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Intra- and intercellular trafficking of the prion protein

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The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' (I found it!) but 'That's funny ...'
Isaac Asimov

I was gratified to be able to answer promptly. I said I don't know.
Mark Twain

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I would like to thank first of all my two supervisors.

Without Chiara's drive, support and most importantly immense patience with me, I wouldn't be writing these acknowledgements for my own thesis, because there wouldn't be any. I know that it wasn't easy for her and she certainly grew a few grey hairs due to me. Sorry for that. On the other hand, we also set free a lot of positive energy, which ended in quite a good job, I think. I truly hope that we will manage to bring the submitted manuscripts to a good end.

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I. Abbrevations and Nomenclatures utilized

I.1: Abbreviations

A: alanine

aa: amino acid

BSE: bovine spongiform encephalopathy

CD: compact disc

CHO: chinese hamster ovary

CJD: Creutzfeldt-Jakob disease

CNS: central nervous system

CWD: Chronic Wasting Disease

D: aspartic acid

DC: dendritic cell

DNA: deoxyribonucleic acid

DRM: detergen resistant membrane

E: glutamic acid

EM: electron microscopy

ER: endoplasmic reticulum

fCJD: familial Creutzfeldt-Jakob disease

FDC: follicular dendritic cell

FFI: Fatal familial insomnia

FSE: feline spongiform encephalopathy

G: glycine

GALT: gut associated lymphatic system

GSS: Gerstmann-Sträussler-Scheinker syndrome

GPI: glycosylphophatidylinositol

GTP: guanosine triphosphate

H: histidine

HIV-1: human immunodeficiency virus 1

HLA: human leukocyte antigen

iCJD: iatrogenic Creutzfeldt-Jakob disease

IHC: immunohistochemistry

IHF: immunohistofluorescence

K: lysine

kDa: kilo Dalton

M: molar

MALT: mucosa associated lymphatic system

MHC: major histocompatibility complex

mRNA: messenger RNA

N: asparagine

NaOH: sodium hydroxide

nm: nanometer

nvCJD: see vCJD

P: proline

PIPLC: phosphatidylinositol-specific phospholipase C

PK: proteinase K

PMCA: protein misfolding cyclic amplification

prion: proteinaceous infectious particles

prnp: prion gene

PrP^{C}: cellular (i.e. wild type) prion protein

PrP^{Sc}: scrapie (i.e. infectious) prion protein

PrPres: resistant prion protein (i.e. resistant to proteinase K digestion)

Q: glutamine

R: arginine

rER: rough endoplasmic reticulum

RNA: ribonucleic acid

S: Svedberg

SDS: sodium dodecyl sulfate

SNS: sympathetic nervous system

sCJD: sporadic Creutzfeldt-Jakob disease

T: threonine

TGN: trans-Golgi-network

TME: transmissible mink encephalopathy

TSE: transmissible spongiform encephalopathy

TNT: tunneling nanotubes

UK: United Kingdom

USA: United States of America

V: valine

vCJD: variant Creutzfeldt-Jakob disease

W: tryptophan

I.2: Nomenclature and definition of the different forms of PrP

PrP is an acronym standing for "protease resistant protein". S. Prusiner coined the term prion (acronym for proteinaceous infectious particle); this term usually describes the infectious agent of the diseases Transmissible Spongiform Encephalopathies (TSEs).

PrP^{C}: Cellular PrP, the wild-type form of PrP, expressed in a wide variety of cells such as neurons, lymphoid system and others

PrP^{Sc}: PrP scrapie, the pathogenic and transmissible isoform of PrP^{C}, causative agent of TSEs

PrPsen: PrP sensitive to treatment with proteases. This term specifies that only a certain percentage of PrP^{Sc} is resistant to treatment with proteases (Tzaban et al., 2002).

PrPres: PrP resistant to digestion with proteinase K (PK), opposite of PrPsen

PrP: Utilized, to indicate the whole pool of PrP-proteins, be it PrP^{C}, PrP^{Sc} or a mixture of both. Also utilized when differentiation between native and pathologic form is impossible.

PrPmut: Derives from mutant PrP and describes a pathologic form of PrP caused by a mutation in the *prnp*-gene, as found in inheritable TSEs in humans.

II. A brief chronology of Transmissible Spongiform Encephalopathies (TSEs)

The first Transmissible Spongiform Encephalopathies (TSEs) were described in the 18th century in sheep (1732) and in goat (1772). Lacking deeper insight, the malady was called scrapie, due to the typical behaviour of the diseased animals, suffering from an intense pruritus and scraping themselves on walls, trees etc (Detwiler, 1992). The first TSE in cattle had already been described in 1883, but was bound to remain a sole and isolated case until the advent of the Bovine Spongiform Encephalopathy (BSE) epidemic, also known as „Mad Cow Disease“, in the 1980s mainly in the United Kingdom. The first cases of human TSEs were described by two German neurologists Creutzfeldt (1885-1964) and Jakob (1884-1931) in the years 1920 and 1921, who also became the eponyms for the most common of the TSEs in humans, the Creutzfeldt-Jakob disease (CJD). An important contribution came from two French scientists in 1936, Cuillé and Chelle, who managed to prove the transmissibility of scrapie by inoculating healthy animals with nervous tissue from diseased animals. In the 1950s-70s Lindenbaum, Zigas and Gajdusek described the ailment Kuru, an endemic disease in the Fore people of Papua New Guinea. Already in 1959, Hadlow reported that scrapie and Kuru have common histopathological and clinical traits, making a link between an animal and a human form of TSE for the first time (Hadlow, 1959; Hadlow, 1995). In the same year, Gajdusek managed to transmit Kuru from brain material of deceased humans to chimpanzees by intracerebral injection, while in the year 1961, Chandler managed to pass scrapie to mice (Chandler, 1961). In 1976 Gajdusek received one of three Nobel Prizes to be given to researchers in the TSE-field.

In 1979 Stanley Prusiner started to work on the concept of an infectious agent completely devoid of DNA and judging from his results, entirely based on protein (Prusiner et al., 1980a; Prusiner et al., 1980b). In 1982, when presenting for the first time his heretical concept of the “prion”, an acronym of “proteinaceous infectious particles“, he surely must have caused a stir in his audience and was awarded the Nobel Prize for his work in 1997. In the early 1980’s, the first CJD-cases caused by transplants are reported in the United States and Australia, as well as cases caused by cadaver-derived growth hormone-treatments in Japan, the United States and France (Billette de Villemeur et al., 1994). The cause being medical interventions, this type of disease was called iatrogenic CJD (iCJD). Starting from 1985, the first BSE-cases were reported in the United Kingdom (UK), the epidemic gaining momentum and peaking in 1992, when

a total of 37000 cattle were found to test positive. Only four years later, in 1996, a link was established between the consumption of BSE-contaminated foodstuff and the emergence of a new form of CJD, termed variant or new variant CJD (vCJD/nvCJD). This news produced a large economic commotion in the UK and Europe leading to embargoes on beef and products deriving from cattle (e.g. cartilage) coming from the UK. From 1996 on, many laboratories showed that the vCJD-agent is the same as found in BSE and that foodstuffs can transfer the infectious particle. Since 2000 the numbers of cases in the UK have subsided (Andrews et al., 2003).

III. Introduction

III.1: On the nature of prions

Prions are able to propagate and multiply, nevertheless the cause for TSE seems to be a very non-conventional pathogen, because it could not be shown to be associated with any form of nucleic acid as a carrier of biological information and therefore does not fit into any classical virological model. Its inability to be inactivated by classical procedures utilized on viruses or bacteria (e.g. heat, irradiation with ultraviolet radiation) led scientists to discard the theory that TSEs were caused by extremely slow viruses and put forward a new "protein-only"-hypothesis, suggesting that prions consist of only an infectious protein. Even today, despite strong support from various laboratories (Castilla et al., 2005a; Legname et al., 2004), the proteinaceous nature of prions is still contested by some researchers (Broxmeyer, 2004; Manuelidis et al., 2007).

III.1.1: Prions are not easily inactivated by ionizing radiation: Is it therefore an infectious protein?

The first researcher to study the unusual biochemical characteristics of prions was Tikvah Alper, who described the unusually small size of prions (Alper et al., 1966). He also found that scrapie-containing brain material remained infectious despite a harsh treatment with ionizing radiation capable of destroying all nucleic acids (DNA and RNA) (Alper et al., 1967; Latarjet et al., 1970).

Contrary to bacteria and viruses, prions are extremely resistant not only to irradiation but resist all the other noxious treatments used in microbiology including humid or dry heat, chemical inactivators such as alcohols or formalin (Fichet et al., 2004; Taylor, 2004) or incubation at 100°C in the presence of sodium dodecyl sulfate (SDS) or urea and variations thereof. Efficacious means of inactivating prions align with procedures aimed at destroying proteins and include phenols, 1N NaOH (for 1 hr at 20°C) (Ernst and Race, 1993)), 4M guanidium hypochlorite or guanidium isothiocyanate, 2,5 % Sodium Hypochlorite (1 hr at 20°C) (Brown et al., 1986), autoclaving with humid heat at 136°C for 18 minutes (Kimberlin et al., 1983) or with dry heat at 160°C for 24 hrs (Dickinson and Taylor, 1978).

Even though Alper's work lead him to conclude that prions might be deprived of nucleic acids, it was Griffith a few years later who proposed that prions are proteins that are capable of adopting an abnormal structure and autoreplicate themselves by imprinting this new structure on to another protein (Griffith, 1967). His innovative and revolutionary theory was negated for a long time, since none of the experts in the field could imagine an infectious particle without storing its information in some form of nucleic acid.

It was only fifteen years later (in 1982), that Stanley Prusiner was able to support the ingenious hypothesis of Griffith by isolating the proteinaceous etiological agent of TSEs termed prions, thus showing that prions are indeed deprived of nucleic acids (Bolton et al., 1982; Prusiner, 1982) and that its infectivity can only be reduced by agents which denature proteins (McKinley et al., 1983).

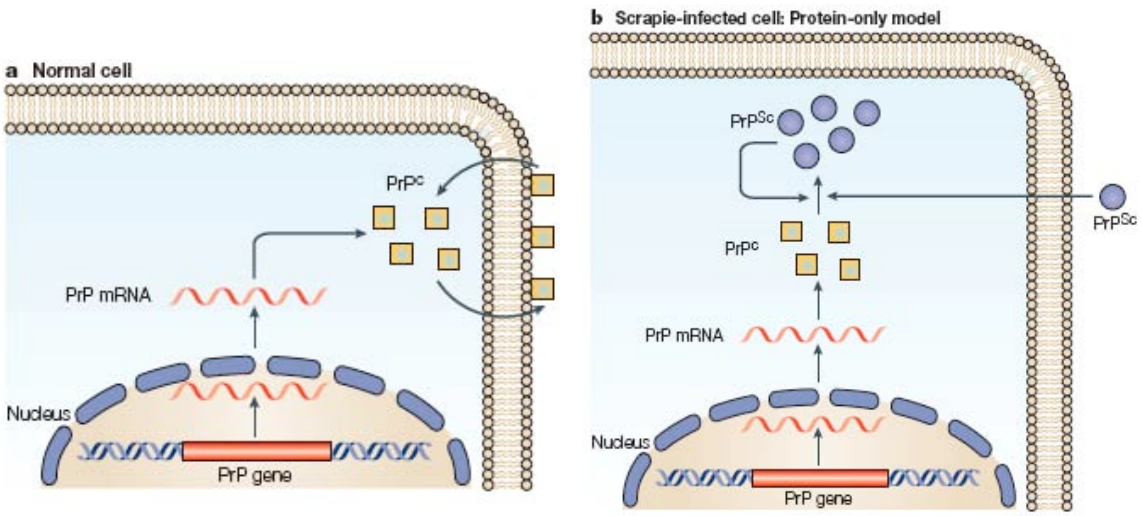

Fig. 1: The "protein-only" hypothesis proposes that the infectious entity in TSEs is an abnormal isoform of PrP^C, termed PrP^{Sc}. PrP^C is an endogenous membrane-bound protein that traffics between the plasma membrane and intracellular vesicles of cells (**A**). Upon exposure to and uptake by cells of exogenous PrP^{Sc}, new PrP^{Sc} is produced through catalytical conversion from the pool of endogenous PrP^C (**B**) (Weissmann, 2004).

According to Prusiner's "protein-only" hypothesis, prions, also termed PrP^{Sc} (Sc for scrapie), are able to replicate by converting the regular endogenous protein PrP^C (C for cellular) into an abnormal toxic form, which accumulates in the cell and produces in due time the amyloid deposits typical for TSEs (see Figure 1).

There are numerous lines of evidence that support the hypothesis put forward by Prusiner and colleagues (Prusiner, 1998):

a) Infectivity co-purifies with an identifiable macromolecule, PrP^{Sc}, which is the pathogenic conformation (partially resistant to proteases, hence also PrPres (res for resistant)) of a normal cellular protein called PrP^{C}.
b) PrP^{Sc} accumulates proportionally with infectious titre without the augmentation of its corresponding mRNA
c) Knock-out mice deprived of their endogenous PrP^{C} are resistant to infection with PrP^{Sc}.
d) Protein-denaturing agents reduce infectivity of infectious samples, while treatments aimed at destroying the RNA/DNA contained in the samples show no reduction in infectivity.
e) Certain mutations in the *prnp*-gene (on chromosome 20 in humans) lead to pathological forms of its corresponding protein, PrP^{C}, and lead to genetically encoded forms of TSEs.

Strong support for the exclusively proteinaceous nature of prions has been recently provided by two different laboratories, that were able to produce infectious prions *in vitro* using different approaches. Legname and coworkers produced infectious prions starting from a recombinant protein, while Castilla and colleagues succeeded to multiply PrP^{Sc}-amounts starting from minimal amounts deriving from infected tissue (Castilla et al., 2005a; Legname et al., 2004). The first group expressed a murine truncated form of PrP^{C} in *E. coli* and managed to polymerize a subset of this into amyloid fibrils. These fibrils were shown to be protease-resistant similar to PrP^{Sc} and induced neurological dysfunction when inoculated into mice (Legname et al., 2004). These results demonstrated that infectious prions can be produced in an *in vitro* system. However it must be mentioned that the mice used for the inoculation assays in this work, were previously shown to spontaneously develop symptoms resembling TSEs (Castilla et al., 2005a; Chiesa et al., 1998; Westaway et al., 1994). Strong arguments in favour of the proteinaceous nature of prions also came from Castilla and colleagues who developed a new procedure to produce *de novo* PrP^{Sc} with cycles of sonication using PrP^{C} as a substrate. They termed this procedure "protein misfolding cyclic amplification" (PMCA, see Paragraph IX) and showed that this *in vitro* produced material was infectious after inoculation in hamsters, which developed symptoms strongly resembling TSEs (Castilla et al., 2005a). Additional surprising support for the protein-only hypothesis came from a rather unexpected research field of biology. Several groups were able to show that the yeast *Saccharomyces cerevisiae* among

others produced anomalous forms of endogenous proteins (e.g. Ure2, Sup35, Rnq1) with a tendency to convert and aggregate, resembling clustered prion-fibrils. Striking structural parallels were recently shown for Sup35 from yeast and prions from humans using X-ray microcrystallography (Sawaya et al., 2007). Furthermore, another research group was able to produce yeast-prions *in vitro* (Brachmann et al., 2005). It is also worthwhile to mention that the aggregates of these proteins are transmitted horizontally by cytoplasmic mixing and mating and some seem to have a deleterious effect on the carrier. Interestingly, these proteins and the phenotypes caused by them in yeast, are considered to be rapid systems of adaptation to changes in the environment of the organism and which, upon misfolding, behave like inheritable genetic elements (reviewed in (Tuite and Cox, 2003)).

III.1.2: Prions: a viral agent?

In spite of the widely accepted theory on the proteinaceous nature of prions, the alternative hypothesis of prions being a slow virus (Eklund et al., 1967; Manuelidis et al., 2007) or a virino (a nucleic acid smaller than a virus, coated with host proteins and therefore not raising an immune response by the infected organism (Dickinson and Outram, 1988)), capable exploiting PrP^{C} as its receptor (Mestel, 1996) (see Fig. 2) still retains ardent supporters today.

The advocates of the viral theory believe that certain, very small viruses can escape inactivation by irradiation and that infectivity is strongly reduced by chemical agents negatively affecting viral core components but not PrP^{Sc} (Manuelidis, 2003). The viral hypothesis would indeed explain some of the characteristics of prion-linked diseases, but all experiments to purify a prion-specific nucleic acid have failed (Lansbury and Caughey, 1996). The Manuelidis group, one of the leading supporters of the viral hypothesis, showed that some nucleic acids copurify with the infectious material found in CJD, and that it is possible to separate the majority of PrP^{Sc} from the infectious fraction in human brain-samples (Akowitz et al., 1993; Sklaviadis et al., 1992).

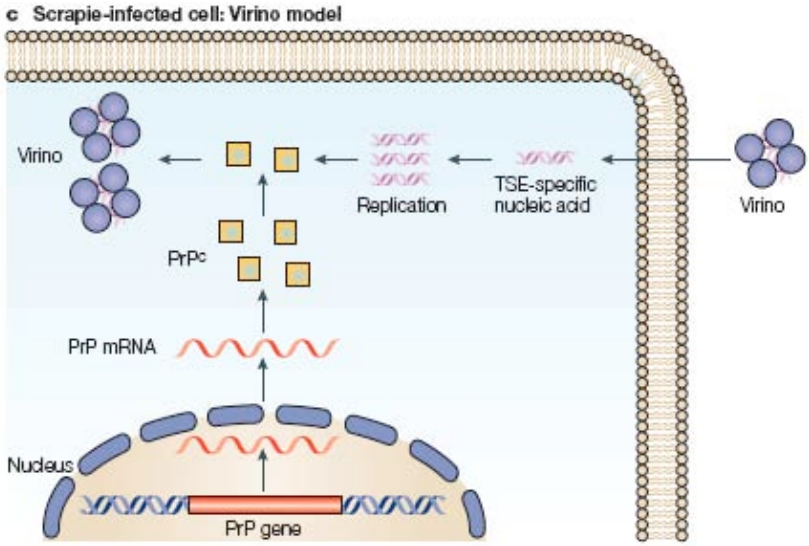

Fig. 2: The virino model. The infectious agent is a TSE-specific nucleic acid packaged in PrP^{Sc}. Upon uptake the nucleic acid is replicated by the host machinery, associates with endogenous PrP^{C}-molecules and converts these to PrP^{Sc}. (from (Weissmann, 2004)).

These studies conclude that the fraction containing the majority of PrP^{Sc} is much less infectious than the fraction containing little PrP^{Sc} and the nucleic acids, suggesting that infectivity is not contained in the protein but in the nucleic acids (Manuelidis et al., 1995). Consequently this group identified the nucleic acids specific for CJD (Manuelidis and Fritch, 1996) and propose that the infectious agent belongs to the class of retroviruses (Dron and Manuelidis, 1996). These results are further corroborated by more recent studies reporting that the rate of conversion of prions is higher in the presence of DNA (Cordeiro et al., 2001) or RNA (Deleault et al., 2003). Additionally, it is postulated that PrP^{C} might have a 3D-structure favourable for binding nucleic acids (Radulescu and Korth, 1996), and it has been recently shown that PrP^{C} interacts with viral RNAs and has chaperoning properties, similar to the nucleocapsid proteins NCP7 of HIV-1 (Gabus et al., 2001a; Gabus et al., 2001b; Moscardini et al., 2002). It was also demonstrated that co-expressed PrP^{C} and HIV-1 interfere with each other: the amount of expressed cellular PrP^{C} negatively influences the amount of produced HIV-1, while the production of HIV-1 leads surprisingly to production of PrPres by an unknown mechanism (Leblanc et al., 2004). The latest results from the same group show that retroviral infection strongly enhanced the release of scrapie infectivity into the supernatant of cultured cells, once more emphasizing the link between viruses and prions (Leblanc et al., 2006).

III.2: TSE in animals

The TSEs are diseases characterized by their specific lesions in the central nervous system (CNS). By nature, both human and animal forms are transmissible, shown in experimental conditions with rodents and primates. In spite of these data, this group of diseases is not regarded to be contagious.

III.2.1: Clinical signs of TSEs

The clinical presentations of these diseases is characterised by a very long asymptomatic incubation period preceding the onset of first symptoms a rapid progression of the disease, leading to neurodegeneration and inevitable death of the organism. The neurodegeneration is associated with very specific clinical symptoms, typical for each type of TSE and include perturbations of the locomotor -- and sensory system, rapid progressive dementia with clinical visual or cerbellar signs and akinetic mutism (i.e. passivity and inability to speak). Behavioural changes in humans manifest early in the disease and may vary but commonly include personality changes, such as psychiatric problems like depression, lack of coordination, in some cases also unsteady gait (ataxia). Patients can also produce uncontrolled jerking movements termed myoclonus and suffer from insomnia, confusion or memory problems (Collinge, 2001).

