_BamHI_Rev_ PET	CGCGGATCCCTAGGGCCGCAGCAGCCCCA GGGCT
3-HuSho_HindII_Forw	CCCAAGCTTATGAACTGGGCACCCGCA
3-HuSho_XbaI_rev	CTAGTCTAGACTAGGGCCGCAGCAGCCC
2-PrP_Cter_Xhol_Rev	CCGCTCGAGTCATCCCACGATCAGGAA
2-ZSho_HindIII_Forw	CCCAAGCTTATGCTGGGCAATCAGAAG
2ZSho-PrP_Rev	GCTTCCCTGCCCGGGATATGAGCCTTTGCT CTGGGA
2-ZSho_PrP_Forw	TCCCAGAGCAAAGGCTCATATCCCGGGCA GGGAAGC

Chemicals and reagents

Acetone	Merck, Darmstadt
Agarose	Serva, Heidelberg
Ampicillin	Boehringer Mannheim, Mannheim
Ammonium persulfate (APS)	USB, Clevelan,OH,USA
Bcto agar	Difco Laboratories, Detroit, MI, USA
Complete Protease-inhibitor	Boehringer Mannheim, Mannheim
Bromophenol blue	Merck, Darmstadt
Sodium deoxycholate	Sigma, Taufkirchen
Deoxynucleoside triphosphate dATP, dCTP, dGTP, dTTP	Sigma, Taufkirchen
Ethanol	Sigma, Taufkirchen
EDTA	USB, Clevelan,OH,USA
Ethidium bromide	Sigma, Taufkirchen
Acetic acid	Merck, Darmstadt
Fetal calf serum (FCS)	Invitrogen, Karlsruhe
Disodium hydrogen phosphate	Merck, Darmstadt

Formamide	Merck, Darmstadt
Gentamicin	Sigma, Taufkirchen
Glutamine	Invitrogen, Karlsruhe
Glutaraldehyde	Sigma, Taufkirchen
Glycerol	USB, Cleveland, OH, USA
Glycine	USB, Cleveland, OH, USA
Urea	Sigma, Taufkirchen
Yeast extract	Difco Laboratories, Detroit, MI, USA
Immersion oil	Merck, Darmstadt
Instant-skimmed milk powder	Uelzena, Uelzen
Potassium acetate	Sigma, Taufkirchen
Potassium chloride	USB, Cleveland, OH, USA
Potassium dihydrogen phosphate	Merck, Darmstadt
Copper sulfate	Sigma, Taufkirchen
L-[35S]-Methionine	AmershamPharmacia Biotech,Freiburg
Lipofectamine reagent	Invitrogen, Karlsruhe
Magnesium chloride	USB, Cleveland, OH, USA
Manganese chloride	Sigma, Taufkirchen
Methanol	Merck, Darmstadt
Sodium chloride	Merck, Darmstadt
Sodium nitrate	Merck, Darmstadt
Diatrizoate sodium	Sigma, Taufkirchen
PBS Dulbecco's -/- Mg/Ca	Invitrogen, Karlsruhe
PBS Dulbecco's +/+ Mg/Ca	Invitrogen, Karlsruhe
Penicillin	Invitrogen, Karlsruhe
Plus reagent	Invitrogen, Karlsruhe
Polyacrylamide/Bisacrylamide (29:1) 40%	Roth, Karlsruhe
Ponceau S	Sigma, Taufkirchen
ProMix 35S-Methionine/Cysteine	AmershamPharmaciaBiotech, Freiburg
Protease-inhibitor mix	Sigma, Taufkirchen
Protein A-agarose	Pierce,Perbio Science, Bonn
Protein A-trisacryl- matrix	Pierce, Perbio Science, Bonn
Protein G-matrix	Pierce, Perbio Science, Bonn
Proteasome inhibitor MG132	Calbiochem, Bad Soden
RediPrimeTM II DNA labeling system	AmershamPharmacia Biotech, Freiburg
Rubidium chloride	Sigma, Taufkirchen
Hydrochloric acid	Merck, Darmstadt
Sarkosyl	USB, Cleveland, OH, USA
SDS	Roth, Karlsruhe
Streptomycin	Invitrogen, Karlsruhe
TEMED	USB, Cleveland, OH, USA
Trichloroacetic acid	Sigma, Taufkirchen
Tris	USB, Cleveland, OH, USA
Triton X-100	USB, Cleveland, OH, USA
Trypan blue	Invitrogen, Karlsruhe

Tunicamycin	Sigma, Taufkirchen
Tween-20	USB, Cleveland, OH, USA
β-Mercaptoethanol	Merck, Darmstadt

Medium

Dulbecco's Modified Eagle's medium (DMEM),	Invitrogen, Karlsruhe
Minimal Essential Medium (MEM),	Invitrogen, Karlsruhe
Minimal Essential Medium, without L-Methionine	Invitrogen, Karlsruhe
OPTIMEM	Invitrogen, Karlsruhe
LB-Medium	1% NaCl 1% Bacto tryptone 0.5% Yeast extract 100 mg / ml Ampicillin and 30 mg / ml Kanamycin (added after autoclaving)
LB-Agar	LB medium + 1.5% Bacto agar 100 μg/ ml Ampicillin and 30 mg / ml Kanamycin (added after autoclaving)

Kits

ECL RPN 2106	Amersham Pharmacia Biotech, Freiburg
Immobilon Western chemiluminescent HRP substrate	Millipore, Schwalb Bach
Protein assay kit	Bio-Rad, München
QIAprep spin plasmid extraction kit Mini / Maxi	Qiagen, Hilden
QIAquick gel extraction kit	Qiagen, Hilden
TNT T7 quick coupled transcription / translation system	Promega, Mannheim

Equipments

Agarose gel electrophoresis	Central workshop, MPI, Martinsried
Mettler Toledo AG285 Analytical Balance	Mettler-Toledo GmbH, Giessen
Incubators	Heraeus, Hanau
X-Omat Kodak film developer, Stuttgart	
X-Omat Film developer	Kodak, Stuttgart
Gel documentation system	MWG Biotech, Ebersberg
Gel dryer SGD300	Savant, Holbrook, NY, USA
GS-6R refrigerated centrifuge with rotor GH3.8	Beckmann, Unterschleissheim
J2-21M refrigerated centrifuge with rotor JA-14	Beckmann, Unterschleissheim
Microscope Axiovert 25, 200M	Carl Zeiss, Göttingen

Axioscope2 plus microscope (Axiovision software)	Carl Zeiss, Göttingen
pH meter	Fisher Scientific, Nidderau
Pipettes (P10, P20, P100, P200)	Abimed Gilson, Langenfeld
Pipette (P1000)	Eppendorf, Hamburg
Polyacrylamide gel electrophoresis	Amersham Biosciences, Freiburg
PCR machine T3 thermocycler	Biometra GmbH, Göttingen
Thermomixer	Eppendorf, Hamburg
Table centrifuge centrifuge 5415C	Eppendorf, Hamburg

Solutions and buffers

APS solution	10% APS in PBS
Blocking milk for Western blot	5% Skimmed milk powder in 1x PBST
Blocking buffer for IF	1% BSA in PBS
Coomassie destaining solution	40% Methanol 7% Acetic acid
DNA sample buffer (6x)	0.25% Bromophenol blue 30% Glycerol
Laemmli sample buffer (2x)	120 mM Tris pH 6.8 2% SDS 20% Glycerol 0.5% Bromophenol blue 2% β-Mercaptoethanol
Lysis buffer	0.5% Triton X-100, 0.5% DOC + Protease inhibitor in PBS
PCR mix	1200 µl of H_2O 200 µl of 10x Pfu / Taq buffer each 2 µl of dNTPs
PBS (cell culture)	Gibco, BRL Life Technologies, Karlsruhe
PBS (10x)	80 g of NaCl 2 g of KCl 14.4 g of Na_2HPO_4 x 2 H_2O 2.4 g of KH_2PO_4 for 1000 ml of H_2O
PBS-T (1x)	1% Tween-20 in 1x PBS
Pfu polymerase buffer (10x)	Promega, Mannheim
Ponceau S staning solution	0.2 g of Ponceau S 5 ml of acetic acid 100 ml of H_2O
Stacking gel buffer for SDS-PAGE	0.5 M Tris, pH 6.8 0.4% SDS

	pH 6.8
Separating gel buffer for SDS-PAGE	1.5 M Tris, pH 8.8 0.4% SDS pH 8.8
Shrimp alkaline phosphatase buffer (10x)	Roche Diagnostics, Mannheim
T4 DNA ligase buffer (10x)	MBI Fermentas, St. Leon-Rot
TAE buffer (50x)	2 M Tris base 57.1 ml of Glacial acetic acid 50 mM Na_2EDTA x $2H_2O$, pH 8.0 add1000 ml of H_2O
TE buffer	10 mM Tris-HCl, pH 7.5 1 mM EDTA, pH 8.0
Western blot transfer buffer	20 mM Tris-Base 150 mM Glycine 0.01% SDS 20% Methanol
Tunicamycin	10 mg/ml in H_2O
Brefeldin A	5mg/ml in Ethanol