III.2.2: The histopathology of TSEs

Autopsy of afflicted animals shows non-inflamed degenerative lesions of the CNS, particularly affecting the cerebellum. Typical for these lesions are vacuoles, conferring a spongiform appearance, with a loss of neurons and astrogliosis as an invariable feature (see Figure 3).

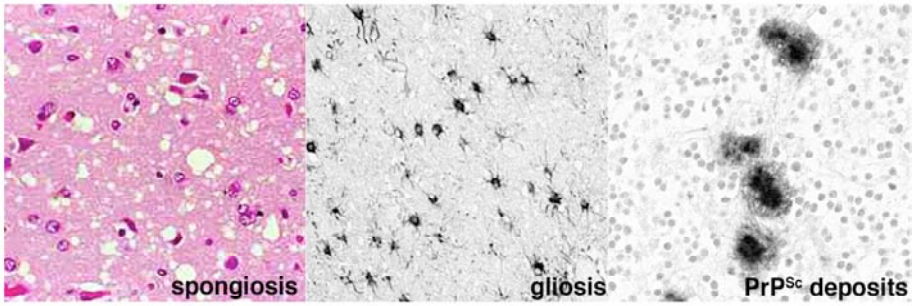

Fig. 3: Characteristic neuropathologies of TSEs. Analysis of grey matter from brain sections of a CJD-victim shows spongiform vacuoles (left, staining with hematoxylin), activation and proliferation of astrocytes (center, IHC with antibody anti-GFAP) and PrP^{Sc}-deposits (left, IHC with antibody anti-PrP) (adapted from (Glatzel and Aguzzi, 2001).

Due to the absence of the immune response, the histological exams do not reveal infiltration of lymphocytes and macrophages. With biochemical techniques it is possible to reveal the presence of PrP^{Sc} (see later). To date diagnostic methods are restricted to the clinical phase and post-mortem diagnostics, but preclinical assessment from blood samples has been recently reported, giving rise to the possibility of early diagnosis of TSE-diseases (Castilla et al., 2005b).

III.2.3: The main forms of TSEs

In the following paragraph an outline of the different TSEs will be given, focusing on the different forms manifesting in various animal hosts, as well as on the different forms found in humans.

III.2.3.1: Scrapie in sheep and goat

III.2.3.1.1: Description

Scrapie in sheep and goats was the first TSE-disease to be described (1732 in England, 1759 in Germany) and was shown to be experimentally transmissible as early as the 1930's by Cuillé and Chelle. The aetiology of scrapie was debated since the beginning of the 19th century but remains unanswered. Some authors suggested spontaneous appearance and concomitant transmissibility, while other supposed it to be caused by

ill-defined pathogens, such as amoeba by Girard in 1830. Due to extensive protective measures preventing introduction of scrapie from imported sheep, Australia and New Zealand are the sole sheep-raising countries considered to be free of scrapie (MacDiarmid, 1996). The disease afflicts animals between two and five years of age and the clinical phase lasts between two and six months (Kimberlin, 1981). The disease has a major economic impact due to its high incidence in the main breeding regions (e.g. the United Kingdom), but no transmission of scrapie to humans has ever been observed (Jeffrey and Gonzalez, 2004). The natural scrapie disease initially manifests itself with mildly impaired social behaviour such as unusual restlessness and nervousness, often only recognized by experienced shepherds (Dickinson, 1976). Later stages are manifesting with either intense pruritus coming from the animal's try to relieve an intense irritation, leading to loss of fur and skin. Despite ataxia, infected sheep will walk long distances to indulge in attacks of scratching, coining in German the name of the disease "traberkrankheit" (trotting disease) (Dickinson, 1976). In the very late stages of scrapie, affected sheep waste away, walk only short distances and are easily agitated by even the mildest stress. Animals have normal appetite, but loose the ability to feed themselves. In goat an additional form manifesting a lethargic phenotype has been reported and is transmissible to sheep (Brugere-Picoux and Chatelain, 1995). The pathogenic agent in affected organisms is distributed in the CNS, the amygdala, the spleen (Race et al., 1998) and the placenta (Onodera et al., 1993). The vertical transmission is probably caused by the presence of scrapie in the placenta (Andreoletti et al., 2000; Andreoletti et al., 2002; Tuo et al., 2002). Recently it was also shown that the accumulation of PrP^{Sc} in neuro-muscular fasci, precedes the occurence of clinical signs but that infectivity associated with the muscle tissue is minimal (Andreoletti et al., 2004).

III.2.3.1.2: Susceptibility

The individual susceptibility of each sheep is defined by a genetic predisposition, caused by the existence of different alleles coding for PrP^{C} (Baylis and Goldmann, 2004). Polymorphisms at amino acid (aa) position 136 (A or V), 154 (R or H) and 171 (Q or R) of PrP^{C} in the animal are directly responsible for its susceptibility. Animals

homozygously expressing the VRQ-combination in the respective positions are, independent of race or geographic position, much more likely to succumb to scrapie, while animals expressing homozygously the ARR-combination and some other aa-substitutions have never been found to be affected by scrapie (Goldmann et al., 1991; Hunter et al., 1996; Hunter et al., 1997; Vaccari et al., 2007). Due to these results, the European Union developed some years ago a programme with the intent to eliminate all VRQ-haplotypes by completely exchanging the stocks with ARR-expressing animals. The long-term success of this plan still needs to be proven, especially since it was shown that the genetic resistance in the ARR-variant sheep could be overcome with high doses of bovine-derived prions (Houston et al., 2003). Additionally, some cases of atypical scrapie (in respect to deposition and glycotype of the scrapie forms detected in brain material) were found in animals considered to be resistant (Benestad et al., 2003). Thus, while genetic susceptibility is clear in the case of the VRQ-haplotype, more research is required in order to determine the full spectrum of genetic susceptibility to scrapie strains in these animals.

III.3: Bovine Spongiform Encephalopathy (BSE)

As previously mentioned, the first cases of BSE occured in the United Kingdom in 1986, peaking in 1992 when about 37000 cattle were tested positive for the disease (Jeffrey and Gonzalez, 2004).

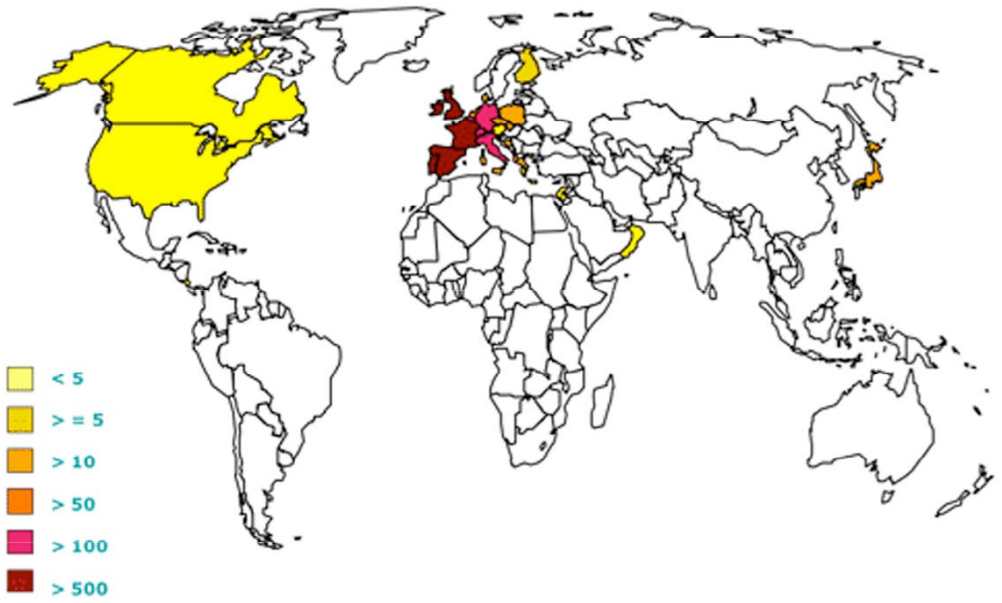

Fig. 4: Distribution of BSE in the world. (from(Belay et al., 2004))

In the time period between 1987 and 1999, approximately 200,000 cattle tested positive for BSE in the UK and the bleak number of 200,000,000 BSE-positive cattle is estimated to have entered the food chain with much less detected outside of the UK (see also Fig. 5).

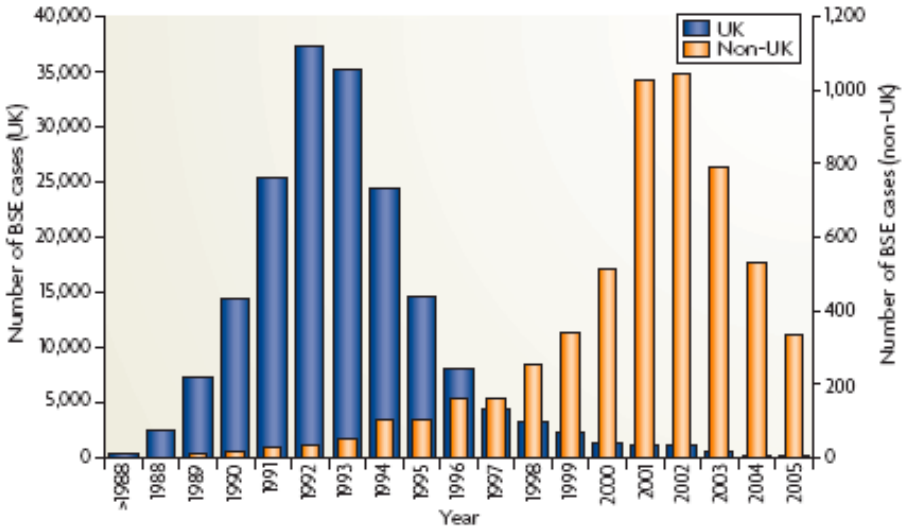

Fig. 5: Numbers of BSE-infected cattle reported in the UK and outside over the years. Shown in blue are the numbers of cattle reported only in the UK, while the numbers of infected cattle from EU-countries excluding the UK as well as Canada, Israel, Japan, Switzerland and USA are indicated in orange (from (Aguzzi et al., 2007)).

Animals show first symptoms at an average age between 3 and 6 years with a striking resemblance to sheep with scrapie, while the clinical phase afterwards is much shorter and lasts 1 to 6 months. They also present an altered behaviour (apprehension and sometimes aggressiveness), ataxia (uncoordinated gait with occasional falling) and abnormal responses to touch and sound (dysesthesia). Epidemiologic studies point to forage mixed with carcass meal as the main culprit for the spread of the BSE-disease (Wilesmith et al., 1992), however the real origins of the disease remain unknown but are supposedly linked to scrapie in sheep (rev. (Balter, 2001)).

The ban of carcass meal-usage in forage, established in 1988 in the UK as a prompt reaction to the outbreak, resulted in a marked decrease of BSE-cases starting from 1993. In spite of these measures, several cases of BSE were reported in cattle born after the ban, suggesting horizontal transmission of infection by other foodstuffs or vertical infection from the infected mothers. Placental transfer has been shown to be inefficient but possible (10% of calves born from infected mothers are infected) (Wilesmith and Ryan, 1997) and to be dependent on the phase of progression of the disease in the mother (Donnelly et al., 1997), while other reports suggest that bovine placenta is not infectious (rev. in (Wrathall, 1997)). However, prions seem to be concentrated merely in the distal ileum and the CNS of infected animals (Aguzzi and Polymenidou, 2004). In light of recent results other ways of transfer of infection could be envisioned: urinary excretion due to nephritis of the kidneys (Seeger et al., 2005) and excretion by the mammary glands in connection with mastitis (Ligios et al., 2005). Recently, it was also reported that prions could persist and remain infectious in soil and aqueous soil extracts for at least 29 months (Seidel et al., 2007), rendering the possibility of infection of new-borns by excreta quite plausible. Susceptibility differences due to genetic factors as shown in sheep, have not been discovered yet. Very recently a group succeeded in producing cattle lacking the prion protein (Richt et al., 2007), which appeared to be physiologically normal. Brain tissue homogenates from these cattle, tested for prion propagation by protein misfolding cyclic amplification (PMCA) were resistant, suggesting that these cattle could be used for making BSE-resistant herds.

III.4: Chronic Wasting Disease (CWD)

CWD is found only in the US and Canada and affects only cervids (i.e. deer, elk and moose) (Baeten et al., 2007; Miller and Williams, 2004). This disease appeared initially in the 1960s in Colorado, but was recognized to belong to the family of TSEs only in 1978. As shown in Figure 6, the cases have a strong geographic focus in the Rocky Mountains of Colorado. CWD is notorious for its rapid spread within herds in North America.

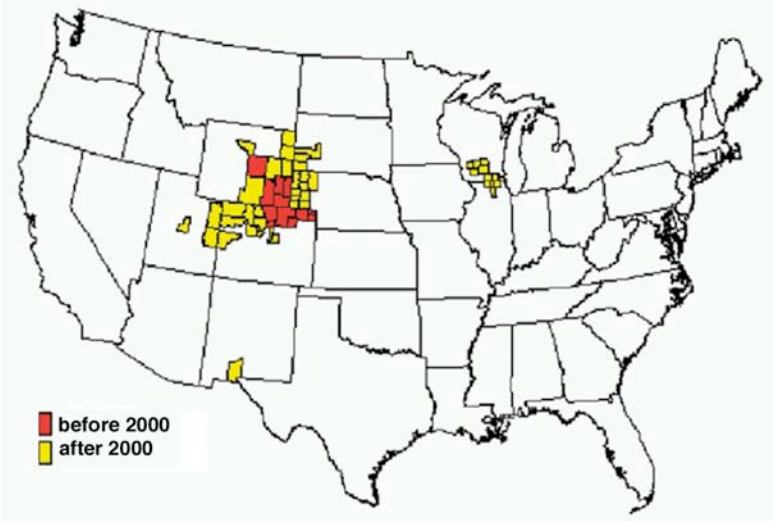

Fig. 6: Distribution and spread in time of CWD in the US. (from (Belay et al., 2004))

The majority of cases develop at 3-4 years of age, and animals succumb to the disease very quickly over the course of 2 weeks to 8 months. The routes of transmission of CWD remain unknown. Experiments with humanized mice (i.e. expressing human PrP^{C}) indicate a species barrier for CWD in humans suggesting that a species barrier for infection of humans with CWD exist (Belay et al., 2004; Kong et al., 2005). Recently caribou were also shown to host the prion protein gene (Happ et al., 2007). Additionally bank voles (*Clethrionomys glareolus),* a wild rodent species, were found to have a high susceptibility to prion infections so that they are currently under investigation as a new experimental system (Cartoni et al., 2005; Nonno et al., 2006; Zanusso et al., 2007).

III.5: Other animal transmissible encephalopathies

Animal prion diseases in addition to those mentioned above have also been reported. Transmissible mink encephalopathy (TME) was the first TSE to be identified in non-domestic animals. Captive mink (Mustela vison) develop TME very rarely and it is believed to be associated to exposure of BSE-contaminated feed (rev. in (Williams and Miller, 2003)). Other very rare TSE-diseases include the feline spongiform encephalopathy of zoological and domestic cats (FSE), TSE in bovids and TSE in non-human primates. All these subforms appear to be linked to the BSE epidemic (rev. in (Sigurdson and Miller, 2003)).

III.6: Human encephalopathies

The human forms of prion related disease have different aetiologies (Table 1). These include sporadic, genetic and acquired subtypes. The sporadic forms were the first to be described in the 1920's by Creutzfeldt and Jakobs and are the most frequent forms. Genetic prion diseases include Creutzfeldt-Jacobs-Disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS) and Fatal Familial Insomnia (FFI). Finally, human TSEs can be acquired by ingestion of contaminated foodstuffs or by iatrogenic infection during surgical procedures (rev. in (Collinge, 2001)).

Type	**Cause**
Aquired forms	
Kuru	ritual cannibalism
iatrogenic CJD	Use of prion-contaminated surgical instruments and prion contaminated transplants (e.g. dura mater, cadaveric growth hormones, etc.)
new variant CJD	probable infection with BSE
Sporadic forms	
sporadic CJD	Spontaneous conversion of PrP^{C} into PrP^{Sc} or somatic mutation
Sporadic Fatal Familial Insomnia	
Familial forms	
CJD	Germinal mutation of the *prnp*-gene
Gerstmann-Sträussler-Scheinker syndrome (GSS)	Germinal mutation of the *prnp*-gene
Fatal Familial Insomnia (FFI)	Mutation D178N of the *prnp*-gene in association with M129 polymorphism

Table 1: Human forms of TSEs.

III.6.1: The sporadic forms

Sporadic CJD represents about 80-85% of all CJD cases. The incidence rate is between 1-1,67/million people per year and is homogenously distributed over the world without any sexual preference and without any apparent link with scrapie or BSE (Johnson and Gibbs, 1998; Ladogana et al., 2005; Linsell et al., 2004). The aetiology is not believed to be infectious, since surgeons, pathologists, abattoir workers, butchers and cooks are not overrepresented among CJD-patients (Harries-Jones et al., 1988). Furthermore, long-term exposure to afflicted individuals does not seem to increase the risk of infection, with only one conjugal case documented (Brown et al., 1998). Lifelong vegetarians have also been reported to develop sporadic CJD (Matthews and Will, 1981), suggesting that development of disease is not linked to exogenous infection by contaminated foodstuffs. The average onset of disease is at 60 years with occasional cases occurring in individuals less than 40 years of age or older than 80 years (Brown et al., 1994). The median time to death in sporadic cases is only 4-5 months (Johnson and Gibbs, 1998). The pathology is limited to the brain and spinal cord. Neuronal loss occurs with vacuolisation in cell bodies and dendrites giving the cerbral cortex a spongiform phenotype with absence of amyloid plaques (Fig. 7).

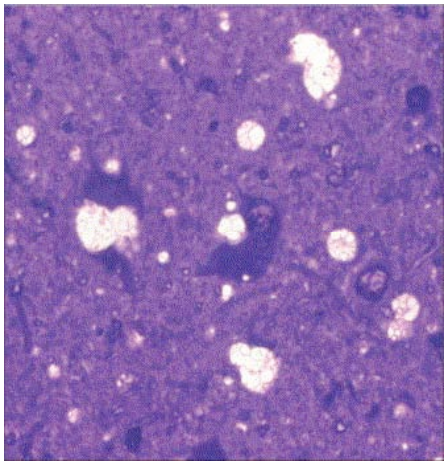

Fig. 7: Histopathology of sporadic CJD in a post-mortem brain biopsy. Note the spongiform vacuolisation of the tissue. Staining with cresyl violet, 300x (from(Johnson, 2005)).

III.6.2: Familial or hereditary prion diseases

Heritable disease represents about 15% of all cases of human TSEs. All hereditary forms are linked to mutations in the *prnp*-gene situated on chromosome 20 in humans. More than 50 mutations have been identified to date and all are inherited in an autosomal-dominant fashion (Fig. 8).

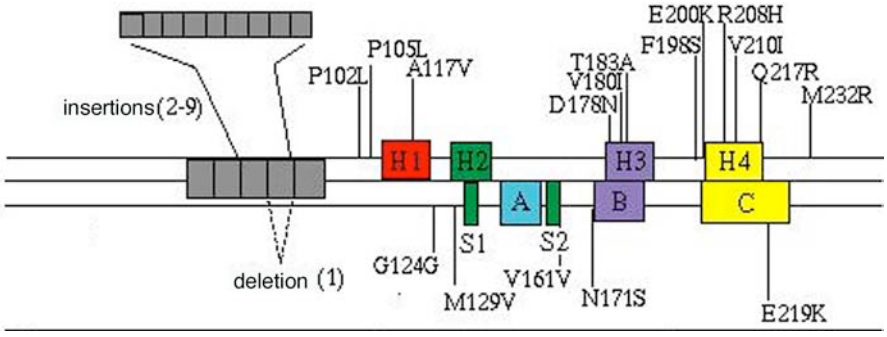

Fig. 8: Overview of most frequent genetic mutations and of polymorphic sites in the human *prnp*-gene (from (Prusiner, 1998)).

The most prominent marker of susceptibility to CJD is a polymorphism at codon 129 of the *prnp*-gene, which contains either methionine or valine. It was reported that more than 80% of patients with sporadic CJD are homozygous for methionine at this site in comparison to only 49% of healthy controls, suggesting an inheritable genetic predisposition for sporadic CJD (Palmer et al., 1991). This polymorphism is also predictive of the subtype of disease, developed by carriers of the mutation D178N

(exchange of aspartic acid to asparagine at aa-position 178): Individuals homozygous for valine at position 129 develop fCJD, while individuals homozygous for methionine at this region develop FFI (Goldfarb et al., 1992; Monari et al., 1994). The reason for this genetic link is still unclear, but it is widely believed that the silent polymorphism at position 129 influences the tertiary structure of the pathologic isoform of PrP. Four of the point mutations found in familial CJD (fCJD), affecting the codon positions: 102, 178, 200 and 210 and insertions of 5-6 octapeptide repeats, account for 95% of all familial cases (Capellari et al., 2005). Onset of disease is usually between 50-65 years of age, while the clinical phase lasts from 1 to 13 months. The most common fCJD mutation occurs at position 200 (Hsiao et al., 1991), followed by lower occurrences at numerous others at positions: 105, 148, 160, 178, 180, 183, 187, 188, 198, 198, 203, 210, 212, 208, 210, 211, 212, 217, 232, 238 (compare with Fig. 8). In terms of onset and progress of disease, forms of familial CJD (fCJD) generally develop earlier and have a longer clinical course than sporadic CJD. Nevertheless this does not apply generally, since the most common fCJD-mutation at codon position 200 for instance, resembles more the pathology of sporadic CJD (Mastrianni et al., 2001).

Quite a few of these mutations give a phenotype distinguishable from fCJD, which resulted in the arborisation of the inheritable TSEs into different subtypes with distinct names.

Gerstmann-Sträussler-Scheinker syndrome (GSS) affects 1 person/10 million per year. The most common mutation occurs at position 102 (Hsiao et al., 1989), while others have been found at codon position 105, 117, 145 and 217 (Fig. 8). GSS is characterised by onset of disease between 20 and 40 years of age and symptoms include progressive cerebellar ataxia, spastic paraparesis with the course of disease being very long (5-11 years). Plaque depositions with microglial cells present therein were also described, but the pattern of depositions varies between families (Liberski and Budka, 2004).

The third subtype, belonging to the inheritable TSEs, is Fatal Familial Insomnia (FFI), which was described for the first time in 1986 (Lugaresi et al., 1986). Doubtless, FFI has the strangest phenotype of inheritable TSEs, since one of its hallmarks is progressive insomnia, accompanied by autonomic dysfunction and dementia. Neuropathology shows loss of neurons and mild astrocytosis in the thalamus and the brainstem accompanied by very little vacuolisation (Montagna et al., 2003). Generally this form of disease is linked to a mutation at position 178 of the *prnp*-gene (see Fig. 8)

and depends upon the homozygous expression of methionine at position 129 (Gambetti et al., 1993; Goldfarb et al., 1992).

Usually, post-mortem specimens of hereditary TSE-patients contain PrP^{Sc}-molecules. In order to study the characteristics of PrP^{Sc} derived from hereditary TSEs, scientists had to transfer these point mutations into animals and cell-systems. Hsiao and colleagues were one of the first to establish the production of transmissible pathologic prions in a murine system. When mice expressed the amino-acid exchange P101L (proline to leucine at aa position 101), they developed a spontaneous neurological disease, reminiscent of the analogous P102L-mutation found in human GSS. Therefore, by genetic engineering, the neurodegenerative process found in humans could be transferred to mice and showed that the aa-exchange suffices to destabilize PrP^{C}-molecules and/or render it susceptible to pathological conversion into PrP^{Sc} (Hsiao et al., 1990). One of the forerunner laboratories for studies of point mutations from inheritable TSE-diseases was the Harris group who used Chinese Hamster Ovary cells (CHO) for their experiments. They found that cells produce PrP^{Sc}-like molecules, when they host aa-mutations analogous to human hereditary TSEs (Daude et al., 1997; Lehmann and Harris, 1995; Lehmann and Harris, 1996a; Lehmann and Harris, 1996b). Numerous mutations found in human hereditary diseases have been studied also in *in vitro*-systems, including those at position 105, 117, 145, 180, 200, 232 as well as various insertions, allowing for the characterization of disease-associated alleles but have also sometimes yielded ambiguous results ((Campana et al., 2006; Daude et al., 1997; Lehmann and Harris, 1995; Lehmann and Harris, 1996a; Lehmann and Harris, 1996b) see also Manuscript 1 and references therein).

III.6.3: Acquired human TSEs

The group of acquired prion diseases contains at least three subgroups with the common denominator that affected people acquire the disease exogenously regardless of PrP haplotype without hosting any genetic mutation in the *prnp*-gene.

The oldest of these diseases documented is kuru, a disease found in a tribe from New Guinea, called the Fore people. Kuru was described in 1957 and is caused by ritual cannibalism (Gajdusek and Zigas, 1957). The typical clinical progression for kuru is progressive cerebellar ataxia, evolving in few months with a very broad incubation

period of 4 to 40 years. Vertical or lateral spread of kuru could not be demonstrated: women with the disease, which delivered and suckled their babies, did not transmit the disease to their offspring. The suppression of cannibalism reduced the numbers of diseased people to a minimum (D'Aignaux et al., 2002).

The second group of acquired TSEs consists of the transmission of hereditary CJD to healthy subjects by medical interventions and are pooled by the name of "iatrogenic CJDs". The accidental transmission occurred by different medical interventions: tissues deriving from undiagnosed CJD-patients such as corneal transplants (Gandhi et al., 1981), dural grafts (Preusser et al., 2006), blood transfusion (Aguzzi and Glatzel, 2006) or cadaver-derived growth hormones (Billette de Villemeur et al., 1994) were transplanted or transfused to non-CJD patients, who subsequently developed CJD. Additionally, contaminated neurosurgical instruments such as metal electrodes and steel wires (Fichet et al., 2004; Flechsig et al., 2001), previously used on CJD-patients and not properly sterilized thereafter, were also suspected for accidental infection of non-CJD patients. The incubation time can be up to 15 years, while the average time between onset and death is 15 months.

The last and possibly most interesting group of this disease belonging to the acquired forms is the variant CJD (vCJD). Described for the first time in 1996 in the UK (Will et al., 1996), the country with the highest incidence, this form counts up to now about 150 reported cases. Afflicted individuals have no mutation in the *prnp*-gene differently from the inheritable forms but host a haplotype resulting in genetic predisposition (see later). The vCJD-form is quite well to discern from sporadic CJD (Fig. 9).

Clinical Features	Variant CJD	Sporadic CJD
Mean age of onset	29 years	60 years
Length of survival	14 months	4 months
Early psychiatric symptoms	Common	Unusual
Painful sensory symptoms	Common	Rare
Later cerebellar ataxia	All	Many
Dementia	Commonly delayed	Typically early
Electroencephalogram	Non-specific slowing	Biphasic and triphasic periodic complexes
MRI*	Signal in pulvinar region of thalamus	Signal in basal ganglion and putamen
Cerebrospinal fluid	14-3-3 concentration high in 50% of patients	14-3-3 concentration high in most patients
Histopathology of brain	Many florid plaques	No amyloid plaques
Immunostaining of tonsils	Positive	Negative
Polymorphism at codon 129	All homozygotes (M/M)	Homozygosity and heterozygosity

Fig. 9: Comparison of characteristics of variant and sporadic CJD. (from (Johnson, 2005)

Patients are young at the onset of disease (average onset is at 29 years), have a significant longer disease course, present florid plaque deposits in the brain and are homozygous for methionine at position 129 in the *prnp*-gene. Of public concern and scientific interest is the last characteristic, since this suggests a genetic susceptibility for succumbing to vCJD.

Because prions from patients with vCJD and prions from BSE-cattle gave in experimentally infected mice similar pathological and biochemical characteristics (e.g. incubation period and localization in brain), researchers concluded that the most likely cause for vCJD in humans was the consumption of BSE-contaminated beef (Bruce et al., 1997; Hill et al., 1997). It is estimated that 750000 BSE-positive cattle were consumed in the years between 1986-1996. Despite this alarmingly high number, mathematical models estimate that the peak of human casualties might have passed already (Valleron et al., 2001). However due to the large number of variables in these models, these estimations are likely inaccurate and should be considered with caution. Fortunately, some of the bleakest predictions basing on the genetic predisposition (Ghani et al., 2000), which prognosed about 136000 human casualties in the UK by 1999, turned out to be wrong (Alperovitch and Will, 2002). Other more conservative models, predicting a number of exceeding not more than 100 casualties seem to be more accurate (d'Aignaux et al., 2001; Ghani et al., 2003; Valleron et al., 2001). Nevertheless cases of positive biopsies from hospitalized non-CJD patients (e.g. from tonsils) suggest a higher number of subclinical infections that may or may not develop into an epidemic of clinical disease in the future (Hilton et al., 2004).

III.6.4: Susceptibility to vCJD

The genetic susceptibility to vCJD is based on the polymorphism at position 129 of the *prnp*-gene. Studies in Caucasians showed that, 50% of people are heterozygous (Met/Val), 40% are homozygous (Met/Met) and only 10% are homozygous Val/Val at this position (Owen et al., 1990). Interestingly the ratios of this polymorphism are quite different when healthy populations are compared to those with disease (Table 2 and (Palmer et al., 1991).

	healthy	CJD	vCJD
Met/Val	50%	10%	0
Met/Met	40%	79%	100%
Val/Val	10%	11%	0

Table 2: Polymorphisms of codon 129 and its effect on CJD/vCJD occurrence. (adapted from (Hill et al., 2003))

Table 2 illustrates that Met/Met homozygosity at position 129 of the *prnp*-gene results in a high genetic predisposition for vCJD and an increased predisposition for other CJD-subtypes with all vCJD cases to date being Met/Met homozygotes at codon 129 (Collinge et al., 1996). Similar results were found in mice expressing the human PrP-protein containing the Met/Met polymorphism, which were the only ones to develop the disease when intracerebrally infected with vCJD (Asante et al., 2002). Altogether, these data suggest that the Met/Met homozygosity at codon 129 favours the development of vCJD and CJD while heterozygosity and Val/Val homozygosity determines resistance against infection by unknown mechanisms.

III.6.5: Physiopathology

One of the hallmarks of the TSEs is the complete absence of toxic effects, e.g. lesions or vacuolization in the periphery of the organism (i.e. aside of the brain). Brain histopathology studies reveal spongiosis, astrocytosis, microgliosis, gliosis and most importantly neuronal loss (see Figure 3). Depending on the disease neuronal loss is linked to amyloid plaque deposits in different regions of the brain (Fig. 10). Additionally, during peripheral invasion of the organism, TSEs do not produce fever or a humoral immune response (Kubler et al., 2003).

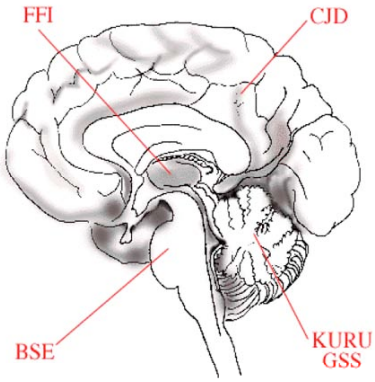

Fig. 10: Scheme of a human brain depicting the regions affected by different TSE-diseases. Kuru and GSS affect brainstem, CJD the cerebral cortex, FFI the thalamus and BSE the medulla oblongata (from S.Prusiner's Nobel prize lecture, 1997 available online at http://nobelprize.org/ nobel_prizes/ medicine/laureates/1997/press-1.gif).

III.7: The cellular PrP (PrP^C)

III.7.1: The *prnp*-gene

In 1985 researchers were surprised to discover that the gene for the believed toxic agent of TSEs was encoded in the host genome, of both healthy and diseased people (Chesebro et al., 1985; Oesch et al., 1985). Since the primary structure encoded by the genes from healthy and diseased animals did not differ, it was suggested that the different properties of PrP^C and PrP^{Sc} derived from post-translational events (Basler et al., 1986). This was substantiated when scrapie-associated fibrils (SAF) were isolated and demonstrated to have the sequence of endogenous PrP (Hope and Chong, 1994). The sequencing of the PrP-gene of numerous mammalian species (including Homo sapiens) revealed an 80% sequence-homology between species as well as high structural homologies, suggesting a strong genetic conservation in evolution (Oesch et al., 1991).

Depending on the species, the gene contains 2 to 3 exons, of which only one is utilized in protein expression (Puckett et al., 1991). Interestingly, the first or the first two small exons are transcribed but not translated. Very little is known about the regulatory elements controlling the expression of the *prnp*-gene, only that expression is controlled by ubiquitous transcription factors (Baybutt and Manson, 1997; Puckett et al., 1991).

III.7.2: Expression of PrP^C

III.7.2.1: The site of expression

The expression of the *prnp*-gene is particularly high in neurons (Brown et al., 1990; Cagampang et al., 1999), the choroid plexus (Brown et al., 1990) and in glial cells (Moser et al., 1995). Studies in hamsters showed its expression in the central nervous system (CNS) is prominent in neocortical neurons, the olfactory bulb, thalamus and hippocampus (Brown et al., 1990) but is also found in the peripheral nervous system, (e.g. dorsal root ganglia, sympathetic ganglia and nerves and afferent nerves in the skin) (Bendheim et al., 1992; Ford et al., 2002). While its expression in synaptic axon endings has been shown by light- and electron microscopy, none was found in the neuronal perikaryon (Sales et al., 1998). Certain dopaminergic neurons were also shown to not express PrP^C (Ford et al., 2002), while all studies found that glial cells have a high expression level of PrP^C. Outside the CNS, haematopoietic cells (except for eosinophils) were found positive for PrP^C-expression (Barclay et al., 2002). Similarly the lymphoreticulum was also shown to be positive for PrP-expression (Lotscher et al., 2003) with particularly high amounts on the surface of lymphocytes and of follicular dendritic cells in lymph nodes, in the spleen and in Peyer's Patches (Cashman et al., 1990; Ford et al., 2002). Additionally murine PrP^C is expressed in the lamina propria of the aerodigestive tract, in the intestinal enterocytes (Morel et al., 2004), in the gut associated lymphatic system (GALT) and mucosa associated lymphatic system (MALT), the intestinal nervous system and its associated Schwann cells (Follet et al., 2002; Ford et al., 2002).

By more sensitive techniques such as Northern - and Western Blot, expression of PrP^C in different organs was shown to occur in the heart, lungs, pancreas, testis and kidneys of rodents (Moudjou et al., 2001; Oesch et al., 1985; Robakis et al., 1986). Expression of PrP^C in the muscle and uterus and to a lesser extent in the liver (Horiuchi et al., 1995) was shown for ovines.

III.7.2.2: The regulation of PrP^C expression

In the course of murine embryonal development, PrP^C-expression starts in the vitelline membrane (i.e. extra-embryonic) at day 6,5 and starting from day 13,5 in neuronal and non-neuronal cells of the embryo (Manson et al., 1992). Particularly high expression was reported during neuritogenesis in the synaptic region (Sales et al., 2002; Sales et al., 1998). Although some studies in human neuroblastomas had shown that the expression of PrP^C is inducible by β-IL1, α-TNF and γ-INF, the majority of cell models suggest a constitutive expression (Satoh et al., 1998). However, its expression has also been linked to the activation state of lymphocytes, because PrP-presence on the cell surface is increased in activated lymphocytes (Cashman et al., 1990). Changes in expression-levels of the protein were not found during the development of prion pathology neither in *ex vivo*-experiments nor in animal systems (Chesebro et al., 1985; Oesch et al., 1985).

III.7.3: The structure of the PrP^C-protein

PrP^C contains 253 aminoacids (aa) in humans, 254 aa in mice and hamsters, 256 aa in sheep and 264 aa in bovines and migrates as a band at approximately 35 kDa in SDS-PAGE (Prusiner, 1991). Its N-terminus contains a 22 aa-long signal peptide, accounting for its translocation into the rough endoplasmatic reticulum (ER). Aminoacids 23-120 comprise a flexible, non-structured region (Donne et al., 1997; Lopez Garcia et al., 2000), followed by 4-8 repetitive octapeptides with the consensus sequence PHGGGWGQ, which are implicated in its interaction with divalent cations. The central region contains a conserved hydrophobic domain (TM1), which in some cases can span the membrane serving as a trans-membrane anchorage, leading to different PrP^C topologies (see paragraph III.13.2.2). Usually the membrane anchor is a glycosylphosphatidylinositol (GPI)-anchor, a post-translational modification, attached after the removal of the C-terminal part of the protein (Fig. 11).

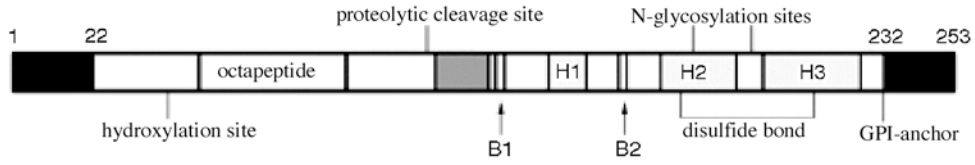

Fig. 11: Primary structure of PrP^C. Schematic diagram of human PrP^C, showing the signal peptide (black, left), the hydrophobic region (grey), the α-helical regions (H1-3) and the β-sheets (B1-2). Also indicated are the N-glycosylation sites (Asn181, Asn 197), a hydroxylation site (Pro44) and the proteolytic cleavage site (Lys112/His113). The cysteines at positions 179 and 214 produce a disulfide bond (adapted from (van Rheede et al., 2003)).

The combined use of the trans-membrane anchorage and the GPI-anchor can result in a form attached to the lumenal side of membranes by its GPI-anchor and with the N-terminal part in the cytoplasm and was linked to pathology (Hegde et al., 1998) (see also paragraph III.13.2.2).

The production of recombinant PrP^C gave to the researchers the opportunity to study the structure of the protein by infrared spectroscopy and circular dichroism (Hornemann et al., 2004). These studies indicated that PrP^C contains a globular structure (core) (see Figs. 11 and 12), characterized by a low content (3%) of β-sheets (two antiparallel β-sheets called B1 and B2 at residues 128-131 and 161-164) (Zahn et al., 2000) and a high content (approximately 40%) of α-helical regions (H1, H2 and H3) situated at positions 144-154, 173-194 and 200-228 (Fig. 12).

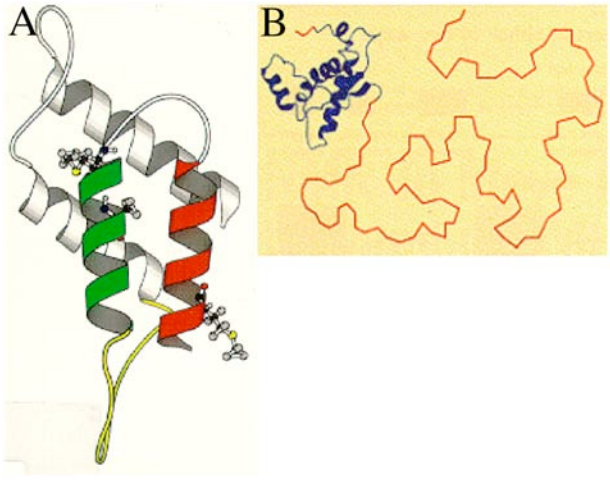

Fig. 12: A. Scheme of the secondary structure of PrP^C. B. Structure of PrP^C in solution obtained by the Wüthrich-laboratory at the Swiss Federal Institute of Technology (Riek et al., 1996). Shown are three α-helices, a β-sheet towards the C-terminal part and the flexible, unstructured segment at the N-terminal part of the protein.

PrP^C contains two complex sialylated oligosaccharides attached to two asparagines (positions 180, 196) in humans. The first site of N-glycosylation is in the second α-helix (H2 in Fig. 11), while the second is in the connector-region between helix 2 and 3. The disulfide bond between α-helices 2 and 3 is indispensable for the stabilization of the tertiary structure (Miura et al., 1996).

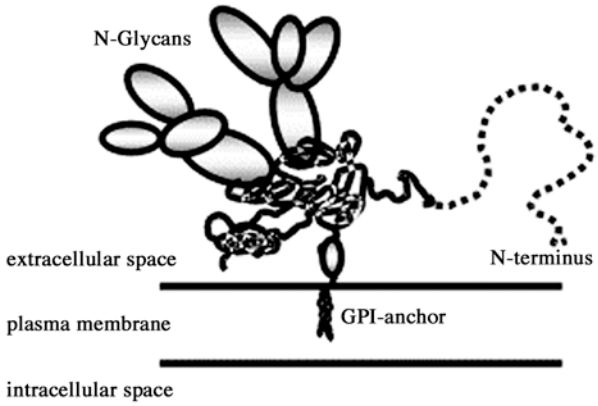

Fig. 13: Schema of the structural domains of PrP^C inserted in the plasma membrane. NMR-studies on recombinant PrP showed that the C-terminal region (aa124-230) is structured, while the N-terminal part is not. The N-glycans can occupy an area bigger than the protein itself, while the GPI-anchor keeps the PrP-protein at a distance of 9-13 Å from the cell membrane.