References

Aguzzi A, Baumann F, Bremer J (2008) The prion's elusive reason for being. *Annual Review of Neuroscience* **31:** 439-477

Aguzzi A, Glatzel M (2004) vCJD tissue distribution and transmission by transfusion--a worst-case scenario coming true? *Lancet* **363:** 411-412

Aguzzi A, Glatzel M, Montrasio F, Prinz M, Heppner FL (2001) Interventional strategies against prion diseases. *Nat Rev Neurosci* **2:** 745-749

Aguzzi A, Weissmann C (1996) Spongiform encephalopathies: a suspicious signature. *Nature* **383:** 666-667

Airaksinen MS, Saarma M (2002) The GDNF family: signalling, biological functions and therapeutic value. *Nat Rev Neurosci* **3:** 383-394

Allen JA, Halverson-Tamboli RA, Rasenick MM (2007) Lipid raft microdomains and neurotransmitter signalling. *Nat Rev Neurosci* **8:** 128-140

Alper T, Cramp WA, Haig DA, Clarke MC (1967) Does the agent of scrapie replicate without nucleic acid? *Nature* **214:** 764-766

Amitsuka H, Kuwahara K, Yoshida T, Tenya K, Sakakibara T, Mihalik M, Menovsky AA (1999) Anomalous transport properties of dilute uranium alloys R1-xUxRu2Si2 (R = Th, Y; x <= 0.07). *Physica B* **259-61:** 412-414

Appel TR, Dumpitak C, Matthiesen U, Riesner D (1999) Prion rods contain an inert polysaccharide scaffold. *Biol Chem* **380:** 1295-1306

Ballif BC, Hornor SA, Jenkins E, Madan-Khetarpal S, Surti U, Jackson KE, Asamoah A, Brock PL, Gowans GC, Conway RL, Graham JM, Jr., Medne L, Zackai EH, Shaikh TH, Geoghegan J, Selzer RR, Eis PS, Bejjani BA, Shaffer LG (2007) Discovery of a previously unrecognized microdeletion syndrome of 16p11.2-p12.2. *Nat Genet* **39:** 1071-1073

Barry AE, Klyubin I, Mc Donald JM, Mably AJ, Farrell MA, Scott M, Walsh DM, Rowan MJ (2011) Alzheimer's Disease Brain-Derived Amyloid-{beta}-Mediated Inhibition of LTP In Vivo Is Prevented by Immunotargeting

Cellular Prion Protein. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **31:** 7259-7263

Basler K, Oesch B, Scott M, Westaway D, Walchli M, Groth DF, McKinley MP, Prusiner SB, Weissmann C (1986) Scrapie and cellular PrP isoforms are encoded by the same chromosomal gene. *Cell* **46:** 417-428

Baumann F, Tolnay M, Brabeck C, Pahnke J, Kloz U, Niemann HH, Heikenwalder M, Rulicke T, Burkle A, Aguzzi A (2007) Lethal recessive myelin toxicity of prion protein lacking its central domain. *Embo Journal* **26:** 538-547

Beck JA, Campbell TA, Adamson G, Poulter M, Uphill JB, Molou E, Collinge J, Mead S (2008) Association of a null allele of SPRN with variant Creutzfeldt-Jakob disease. *J Med Genet* **45:** 813-817

Belay ED, Maddox RA, Williams ES, Miller MW, Gambetti P, Schonberger LB (2004) Chronic wasting disease and potential transmission to humans. *Emerg Infect Dis* **10:** 977-984

Bendheim PE, Brown HR, Rudelli RD, Scala LJ, Goller NL, Wen GY, Kascsak RJ, Cashman NR, Bolton DC (1992) Nearly ubiquitous tissue distribution of the scrapie agent precursor protein. *Neurology* **42:** 149-156

Beraldo FH, Arantes CP, Santos TG, Machado CF, Roffe M, Hajj GN, Lee KS, Magalhaes AC, Caetano FA, Mancini GL, Lopes MH, Americo TA, Magdesian MH, Ferguson SS, Linden R, Prado MA, Martins VR (2011) Metabotropic glutamate receptors transduce signals for neurite outgrowth after binding of the prion protein to laminin gamma1 chain. *FASEB J* **25:** 265-279

Bernoulli C, Siegfried J, Baumgartner G, Regli F, Rabinowicz T, Gajdusek DC, Gibbs CJ, Jr. (1977) Danger of accidental person-to-person transmission of Creutzfeldt-Jakob disease by surgery. *Lancet* **1:** 478-479

Bespalov MM, Saarma M (2007) GDNF family receptor complexes are emerging drug targets. *Trends Pharmacol Sci* **28:** 68-74

Bieschke J, Weber P, Sarafoff N, Beekes M, Giese A, Kretzschmar H (2004) Autocatalytic self-propagation of misfolded prion protein. *Proc Natl Acad Sci U S A* **101:** 12207-12211

Blum R, Konnerth A (2005) Neurotrophin-mediated rapid signaling in the central nervous system: Mechanisms and functions. *Physiology* **20:** 70-78

Bockman JM, Kingsbury DT, McKinley MP, Bendheim PE, Prusiner SB (1985) Creutzfeldt-Jakob disease prion proteins in human brains. *N Engl J Med* **312:** 73-78

Bockman JM, Prusiner SB, Tateishi J, Kingsbury DT (1987) Immunoblotting of Creutzfeldt-Jakob disease prion proteins: host species-specific epitopes. *Ann Neurol* **21:** 589-595

Bolton B (1982) Issues in validity research on the 16 PF. *Psychol Rep* **50:** 1077-1078

Bolton DC, Meyer RK, Prusiner SB (1985) Scrapie PrP 27-30 is a sialoglycoprotein. *J Virol* **53:** 596-606

Borchelt DR, Taraboulos A, Prusiner SB (1992) Evidence for Synthesis of Scrapie Prion Proteins in the Endocytic Pathway. *Journal of Biological Chemistry* **267:** 16188-16199

Brandner S, Isenmann S, Raeber A, Fischer M, Sailer A, Kobayashi Y, Marino S, Weissmann C, Aguzzi A (1996) Normal host prion protein necessary for scrapie-induced neurotoxicity. *Nature* **379:** 339-343

Brown DR, Qin K, Herms JW, Madlung A, Manson J, Strome R, Fraser PE, Kruck T, von Bohlen A, Schulz-Schaeffer W, Giese A, Westaway D, Kretzschmar H (1997a) The cellular prion protein binds copper in vivo. *Nature* **390:** 684-687

Brown DR, Schulz-Schaeffer WJ, Schmidt B, Kretzschmar HA (1997b) Prion protein-deficient cells show altered response to oxidative stress due to decreased SOD-1 activity. *Exp Neurol* **146:** 104-112

Brown DR, Wong BS, Hafiz F, Clive C, Haswell SJ, Jones IM (1999) Normal prion protein has an activity like that of superoxide dismutase. *Biochem J* **344 Pt 1:** 1-5

Brown P (1992) The phenotypic expression of different mutations in transmissible human spongiform encephalopathy. *Rev Neurol (Paris)* **148:** 317-327

Brown P, Coker-Vann M, Pomeroy K, Franko M, Asher DM, Gibbs CJ, Jr., Gajdusek DC (1986) Diagnosis of Creutzfeldt-Jakob disease by Western blot identification of marker protein in human brain tissue. *N Engl J Med* **314:** 547-551

Bruce ME, Will RG, Ironside JW, McConnell I, Drummond D, Suttie A, McCardle L, Chree A, Hope J, Birkett C, Cousens S, Fraser H, Bostock CJ (1997) Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent. *Nature* **389:** 498-501

Bueler H, Aguzzi A, Sailer A, Greiner RA, Autenried P, Aguet M, Weissmann C (1993) Mice devoid of PrP are resistant to scrapie. *Cell* **73:** 1339-1347

Bueler H, Fischer M, Lang Y, Bluethmann H, Lipp HP, DeArmond SJ, Prusiner SB, Aguet M, Weissmann C (1992) Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein. *Nature* **356:** 577-582

Burns CS, Aronoff-Spencer E, Legname G, Prusiner SB, Antholine WE, Gerfen GJ, Peisach J, Millhauser GL (2003) Copper coordination in the full-length, recombinant prion protein. *Biochemistry* **42:** 6794-6803