III.7.4: Possible function(s) of PrP^C

In spite of the numerous studies on molecules interacting with PrP^C (discussed below), its function still remains enigmatic and is subject of ongoing debate. The protein has been proposed to be involved in the metabolism or uptake of copper- or zinc-ions (Brown, 2003; Cui et al., 2003; Pauly and Harris, 1998; Watt and Hooper, 2003), protection against oxidative stress (Brown, 2003; Cui et al., 2003), cell-signalling transduction (Chiarini et al., 2002; Mouillet-Richard et al., 2000), synaptic transmission and excitability of membranes (Collinge et al., 1994; Mallucci et al., 2002), neuritogenesis (Graner et al., 2000a), apoptosis (Solforosi et al., 2004), transendothelial migration (Viegas et al., 2006) and very recently in the regulation of β-site APP cleaving enzyme (BACE1) (Parkin et al., 2007) and in the signal-transduction of nociceptive stimuli (Meotti et al., 2007).

III.7.4.1: $PrP^{-/-}$ knock-out mice and the possible role of PrP^{C} in synaptic signalling

Mice with no expression of the *prnp*-gene, (i.e. knock-outs at the germinal level (Bueler et al., 1992; Manson et al., 1994) or conditional knock-outs with an induced *prnp*-inactivation after birth (Mallucci et al., 2002)) are perfectly healthy animals and do not present obvious phenotypes, aside from being resistant to infection with prions (Prusiner, 1998). This suggests that the function of PrP^{C} is dispensable for normal function, which seems contradictory to its high genetic conservation over several phylii as well as the high degree of conservation of its tertiary structure (Premzl et al., 2004). Therefore, it is believed that the function of PrP^{C} is redundant and might be compensated by other proteins. Nevertheless some subtle differences in the phenotype of conditional knock-out mice have been described: these include alterations in the circadian rhythm and sleep regulation (Tobler et al., 1996), electrophysiological changes in the pyramidal cells of the hippocampus (Colling et al., 1997; Mallucci and Collinge, 2004) weakened GABA (gamma-aminobutyric acid type A) receptor-mediated fast inhibition as well as aberrant long-term potentiation of neuronal synapses (Collinge et al., 1994). These findings lead to the hypothesis that PrP^{C} might be involved in synaptic function such as signalling/activity of neurons. Whether this is really due to the presence of PrP^{C} in neurons is debated since there are reports of normal functionality of the hippocampus even in mice deficient for the prion protein (Lledo et al., 1996).

III.7.4.2: The possible involvement in the metabolism of copper and oxidative stress

In its flexible N-terminal part, PrP^{C}-protein contains a repetition of octapeptides rich in histidines, shown to bind divalent cations, e.g. Zn^{2+}, Co^{2+}, Mn^{2+} and especially Cu^{2+} (Kramer et al., 2001; Miura et al., 1999; Viles et al., 1999; Whittal et al., 2000) (see also Figs. 12 and 14). Another site, binding Cu^{2+} with high affinity, was found in the human PrP^{C}-form within aa's 96-111. These binding sites were proposed to be involved in the stabilization of the protein when performing diverse functions (Miura et al., 1996; Wong et al., 2000) or as an ionic receptor responsible for the uptake of divalent ions, especially copper (Prado et al., 2004) (Fig. 14). The implication in the uptake of copper,

was deduced from the finding that binding of Cu^{2+} stimulates the endocytosis of the PrP^C-protein and thereby also the endocytosis of the extracellular ions (Lee et al., 2001; Pauly and Harris, 1998; Perera and Hooper, 2001). Consequently it was shown that knock-out mice had two times less copper than control mice in presynaptic vesicles, suggesting that the protein could be involved in the regulation of copper-uptake in neuronal synapses (Kretzschmar et al., 2000). Recently, it was also shown that the presence of extracellular copper leads to the dynamic down regulation of PrP^C, emphasizing its possible role in copper-homeostasis (Nishimura et al., 2004; Toni et al., 2005). Because copper and zinc regulate the activity of the superoxide dismutase (SOD), an enzyme involved in protection from oxidative stress (Milhavet and Lehmann, 2002) the interaction of PrP^C with copper also hints at another function in reactive oxygen species (ROS) metabolism. Mice lacking expression of PrP^C, were shown to have a reduced SOD activity in cerebral tissues (Brown and Besinger, 1998) and were more susceptible to oxidative stress (Wong et al., 2000), suggesting that PrP^C could play a role in the regulation of SOD-activity or even have SOD-activity itself (Brown et al., 1999). Evidence suggests that the N-terminal part of PrP^C is responsible for the regulation of SOD-activity (Sakudo et al., 2005; Sakudo et al., 2003).

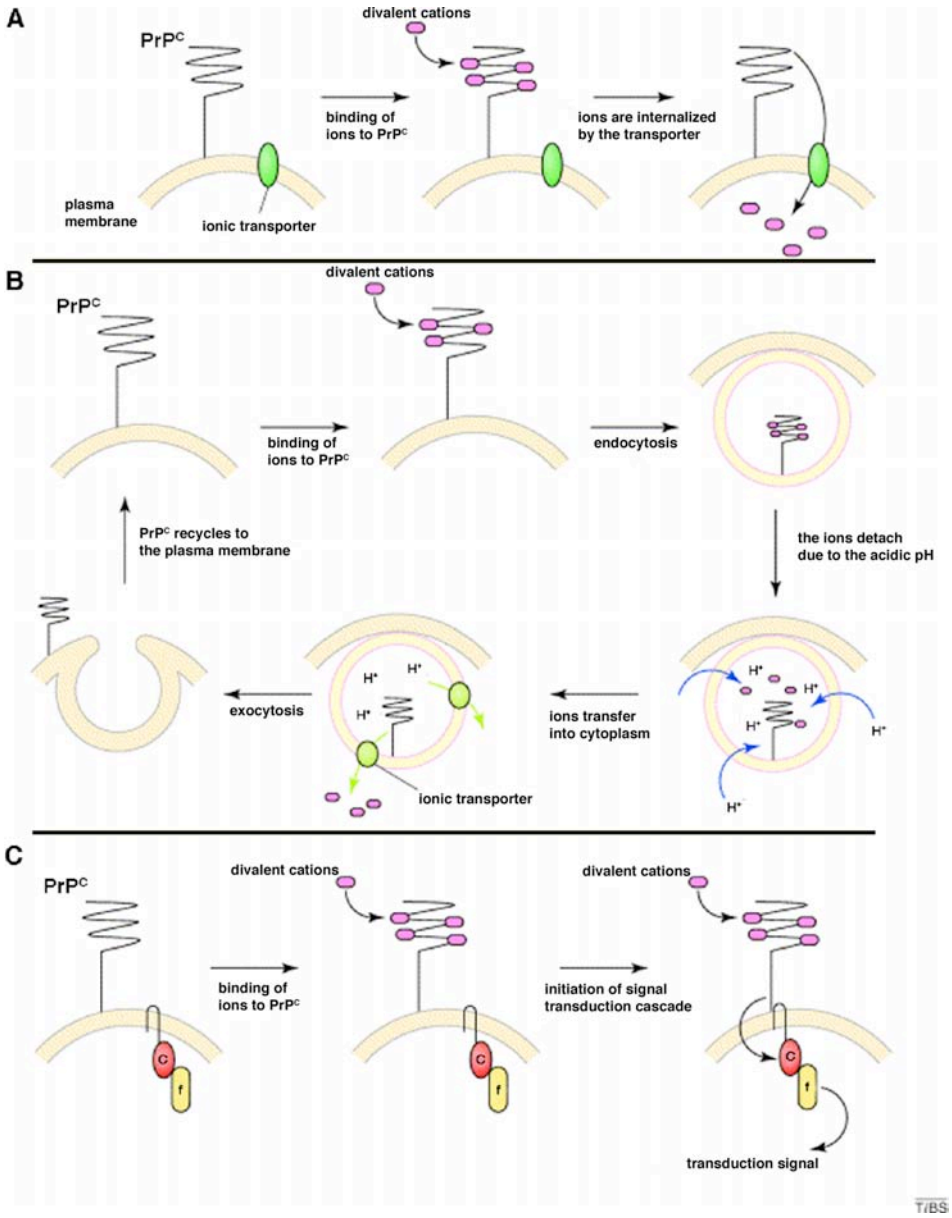

Fig. 14: Models of how PrP^C could interact with divalent ions.

A. PrP^C acts as a receptor for divalent ions. Situated on the cell surface, PrP^C binds divalent ions from the extracellular space and assists in their intracellular sequestration by transferring these to specialized membrane transporters. **B.** PrP^C is directly involved in intracellular uptake of Cu^{2+}. After binding divalent ions, PrP^C is endocytosed and upon acidification in the endosomal pathway releases the divalent ions intracellularly. Next, specific transporters transfer the ions from the endosomal compartment, while PrP^C returns to the cell surface for a new cycle. **C.** PrP^C acts as a sensor for divalent ions. Too high concentrations of extracellular ions leads to a transduction signal which allows the cell to respond to the toxic levels of these ions. (adapted from (Watt and Hooper, 2003))

III.7.4.3: Implication of PrP^C in signal transduction, cell adhesion and neuronal survival

Numerous GPI-anchored proteins are situated in specific membrane subdomains called lipid rafts (see paragraph III.14.1.3) in which they participate in various signal transduction cascades (Simons and Toomre, 2000). The facts that PrP^C is inserted into the plasma membrane, its presence in rafts and the accessibility of its N-terminus to ligands in the extracellular space could give this protein a role in cell-signalling cascades. A study on 1C11 cells (neuronal precursors, differentiable into either serotoninergic or noradrenergic neurons), showed that the activation of surface PrP^C by cross-linking with antibodies, induced the dephosphorylation (probably mediated by caveolin 1) and hence activation of Fyn, a tyrosine-kinase from the Src-family (Mouillet-Richard et al., 2000). The same group reported links between the activation of PrP^C and the PKA-enzyme, the PI-3-Kinases/Akt and the MAPK/ERK-kinases (Schneider et al., 2003). Another group showed that PrP^C interacts with Grb2 and synapsin Ib (Spielhaupter and Schatzl, 2001), both of which are involved in signal transduction in neurons. The finding that the expression of PrP^C during development is tightly controlled in terms of time and localization on growing axons suggests its implication in the development of axons and synapses (Hajj et al., 2007; Sales et al., 2002).

On the other hand, PrP^C could be involved in intercellular communication (Chen et al., 2003) and cell-adhesion (Mange et al., 2002). It was reported that PrP^C could bind extracellular proteins like laminin (Graner et al., 2000a; Graner et al., 2000b) and N-CAM (Schmitt-Ulms et al., 2001), which play a role in the proliferation and differentiation but also in neuronal death. Due to its interaction with N-CAM, PrP^C can promote the growth of neurites through a signal-transduction cascade involving the molecule Fyn (Santuccione et al., 2005). Most recently binding of PrP^C to E-, P- and L-selectins, molecules important for migration and adhesion, was also shown (Li et al., 2007a).

The PrP^C molecule also binds to the 67 kDa laminin receptor (LRP) (Rieger et al., 1997) and to its 37 kDa-precursor (Gauczynski et al., 2001) and it was also shown that PrP^{Sc}-propagation in neuronal cells requires the presence of the 37 kDa/67 kDa laminin receptor (Leucht et al., 2003).

A number of groups have also reported a role of PrP^C in neuronal survival. Nevertheless, the role of PrP^C in this process is hotly debated: some argue for a pro-apoptotic role in neuronal models while others favour a protective role for PrP^C. Neuronal cell culture models showed that the presence of PrP^C made neurons more susceptible to staurosporine induced death involving caspase 3 activation and apoptosis (Paitel et al., 2002; Paitel et al., 2004). More recent studies showed also that the cross-linking of PrP^C by antibodies produced a pro-apoptotic cascade *in vivo* (Solforosi et al., 2004).

On the other hand evidence for a neuroprotective role of PrP is suggested by data showing interaction of PrP^C with Stress-inducible protein 1 (Zanata et al., 2002). The neuroprotective role probably involves signalling by cyclic adenosine monophosphate (cAMP)/ and protein kinase A (PKA) (Chiarini et al., 2002). Another putative interactor was shown to be Bcl-2 (Kurschner and Morgan, 1995) an interaction which protected hippocampal neurons from death by serum deprivation (Kuwahara et al., 1999) and from apoptotic death mediated by Bax (Bounhar et al., 2006; Roucou et al., 2003).

In the light of these abovementioned numerous proposed roles for PrP^C it is difficult to discern a clear function, especially considering that knockout mice do only present subtle behavioural changes. Further analysis of these mice and more detailed studies, possibly using RNAi and high-throughput screening approaches, might be able to shed some light on the enzymatic function of PrP^C.

III.8: The pathological prion protein: PrP^{Sc}

III.8.1: The structure of PrP^{Sc}

Studies utilizing circular dichroism and nuclear magnetic resonance (NMR) revealed that PrP^{Sc} contains a significantly higher amount of β-sheet content than PrP^{C} (42% in PrP^{Sc} and 3% in PrP^{C}) (see also Fig. 12). Recent studies propose a model in which PrP^{Sc}-polymers produce a structure with β-sheets derived from three PrP^{Sc}-molecules on the inside of aggregates and all α-helices oriented to the outside (Fig. 15 and reviewed in (Riesner, 2003)).

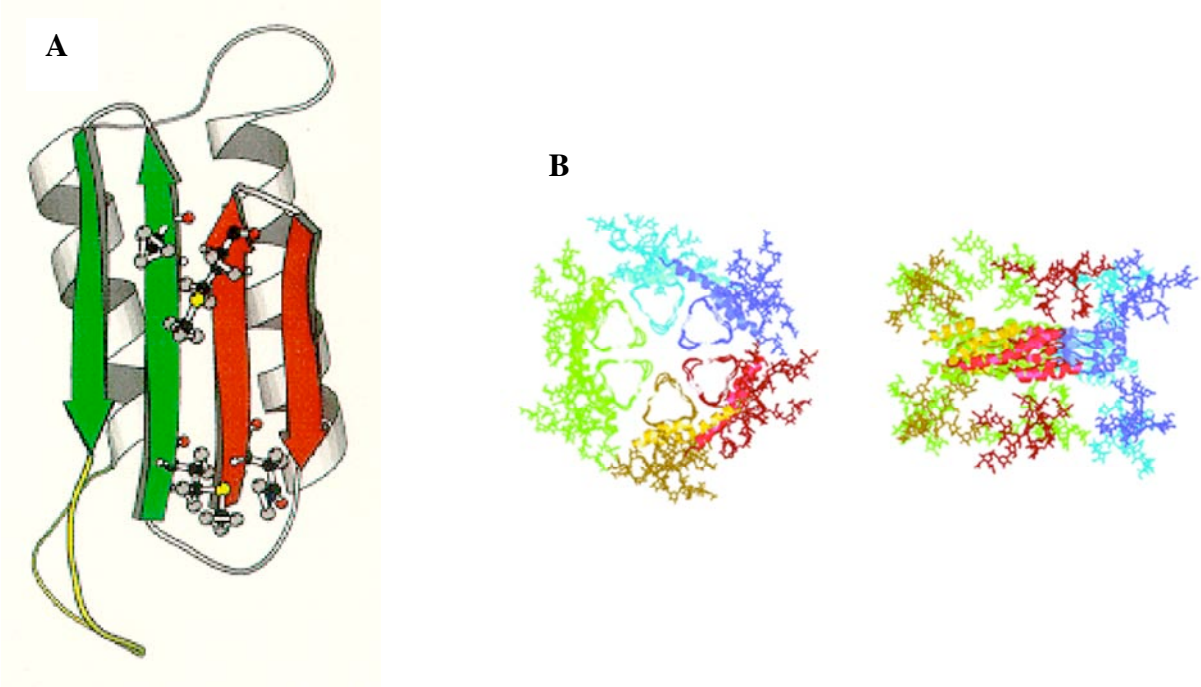

Fig. 15: A. Scheme of PrP^{Sc} enriched in β-sheets depicted in green and red. **B.** Structure of PrP^{Sc}-oligomers, received from the reconstruction of electron microscopic analysis of two-dimensional crystals (from (Riesner, 2003))

III.8.2: The physico-chemical traits of PrP^{Sc}

The particular conformation of PrP^{Sc} confers characteristics very different to its normally folded counterpart:

In contrast to PrP^{C}, the solubility of PrP^{Sc} is diminished in non-ionic detergent (Caughey and Raymond, 1991; Meyer et al., 1986), a trait, which renders its purification and crystallographic study very difficult (Table 3).

Protein	PrP^{Sc}	PrP^{C}
Structure	globular	stretched
Resistance to proteases	yes	no
Production of fibrils	yes	no
Turnover of protein	days	hours

Table 3: Comparison of PrP^{Sc} and PrP^{C}

The high content of β-sheets of PrP^{Sc} renders the protein more stable and resistent to digestion by proteases, especially by proteinase K (PK). In contrast to PrP^{C}, which is completely digested by PK, only the N-terminal part of PrP^{Sc} (specifically the first 67 aa) are hydrolyzed. This hydrolysis leaves a large part of the protein undigested and results in an electrophoretic product that migrates now at the characteristic height of 27-30 kDa and is therefore called PrPres (res for resistant to digestion) or PrP 27-30. This intrinsic resistance to digestion with PK is a hallmark of PrP^{Sc} that distinguishes it from PrP^{C} (Fig. 16).

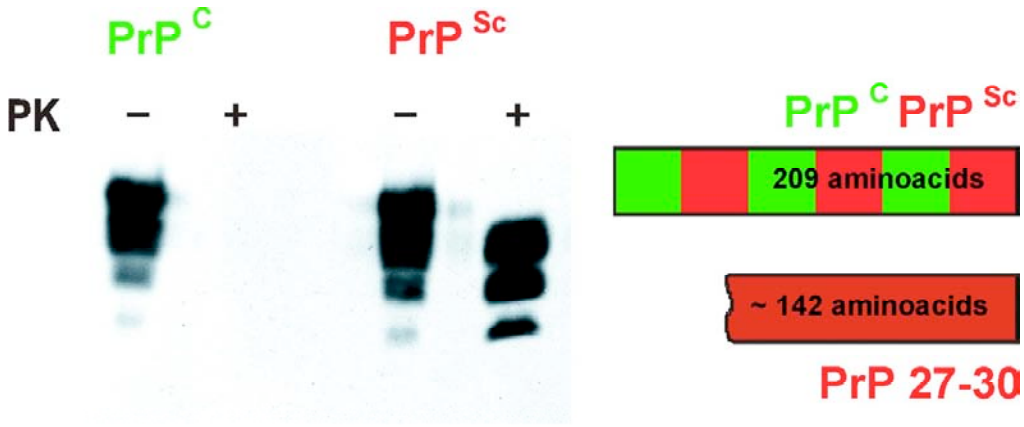

Fig. 16: PrP^{Sc} is partly resistant to digestion with PK. Upon addition of PK (+PK) a N-terminal truncated PrP-form remains undigested in the case of PrP^{Sc}, migrating on a polyacrylamide gel at a height of 27-30 kDa. Please note that while PrP^{C} is completely digested, PrP^{Sc} gives three bands, which remain nearly undiminished in terms of intensity but shifted to a lower molecular weight. The three distinct bands represent the three glycosylated forms of PrP (un-, mono and diglycosylated). (from (Riesner, 2003))

III.9: The conformational change *in vivo*

Two models were proposed to explain how PrP^{Sc} is propagated by conversion of endogenous PrP^{C} (Fig. 17 and (Come et al., 1993; Harper and Lansbury, 1997; Prusiner, 1991; Prusiner, 1998)).

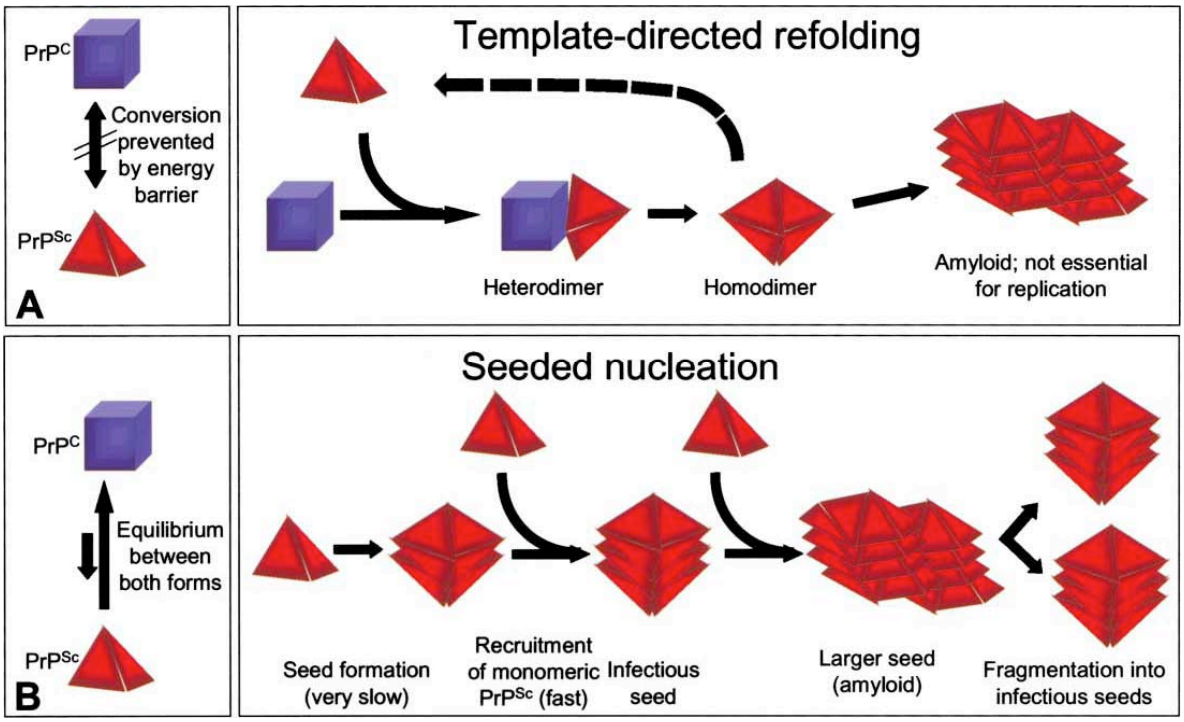

Fig. 17: Models for the conformational conversion of PrP^{C} to PrP^{Sc}. A. Depicted in A is the "template-directed refolding model" also termed "template assistance", which hypothesizes an interaction between the exogenously introduced PrP^{Sc} and the endogenously produced PrP^{C}. This interaction induces PrP^{C} to adopt the malconformation of its interactor and turn itself into PrP^{Sc}. A high energetic barrier prevents the spontaneous transconformation of PrP^{C} to PrP^{Sc}. **B.** The "seeded nucleation" model also termed "nucleation-polymerization model" proposes a reversible thermodynamic equilibrium between PrP^{C} and PrP^{Sc} in cells. Only when PrP^{Sc} monomers organize themselves into a so-called "seed" do PrP^{Sc}-molecules reach an increased stability and become capable of recruiting new PrP^{Sc} monomers in order to form an amyloidal structure. The fragmentation of aggregates of PrP^{Sc} increases the number of seed-nuclei, which once again can recruit, and stabilize PrP^{Sc}-monomers and thereby achieve a replication of the agent (from (Aguzzi and Polymenidou, 2004)).

The "refolding" or "template-directed" model (Fig. 17, A) proposes that PrP^{Sc} is a template or matrix for the conversion of PrP^{C} into new PrP^{Sc} monomers (Prusiner, 1991). On the other hand the "seeded nucleation model" (Fig. 17, B), posits that PrP can spontaneously adopt different conformations (those of PrP^{C} or PrP^{Sc}) that exist in

equilibrium. Under physiological conditions the PrP^{C}-conformation is highly favoured, due to the high activation energy, which is necessary for the acquisition and maintenance of the PrP^{Sc}-conformation (Come et al., 1993; Harper and Lansbury, 1997). The presence of a PrP^{Sc}-aggregate shifts this equilibrium towards PrP^{Sc} by stabilizing this conformational form and achieves a rapid accumulation of new monomers to the aggregate (Jarrett and Lansbury, 1992).

This hypothesis fits well with the experimental finding that the conversional activity and infectivity are specifically associated with the aggregates and not with the monomers of PrP^{Sc} (Weissmann, 2004). Indeed, it was reported that disaggregation and denaturation of PrP^{Sc} (but not the reversible loss of conformation) coincided with the loss of conversional activity and infectivity (Morillas et al., 2001). As a consequence, while the process of spontaneous misfolding of PrP^{C} might be a slow event, the pathological conversion with the help of a PrP^{Sc}-molecule would be a quick process, suggesting that it is the aggregates, which induce the formation of prions. The occasional fragmentation of aggregates could also explain the exponential growth of PrP^{Sc} during infection (Orgel, 1996). In addition more recently it has been proposed that oligomeric PrP^{Sc} might be just as infectious and toxic as bigger aggregates (Chiesa and Harris, 2001; Novitskaya et al., 2006; Silveira et al., 2005; Simoneau et al., 2007).

It is possible that in the case of sporadic CJD and genetic TSEs, mutations in the gene *prnp* generate a protein whose structure is more favourable to conversion, while in the infectious form it is the contact with exogenous PrP^{Sc}, which leads to progressive misfolding.

Elegant *in vivo* and *in vitro* studies have shown that sequence homology plays a very relevant role for pathological conversion of PrP^{C} to PrP^{Sc}. Mice co-expressing a murine and a hamster prion-form, but infected with mouse-prions produced only PrP^{Sc} containing mouse-prions, while infection of littermates with hamster-prions resulted in the production of PrP^{Sc} containing only hamster-prions (Prusiner et al., 1990). Furthermore, co-expression of a nonconvertible PrP-deletion mutant in scrapie-producing MNB-cells lead to a reduction of the infectious protein in these cells (Holscher et al., 1998) underlining that protein homology is required for the process of conversion and infection.

Another variable influencing the conversion reaction is the speed of synthesis of PrP^{Sc} from PrP^{C}: continuous exposure of infected scN2a cells to phosphatidylinositol-specific phospholipase C (PIPLC), an enzyme which cleaves all GPI-anchored proteins from the

cell membrane (including PrP^{C} but not PrP^{Sc}) resulted in depletion of membranous PrP^{C}, and leading to the cure of these cells (Enari et al., 2001). Charles Weissmann put these findings into perspective in his model of "dynamic susceptibility", in which he proposes that the capacity of cells to sustain and propagate infection by prions depends on equilibrium between synthesis and degradation of PrP^{Sc} (Weissmann, 2004).
According to this model, if the rate of synthesis of PrP^{Sc} is not at least equal to or twice superior to its rate of degradation, prions will be eliminated from infected cells. Only when the synthesis rate is two times higher than its degradation rate, can it overcome its dilution by cell division and manage to accumulate in cells. Prions manage to propagate only by keeping this delicate balance. This model explains why certain cell systems are not susceptible to prion infections (Race et al., 1987; Rubenstein et al., 1984; Schatzl et al., 1997; Vilette et al., 2001; Vorberg et al., 2004a; Vorberg et al., 2004b) however this does not explain why some cell lines are immune to some prion strains but susceptible to others (Bosque and Prusiner, 2000; Nishida et al., 2000; Race et al., 1987).

III.10: Prion strains and the species barrier phenomenon

By the 1920's the existence of two different scrapie-forms in sheep was described: one was called the "nervous" form while the other was termed the "pruritic" form. Even though the existence of prion strains is now quite well established (Bruce, 1993), this remains one of the most poorly understood phenomena of prion research and is especially hard to reconcile with the protein-only hypothesis. Work with different prion strains began in the early 1970's (Dickinson et al., 1968), by infecting laboratory animals with infectious material derived from wild animals. This technique quickly established itself as the standard procedure for the study, propagation and ultimately characterization of prion strains: Wild-type prion strain isolates are inoculated into murine animal models and are successively passaged to genetically identical animals utilizing the same amount of infectious agent, until the properties of the strain stabilizes in this new host species.

The criteria serving to define a new prion strain are:

i) The time of incubation of the strain once stabilized. This information is linked to the nature of the strain, the genetic predisposition of the host mice and the amount of the inoculum.

ii) The lesion profile caused by the infection in the brain of the host animal. This corresponds to its spatial distribution (and deposition) in the brain, as well as to the severity of the vacuolization. It is worthwhile to mention that the same isolate can lead to the isolation of different strains. The most interesting example of this was found when an isolate from a mink hosting transmissible mink encephalopathy (TME) was inoculated into Syrian golden hamsters. Infection of littermate hamsters resulted in two clearly distinguishable syndromes, termed hyper (HY) and drowsy (DY). These two strains are distinguishable by several key features: incubation period (HY: 65 days; DY: 168), clinical signs (HY: hyperaesthesia; DY: lethargy), titres of infection (HY: $10^{9,5}$ LD50/g of tissue; $10^{7,4}$ LD50/g of tissue) and pathogenesis (only the DY strain retained virulenece in mink) (Bessen and Marsh, 1992).

These types of studies also enabled researchers to find similarities between BSE and new variant CJD (nvCJD), supporting the notion that these two strains are closely related and giving the opportunity to distinguish them from sporadic CJD (Bruce et al., 1997).

Nevertheless this approach also has some disadvantages. First, it is time consuming, since some strains require passaging for a few years until properly stabilized. Secondly, it is costly, since hundreds of mice have to be kept at a high security level and third it is also complex, since the isolation of strains and analysis of their properties are challenging tasks not necessarily giving consistent results.

Alternative methods, basing on a biochemical approach (see below), aim at a much faster and easier characterization of the different prion species.

III.10.1: Molecular features of different prion strains

In the absence of nucleic acids coding for strain specifity, researchers hypothesized that strain characteristics were encoded in the different conformational isoforms of PrP^{Sc} (Weissmann, 2004). PrP^{Sc} can show characteristic biochemical variations depending on the strain. Additionally, biochemical analysis of cellular extracts or of infected tissue

digested with proteinase K reveals different biochemical profiles for PrP^{Sc}, depending on the source of origin. These profiles enabled the classification of different prion species. For instance, in the case of human TSEs, this analysis allowed researchers to distinguish four groups (type I-IV) of human prions depending on their respective electrophoresis profiles (Fig. 18) (Collinge et al., 1996). Type I gives a dominant monoglycosylated band at approximately 30 kDa while type II reveals a non-glycosylated band at 21 kDa and a monoglycosylated band slightly lower than the corresponding band for type I. Type III, displays prominent mono- and unglycosylated bands just as type II, however bands show differences in electrophoretic mobility. Finally, type IV has a strong diglycosylated bands and only minor amounts of the other two.

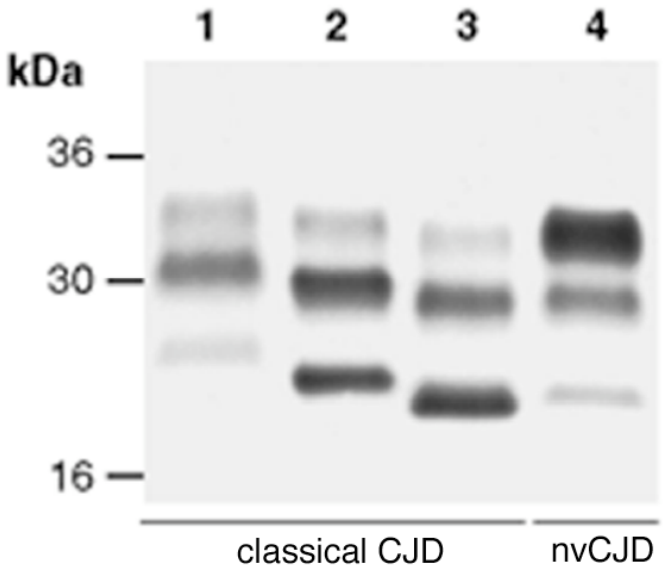

Fig. 18: Differentiation of PrP^{Sc} type I-IV in human TSEs. A Western blot is shown revealed with the monoclonal antibody 3F4 and depicts type I-IV of PrP^{Sc} from human TSEs. Type I-III were isolated from patients with classical CJDs, i.e. sporadic or iatrogenic. Type IV is unique to patients who succumbed to the new variant CJD-form (nvCJD) (adapted from (Collinge et al., 1996)).

Interestingly, the type IV profile was also found in brains from cattle, macaques and mice infected with the BSE-strain or with the nvCJD strain (Hill et al., 1997). The resemblance of these two strains and their conservation when raised in different animals lends strong support to the notion that nvCJD originates from the bovine agent BSE (Bruce et al., 1997; Hill et al., 1997).

The classification of human CJDs into four types is probably not exhaustive, since a fifth and a sixth type of PrP^{Sc} have recently been described (Gambetti et al., 2003; Zanusso et al., 2001). Furthermore biochemical characterization is still hotly debated since some researchers believe that the differences partly derive from pH-differences during tissue/sample preparations (Cali et al., 2006).

Different biochemical profiles that distinguish different prion strains can also be applied to other species. For instance, even the two species HY and DY found in Syrian hamster (described above) present different biochemical characteristics. After PK-digestion the non-glycosylated band of PrP^{Sc} migrated at 21 kDa for HY and at 19 kDa for DY (Bessen and Marsh, 1992). These prion strain-differences were recently also demonstrated for BSE-derived prions, suggesting that various BSE-strains may coexist in cattle (Beringue et al., 2006).

Another criterion used for distinguishing strains is the degree of resistance of PrP^{Sc} to PK-digestion (Kuczius and Groschup, 1999). It is believed that the resistance to digestion derives from differences in structure and folding of PrP^{Sc}, resulting in different accessibility of proteolytic enzymes to prion aggregates.

A test allows for prion strain classification based on their conformational stability. This approach, termed CDI (conformation-dependent immunoassay) was utilized on prions derived from hamsters, allowed the classification of eight different strains in four distinct subgroups. The CDI-test measures the binding of PrP^{Sc}-specific antibodies recognizing different epitopes that are not accessible in the protein's native conformation. The exposure of the protein to a chaotropic agent results in its denaturation and exposes previously hidden epitopes with a kinetic that is specific for each of the tested prion strains. Therefore, this test allowed the establishment of a "biochemical map" for each of the prion strains based on the different affinities of the specific antibodies (Peretz et al., 2001; Safar et al., 1998). The ideal test for the prion strain-hypothesis was to see whether strains conserved their characteristics after *in vivo* or *in vitro* passaging (Fig. 19). Indeed, at least in some cases, glycosylation and conformational differences were preserved after transmission into mice (Telling et al., 1996) or after *de novo* formation in a cell-free system (Bessen et al., 1995; Eiden et al., 2006; Iniguez et al., 2000). The existence of different prion-strains was an unanticipated aspect of TSE-diseases. The difference of strains appears to be enciphered in the conformational variants of PrP^{Sc} and not by nucleic acid sequence as with traditional pathogens such as bacteria and viruses. Although technically challenging, researchers

have developed tools for characterizing prion strain varieties and allow the identification of the likely origin of infectious material. Development of more rapid and sensitive biochemical strain typing methodologies should prove useful for identifying subclinical disease as well as tracking infectious outbreaks of TSEs in agriculture and in natural environments.

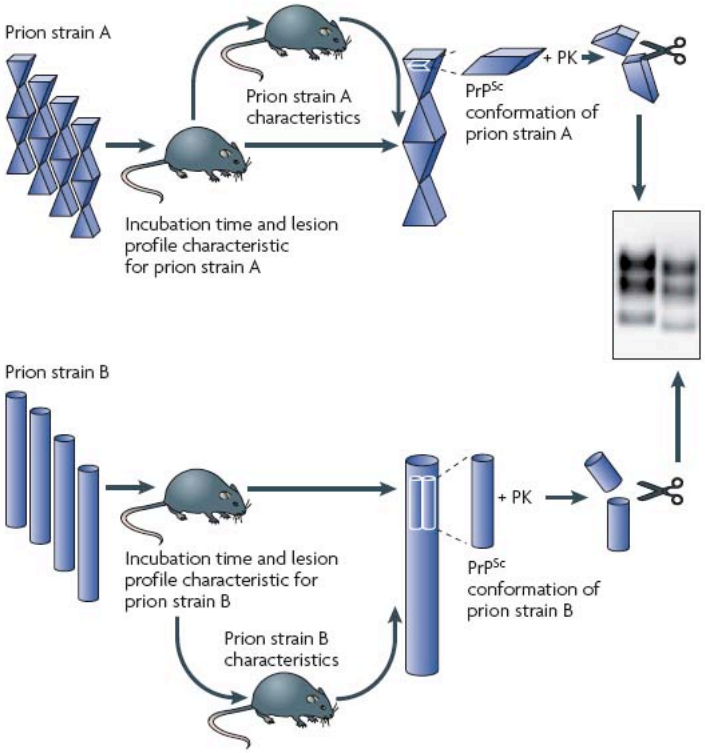

Fig. 19: Prion strain variation and preservation of characteristics upon serial passages in hosts. Transmission of different prion strains to animals results in an incubation time and lesion profile specific for each strain. Additionally, biochemical features persist upon serial passage to animals, as tested by PK-digestion and electrophoresic mobility assays (adapted from (Aguzzi et al., 2007)).

III.10.2: Interspecies transmission of prions and the species barrier

As just discussed, one of the characteristics of prions is the existence of different strains (see Fig. 19), having distinct incubation times and distinct neuropathological characteristics (Bruce, 1993; Bruce, 2003; Bruce et al., 1994). Yet, the finding that interspecies transmission of prions is less efficient (and sometimes even impossible) than intraspecies transmission and that different attack rates (i.e. efficiency of infection)

exist for different prion-strains, suggested the existence of a species barrier (Bruce, 1993; Bruce et al., 1994).

The species barrier is characterized by an adaptation of the inoculated strain in the new host, which is measured by its increasing penetrance (i.e. number of animals developing symptoms upon inoculation) and by the reduction of incubation time after serial passages in the new host (Fig. 20). In some cases the initial species barrier can be overcome by increasing the amount of PrP^{Sc} administered.

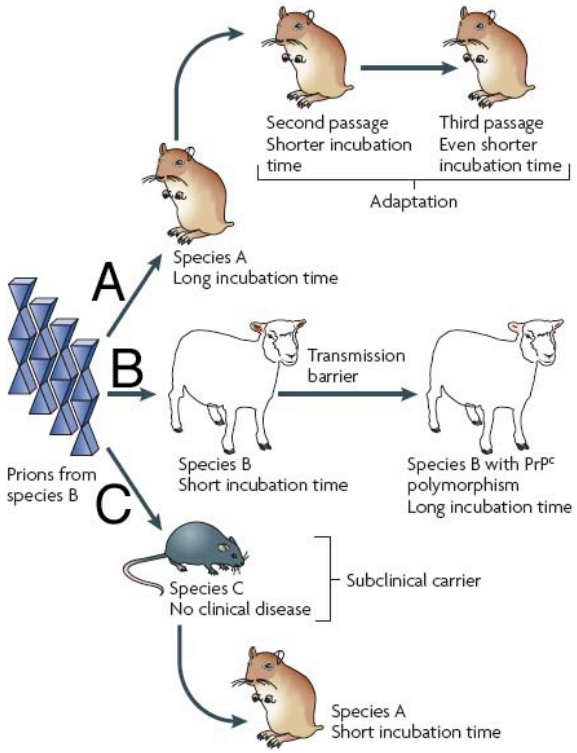

Fig. 20: The species barrier phenomenon. Transspecies infection of prions results in prolonged incubation times or the complete abrogation of disease transmissions. The shortening of incubation times after serial passages is called adaptation (A). Polymorphisms of the *prnp* gene represent another kind of hurdle and are termed transmission barriers (B). Some species barriers are insurmountable as a consequence some species do not show any clinical disease (C). Their brain isolates remain nevertheless infectious for the original host species, indicating the existence of subclinical carriers (adapted from (Aguzzi et al., 2007)).

The finding that only some prion strains can be transmitted in hosts of different species (e.g. to the murine system) indicates that the stringency of the interspecific barrier varies depending on the strain itself (Hill and Collinge, 2004). This was manifested by reports, showing that only 1/3 of naturally occurring scrapie strains were transmissible

to C57/BL/6 mice (Bruce et al., 2002; Hunter et al., 1997). The same was reported for human strains, which were found to be only partly transmissible to mice, while others were not (e.g. strains found in sporadic CJD (sCJD)) (Hill et al., 1997). In order to overcome species barriers a high homology between the PrP^{C}-proteins of the two animals plays a relevant role. A report from Prusiner et al., showed this very conclusively: wild-type mice expressing natural endogenous murine PrP^{C}, proved to be resistant to infection with a prion strain adapted to hamster (263K) but sensitive to the infection with murine-adapted prion strains. Only when mice were engineered to express hamster PrP^{C} instead of murine PrP^{C}, did they succumb to infection by the hamster prion strains (Prusiner et al., 1990). The importance of the contribution of PrP-homology to the species barrier is exemplified by the finding that only one amino acidic mutation can protect of interspecies transmission in *in vivo* assays (Priola and Chesebro, 1995) and *in vitro* conversion assays (Priola et al., 2001). These initial findings, both from mice and in cell culture, were extended further to humans, bovines, ovines and cervids by other laboratories (Browning et al., 2004; Crozet et al., 2001; Scott et al., 1997; Telling et al., 1995; Vilette et al., 2001).