Caetano FA, Beraldo FH, Hajj GN, Guimaraes AL, Jurgensen S, Wasilewska-Sampaio AP, Hirata PH, Souza I, Machado CF, Wong DY, De Felice FG, Ferreira ST, Prado VF, Rylett RJ, Martins VR, Prado MA (2011) Amyloid-beta oligomers increase the localization of prion protein at the cell surface. *Journal of neurochemistry* **117:** 538-553

Calzolai L, Lysek DA, Perez DR, Guntert P, Wuthrich K (2005) Prion protein NMR structures of chickens, turtles, and frogs. *Proc Natl Acad Sci U S A* **102:** 651-655

Cao H, Bangalore L, Dompe C, Bormann BJ, Stern DF (1992) An extra cysteine proximal to the transmembrane domain induces differential cross-linking of p185neu and p185neu. *The Journal of biological chemistry* **267:** 20489-20492

Caughey B, Raymond GJ (1991) The Scrapie-Associated Form of Prp Is Made from a Cell-Surface Precursor That Is Both Protease-Sensitive and Phospholipase-Sensitive. *Journal of Biological Chemistry* **266:** 18217-18223

Chandler RL (1961) Encephalopathy in mice produced by inoculation with scrapie brain material. *Lancet* **1:** 1378-1379

Chesebro B, Race R, Kercher L (2005a) Scrapie pathogenesis in brain and retina: effects of prion protein expression in neurons and astrocytes. *J Neurovirol* **11:** 476-480

Chesebro B, Trifilo M, Race R, Meade-White K, Teng C, LaCasse R, Raymond L, Favara C, Baron G, Priola S, Caughey B, Masliah E, Oldstone M (2005b) Anchorless prion protein results in infectious amyloid disease without clinical scrapie. *Science* **308:** 1435-1439

Christensen HM, Harris DA (2009) A deleted prion protein that is neurotoxic in vivo is localized normally in cultured cells. *J Neurochem* **108:** 44-56

Chung E, Ji Y, Sun Y, Kascsak RJ, Kascsak RB, Mehta PD, Strittmatter SM, Wisniewski T (2010) Anti-PrPC monoclonal antibody infusion as a novel treatment for cognitive deficits in an Alzheimer's disease model mouse. *BMC Neurosci* **11:** 130

Cohen FE, Pan KM, Huang Z, Baldwin M, Fletterick RJ, Prusiner SB (1994) Structural Clues to Prion Replication. *Science* **264:** 530-531

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

Collinge J, Asante EA, Li YG, Gowland I, Jefferys JGR (2004) Pathogenic human prion protein rescues PrP null phenotype in transgenic mice. *Neuroscience Letters* **360:** 33-36

Collinge J, Sidle KC, Meads J, Ironside J, Hill AF (1996) Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD. *Nature* **383:** 685-690

Collinge J, Whittington MA, Sidle KC, Smith CJ, Palmer MS, Clarke AR, Jefferys JG (1994) Prion protein is necessary for normal synaptic function. *Nature* **370:** 295-297

Corley SM, Gready JE (2008) Identification of the RGG box motif in Shadoo: RNA-binding and signaling roles? *Bioinform Biol Insights* **2:** 383-400

Couvineau A, Fabre C, Gaudin P, Maoret JJ, Laburthe M (1996) Mutagenesis of N-glycosylation sites in the human vasoactive intestinal peptide 1 receptor. Evidence that asparagine 58 or 69 is crucial for correct delivery of the receptor to plasma membrane. *Biochemistry* **35:** 1745-1752

Creutzfeldt HG (1920) A peculiar localised disease of the central nervous system (Preliminary announcement). *Z Gesamte Neurol Psy* **57:** 1-18

Creutzfeldt HG (1921) The new findings of the brain-anatomical (histo-pathological) research for mental illnesses. *Deut Med Wochenschr* **47:** 1589-1590

Cuille J, Chelle PL (1938) Is the trembling of sheep detemined by a filtrable virus? *Cr Hebd Acad Sci* **206:** 1687-1688

Cunningham O, Andolfo A, Santovito ML, Iuzzolino L, Blasi F, Sidenius N (2003) Dimerization controls the lipid raft partitioning of uPAR/CD87 and regulates its biological functions. *EMBO J* **22:** 5994-6003

Curtis J, Errington M, Bliss T, Voss K, MacLeod N (2003) Age-dependent loss of PTP and LTP in the hippocampus of PrP-null mice. *Neurobiol Dis* **13:** 55-62

Daude N, Wohlgemuth S, Rogaeva E, Farid AH, Heaton M, Westaway D (2009a) Frequent missense and insertion/deletion polymorphisms in the ovine Shadoo gene parallel species-specific variation in PrP. *PLoS One* **4:** e6538

Davanipour Z, Goodman L, Alter M, Sobel E, Asher D, Gajdusek DC (1984) Possible modes of transmission of Creutzfeldt-Jakob disease. *N Engl J Med* **311:** 1582-1583

Davis D, Liu X, Segaloff DL (1995) Identification of the sites of N-linked glycosylation on the follicle-stimulating hormone (FSH) receptor and assessment of their role in FSH receptor function. *Mol Endocrinol* **9:** 159-170

Della-Bianca V, Rossi F, Armato U, Dal-Pra I, Costantini C, Perini G, Politi V, Della Valle G (2001) Neurotrophin p75 receptor is involved in neuronal damage by prion peptide-(106-126). *J Biol Chem* **276:** 38929-38933

Donne DG, Viles JH, Groth D, Mehlhorn I, James TL, Cohen FE, Prusiner SB, Wright PE, Dyson HJ (1997) Structure of the recombinant full-length hamster prion protein PrP(29-231): the N terminus is highly flexible. *Proc Natl Acad Sci U S A* **94:** 13452-13457

Duffy P, Wolf J, Collins G, DeVoe AG, Streeten B, Cowen D (1974) Letter: Possible person-to-person transmission of Creutzfeldt-Jakob disease. *N Engl J Med* **290:** 692-693

Dumpitak C, Beekes M, Weinmann N, Metzger S, Winklhofer KF, Tatzelt J, Riesner D (2005) The polysaccharide scaffold of PrP 27-30 is a common compound of natural prions and consists of alpha-linked polyglucose. *Biol Chem* **386:** 1149-1155

Eisenhaber B, Bork P, Eisenhaber F (2001) Post-translational GPI lipid anchor modification of proteins in kingdoms of life: analysis of protein sequence data from complete genomes. *Protein Eng* **14:** 17-25

Fevrier B, Vilette D, Archer F, Loew D, Faigle W, Vidal M, Laude H, Raposo G (2004) Cells release prions in association with exosomes. *Proc Natl Acad Sci U S A* **101:** 9683-9688

Fischer M, Rulicke T, Raeber A, Sailer A, Moser M, Oesch B, Brandner S, Aguzzi A, Weissmann C (1996) Prion protein (PrP) with amino-proximal deletions restoring susceptibility of PrP knockout mice to scrapie. *EMBO J* **15:** 1255-1264

Freir DB, Nicoll AJ, Klyubin I, Panico S, Mc Donald JM, Risse E, Asante EA, Farrow MA, Sessions RB, Saibil HR, Clarke AR, Rowan MJ, Walsh DM, Collinge J (2011) Interaction between prion protein and toxic amyloid beta assemblies can be therapeutically targeted at multiple sites. *Nat Commun* **2:** 336

Gajdusek DC (1973) Kuru and Creutzfeldt-Jakob disease. Experimental models of noninflammatory degenerative slow virus disease of the central nervous system. *Ann Clin Res* **5:** 254-261

Gajdusek DC, Gibbs CJ, Alpers M (1966) Experimental transmission of a Kuru-like syndrome to chimpanzees. *Nature* **209:** 794-796

Gauczynski S, Peyrin JM, Haik S, Leucht C, Hundt C, Rieger R, Krasemann S, Deslys JP, Dormont D, Lasmezas CI, Weiss S (2001) The 37-kDa/67-kDa laminin receptor acts as the cell-surface receptor for the cellular prion protein. *EMBO J* **20:** 5863-5875

Gerstmann J, Straussler E, Scheinker I (1935) On a peculiar hereditary-congenital disease of the central nervous system - Along with an article on the question of premature ageing. *Z Gesamte Neurol Psy* **154:** 736-762

Gibbs CJ, Jr., Gajdusek DC, Asher DM, Alpers MP, Beck E, Daniel PM, Matthews WB (1968) Creutzfeldt-Jakob disease (spongiform encephalopathy): transmission to the chimpanzee. *Science* **161:** 388-389