Nevertheless, it must be stressed that sequence homology between the inoculum and the host PrP^{C}-form is required but not always sufficient for species barrier crossing. Transgenic mice expressing the human version of PrP^{C} were very poorly infected with prion-strain isolates from nvCJD (Vilotte and Laude, 2002), suggesting that additional factors, possibly interacting with PrP^{C}, are implicated in establishing the species barrier. The search for these putative interactors proved to be a non-trivial question, because only PrP^{C} was found to interact strongly and co-precipitate with PrP^{Sc} in cell lysates, thus suggesting that the interaction with other participants, aiding in the process of transconformation, might be very transient (Horiuchi and Caughey, 1999).

Kimberlin and colleagues proved that a prion strain can be transmitted to other species, despite sequence differences between the two host PrP-forms (Kimberlin et al., 1987), contradicting the hypothesis of the sequence homology requirements and possibly implying that PrP molecules with different amino acidic sequence could acquire the same conformation. This apparently contradictory finding underlines that the species barrier phenomenon as it is established today is more complex and only superficially understood making additional research in this field necessary.

III.11: In vitro conversion of PrP^{C} to PrP^{Sc}

Final proof for the much-contested protein-only hypothesis first put forward by Stanley Prusiner would be an experiment demonstrating conformational conversion of PrP^{C} to PrP^{Sc} in an *in vitro* cell-free system. Although many studies tackled this question of conformational transition (reviewed in (Riesner, 2003)) and have managed to elucidate some aspects of prion replication, all have failed to conclusively produce infectivity *in vitro*. One of the first attempts aimed at studying the refolding process of infectious natural PrPres 27-30 with varying concentrations of detergents at a neutral pH (Riesner et al., 1996). Conversion of PrP^{Sc} into an α-helical, non-infectious and oligomeric protein and even a rapid reconversion into a β-sheet-enriched, aggregated and partially PK-resistant form was reported (Post et al., 1998). However, although this latter form displayed the main characteristics of PrP^{Sc}, it was no longer infectious, suggesting that merely conformational conversion is not sufficient for establishing infectivity. Nevertheless, these experiments showed that the conversion process consisted of three main steps: a rapid switch from α-helices to β-sheets and dimerization within the first minute followed by slow formation of larger oligomers after 20 minutes and ultimately the production of proteolysis-resistant, large multimers after several hours. Several groups demonstrated that denaturing agents used at acidic pH could promote the production of the scrapie-conformation and that at acidic pH the β-sheet-enriched form is more stable and aggregation is favoured (Baskakov et al., 2001; Hornemann and Glockshuber, 1998; Morillas et al., 1999). All these reports agreed on the fact that PrP^{Sc}-conformation is thermodynamically more stable at pH 7 and below (i.e. acidic), and that the entire conformational transition process is slow (days to weeks) (Riesner, 2003). Other groups have tried to produce prions in cell-culture systems, either by co-expression of PrP^{C} with hereditary pathological mutants (Chiesa et al., 1998; Lehmann and Harris, 1996a; Lehmann and Harris, 1996b) or with refolding assays of recombinant PrP-proteins (Baskakov et al., 2000; Jackson et al., 1999; Lee and Eisenberg, 2003; Zhang et al., 1995), however the results proved to be either negative or inconclusive.

One of the first assays to convert mammalian prions in a cell-free system consisting of purified constituents succeeded in showing that purified PrP^{Sc} could catalyze the PrP^{C}-to-PrP^{Sc} conversion process. Unfortunately, when compared to the amount of template used, very little product was obtained and rendered the assessment of its infectious proprieties impossible (Kocisko et al., 1994). Interestingly it was found that chaperones

like GroEL or Hsp104 (DebBurman et al., 1997) as well as elevated temperature in combination with sulphated glycans like heparan sulphates or pentosan polysulphates could all positively influence the conversion process (Wong et al., 2001).

Recently, two reports from this field have received much attention:

In the first report, Legname and coworkers showed that recombinantly produced mouse prions consisting of aa89-230 polymerized into amyloid fibrils and produced neurological dysfunction after intracerebral inoculation in mice expressing the same truncated PrP-form. Additionally, brain material from these mice proved to be infectious to wild-type FVB mice in a second passage (Legname et al., 2004). This work showed for the first time the infectious properties of *in vitro*-produced, recombinant protein, supporting the protein-only hypothesis. Nevertheless, one cannot rule out that some co-purifying factor from bacteria contributed to the conversion process and it is worth mentioning that the transgenic mice used in this study were reported by others to develop spontaneous prion diseases after long observation times (Chiesa et al., 1998; Westaway et al., 1994).

In a second report Castilla and colleagues presented an optimized protocol for converting PrP^{C} to PrP^{Sc} in a cell-free system. In this report, addition of minute amounts of PrP^{Sc} to a large pool of PrP^{C}-template followed by repeated sonication and incubation steps (termed PMCA for protein misfolding cyclic amplification, Fig. 21) produced large amounts of PrP^{Sc} (Castilla et al., 2005a; Saborio et al., 2001). The misfolded protein produced by this process, resembled in its structural and biochemical properties the hamster brain template and proved to be infectious in wild-type hamsters. Furthermore, the pathology of the hamsters infected with this material was identical to that of animals inoculated with the original template. These data are up to now the most conclusive evidence of *in vitro* generation of prions, and therefore strongly support the protein-only hypothesis.

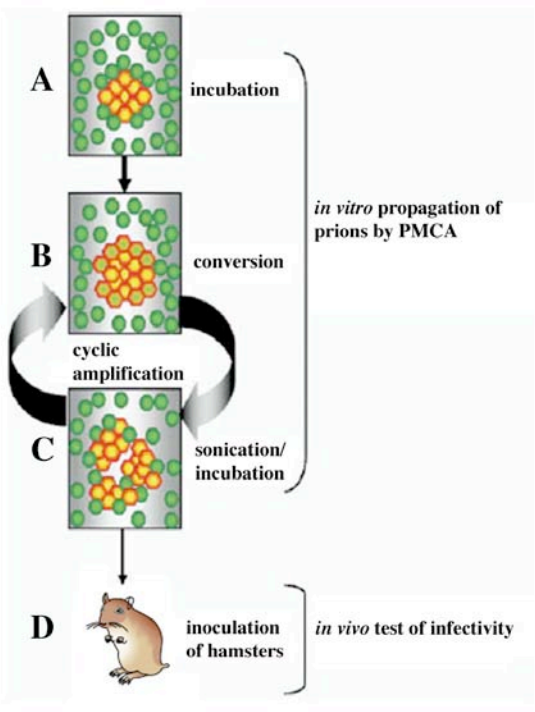

Fig. 21: The Protein Misfolding Cyclic Amplification (PMCA) as a means for *in vitro* conversion and amplification. A small amount of PrP^{Sc}-template (yellow dots) is added to a large pool of PrP^{C}-substrate (green dots) (A). Multiple sonication and incubation-steps allow for increased conversion of PrP^{C}-molecules to PrP^{Sc} (B and C). Inoculation into permissive host verifies the infectivity of *in vitro* produced PrP^{Sc} (D) (adapted from (Saborio et al., 2001)).

In spite of these impressive results the involvement of other factors coming from the utilized brain homogenates cannot be excluded.

The idea that some additional co-factors might be participating in the conversion process is not surprising since it was shown that addition of mammalian RNA preparations to the PMCA-process increased the stoichiometric transformation rate of PrP^{C} to PrP^{Sc} in vitro (Deleault et al., 2003).

Very recently spontaneous *de novo* generation of PrP^{Sc} molecules was shown (Deleault et al., 2007). In these experiments prions formed spontaneously at low frequency in the presence of polyanionic components such as poly(A) RNA-substrates and could thereafter be amplified by PMCA. Accidental contamination was ruled out by utilizing only new or prion-free equipment and source materials in a prion-free laboratory. The produced prions were infectious and lethal when inoculated intracerebrally in wild-type hamsters (Deleault et al., 2007).

III.12: Possible interactors for the PrP-proteins

The existence of potential cofactors participating in the conversion process were initially invoked to explain results obtained from transmission assays of human prions to transgenic mice (Telling et al., 1995) and also in order to explain the protective effect of some PrP-polymorphisms found in humans and ovines (Shibuya et al., 1998). Telling and colleagues observed that mice co-expressing a human and a murine version of PrP^{C} were resistant to infection by human prions, while mice expressing either the human PrP^{C} or a chimeric PrP-version (termed Mhu2M and containing the murine C-terminal part attached to the N-terminal human part) were susceptible to infection. From these results they concluded that the murine allele inhibited the transmission of human prions, but had only a minor effect on the conversion of the chimeric protein (Telling et al., 1995). These findings lead to the hypothesis that a murine host protein (termed Protein X) is indispensable for the conversion process but binds with a higher affinity to the murine PrP^{C}-protein than to the co-expressed human PrP^{C}-form. Therefore human PrP^{C} would not be available for the conversion process by inoculated human PrP^{Sc}. Because only a diminished resistance could be seen when infecting animals expressing the chimeric protein, it was concluded that Protein X would interact with the C-terminus of murine PrP^{C}.

Successively, putative interaction sites between Protein X and PrP^{C} were described. Because substitutions at aa-sites 167, 171, 214 and 218 of PrP^{C} rendered this protein resistant to conversion by PrP^{Sc} it was proposed that these mutations abrogate or diminish the interaction between Protein X and PrP-proteins (Perrier et al., 2002). However, in spite of intense research, the putative chaperone of prion-conversion, Protein X remained elusive (Fig. 22).

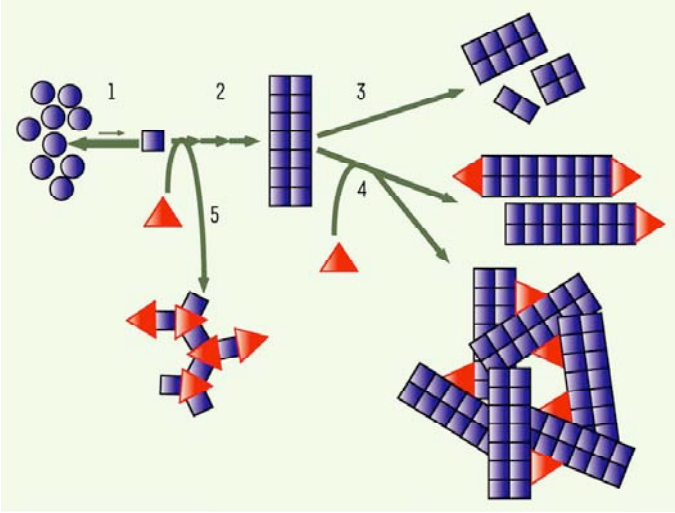

Fig. 22: The potential role of chaperones in the aggregation process of prions. Molecular chaperones (red triangles) could influence the equilibrium between PrP^{C} (blue dots) and PrP^{Sc} (blue rectangles) by catalyzing the production of amyloids (1) or sequestering free PrP^{Sc}-oligomers (5) and thereby achieving a steady production of these molecules. Additionally they could assist in breaking up amyloidal multimers (3) or by stabilizing amyloidal structures and fibrils (4) (from (Bousset and Melki, 2005)).

The hypothesis of an interactor also received support from the field of cell biology. Endocytosis studies on the PrP-protein from chicken (chPrP) showed that it was dependent on clathrin-coated pits for recycling from the plasma membrane into the cell. Since chPrP does not contain any cytoplasmic domain to interact directly with the intracellular endocytosis machinery, it was suggested that some additional transmembrane protein containing an internalization signal for coated pits could guide chPrP on its endocytic path into coated pits (Shyng et al., 1994). More recently, several studies reported the interaction of PrP^{C} with a wide variety of proteinaceous and non-proteinaceous molecules possibly involved in the PrP^{C} to PrP^{Sc} conversion process (summarized in Table 4). Molecules that were suggested to promote conversion include: nucleic acids (RNA as well as DNA) (Cordeiro et al., 2001; Deleault et al., 2003), glycosaminoglycans (GAGs) and sulphated glycans (Pan et al., 2002; Priola and Caughey, 1994; Wong et al., 2001) and lipids (Kazlauskaite and Pinheiro, 2005; Kazlauskaite et al., 2003; Sanghera and Pinheiro, 2002).

Table 4. Molecules to interact with PrP.				
Name of molecule	**Proposed function/known function**	**Interacting with which isoform of PrP**	**Exp. system tested**	**Intracellular compartment**
Bip	chaperone	PrP^{C}/PrPm	cells	ER
Calnexin	chaperone	PrP^{C}	cells	ER
Calreticulin	chaperone	PrP^{C}	cells	ER
Hsp60	aggregation	recPrP/ PrP^{C}	cDNA library, *in vitro*	unknown
ST1	signal transduction, neuroprotection	PrP^{C}	cells, *in vitro*	PM
nucleic acids	chaperone	PrP^{C}	celluls, *in vitro*	nucleus
lipids	chaperone/aggregation	α-/β-PrP/ PrP^{C}/PrP^{Sc}/ PrPm	artificial microsomes	rafts/membrane
Laminin	neurite growth	PrP^{C}	cell membranes	PM
GAG	internalization	recPrP/PrP^{C}	cells, *in vitro*	PM
37- / 67-kDa rec Laminin	internalization	PrP^{C}	cells, *in vitro*	PM
Aplp1	unknown	PrP^{C}	cells	PM
Caveolin1	signal transduction, internalization, concentration	PrP^{C}	cDNA library, *in vitro*	PM
p75-NTR	apoptosis, internalization, transport	PrPfrag	cells	PM, caveolae
CK2	modulator, internalization, concentration	recPrP	cells	caveolae
N-CAM	neurite growth, internalization,	PrP^{C}	*in vitro*	PM, caveolae
Pint1	signal transduction, internalization, transport	recPrP/ PrP^{C}	cells	unknown
Flotillins	signal transduction, raft carrier	PrP^{C}	cells	rafts
Grb-2	internalization, concentration	recPrP/PrP^{C}	cells	vesicles
Synapsin 1b	signal transduction, internalization, concentration	recPrP /PrP^{C}	cells	vesicles
Plasminogen	activator of plasminogen	PrP^{Sc}	cells	rafts/extracellularly
Bcl-2	unknown	PrP^{C}	*in vitro*	unknown
Nrf2	unknown	PrP^{C}	cells	unknown
GFAP	unknown	PrP^{C}/PrP^{Sc}	*in vitro*, brain extracts	unknown

Identifying the minimal requirements for PrP^{C} to PrP^{Sc} conversion is complicated also because many different intracellular compartments seem to be involved in prion-production (Campana et al., 2005; Harris, 2003). Therefore different molecules may interact with PrP^{C} or PrP^{Sc} at different intracellular sites. As summarized in Table 4, numerous potential interactors of PrP^{C} have been found at the level of the endoplasmatic reticulum (ER), in endosomes or lysosomes and on the plasma membrane (PM). The sheer number of potential interactors suggests that a macromolecular complex rather than one single factor would be involved in the internalization and/or conversion of the PrP-proteins and leaves many unanswered questions on the nature and function of this complex.

III.13: Pathogenesis and cytotoxicity

Why PrP^{Sc} harms the central nervous system producing neuropathological lesions and neuronal death remains obscure, in spite of intense research for almost ninety years (reviewed in (Chiesa and Harris, 2001; Roucou and LeBlanc, 2005; Tatzelt and Schatzl, 2007)). The roles of PrP^{C} or PrP^{Sc} in neurodegeneration are still undefined and it is still unclear whether other forms of PrP—possibly of transient nature, could be involved in cytotoxicity.

III.13.1: The role of PrP^{Sc}

In an oversimplified view one might say that the appearance of PrP^{Sc} correlates (spatially and temporally) with neurodegeneration (Jeffrey et al., 2001; Parchi et al., 1996). However, upon closer inspection the picture gets less clear and shows exceptions to the rule. In some situations preclinical symptoms (suggesting initial deterioration of the CNS) were described in diseased animals that contained little to no amounts of PrP^{Sc} in the brain. For instance C57BL/6 mice intracerebrally inoculated with BSE-derived prions developed pathology even though only 55% produced PrP^{Sc} as tested by western blot and immunohistochemistry (Lasmezas et al., 1997). Similar results were reported for mice infected with CJD-derived prions (Manson, 1999). Another study reported that

mice overexpressing PrP^{C} and inoculated with low prion doses showed only preclinical symptoms, never progressing to terminal disease but containing high amounts of PrP^{Sc} deposits in the brain (Thackray et al., 2002). The same was seen when mice were infected with a specific hamster prion strain (Sc237). The animals showed accumulation of PrP^{Sc} in the brain but remained asymptomatic and lived as long as the control animals (also called a subclinical form of the disease, see Fig. 20) (Hill and Collinge, 2002; Hill et al., 2000). Altogether these observations could lead to the following hypothesis: (a) PrP^{Sc} is not the sole factor responsible for cytotoxicity or (b) the production of PrP^{Sc} is actually a means of neurons to protect themselves, as had been suggested for the amyloid production in Alzheimer or Parkinson disease (Rao et al., 2006).

Therefore it is probable that PrP^{Sc} is not the molecule causing neurotoxicity, but may be only an inert by-product of a reaction producing another neurotoxic component. Researchers proposing this view believe that the neurotoxic components are the oligomeric PrP^{Sc}-forms (Bucciantini et al., 2002; Novitskaya et al., 2006; Simoneau et al., 2007). This is yet another hotly debated topic in prion-research, since others believe that oligomers fulfil a protective role, sequestering the monomeric PrP^{Sc}-forms, which in their opinion are the true culprits of neurotoxicity (Chesebro et al., 2005).

III.13.2: A possible role for PrP^{C} in cytotoxicity

III.13.2.1: Cytoplasmic intermediates of PrP^{C} involved in toxicity

When misfolded many proteins (such as PrP), which traffic through the endoplasmic reticulum (ER), are retrogradely transported to the cytosol in order to be degraded by the proteasome in a process known as Endoplasmic Reticulum (ER)-associated protein degradation (ERAD). A growing number of reports show that perturbing proteasome activity (due to aging, cellular stress or the like) or inducing ER-stress initiates the accumulation of cytosolic forms of PrP, which turned out to be toxic in neurons (Fig. 23) (Orsi et al., 2006). Ma et al. could show *in vitro* that the increase of cytosolic PrP-forms produces a toxic effect in cultured cells and causes severe ataxia and cerebellar degeneration in transgenic mice (Ma et al., 2002). It is important to mention that the PrP-forms described in this report differed from PrP^{Sc}-molecules insofar as this cytosolic and proteasome-resistant form retains its secretory leader peptide and does not acquire a glycosylphosphatidylinositol (GPI)-anchor, suggesting that the molecules

never enter the endoplasmatic reticulum (Drisaldi et al., 2003). Furthermore, it was shown that this cytosolic PrP-form might convert PrP^C-molecules in an autocatalytic manner, thereby sustaining further accumulation in the cytosol (Ma and Lindquist, 2002). What is puzzling about this cytosolic form is its toxic effect in neurons but the complete absence of toxicity in fibroblast.