Gomi H, Yokoyama T, Fujimoto K, Ikeda T, Katoh A, Itoh T, Itohara S (1995) Mice devoid of the glial fibrillary acidic protein develop normally and are susceptible to scrapie prions. *Neuron* **14:** 29-41

Gordon WS (1946) Advances in veterinary research. *Vet Rec* **58:** 516-525

Griffith JS (1967) Self-replication and scrapie. *Nature* **215:** 1043-1044

Haigh CL, Lewis VA, Vella LJ, Masters CL, Hill AF, Lawson VA, Collins SJ (2009) PrPC-related signal transduction is influenced by copper, membrane integrity and the alpha cleavage site. *Cell Res* **19:** 1062-1078

Hamir AN, Kunkle RA, Cutlip RC, Miller JM, O'Rourke KI, Williams ES, Miller MW, Stack MJ, Chaplin MJ, Richt JA (2005) Experimental transmission of chronic wasting disease agent from mule deer to cattle by the intracerebral route. *J Vet Diagn Invest* **17:** 276-281

Hamir AN, Kunkle RA, Miller JM, Greenlee JJ, Richt JA (2006) Experimental second passage of chronic wasting disease (CWD(mule deer)) agent to cattle. *J Comp Pathol* **134:** 63-69

Hamir AN, Miller JM, Kunkle RA, Hall SM, Richt JA (2007) Susceptibility of cattle to first-passage intracerebral inoculation with chronic wasting disease agent from white-tailed deer. *Vet Pathol* **44:** 487-493

Harrison PM, Khachane A, Kumar M (2010) Genomic assessment of the evolution of the prion protein gene family in vertebrates. *Genomics* **95:** 268-277

Hartsoug.Gr, Burger D (1965) Encephalopathy of Mink .I. Epizootiologic and Clinical Observations. *Journal of Infectious Diseases* **115:** 387-&

Hatanaka M, Seya T, Miyagawa S, Matsumoto M, Hara T, Tanaka K, Shimizu A (1998) Cellular distribution of a GPI-anchored complement regulatory protein CD59: homodimerization on the surface of HeLa and CD59-transfected CHO cells. *J Biochem* **123:** 579-586

He J, Xu J, Castleberry AM, Lau AG, Hall RA (2002) Glycosylation of beta(1)-adrenergic receptors regulates receptor surface expression and dimerization. *Biochem Biophys Res Commun* **297:** 565-572

Hill AF, Desbruslais M, Joiner S, Sidle KC, Gowland I, Collinge J, Doey LJ, Lantos P (1997) The same prion strain causes vCJD and BSE. *Nature* **389:** 448-450, 526

Jakob A (1921) Unusual diseases of the central nervous system with striking anatomic results (Spastic pseudosclerosis - Encephalomyelopathy with disseminated focal degeneration). *Z Gesamte Neurol Psy* **64:** 147-228

Jarrett JT, Lansbury PT, Jr. (1993) Seeding "one-dimensional crystallization" of amyloid: a pathogenic mechanism in Alzheimer's disease and scrapie? *Cell* **73:** 1055-1058

Jiayu W, Zhu H, Ming X, Xiong W, Songbo W, Bocui S, Wensen L, Jiping L, Keying M, Zhongyi L, Hongwei G (2009) Mapping the interaction site of prion protein and Sho. *Mol Biol Rep*

Jin T, Gu Y, Zanusso G, Sy M, Kumar A, Cohen M, Gambetti P, Singh N (2000) The chaperone protein BiP binds to a mutant prion protein and mediates its degradation by the proteasome. *J Biol Chem* **275:** 38699-38704

Johnson RT (2005) Prion diseases. *Lancet Neurol* **4:** 635-642

Johnson RT, Gibbs CJ, Jr. (1998) Creutzfeldt-Jakob disease and related transmissible spongiform encephalopathies. *N Engl J Med* **339:** 1994-2004

Kanaani J, Prusiner SB, Diacovo J, Baekkeskov S, Legname G (2005) Recombinant prion protein induces rapid polarization and development of synapses in embryonic rat hippocampal neurons in vitro. *J Neurochem* **95:** 1373-1386

Kessels HW, Nguyen LN, Nabavi S, Malinow R (2010) The prion protein as a receptor for amyloid-beta. *Nature* **466:** E3-4; discussion E4-5

Khosravani H, Zhang Y, Tsutsui S, Hameed S, Altier C, Hamid J, Chen L, Villemaire M, Ali Z, Jirik FR, Zamponi GW (2008) Prion protein attenuates excitotoxicity by inhibiting NMDA receptors. *J Cell Biol* **181:** 551-565

Kim JI, Cali I, Surewicz K, Kong Q, Raymond GJ, Atarashi R, Race B, Qing L, Gambetti P, Caughey B, Surewicz WK (2010) Mammalian prions generated from bacterially expressed prion protein in the absence of any mammalian cofactors. *J Biol Chem* **285:** 14083-14087

Kim YJ, Johnson KR, Wheelock MJ (2005) N-cadherin-mediated cell motility requires cis dimers. *Cell Commun Adhes* **12:** 23-39

Kirkwood JK, Wells GA, Wilesmith JW, Cunningham AA, Jackson SI (1990) Spongiform encephalopathy in an arabian oryx (Oryx leucoryx) and a greater kudu (Tragelaphus strepsiceros). *Vet Rec* **127:** 418-420

Klatzo I, Gajdusek DC, Zigas V (1959) Pathology of Kuru. *Lab Invest* **8:** 799-847

Klein TR, Kirsch D, Kaufmann R, Riesner D (1998) Prion rods contain small amounts of two host sphingolipids as revealed by thin-layer chromatography and mass spectrometry. *Biol Chem* **379:** 655-666

Kondo K, Kuroiwa Y (1982) A case control study of Creutzfeldt-Jakob disease: association with physical injuries. *Ann Neurol* **11:** 377-381

Kostrewa D, Brockhaus M, D'Arcy A, Dale GE, Nelboeck P, Schmid G, Mueller F, Bazzoni G, Dejana E, Bartfai T, Winkler FK, Hennig M (2001) X-ray structure of junctional adhesion molecule: structural basis for homophilic adhesion via a novel dimerization motif. *EMBO J* **20:** 4391-4398

Kretzschmar HA, Stowring LE, Westaway D, Stubblebine WH, Prusiner SB, Dearmond SJ (1986) Molecular cloning of a human prion protein cDNA. *DNA* **5:** 315-324

Kurschner C, Morgan JI (1995) The cellular prion protein (PrP) selectively binds to Bcl-2 in the yeast two-hybrid system. *Brain Res Mol Brain Res* **30:** 165-168

Lampo E, Van den Broeck W, Willemarck N, Van Poucke M, Casteleyn CR, De Spiegelaere W, Van Zeveren A, Peelman LJ (2010) Distribution of the Shadoo protein in the ovine brain assessed by immunohistochemistry. *Res Vet Sci*

Lauren J, Gimbel DA, Nygaard HB, Gilbert JW, Strittmatter SM (2009) Cellular prion protein mediates impairment of synaptic plasticity by amyloid-beta oligomers. *Nature* **457:** 1128-1132

Le Pichon CE, Valley MT, Polymenidou M, Chesler AT, Sagdullaev BT, Aguzzi A, Firestein S (2009) Olfactory behavior and physiology are disrupted in prion protein knockout mice. *Nat Neurosci* **12:** 60-69

Lee KS, Linden R, Prado MA, Brentani RR, Martins VR (2003) Towards cellular receptors for prions. *Rev Med Virol* **13:** 399-408

Leggett MM, Dukes J, Pirie HM (1990) A spongiform encephalopathy in a cat. *Vet Rec* **127:** 586-588

Legname G, Baskakov IV, Nguyen HO, Riesner D, Cohen FE, DeArmond SJ, Prusiner SB (2004) Synthetic mammalian prions. *Science* **305:** 673-676

Li A, Christensen HM, Stewart LR, Roth KA, Chiesa R, Harris DA (2007a) Neonatal lethality in transgenic mice expressing prion protein with a deletion of residues 105-125. *EMBO J* **26:** 548-558

Li AM, Barmada SJ, Roth KA, Harris DA (2007b) N-terminally deleted forms of the prion protein activate both bax-dependent and bax-independent neurotoxic pathways. *Journal of Neuroscience* **27:** 852-859

Linden R, Martins VR, Prado MA, Cammarota M, Izquierdo I, Brentani RR (2008) Physiology of the prion protein. *Physiol Rev* **88:** 673-728

Llewelyn CA, Hewitt PE, Knight RS, Amar K, Cousens S, Mackenzie J, Will RG (2004) Possible transmission of variant Creutzfeldt-Jakob disease by blood transfusion. *Lancet* **363:** 417-421

Lloyd SE, Grizenkova J, Pota H, Collinge J (2009) Shadoo (Sprn) and prion disease incubation time in mice. *Mamm Genome* **20:** 367-374

Locht C, Chesebro B, Race R, Keith JM (1986) Molecular cloning and complete sequence of prion protein cDNA from mouse brain infected with the scrapie agent. *Proc Natl Acad Sci U S A* **83:** 6372-6376

Lukasiewicz R, Nolen B, Adams JA, Ghosh G (2007) The RGG domain of Npl3p recruits Sky1p through docking interactions. *Journal of Molecular Biology* **367:** 249-261

Maglio LE, Perez MF, Martins VR, Brentani RR, Ramirez OA (2004) Hippocampal synaptic plasticity in mice devoid of cellular prion protein. *Brain Res Mol Brain Res* **131:** 58-64

Maley F, Trimble RB, Tarentino AL, Plummer TH, Jr. (1989) Characterization of glycoproteins and their associated oligosaccharides through the use of endoglycosidases. *Anal Biochem* **180:** 195-204

Mallucci G, Dickinson A, Linehan J, Klohn PC, Brandner S, Collinge J (2003) Depleting neuronal PrP in prion infection prevents disease and reverses spongiosis. *Science* **302:** 871-874

Mallucci GR, Ratte S, Asante EA, Linehan J, Gowland I, Jefferys JG, Collinge J (2002) Post-natal knockout of prion protein alters hippocampal CA1 properties, but does not result in neurodegeneration. *EMBO J* **21:** 202-210

Manuelidis L (1985) Creutzfeldt-Jakob Disease Prion Proteins in Human Brains. *New Engl J Med* **312:** 1643-1644

Manuelidis L, Tesin DM, Sklaviadis T, Manuelidis EE (1987) Astrocyte gene expression in Creutzfeldt-Jakob disease. *Proc Natl Acad Sci U S A* **84:** 5937-5941

Manuelidis L, Valley S, Manuelidis EE (1985) Specific proteins associated with Creutzfeldt-Jakob disease and scrapie share antigenic and carbohydrate determinants. *Proc Natl Acad Sci U S A* **82:** 4263-4267

Martins VR, Graner E, Garcia-Abreu J, de Souza SJ, Mercadante AF, Veiga SS, Zanata SM, Neto VM, Brentani RR (1997) Complementary hydropathy identifies a cellular prion protein receptor. *Nat Med* **3:** 1376-1382

Mayor S, Riezman H (2004) Sorting GPI-anchored proteins. *Nat Rev Mol Cell Biol* **5:** 110-120

McLennan NF, Brennan PM, McNeill A, Davies I, Fotheringham A, Rennison KA, Ritchie D, Brannan F, Head MW, Ironside JW, Williams A, Bell JE (2004) Prion protein accumulation and neuroprotection in hypoxic brain damage. *American Journal of Pathology* **165:** 227-235

Mead S (2006) Prion disease genetics. *Eur J Hum Genet* **14:** 273-281

Meggio F, Negro A, Sarno S, Ruzzene M, Bertoli A, Sorgato MC, Pinna LA (2000) Bovine prion protein as a modulator of protein kinase CK2. *Biochem J* **352 Pt 1:** 191-196

Meyer RK, Lustig A, Oesch B, Fatzer R, Zurbriggen A, Vandevelde M (2000a) A monomer-dimer equilibrium of a cellular prion protein (PrPC) not observed with recombinant PrP. *The Journal of biological chemistry* **275:** 38081-38087

Meyer RK, Lustig A, Oesch B, Fatzer R, Zurbriggen A, Vandevelde M (2000b) A monomer-dimer equilibrium of a cellular prion protein (PrPC) not observed with recombinant PrP. *J Biol Chem* **275:** 38081-38087

Miesbauer M, Bamme T, Riemer C, Oidtmann B, Winklhofer KF, Baier M, Tatzelt J (2006) Prion protein-related proteins from zebrafish are complex glycosylated and contain a glycosylphosphatidylinositol anchor. *Biochem Biophys Res Commun* **341:** 218-224

Milligan G (2004) G protein-coupled receptor dimerization: function and ligand pharmacology. *Molecular pharmacology* **66:** 1-7

Mitteregger G, Vosko M, Krebs B, Xiang W, Kohlmannsperger V, Nolting S, Hamann GF, Kretzschmar HA (2007) The role of the octarepeat region in neuroprotective function of the cellular prion protein. *Brain Pathol* **17:** 174-183

Miyazawa K, Manuelidis L (2010) Agent-specific Shadoo responses in transmissible encephalopathies. *J Neuroimmune Pharmacol* **5:** 155-163

Mouillet-Richard S, Ermonval M, Chebassier C, Laplanche JL, Lehmann S, Launay JM, Kellermann O (2000) Signal transduction through prion protein. *Science* **289:** 1925-1928

Munter LM, Voigt P, Harmeier A, Kaden D, Gottschalk KE, Weise C, Pipkorn R, Schaefer M, Langosch D, Multhaup G (2007) GxxxG motifs within the amyloid precursor protein transmembrane sequence are critical for the etiology of Abeta42. *The EMBO journal* **26:** 1702-1712

Nicolas O, Gavin R, del Rio JA (2009) New insights into cellular prion protein (PrPc) functions: the "ying and yang" of a relevant protein. *Brain Res Rev* **61:** 170-184

Oesch B, Teplow DB, Stahl N, Serban D, Hood LE, Prusiner SB (1990) Identification of cellular proteins binding to the scrapie prion protein. *Biochemistry* **29:** 5848-5855

Oesch B, Westaway D, Walchli M, McKinley MP, Kent SB, Aebersold R, Barry RA, Tempst P, Teplow DB, Hood LE, et al. (1985) A cellular gene encodes scrapie PrP 27-30 protein. *Cell* **40:** 735-746

Overton MC, Blumer KJ (2000) G-protein-coupled receptors function as oligomers in vivo. *Curr Biol* **10:** 341-344

Overton MC, Blumer KJ (2002) The extracellular N-terminal domain and transmembrane domains 1 and 2 mediate oligomerization of a yeast G protein-coupled receptor. *J Biol Chem* **277:** 41463-41472

Paladino S, Sarnataro D, Pillich R, Tivodar S, Nitsch L, Zurzolo C (2004) Protein oligomerization modulates raft partitioning and apical sorting of GPI-anchored proteins. *J Cell Biol* **167:** 699-709

Pan KM, Baldwin M, Nguyen J, Gasset M, Serban A, Groth D, Mehlhorn I, Huang Z, Fletterick RJ, Cohen FE, et al. (1993) Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins. *Proc Natl Acad Sci U S A* **90:** 10962-10966

Pauly PC, Harris DA (1998) Copper stimulates endocytosis of the prion protein. *J Biol Chem* **273:** 33107-33110

Peden AH, Head MW, Ritchie DL, Bell JE, Ironside JW (2004) Preclinical vCJD after blood transfusion in a PRNP codon 129 heterozygous patient. *Lancet* **364:** 527-529

Perera WS, Hooper NM (2001) Ablation of the metal ion-induced endocytosis of the prion protein by disease-associated mutation of the octarepeat region. *Curr Biol* **11:** 519-523

Plummer TH, Jr., Elder JH, Alexander S, Phelan AW, Tarentino AL (1984) Demonstration of peptide:N-glycosidase F activity in endo-beta-N-acetylglucosaminidase F preparations. *J Biol Chem* **259:** 10700-10704

Premzl M, Gready JE, Jermiin LS, Simonic T, Marshall Graves JA (2004) Evolution of vertebrate genes related to prion and Shadoo proteins--clues from comparative genomic analysis. *Mol Biol Evol* **21:** 2210-2231

Premzl M, Sangiorgio L, Strumbo B, Marshall Graves JA, Simonic T, Gready JE (2003) Shadoo, a new protein highly conserved from fish to mammals and with similarity to prion protein. *Gene* **314:** 89-102

Prince LA, Mann D, Reilly T (2006) Creutzfeldt-Jakob disease: an emergency department presentation of a rare disease. *J Emerg Med* **31:** 41-44

Priola SA, Caughey B, Wehrly K, Chesebro B (1995a) A 60-kDa prion protein (PrP) with properties of both the normal and scrapie-associated forms of PrP. *J Biol Chem* **270:** 3299-3305

Prusiner SB (1982) Novel proteinaceous infectious particles cause scrapie. *Science* **216:** 136-144

Prusiner SB (1989) Scrapie prions. *Annu Rev Microbiol* **43:** 345-374

Prusiner SB, McKinley MP, Bowman KA, Bolton DC, Bendheim PE, Groth DF, Glenner GG (1983) Scrapie prions aggregate to form amyloid-like birefringent rods. *Cell* **35:** 349-358