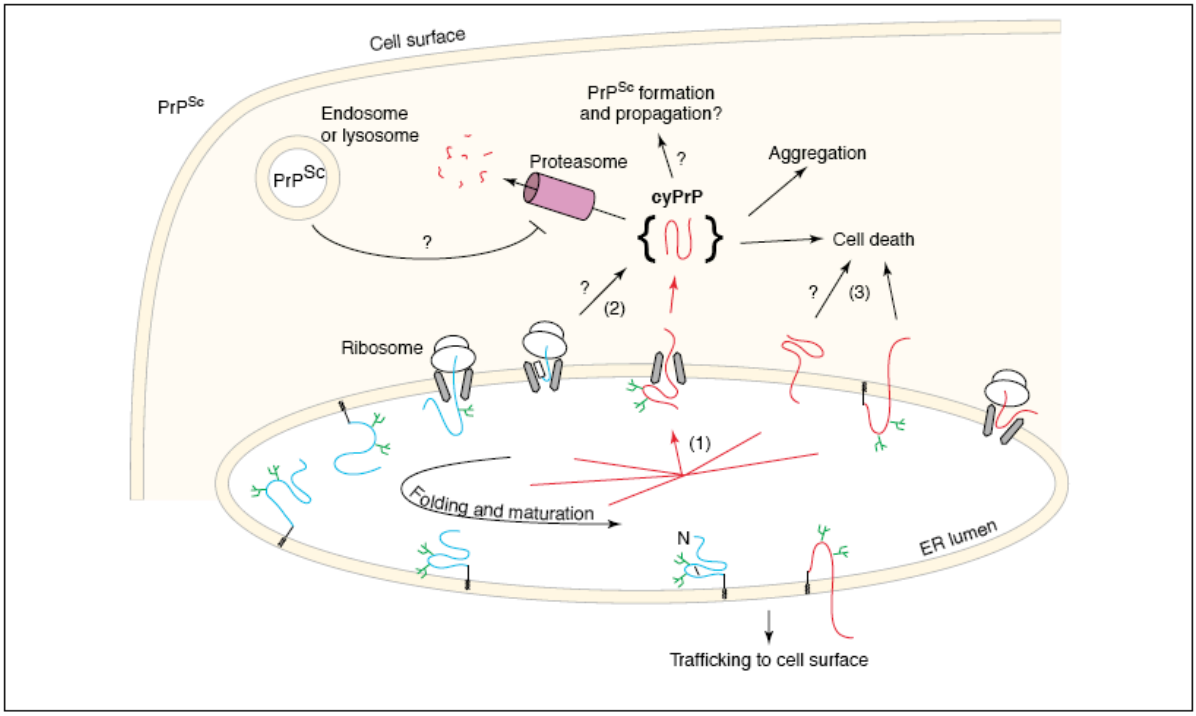

Fig. 23: Cytosolic PrP (cyPrP): a key player in neurotoxicity? The vast majority of PrP^C is translocated into the ER and correctly subjected to post-translational modifications (e.g. addition of sugar-residues (green)) and correctly inserted into the membrane (blue). Some minor population of PrP (red) may enter the cytoplasm by different routes: retro translocation of incorrectly folded forms (1), aborted translocation into the ER (2) or generation of transmembrane forms leading to cell death (3). This transient (in parentheses) cytosolic form (cyPrP), which is usually quickly destroyed by the proteasome, could prolong its stay in the cytosol by forming protease-resistant PrP^{Sc} or aggregation and thereby exert a toxic effect in the cell (adapted from (Hegde and Rane, 2003)).

Cytosolic forms of PrP-proteins have also been reported *in vivo* in mouse hippocampus, neocortex and thalamus as described by Mironov et al. using electronic microscopy (EM). However cytotoxicity was not the focus of this work (Mironov et al., 2003). Additionally, abnormally folded, cytosolic PrP^C was reported to occur upon treatment of cells with cyclosporin A (CsA) (Cohen and Taraboulos, 2003). Cyclosporin A is usually utilized as an immunosuppressant and inhibits the cyclophilin family of peptidylprolyl cis-trans isomerases (PPIases). Cells treated with CsA accumulated proteasome resistant PrP-species, that were deposited in cytosolic structures termed aggresomes and

reminiscent of protein deposits found in some GSS-linked hereditary forms of TSE-diseases (Mishra et al., 2003).

Nevertheless, these findings are somewhat contested by some recent publications, which questioned the neurotoxicity of these cytosolic PrP^{C}-forms, since induction of apoptosis upon proteasomal inhibition occurred even in neurons not expressing PrP, showing that this process is not necessarily PrP-protein related (Fioriti et al., 2005). Additionally, increase of the cytosolic forms was readily induced in cell lines and primary neurons with drugs simulating ER-stress by multiple pathways (such as thapsigargin, tunicamycin and brefeldin A) but no clear findings of cytotoxicity were reported (Orsi et al., 2006). Another group showed that cells overexpressing PrP^{C} produced cytosolic PrP^{C}-aggregates when the proteasome was mildly inhibited. Surprisingly this seemed to have a neuroprotective effect on a healthy immortalized neuronal cell-line (GT-1), while turning proapoptotic and therefore neurotoxic in the PrP^{Sc}-infected version of these cells (scGT-1) (Kristiansen et al., 2005). In sum this suggests that the neurotoxic effects described above are probably not linked to the aberrant cytosolic localization of PrP-proteins but rather to the context in which this happens: Healthy cells do not encounter any disadvantages or might even be protected by cytosolic PrP-forms, while infected cells induce proapoptotic pathways thereby leading to increased cell-death. The reasons for these mechanisms are not understood and need further investigation.

III.13.2.2: Transmembrane forms of PrP^{C} involved in toxicity

PrP^{C} is a protein that is very heterogeneous in its appearance. In addition to the GPI-anchored form (by definition fully translocated into the ER-lumen), two other forms have been reported to exist. These two forms have a transmembrane topology and are called ^{Ctm}PrP and ^{Ntm}PrP (the first having the C-terminus inside the ER-lumen, the latter with the N-terminus inside) (Fig. 24) and were reported to represent approximately 10% of total PrP^{C}. These two forms can be produced due to a hydrophobic domain (TM1, aa111-134), which competes with the N-terminal signal peptide for chaperone binding in the ER. If the chaperones do not intervene after the synthesis of the N-terminus, ^{Ntm}PrP and ^{Ctm}PrP-forms can be produced by insertion of TM1 into the ER-membrane.

Both transmembrane forms utilize TM1 for insertion into the membrane, but have a different orientation after insertion. The N-terminus of the ^{Ntm}PrP-form is exposed into the ER-lumen and is neither glycosylated nor GPI-anchored (Hegde et al., 1998). The topology of the ^{Ctm}PrP-form is such that its C-terminus is exposed to the ER-lumen receiving a GPI-anchor and retains its signal peptide ((Stewart et al., 2001; Stewart and Harris, 2001).

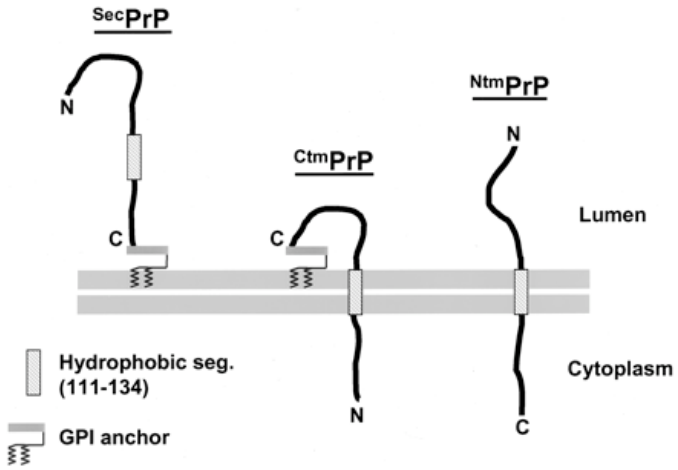

Fig. 24: Schematic of the three topological forms of PrP^C. The two transmembrane forms are named for the C- or N-terminal part exposed to the lumen of the ER; the GPI-anchored form is called ^{sec}PrP by some scientists because of its ability to be shed into the cell supernatant by a process termed GPI-shedding (from (Stewart and Harris, 2001)).

The transmembrane forms represent about 10% of the cellular PrP^C-population under normal conditions, but were found to be present in higher amounts in some hereditary TSEs. While the ^{Ntm}PrP-form was only described in cell culture systems, two mutations within TM1 associated with human hereditary TSE-diseases (P105L and A117V) and were found to augment the production of the ^{Ctm}PrP-form (Hegde et al., 1998; Stewart et al., 2005). Transgenic mice expressing a PrP-form exclusively in the ^{Ctm}PrP-orientation were reported to develop fatal neurological illnesses reminiscent of TSEs; interestingly pathology was dependent on co-expression of wild-type PrP^C (Stewart et al., 2005). This information combined with *post mortem* data from A117V-mutation carriers (Hegde et al., 1998) lead to the hypothesis that the ^{Ctm}PrP-form is involved in neurodegenerative TSE-diseases.

III.13.2.3: Mutated PrP^C-forms involved in toxicity

Some mutations of PrP^C (P101L, PG14, A117V) proved to be neurotoxic in mice but never showed infectious traits (Chiesa et al., 2000; Chiesa et al., 1998; Hsiao et al., 1990). These mice showed spontaneous neurodegeneration strongly reminiscent of the human pathologies. The finding that development of neurodegeneration is not linked to the infectious element strongly suggests that the two forms are not necessarily the same biochemical entity.

Some TSE-linked mutations produce certain metabolic anomalies as found in cell culture studies. Among others characteristics, these mutated forms were shown to become insoluble and resistant to mild protease-digestion (in contrast to PrP^C), were transported in lesser amounts to the plasma membrane (PM) and were retained in intracellular organelles including the ER and the Golgi-compartment (Ivanova et al., 2001; Lehmann and Harris, 1996a; Lehmann and Harris, 1996b; Lorenz et al., 2002; Stewart et al., 2001; Stewart et al., 2005; Wegner et al., 2002).

Interestingly, many studies demonstrated a strong requirement for co-expressed PrP^C, for the development of toxic effects. Brandner and colleagues showed that only PrP^C-overexpressing nervous tissue, homografted into $PrP^{0/0}$-mice, developed histopathological alterations upon infection (Brandner et al., 1996). Another approach chosen by Malucci and coworkers, demonstrated that interrupting the expression of PrP^C post-infection could stop the progress of the infection, prevent neuronal loss and even reverse mild neurophysiologic dysfunctions caused by the disease (Mallucci et al., 2002; Mallucci et al., 2007).

The origin of PrP^C-neurotoxicity could also be mediated by its role as a signal cascade transducer: Antibody-induced crosslinking of PrP^C situated on the plasma membrane of hippocampal and cerebellar neurons triggers extensive and rapid neuronal death by apoptosis (Solforosi et al., 2004).

III.14: Intracellular trafficking of the PrP-proteins

III.14.1: The cell-biology of PrP^C

III.14.1.1: Biosynthesis in the ER

This aspect in prion research has been the subject of numerous publications utilizing a wide variety of cell-types, such as neuronal cell lines (Beranger et al., 2002; Magalhaes et al., 2002; Marella et al., 2002; Nunziante et al., 2003; Shyng et al., 1993; Walmsley et al., 2003), epithelial cell lines (Ivanova et al., 2001; Morel et al., 2004; Peters et al., 2003; Sarnataro et al., 2004; Sarnataro et al., 2002; Vilette et al., 2001) and primary neurons (Fioriti et al., 2005; Madore et al., 1999).

Like for other GPI-anchored proteins, PrP^C is synthesized on the rough Endoplasmic Reticulum (RER), transits through the Golgi apparatus where it undergoes the posttranslational modifications and is finally delivered to the plasma membrane (reviewed in (Harris, 2003)). The N-terminal signal peptide is cut off following translation into the ER lumen. Subsequently oligosaccharides rich in mannose are added (Caughey et al., 1989) onto two asparagines (aa181 and 197 in humans) of the nascent protein (Haraguchi et al., 1989). At this stage, the oligosaccharides of PrP^C are still sensitive to digestion by endoglycosidase H (EndoH), a characteristic of immature, core-glycosylated molecules. On its way through the RER, a disulphide bond is formed between the two cysteine-residues (aa179 and 214 in humans), a prerequisite for proper folding and trafficking (Yanai et al., 1999). Finally, the C-terminal hydrophobic domain is cut off by a transamidase also catalyzing the addition of a GPI-anchor onto a serine (aa 231) (Stahl et al., 1990; Walmsley et al., 2001). During the course of its biosynthesis at the RER, about 10% of translated proteins are folded incorrectly (Lehmann et al., 1999; Ma and Lindquist, 2002) and are degraded by the proteasome through the ERAD-pathway (Endoplasmic Reticulum (ER)-associated protein degradation) (Ma and Lindquist, 2002; Wang et al., 2005; Yedidia et al., 2001). As previously mentioned proteasome inhibition leads to accumulation of cytosolic forms of PrP^C and can result in neurotoxicity (Ma and Lindquist, 2002; Ma et al., 2002). Nevertheless, the involvement of the proteasome in the destruction of PrP^C (and some of its mutant forms) is still debated. In contrast to earlier studies, which suggested the involvement of the proteasome, more recent publications show that neither PrP^C nor its mutants are major

substrates of the proteasomal pathway. Indeed it appears that only a minor amount of PrP was unable to translocate into the ER (possibly caused by overexpression in the system tested), gets ubiquitinated and is thereafter destined for destruction by the proteasome in the cytosol (Campana et al., 2006; Drisaldi et al., 2003; Fioriti et al., 2005). Also occurring in the ER is the insertion of PrP^{C} into the membrane which gives rise to the existence of different topological forms of PrP^{C} (see III.13.2.2).
During the passage of PrP^{C} through the Golgi apparatus, the two core-oligosaccharides mature by the addition of sialic acids, rendering them insensitive to EndoH treatment and sensitive to treatment with neuraminidases. The electrophoretic migration pattern of PrP^{C} on gels reveals three characteristic bands corresponding to its three main glycosylation states: the non-glycosylated protein (at a height of 25 kDa), the mono-glycosylated protein (28 kDa) and the mature, di-glycosylated protein (33 kDa). Under some circumstances a fourth band can be observed which represents the di-glycosylated but immature protein-form (Sarnataro et al., 2004; Sarnataro et al., 2002). Additionally, the relative abundance of un-, mono- and di-glycosylated forms can vary with respect to one another (Moudjou et al., 2001). These characteristics have been useful for strain typing (see paragraph III.10).

III.14.1.2: The role of PrP-glycosylation in conformational stability and in intracellular trafficking

Several lines of investigation suggest that glycosylation of the PrP-protein could play a role in its conformational stability. Computational modelling-studies revealed that the sugars at the second glycosylation-site in particular assist indirectly in stabilizing PrP-conformation, by reducing the mobility of the surrounding water molecules (Zuegg and Gready, 2000). Support for these findings came from studies with fragments of human PrP (aa175-195), showing that once glycosylated, these fragments have significantly reduced tendencies to produce fibrils (Bosques and Imperiali, 2003). Furthermore, cells treated with tunicamycin, a drug that inhibits glycosylation during *de novo*-synthesis of proteins, accumulated increased amounts of malconformed PrP^{C}-forms, with characteristics reminiscent of PrP^{Sc}, indicating the importance of the glycosyl-residues for the correct folding of the PrP-protein (Lehmann and Harris, 1997). *In vitro* results

also showed a facilitated misfolding of un-glycosylated PrP-protein (Priola and Lawson, 2001).

However *in vivo* results contradict these findings. Transgenic mice engineered by site-directed mutagenesis to produce either an un-glycosylated PrP^{C}-form, or mono-glycosylated forms with sugars only at either the first or the second glycosylation site were not affected with respect to PrP maturation and stability. Additionally, these animals did not develop any spontaneous pathology nor was PrP^{Sc}-like protein found in the brain of mice expressing only the un-glycosylated PrP-form (Cancellotti et al., 2005), a result which is at odds with the *in vitro* findings and warrants further investigation.

Glycosylations were also shown to play a role in the trafficking of the PrP-protein. Cell-culture experiments with glycosylation mutants similar to the ones described above in mice, revealed that mutations in both glycosylations sites or at position aa 181 affected the trafficking of PrP^{C} such that increased retention of the protein inside of the ER and Golgi apparatus was observed. In contrast, abrogation of glycosylation at position aa197 did not affect its transport to the plasma membrane (Cancellotti et al., 2005; Lehmann and Harris, 1997; Sarnataro et al., 2004).

Additionally, the glycosylation of PrP is believed to be involved in the tropism of different prion strains for different cerebral zones (DeArmond et al., 1997). The conversion-process affects all different glycoforms but with different efficiencies. This lead to the hypothesis that glycosylation might determine the diverging characteristics of prion strains by affecting their respective protein conformations (see paragraph III.10).

III.14.1.3: The association of PrP-proteins with membrane lipid microdomains

Like other GPI-anchored proteins, PrP^{C} is enriched in specific detergent-resistant membrane domains (DRMs), termed lipid rafts (Gorodinsky and Harris, 1995; Taraboulos et al., 1995).

III.14.1.3.1: The concept of lipid rafts

Based on the fluid mosaic model from Singer and Nicholson (Singer and Nicolson, 1972), a series of experimental results indicated that the plasma membrane is a mosaic of lipids and proteins organized in a bilayer, exhibiting high lateral mobility in a two-dimensional plane (reviewed in (Vereb et al., 2003)). The concept of lipid rafts, restricted domains inside the membrane, (Simons and Ikonen, 1997) proposes that rafts represent small domains sequestering lipids with specific characteristics, resulting in a non-homogeneous plasma membrane organized in discrete compartments in the plane of the bilayer. These lipid-based assemblies derive from the lateral segregation of glycosphingolipids and sphingolipids due to their long and saturated fatty acid chains, resulting in a more ordered state. These structures are believed to be dynamic and to also contain specific membrane proteins with higher affinities for these domains (Edidin, 2003; Simons and Ikonen, 1997) (Fig. 25). By utilizing a biochemical approach, based on the extraction in cold detergents (see below), it has been shown that detergent resistant membranes (DRMs) are characteristically enriched in glycosphingolipids, cholesterol and particular types of proteins, which seem to have an increased affinity for the lipids of the rafts. Some examples of proteins enriched in DRMs are given here: GPI-anchored proteins, proteins with lipid acyl chains as tyrosine kinases of the Src-family, palmitoylated proteins and some transmembrane proteins like the β-secretases and BACE (Brown and Rose, 1992) (Fig. 25). Presently, diverse hypotheses on the nature and size of rafts exist (reviewed in (Edidin, 2003; Zurzolo et al., 2003)). Simons and Ikonen proposed rafts to be small structures of about 50 nm (Simons and Ikonen, 1997); due to fine tuned techniques today's estimations are even smaller but also more divergent (5-350 nm) (Nohe et al., 2006; Yethiraj and Weisshaar, 2007). Alternatively, the lipid shell hypothesis models rafts as proteins, which nucleate a "shell" of lipids around themselves (Anderson and Jacobson, 2002). In the same line it has been proposed that rafts are derived by the clustering of specific lipids in restricted areas as result of the clustering of raft associated proteins (Helms and Zurzolo, 2004; Paladino et al., 2004).

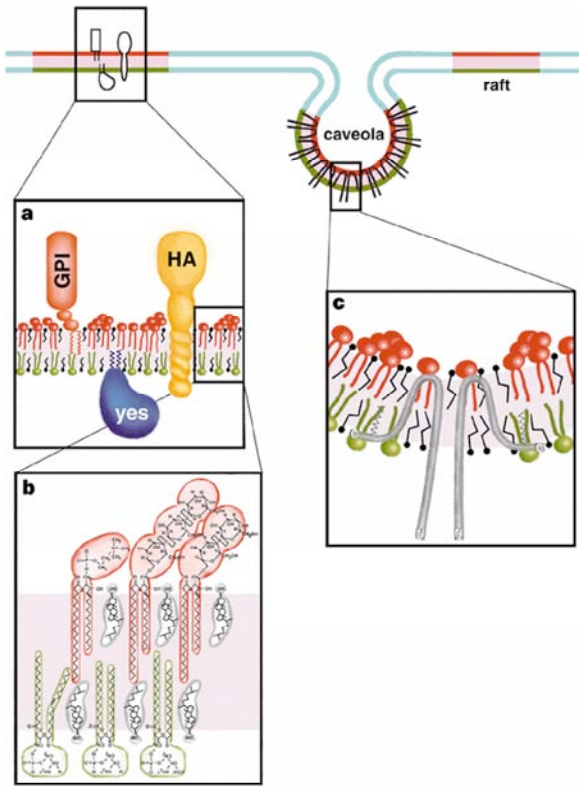

Fig. 25: Schematic of the organizations of rafts and caveolae in the plasma membrane. The membrane (blue) contains subdomains, termed rafts (red) in which unsaturated phosphatidylcholine is mainly on the exoplasmic side of the membrane and which also contain a higher amount of cholesterol. **a)** Lipid Rafts contain GPI-anchored membrane proteins, which are attached to the exoplasmic side of the plasma membrane, then proteins which are attached by acyl chains (the kinase Yes from the Src-family serves here as an example) and also proteins which contain a long hydrophobic transmembrane domain (the protein hemagglutinin of the influenza virus is shown here). **b)** The bi-layer of lipid rafts is also asymmetric with respect to the outer and inner leaflets. Sphingomyelin and glycosphingolipids (all red) are enriched in the outer layer, while glycerolipids such as phosphatidylserine and phosphatidyl ethanolamine (all green) are found in the cytoplasmic layer. Cholesterol (grey) is present in both layers and intercalates between the other elements. **c)** Caveolae form from rafts where molecules auto-associate with caveolin, which forms an invagination. Binding of cholesterol and the acylation of C-terminal cysteines mediate the rafts-interactions (from (Simons and Ikonen, 1997)).