Prusiner SB, McKinley MP, Groth DF, Bowman KA, Mock NI, Cochran SP, Masiarz FR (1981) Scrapie agent contains a hydrophobic protein. *Proc Natl Acad Sci U S A* **78:** 6675-6679

Quaglio E, Chiesa R, Harris DA (2001) Copper converts the cellular prion protein into a protease-resistant species that is distinct from the scrapie isoform. *J Biol Chem* **276:** 11432-11438

Rambold AS, Miesbauer M, Olschewski D, Seidel R, Riemer C, Smale L, Brumm L, Levy M, Gazit E, Oesterhelt D, Baier M, Becker CF, Engelhard M, Winklhofer KF, Tatzelt J (2008a) Green tea extracts interfere with the stress-protective activity of PrP and the formation of PrP. *J Neurochem* **107:** 218-229

Rambold AS, Miesbauer M, Rapaport D, Bartke T, Baier M, Winklhofer KF, Tatzelt J (2006) Association of Bcl-2 with misfolded prion protein is linked to the toxic potential of cytosolic PrP. *Mol Biol Cell* **17:** 3356-3368

Rambold AS, Muller V, Ron U, Ben-Tal N, Winklhofer KF, Tatzelt J (2008b) Stress-protective signalling of prion protein is corrupted by scrapie prions. *EMBO J* **27:** 1974-1984

Rangel A, Burgaya F, Gavin R, Soriano E, Aguzzi A, Del Rio JA (2007) Enhanced susceptibility of Prnp-deficient mice to kainate-induced seizures, neuronal apoptosis, and death: Role of AMPA/kainate receptors. *J Neurosci Res* **85:** 2741-2755

Resenberger UK, Harmeier A, Woerner AC, Goodman JL, Muller V, Krishnan R, Vabulas RM, Kretzschmar HA, Lindquist S, Hartl FU, Multhaup G, Winklhofer KF, Tatzelt J (2011) The cellular prion protein mediates neurotoxic signalling of beta-sheet-rich conformers independent of prion replication. *EMBO J*

Rieger R, Edenhofer F, Lasmezas CI, Weiss S (1997) The human 37-kDa laminin receptor precursor interacts with the prion protein in eukaryotic cells. *Nat Med* **3:** 1383-1388

Riek R, Hornemann S, Wider G, Billeter M, Glockshuber R, Wuthrich K (1996) NMR structure of the mouse prion protein domain PrP(121-321). *Nature* **382:** 180-182

Riek R, Hornemann S, Wider G, Glockshuber R, Wuthrich K (1997) NMR characterization of the full-length recombinant murine prion protein, mPrP(23-231). *FEBS Lett* **413:** 282-288

Robbins PW, Trimble RB, Wirth DF, Hering C, Maley F, Maley GF, Das R, Gibson BW, Royal N, Biemann K (1984) Primary structure of the Streptomyces enzyme endo-beta-N-acetylglucosaminidase H. *J Biol Chem* **259:** 7577-7583

Roffe M, Beraldo FH, Bester R, Nunziante M, Bach C, Mancini G, Gilch S, Vorberg I, Castilho BA, Martins VR, Hajj GN (2010) Prion protein interaction with stress-inducible protein 1 enhances neuronal protein synthesis via mTOR. *Proc Natl Acad Sci U S A* **107:** 13147-13152

Russo D, Chazenbalk GD, Nagayama Y, Wadsworth HL, Rapoport B (1991) Site-directed mutagenesis of the human thyrotropin receptor: role of asparagine-linked oligosaccharides in the expression of a functional receptor. *Mol Endocrinol* **5:** 29-33

Safar JG, Kellings K, Serban A, Groth D, Cleaver JE, Prusiner SB, Riesner D (2005) Search for a prion-specific nucleic acid. *Journal of Virology* **79:** 10796-10806

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988) Primer-Directed Enzymatic Amplification of DNA with a Thermostable DNA-Polymerase. *Science* **239:** 487-491

Sakaguchi S (2007) Molecular biology of prion protein and its first homologous protein. *J Med Invest* **54:** 211-223

Sakthivelu V, Seidel RP, Winklhofer KF, Tatzelt J (2011) Conserved stress-protective activity between prion protein and shadoo. *J Biol Chem* **286**: 8901-8908

Sanger F, Nicklen S, Coulson AR (1977) DNA Sequencing with Chain-Terminating Inhibitors. *P Natl Acad Sci USA* **74:** 5463-5467

Santuccione A, Sytnyk V, Leshchyns'ka I, Schachner M (2005) Prion protein recruits its neuronal receptor NCAM to lipid rafts to activate p59fyn and to enhance neurite outgrowth. *J Cell Biol* **169:** 341-354

Scharfman HE, McNamara JO (2010) Temporal lobe epilepsy and the BDNF receptor, TrkB. *Epilepsia* **51:** 46-46

Schneider B, Mutel V, Pietri M, Ermonval M, Mouillet-Richard S, Kellermann O (2003) NADPH oxidase and extracellular regulated kinases 1/2 are targets of prion protein signaling in neuronal and nonneuronal cells. *Proc Natl Acad Sci U S A* **100:** 13326-13331

Shental-Bechor D, Levy Y (2008) Effect of glycosylation on protein folding: a close look at thermodynamic stabilization. *Proc Natl Acad Sci U S A* **105:** 8256-8261

Shmerling D, Hegyi I, Fischer M, Blattler T, Brandner S, Gotz J, Rulicke T, Flechsig E, Cozzio A, von Mering C, Hangartner C, Aguzzi A, Weissmann C (1998) Expression of amino-terminally truncated PrP in the mouse leading to ataxia and specific cerebellar lesions. *Cell* **93:** 203-214

Shorter J, Lindquist S (2005) Prions as adaptive conduits of memory and inheritance. *Nat Rev Genet* **6:** 435-450

Shyu WC, Lin SZ, Chiang MF, Ding DC, Li KW, Chen SF, Yang HI, Li H (2005) Overexpression of PrPC by adenovirus-mediated gene targeting reduces ischemic injury in a stroke rat model. *J Neurosci* **25:** 8967-8977

Simons K, Toomre D (2000) Lipid rafts and signal transduction. *Nat Rev Mol Cell Biol* **1:** 31-39

Smirnovas V, Baron GS, Offerdahl DK, Raymond GJ, Caughey B, Surewicz WK (2011) Structural organization of brain-derived mammalian prions examined by hydrogen-deuterium exchange. *Nat Struct Mol Biol* **18:** 504-506

Solomon IH, Huettner JE, Harris DA (2010) Neurotoxic Mutants of the Prion Protein Induce Spontaneous Ionic Currents in Cultured Cells. *Journal of Biological Chemistry* **285:** 26719-26726

Soto C, Saborio GP, Anderes L (2002) Cyclic amplification of protein misfolding: application to prion-related disorders and beyond. *Trends Neurosci* **25:** 390-394

Sparkes RS, Simon M, Cohn VH, Fournier RE, Lem J, Klisak I, Heinzmann C, Blatt C, Lucero M, Mohandas T, et al. (1986) Assignment of the human and mouse prion protein genes to homologous chromosomes. *Proc Natl Acad Sci U S A* **83:** 7358-7362

Spielhaupter C, Schatzl HM (2001) PrPC directly interacts with proteins involved in signaling pathways. *J Biol Chem* **276:** 44604-44612

Spudich A, Frigg R, Kilic E, Kilic U, Oesch B, Raeber A, Bassetti CL, Hermann DM (2005) Aggravation of ischemic brain injury by prion protein deficiency: Role of ERK-1/-2 and STAT-1. *Neurobiology of Disease* **20:** 442-449

Steele AD, Emsley JG, Ozdinler PH, Lindquist S, Macklis JD (2006) Prion protein (PrPc) positively regulates neural precursor proliferation during developmental and adult mammalian neurogenesis. *Proc Natl Acad Sci U S A* **103:** 3416-3421

Stockel J, Safar J, Wallace AC, Cohen FE, Prusiner SB (1998) Prion protein selectively binds copper(II) ions. *Biochemistry* **37:** 7185-7193

Strumbo B, Ronchi S, Bolis LC, Simonic T (2001) Molecular cloning of the cDNA coding for Xenopus laevis prion protein. *FEBS Lett* **508:** 170-174

Strumbo B, Sangiorgio L, Ronchi S, Gready JE, Simonic T (2006) Cloning and analysis of transcripts and genes encoding fish-specific proteins related to PrP. *Fish Physiol Biochem* **32:** 339-353

Tarentino AL, Gomez CM, Plummer TH, Jr. (1985) Deglycosylation of asparagine-linked glycans by peptide:N-glycosidase F. *Biochemistry* **24:** 4665-4671

Tarentino AL, Plummer TH, Jr. (1987) Peptide-N4-(N-acetyl-beta-glucosaminyl) asparagine amidase and endo-beta-N-acetylglucosaminidase from Flavobacterium meningosepticum. *Methods Enzymol* **138:** 770-778

Tateishi J, Kitamoto T (1995) Inherited prion diseases and transmission to rodents. *Brain Pathol* **5:** 53-59

Tateyama M, Abe H, Nakata H, Saito O, Kubo Y (2004) Ligand-induced rearrangement of the dimeric metabotropic glutamate receptor 1alpha. *Nat Struct Mol Biol* **11:** 637-642

Tatzelt J, Maeda N, Pekny M, Yang SL, Betsholtz C, Eliasson C, Cayetano J, Camerino AP, DeArmond SJ, Prusiner SB (1996) Scrapie in mice deficient in apolipoprotein E or glial fibrillary acidic protein. *Neurology* **47:** 449-453

Tatzelt J, Winklhofer KF (2004) Folding and misfolding of the prion protein in the secretory pathway. *Amyloid* **11:** 162-172

Tobler I, Gaus SE, Deboer T, Achermann P, Fischer M, Rulicke T, Moser M, Oesch B, McBride PA, Manson JC (1996) Altered circadian activity rhythms and sleep in mice devoid of prion protein. *Nature* **380:** 639-642

Tompa P, Fuxreiter M, Oldfield CJ, Simon I, Dunker AK, Uversky VN (2009) Close encounters of the third kind: disordered domains and the interactions of proteins. *Bioessays* **31:** 328-335

Toni M, Spisni E, Griffoni C, Santi S, Riccio M, Lenaz P, Tomasi V (2006) Cellular prion protein and caveolin-1 interaction in a neuronal cell line precedes Fyn/Erk 1/2 signal transduction. *J Biomed Biotechnol* **2006:** 69469

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* **76:** 4350-4354

Uppington KM, Brown DR (2008) Resistance of cell lines to prion toxicity aided by phospho-ERK expression. *J Neurochem* **105:** 842-852

Van den Brulle J, Fischer M, Langmann T, Horn G, Waldmann T, Arnold S, Fuhrmann M, Schatz O, O'Connell T, O'Connell D, Auckenthaler A, Schwer H (2008) A novel solid phase technology for high-throughput gene synthesis. *Biotechniques* **45:** 340-343

Vassallo N, Herms J, Behrens C, Krebs B, Saeki K, Onodera T, Windl O, Kretzschmar HA (2005) Activation of phosphatidylinositol 3-kinase by cellular prion protein and its role in cell survival. *Biochem Biophys Res Commun* **332:** 75-82

Vella LJ, Sharples RA, Lawson VA, Masters CL, Cappai R, Hill AF (2007) Packaging of prions into exosomes is associated with a novel pathway of PrP processing. *J Pathol* **211:** 582-590

Viles JH, Cohen FE, Prusiner SB, Goodin DB, Wright PE, Dyson HJ (1999) Copper binding to the prion protein: structural implications of four identical cooperative binding sites. *Proc Natl Acad Sci U S A* **96:** 2042-2047

Wadsworth JD, Collinge J (2007) Update on human prion disease. *Biochim Biophys Acta* **1772:** 598-609

Waggoner DJ, Drisaldi B, Bartnikas TB, Casareno RL, Prohaska JR, Gitlin JD, Harris DA (2000) Brain copper content and cuproenzyme activity do not vary with prion protein expression level. *J Biol Chem* **275:** 7455-7458

Wang F, Wang X, Yuan CG, Ma J (2010) Generating a prion with bacterially expressed recombinant prion protein. *Science* **327:** 1132-1135

Wang H, Wan J, Wang W, Wang D, Li S, Liao P, Hao Z, Wu S, Xu J, Li N, Ouyang H, Gao H (2011) Overexpression of Shadoo protein in transgenic mice does not impact the pathogenesis of scrapie. *Neurosci Lett* **496:** 1-4

Watts JC, Drisaldi B, Ng V, Yang J, Strome B, Horne P, Sy MS, Yoong L, Young R, Mastrangelo P, Bergeron C, Fraser PE, Carlson GA, Mount HT, Schmitt-Ulms G, Westaway D (2007) The CNS glycoprotein Shadoo has PrP(C)-like protective properties and displays reduced levels in prion infections. *EMBO J* **26:** 4038-4050

Watts JC, Huo H, Bai Y, Ehsani S, Jeon AH, Shi T, Daude N, Lau A, Young R, Xu L, Carlson GA, Williams D, Westaway D, Schmitt-Ulms G (2009) Interactome analyses identify ties of PrP and its mammalian paralogs to oligomannosidic N-glycans and endoplasmic reticulum-derived chaperones. *PLoS Pathog* **5:** e1000608

Weber P, Giese A, Piening N, Mitteregger G, Thomzig A, Beekes M, Kretzschmar HA (2007) Generation of genuine prion infectivity by serial PMCA. *Vet Microbiol* **123:** 346-357

Weise J, Sandau R, Schwarting S, Crome O, Wrede A, Schulz-Schaeffer W, Zerr I, Bahr M (2006) Deletion of cellular prion protein results in reduced Akt activation, enhanced postischemic caspase-3 activation, and exacerbation of ischemic brain injury. *Stroke* **37:** 1296-1300

Weissmann C (1994) Molecular biology of prion diseases. *Trends Cell Biol* **4:** 10-14

Westaway D, Daude N, Wohlgemuth S, Harrison P (2011) The PrP-Like Proteins Shadoo and Doppel. *Top Curr Chem*

Westergard L, Christensen HM, Harris DA (2007) The cellular prion protein (PrP(C)): its physiological function and role in disease. *Biochim Biophys Acta* **1772:** 629-644

Wheatley M, Hawtin SR (1999) Glycosylation of G-protein-coupled receptors for hormones central to normal reproductive functioning: its occurrence and role. *Hum Reprod Update* **5:** 356-364

Whittington MA, Sidle KCL, Gowland I, Meads J, Hill AF, Palmer MS, Jefferys JGR, Collinge J (1995) Rescue of Neurophysiological Phenotype Seen in Prp Null Mice by Transgene Encoding Human Prion Protein, (Vol 9, Pg 197, 1995). *Nature Genetics* **9:** 451-451

Will RG, Ironside JW, Zeidler M, Cousens SN, Estibeiro K, Alperovitch A, Poser S, Pocchiari M, Hofman A, Smith PG (1996) A new variant of Creutzfeldt-Jakob disease in the UK. *Lancet* **347:** 921-925

Wille H, Michelitsch MD, Guenebaut V, Supattapone S, Serban A, Cohen FE, Agard DA, Prusiner SB (2002) Structural studies of the scrapie prion protein by electron crystallography. *Proc Natl Acad Sci U S A* **99:** 3563-3568

Williams ES, Miller MW (2002) Chronic wasting disease in deer and elk in North America. *Rev Sci Tech* **21:** 305-316

Williams ES, Young S (1992) Spongiform encephalopathies in Cervidae. *Rev Sci Tech* **11:** 551-567

Winklhofer KF, Heller U, Reintjes A, Tatzelt J (2003a) Inhibition of complex glycosylation increases the formation of PrPsc. *Traffic* **4:** 313-322

Winklhofer KF, Heske J, Heller U, Reintjes A, Muranyi W, Moarefi I, Tatzelt J (2003b) Determinants of the in vivo folding of the prion protein - A bipartite function of helix 1 in folding and aggregation. *Journal of Biological Chemistry* **278:** 14961-14970

Winklhofer KF, Heske J, Heller U, Reintjes A, Muranyi W, Moarefi I, Tatzelt J (2003c) Determinants of the in vivo folding of the prion protein. A bipartite function of helix 1 in folding and aggregation. *J Biol Chem* **278:** 14961-14970

Winklhofer KF, Tatzelt J, Haass C (2008) The two faces of protein misfolding: gain- and loss-of-function in neurodegenerative diseases. *EMBO J* **27:** 336-349

Wong BS, Liu T, Li R, Pan T, Petersen RB, Smith MA, Gambetti P, Perry G, Manson JC, Brown DR, Sy MS (2001) Increased levels of oxidative stress markers detected in the brains of mice devoid of prion protein. *J Neurochem* **76:** 565-572

Wopfner F, Weidenhofer G, Schneider R, von Brunn A, Gilch S, Schwarz TF, Werner T, Schatzl HM (1999) Analysis of 27 mammalian and 9 avian PrPs reveals high conservation of flexible regions of the prion protein. *Journal of molecular biology* **289:** 1163-1178

Wroe SJ, Pal S, Siddique D, Hyare H, Macfarlane R, Joiner S, Linehan JM, Brandner S, Wadsworth JD, Hewitt P, Collinge J (2006) Clinical presentation and pre-mortem diagnosis of variant Creutzfeldt-Jakob disease associated with blood transfusion: a case report. *Lancet* **368:** 2061-2067

Young R, Passet B, Vilotte M, Cribiu EP, Beringue V, Le Provost F, Laude H, Vilotte JL (2009) The prion or the related Shadoo protein is required for early mouse embryogenesis. *FEBS Lett* **583:** 3296-3300

Zanata SM, Lopes MH, Mercadante AF, Hajj GN, Chiarini LB, Nomizo R, Freitas AR, Cabral AL, Lee KS, Juliano MA, de Oliveira E, Jachieri SG, Burlingame A, Huang L, Linden R, Brentani RR, Martins VR (2002) Stress-inducible protein 1 is a cell surface ligand for cellular prion that triggers neuroprotection. *EMBO J* **21:** 3307-3316

Zhang CC, Steele AD, Lindquist S, Lodish HF (2006) Prion protein is expressed on long-term repopulating hematopoietic stem cells and is important for their self-renewal. *Proc Natl Acad Sci U S A* **103:** 2184-2189

Zhou F, Xu W, Hong M, Pan Z, Sinko PJ, Ma J, You G (2005) The role of N-linked glycosylation in protein folding, membrane targeting, and substrate binding of human organic anion transporter hOAT4. *Mol Pharmacol* **67:** 868-876

Abbreviations

APS	Ammonium persulfate
aa	Amino acids
ATP	Adenosine triphosphate
BAX	Bcl-associated X protein
Bcl-2	B-cell lymphoma 2
BiP	Immunoglobulin heavy chain binding protein
bp	Base pairs
BSA	Bovine serum albumin
BSE	Bovine spongiform encephalopathy
^{o}C	Celsius
CHO	N-linked glycosylation
CJD	Creutzfeldt-Jakob disease
CNS	Central nervous system
CWD	Chronic wasting disease
Da	Dalton
DAPI	4',6-diamidino-2 phenylindoldihydrochlorid
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
dGTP	Deoxyguanosine triphosphate
dNTP	Deoxynucleotide triphosphates
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOC	Deoxycholate
DRM	Detergent-resistant membranes
DTT	Dithiothreitol
ECL	Enhances chemiluminescence
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
Endo H	Endoglycosidase H (Endo-β-N-acetylglucosaminidase H)
ER	Endoplasmic reticulum
FCS	Fetal calf serum
FFI	Fatal familial insomnia
FSE	Feline spongiform encephalopathy
g	Standard gravity
GPI	Glycosyl-inositol-Phophatidyl
GSS	Gerstmann-Sträussler-Scheinker syndrome
h	Hour
HD	Hydrophobic domain
HRP,	Horseradish peroxidase
Hsp	Heat shock protein
IF	Immunofluorescence
IP	Immunoprecipitation
kb	Kilo base pairs

kDa	Kilodaltons
S-S	Disulfide bond
M	Molar
MEM	Minimum essential medium
ml	Milliliter
mm	Millimolar
Min	Minutes
Mg	Milligram
MG132	N-(benzyloxycarbonyl) leucinylleucinylleucinal
NMDAR	N-Methyl-D-Aspartate receptor
NMR	Nuclear magnetic resonance spectroscopy
OR	Octa repeat
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PIPLC	Phosphatidylinositol-specific phospholipase C
PK	Proteinase K
PNGase F	Peptide: *N*-glycosidase F
PrP	Prion protein
PrP^C	Cellular prion protein
PrP^{Sc}	Scrapie prion protein
pH	Negative logarithm of the H_3O^+- ion concentration
Ponceau S	3-hydroxy-4-[2-sulfo-4-(4-sulfonatophenylazo)-phenylazo] - 2.7 naphthalene disulfonic acid
SAP	Shrimp alkaline phosphatase
SDS	Sodium dodecyl sulfate
Sho	Shadoo
SOD	Superoxide dismutase
TEMED	N, N, N ', N'-tetramethylethylenediamine
TCA	Trichloroacetic acid
TE	Tris-EDTA
Tris	Tris(hydroxymethyl)aminomethane
Triton X-100	t-octylphenoxypolyethoxyethanol
TSE	Transmissible spongiform encephalopathies
Tween 20	Polyoxyethylen-Sorbitan-Monolaurate
U	Enzyme activity, reaction of 1 mmol substance / min
V	Volt
wt	Wild type

Curriculum vitae

Personal Details

Name	Vignesh Sakthivelu
Date of Birth	07th July 1981 in Coimbatore, India
Father's Name	Mr. Sakthivelu Lingae Gowda
Mother's Name	Mrs. Santhi Sakthivelu
Address	Gmunderstr 7, 81379 München
Marital Status	Single
Nationality	Indian

Education

Oct 2007 - present	**Laboratory for Neurodegenerative Disease Research, Neurobiochemistry, Ludwig-Maximilians-University Munich, Germany** PhD project: "Functional characterization of Shadoo, a PrP-like protein with neuroprotective activity". Supervisor: Prof. Dr. Jörg Tatzelt, Ph.D.
Sep 2003 – Feb 2007	**Bioorganic and Neurochemistry Lab, Central Leather Research Institute, Chennai, Tamil Nadu, India.** **Research Scholar.** Project title: "The neuroprotective efficacy of alpha-crystallin against acute inflammation in mice". Supervisor: Dr.R.Jayakumar, Ph.D.
Jun 2001- Apr 2003	**M.Sc., (Master of Science) in Biotechnology, Periyar University, Salem, Tamil Nadu, India.**
Jun 1998- Apr 2001	**B.Sc., (Bachelor of Science) in Biochemistry, Bharathiyar University, Coimbatore, Tamil Nadu, India.**
Jun 1997- Apr 1998	**HSC (Higher Secondary Course), MSSD Higher Secondary School, Coimbatore, Tamil Nadu, India.**
Jun 1995- Apr 1996	**SSLC (Secondary School Leaving Certificate), SFV Govt. High School, Coimbatore, Tamil Nadu, India**

Publications

Sakthivelu V, Seidel RP, Winklhofer KF, Tatzelt J (2011) Conserved stress-protective activity between prion protein and shadoo. J Biol Chem 286: 8901-8908.

Gunasingh MJ, Philip JE, Ashok BS, Kirubagaran R, Jebaraj WC, Davis GD, **Vignesh S,** Dhandayuthapani S, Jayakumar R (2008) Melatonin prevents amyloid protofibrillar induced oxidative imbalance and biogenic amine catabolism. *Life Sci* 83: 96-102.

Jesudason EP, Masilamoni JG, Ashok BS, Baben B, Arul V, Jesudoss KS, Jebaraj WC, Dhandayuthapani S, **Vignesh S,** Jayakumar R (2008) Inhibitory effects of short-term administration of DL-alpha-lipoic acid on oxidative vulnerability induced by Abeta amyloid fibrils (25-35) in mice. *Mol Cell Biochem* 311: 145-156.

Masilamoni JG, Jesudason EP, Dhandayuthapani S, Ashok BS, **Vignesh S,** Jebaraj WC, Paul SF, Jayakumar R (2008) The neuroprotective role of melatonin against amyloid beta peptide injected mice. *Free Radic Res* 42: 661-673.

Masilamoni JG, **Vignesh S,** Kirubagaran R, Jesudason EP, Jayakumar R (2005) The neuroprotective efficacy of alpha-crystallin against acute inflammation in mice. Brain Res Bull 67: 235-241.

Conferences and workshops attended

Graduate Retreat Neurodegenerative Disease Research
Fraueninsel, Chiemsee, Germany, Oct. 28-30 2007.
Talk: **Characterization of the physiological function of mammalian prion protein**

Ringberg Symposium: Molecular Mechanisms of Prion diseases and Parkinson's disease
Ringberg Castle, Rottach-Egern, Germany, March 5-8 2008

Prion 2009- Transmissible Spongiform Encephalopathies
Porto Carras Grand Resort, Chalkidiki, Greece, 23-25 September 2009

Workshop: Young Researchers Event: Training in scientific communication. Organized by Neuroprion in NIKITI - ELIA BEACH, Greece, 19-22 September 2009

Stipends

PhD fellowship awarded by **DAAD** (German Academic Exchange Service, Germany) from Jun-2007- Mar 2011.

Travel grant awarded to attend the Neuroprion Young Researchers Training Event in Greece from 19-22 Sep 2009.