III.14.1.3.2: Detergent resistant membranes (DRMs) and rafts in living cells

One of the main methods for isolating and defining the composition of rafts in cell membranes was their resistance to extraction with non-ionic detergents as Triton X-100 at cold temperatures followed by buoyancy in sucrose gradients (Edidin, 2003; Zurzolo

et al., 2003). Utilizing these criteria, researchers could show that some proteins and lipids from both artificial membranes and cells were resistant to the action of detergents. It is important to stress that DRMs isolated by this technique are not equal to lipid rafts in native membranes, which appear to be much more complex in terms of their composition, content and organization and therefore only serve as an approximation (Edidin, 2003; Zurzolo et al., 2003). In fact many researchers caution against or even oppose the equation of DRMs with discrete membrane microdomains known as lipid rafts (Brown and London, 1998; Heerklotz et al., 2003; Lichtenberg et al., 2005). Since detergents influence the interactions between proteins and lipids obtained experimentally from DRMs, these reveal some biochemical characteristics but cannot provide any information about the organization of these components in living cells. Nonetheless, when complemented with other approaches, the criterion detergent resistance serves as a useful means for receiving hints at the composition and organization of membrane microdomains and is by now an accepted technique in molecular biology (London, 2005; Schuck et al., 2003). Due to their elusive existence, but also due to their small size and dynamic structure (reviewed in (London, 2005; Shaw, 2006)) researchers began to develop additional techniques for studying the lipids and proteins of lipid rafts with respect to their function and dynamics in living cells (reviewed in (Helms and Zurzolo, 2004)). Today, quantitative and dynamic measurements can be additionally done by techniques such as electron microscopy, single particle tracking, optical tweezers, FRAP (fluorescence recovery after photobleaching) and FRET (fluorescence resonance energy transfer).

III.14.1.3.3: The presence of PrP^C in DRMs and the nature of their interaction

PrP^C is localized in DRMs (Gorodinsky and Harris, 1995; Sarnataro et al., 2004; Taraboulos et al., 1995) and its association to DRMs has been studied in neurons (Madore et al., 1999) (Fig. 26).

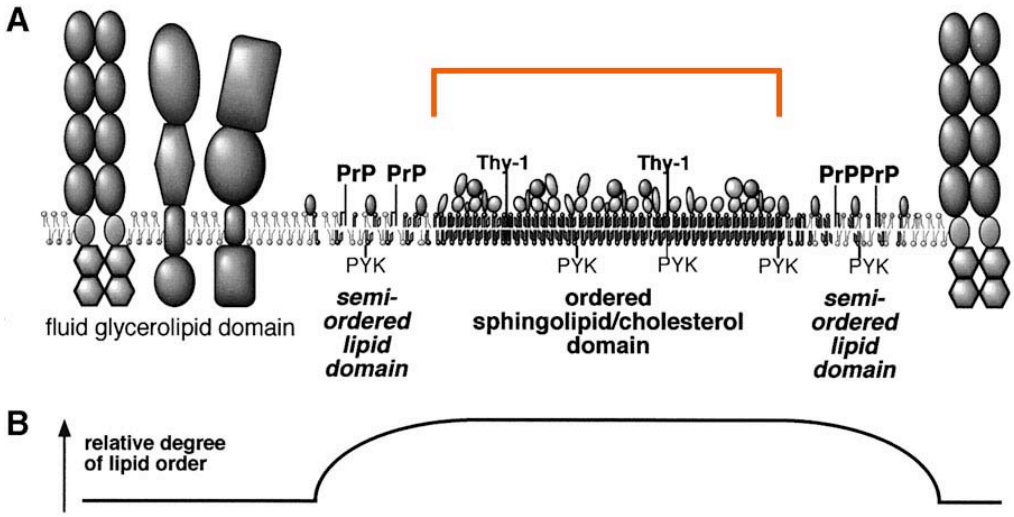

Fig. 26: Model explaining how graded ordering of lipids due to phase partitioning, affects the localization of proteins in the surface membrane. A) Diagram of a cellular plasma membrane with the extracellular part highest. Sphingolipids (straight lipid chains, underneath red bracket) form a highly ordered domain, which contains proteins such as Thy-1. This highly ordered island is surrounded by less ordered membrane containing fluid glycerolipids with unsaturated (kinked) lipid chains and transmembrane proteins. Between the fully-fluid domain on the outside and the fully-ordered part on the inside is a phase of transition termed semi-ordered lipid domain, which also contains PrP^C. **B)** Hypothesised relative degree of lipid order (in arbitrary units) corresponding to the membrane shown in A (adapted from (Madore et al., 1999)).

In contrast to other GPI-anchored proteins, the association of PrP^C to rafts can not only be mediated by the GPI-anchor (Kaneko et al., 1997; Taraboulos et al., 1995) but also by the N-terminal region of its ectodomain (Campana et al., 2007; Walmsley et al., 2003). Different studies also showed that the loss of the GPI-anchor does not abrogate the affinity of PrP^C for cholesterol and sphingolipids (Baron and Caughey, 2003; Campana et al., 2007; Mahfoud et al., 2002; Sanghera and Pinheiro, 2002; Walmsley et al., 2003). When formulating the lipid raft-hypothesis it was proposed that lipid rafts could be vehicles to transport GPI-anchored proteins to the plasma membrane (Simons and Ikonen, 1997). This does not seem to be the case for PrP^C, since destabilizing its association with these microdomains does not affect its exocytic transport or its polarized distribution in the plasma membrane (Campana et al., 2006; Sarnataro et al., 2002). Recent results from our laboratory suggest that rafts have a role in PrP^C-endocytosis (see paragraph III.14.1.5.1) but also in the maturation and correct folding of this protein (Campana et al., 2006; Sarnataro et al., 2004). It appears that the nascent PrP^C-protein associates with cholesterol-enriched rafts earlier than other GPI-anchored

proteins, during its synthesis at the rough endoplasmatic reticulum (RER). This association is obligate for its correct folding, since cholesterol depletion increased the misfolding of the protein. Additionally, cholesterol and sphingolipids have different roles during the maturation of PrP^C, since only cholesterol depletion slowed down its maturation rate and increased its misfolding (Sarnataro et al., 2004). The role of cholesterol in folding of PrP^C may be direct or indirect: (a) cholesterol is an integral part of lipid rafts, and the microdomain-environment is needed for the correct folding of PrP^C or (b) cholesterol is directly participating in the folding process as a lipochaperone (Bogdanov and Dowhan, 1999; Campana et al., 2006; Campana et al., 2005; Sanders and Nagy, 2000).

III.14.1.4: The distribution of PrP^C on the plasma membrane

The distribution of PrP^C on the plasma membrane of polarized cells depends on the cell type. In some cellular models, such as in primary cerebellar granule neurons (CGN) from wild-type mice (Madore et al., 1999), transgenic for ovine PrP^C (Cronier et al., 2004) or expressing pathogenic forms of murine PrP^C (Fioriti et al., 2005) the protein appears to be homogeneously dispersed on the cell body as well as on the neurites and axons. Contrasting this are findings from experiments utilizing primary hippocampal neurons where PrP^C localizes to axons in mature neurons (Galvan et al., 2005). Also in polarized epithelial cell lines like MDCK, FRT and Caco-2/TC7 as well as in primary enterocytes localization of the protein occured to a greater degree on the basolateral side (Morel et al., 2004; Sarnataro et al., 2004; Sarnataro et al., 2002). However the localization of PrP^C could be dependent on the cell system or on the origin of the expressed protein since human PrP^C was recently found to be apically-situated in MDCK and Caco-2 cells (De Keukeleire et al., 2007).

III.14.1.5: Internalization and recycling

Neosynthesized PrP^C reaches the plasma membrane of the murine neuroblastoma N2a cells in approximately one hour (Borchelt et al., 1990). One hour is also required for the

internalization and continuous recycling of the protein (Harris, 2003). Others have shown that the internalization process is inducible by the addition of zinc or copper ions, leading to the hypothesis that PrP^C could serve either as a receptor for the uptake of these ions (Lee et al., 2001; Pauly and Harris, 1998; Watt and Hooper, 2003) or be involved in signal transduction processes caused by these ions (Mouillet-Richard et al., 2000). It is still unclear whether the mechanism of ion-uptake utilizes the same molecular machinery as for its constitutive endocytosis (Lee et al., 2001; Magalhaes et al., 2002; Pauly and Harris, 1998).
The mechanism for PrP^C-internalization is currently debated and seems to use different pathways depending on the cell types utilized (see below).

III.14.1.5.1: Overview of the different mechanisms of endocytosis

The endocytosis of plasma membrane components can be accomplished by different molecular mechanisms mainly distinguishable by their dependence or independence from clathrin, one of the main proteins involved in endocytosis (Ehrlich et al., 2004; Kirchhausen, 2000; Robinson, 2004).
The endocytosis by clathrin coated invaginations represents a lipid-raft independent and selective cellular mechanism used for diverse functions such as the internalization of nutrients, transduction of signals or recycling of synaptic vesicles. The mechanism utilizes clathrin-lattices on the inside of the endocytosed membrane (hence clathrin coated pits) to induce membrane curvature, which quickly disperses upon vesicle-formation due to the involvement of molecules such as Hsc70 and auxilin (Fotin et al., 2004).
The clathrin-independent mechanisms are less well characterized; however the best known among them is the endocytosis involving caveolae. These invaginations are flask-shaped and resemble small caves (hence the Latin-derived name caveolae) of 50-100nm in length, initially identified by electron microscopy in a wide variety of cells and tissues (Stan et al., 1997) and are often exploited by pathogens to enter and infect cells (van der Goot and Harder, 2001). Caveolae were shown to fractionate as detergent resistant microdomains (DRM). In contrast to those DRMs previously described

enriched in GPI-anchored proteins, they contain caveolin-proteins, which serve as a marker for this subgroup of DRMs (Schnitzer et al., 1995).
The invaginations of caveolae are probably directed by the polymerization of the caveolin-protein family, which consists of three members, type 1, 2 and 3. In particular Caveolin-1 seems to have an obligate role in the formation-process, since its absence also causes the abrogation of caveolae in cells (Fra et al., 1995; Schnitzer et al., 1995).
GPI-anchored proteins can be internalized by diverse mechanisms. The majority of them are constitutively internalized by a clathrin independent, dynamin- and lipid rafts-dependent mechanism (Mayor and Riezman, 2004). In contrast to this, cross-linked GPI-anchored proteins can be internalized by caveolae. When interacting with transmembrane proteins possessing a signal for clathrin-dependent endocytosis, GPI-anchored proteins also utilize the clathrin-dependent pathway (Mayor and Riezman, 2004).

III.14.1.5.2: Mechanisms of endocytosis of PrP^C

Despite its association with rafts, clathrin-dependent endocytosis of PrP^C was shown in neuroblastoma cells (Martins et al., 1997; Shyng et al., 1995; Sunyach et al., 2003), neurons (Sunyach et al., 2003) and in epithelial cells cells (Shyng et al., 1994) (Fig. 27). Utilizing electron microscopy, PrP^C has also been described in clathrin-coated vesicles (Madore et al., 1999; Shyng et al., 1995; Sunyach et al., 2003). When cells are incubated with a hypertonic sugar solution (used to destroy clathrin lattices) inhibition of PrP^C endocytosis can be accomplished (Shyng et al., 1995).
Since PrP^C, as a GPI-anchored protein, does not possess a cytoplasmic domain to interact with clathrin-molecules or with adaptor molecules (Sunyach et al., 2003), other membrane-associated proteins were recently proposed to serve as a mediator of the signal, including formyl-peptide-receptor-like-1 (FPRL1) (Brandenburg et al., 2007), low-density lipoprotein receptor-related protein 1 (LRP1) (Taylor and Hooper, 2007) and apolipoprotein E (Gao et al., 2006).

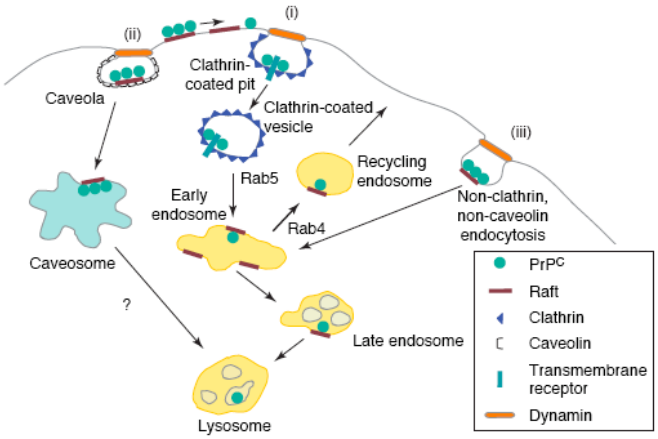

Fig. 27: Mechanisms of internalization of PrP^C. The main mechanism for PrP^C internalization is clathrin-dependent and lipid raft-independent (i) involving Rab5-positive vesicles for endocytosis and Rab4 for recycling. In addition, caveolin-mediated endocytosis has been shown in glial cells (ii). A third way of endocytosis, clathrin- and caveolin-independent but lipid raft-dependent has also been proposed (iii) (adapted from (Campana et al., 2005)).

The N-terminal region of PrP^C seems to be indispensable for its localization in clathrin-coated vesicles and therefore for its internalization, since deletion of this region gives a strong reduction in the internalization-rate (Shyng et al., 1995). Therefore it was suggested that this region could interact with proteins guiding PrP^C into clathrin-coated vesicles.

In contrast to this, other reports show that caveolae or caveolae-like domains are utilized for PrP^C internalization (Marella et al., 2002; Peters et al., 2003; Vey et al., 1996) (Fig. 27) as has already been described for other GPI-anchored proteins (Mayor et al., 1994). In CHO-cells expressing caveolin-1, PrP^C is found to be enriched in caveolae, both at the plasma membrane or in the trans-Golgi network (TGN) but is surprisingly absent from clathrin-coated vesicles (Peters et al., 2003). Recent data from our laboratory show that rafts and clathrin but not caveolae are utilized for internalization in polarized epithelial cells (Sarnataro et al., in preparation). Additionally it was shown in mature primary hippocampal neurons that PrP^C internalization is dependent on DRMs and cholesterol (Galvan et al., 2005).

Altogether, it appears that PrP^C can be internalized by at least two pathways, clathrin-dependent and raft-dependent endocytosis. Another possibility is that PrP might be internalized by a pathway that is both raft- and clathrin-dependent, as previously shown

for several bacterial toxins and surface receptors (Abrami et al., 2003; Puri et al., 2005; Shogomori and Futerman, 2001; Stoddart et al., 2002).

In the course of its endocytosis, PrP^C pinches off from the plasma membrane, reaches early endosomes, either recycles back through recycling endosomes to the plasma membrane or proceeds to late endosomes and lysosomes (Brown and Harris, 2003; Lee et al., 2001; Magalhaes et al., 2002; Marella et al., 2002; Peters et al., 2003) (Fig. 27). Independent of the mechanism utilized for endocytosis (either rafts- or clathrin-dependent), PrP^C arrives in late endosomes and, if not recycled to the plasma membrane, is degraded in lysosomes. However, it has also been shown that PrP^C could be relocalized to the trans-Golgi-network and even to the endoplasmatic reticulum (Beranger et al., 2002) in the presence of dominant-negative Rab4 or constitutively active Rab6. Very recently, one group reported that a truncated form of PrP^C (aa 23-230) accumulated in the nucleus interacting with chromatin in living cells and to not be cytotoxic (Crozet et al., 2001).

In conclusion PrP^C seems to enter many different organelles during its intracellular trafficking and this appears to be a fine-tuned mechanism, which can be disturbed with the overexpression of certain Rab-proteins (e.g. Rab4 or Rab6) in cells.

III.14.6: Proteolytic cleavage and release by GPI-shedding

In the course of its internalization, PrP^C can be submitted to several proteolytic events, producing C-terminal fragments of 27, 22 and 18 kDa in size (highly-glycosylated, intermediate and un-glycosylated forms respectively) (Chen et al., 1995; Mishra et al., 2002; Shyng et al., 1993). Additionally 1-5% of the protein can be cut near the GPI-anchor while situated on the plasma membrane and therefore liberated into the extracellular medium, by a process called GPI-shedding. This is probably mediated by phospholipases or copper-dependent proteases (Parkin et al., 2004; Toni et al., 2005). The relevance of these processes is not well understood and needs further investigation.

III.14.2: The cell biology of PrP^{Sc}

Establishing a system, permitting prion propagation in cell culture, gave researchers the opportunity to study the biosynthesis of PrP^{Sc}, the mechanisms of infection and the conversion-process (Campana et al., 2005; Harris, 1999a; Solassol et al., 2003) as well as the discovery of potential therapeutic molecules (Beringue et al., 2004; Gilch et al., 2007; Touil et al., 2006). The few cell lines, utilized in prion-research today, are mainly of neuronal origin: N2a-cells are murine neuroblasts (Butler et al., 1988; Race et al., 1987) while GT-1-cells derive from murine hypothalamus (Schatzl et al., 1997) and SMB-cells are unspecified scrapie mouse brain-cells (Clarke and Haig, 1970). An exception to the rule is an epithelial cell line derived from rabbit ectopically expressing ovine PrP^{C} (Vilette et al., 2001). Unfortunately only a few experimental prion-strains can be propagated in these lines. Additionally these cell lines do not show any cytotoxicity, which on one hand allows working with chronically infected cultures but on the other hand is not quite adequate for studying the neurotoxic mechanisms of TSEs. Until lately, no cellular system proved capable of replicating and propagating prions originating from natural prion strains.

Another common tool in today's molecular biology are antibody-based techniques. Production of PrP^{Sc}-specific antibodies (i.e. discerning between the cellular form of the PrP^{C}-protein and the malconformed counterpart) was not very successful and appears to be restricted in their use (Korth et al., 1997), therefore making advances in research on PrP^{Sc} slow. To circumvent this, animals/cell systems with knocked-out PrP^{C} had to be created on one side (Bueler et al., 1992; Manson et al., 1994; Sakaguchi et al., 1996) and protocols for augmenting the immunoreactivity in immunofluorescence-experiments had to be established on the other side (Taraboulos et al., 1990). Due to these technical problems, the study of the cell biology of PrP^{Sc} is restricted to biochemical analysis and microscopy with little sensitivity; therefore results from these experiments must be interpreted with caution (rev. in (Campana et al., 2005)).

III.14.2.1: Synthesis and subcellular localization of PrP^{Sc}

As shown by a study with N2a cells, the synthesis kinetics of PrP^{C} and PrP^{Sc} are different. PrP^{C} is synthesized and degraded rapidly (half life of 5 hours), while PrP^{Sc} is more slowly synthesized (15 hours) (Borchelt et al., 1990) with a half-life longer than 24 hours (Caughey and Raymond, 1991). Additionally, PrP^{Sc} accumulated inside of infected cells as shown by several reports (Borchelt et al., 1990; Caughey and Raymond, 1991; Taraboulos et al., 1992). Using both biochemical and immunofluorescent techniques, PrP^{Sc} was revealed in the Golgi apparatus (Taraboulos et al., 1990) and by electron microscopy (EM) in the endolysosomal compartment (Arnold et al., 1995; McKinley et al., 1991; Mironov et al., 2003). This intracellular distribution pattern corresponds to findings in post mortem brain-samples from CJD-patients and from mice infected with scrapie (Grigoriev et al., 1999; Laszlo et al., 1992). Minor amounts of PrP^{Sc} were also found on the plasma membrane of cells (Caughey and Raymond, 1991; Jeffrey et al., 1992; Vey et al., 1996). Nevertheless its membrane attachment seems to be different to PrP^{C}, since PrP^{Sc} is inefficiently released by the enzyme phosphatidylinositol-specific phospholipase C (PIPLC), contrasting to the findings obtained with PrP^{C} (Borchelt et al., 1990; Caughey et al., 1990; Stahl et al., 1990). Since the anchor of PrP^{Sc} becomes fully susceptible to PIPLC when the protein is denatured in SDS, researchers suggest that the aggregation state or the association with other molecules renders the GPI-anchor physically inaccessible to cleavage (Narwa and Harris, 1999).

PrP^{Sc} was also revealed in the nucleus of some chronically infected cells and interacting there with chromatin in living cells (Mange et al., 2004) as recently reported for a truncated PrP^{C}-form (Crozet et al., 2006). The physiopathological implications from these findings are not yet understood.

As a conclusion, it is clear, that the synthesis and half-life appear to differ between PrP^{C} and PrP^{Sc}, suggesting that the change in conformation also modifies these traits. Additionally, in spite of its partial membrane localization, PrP^{Sc} appears to aggregate differently or to associate with other molecules, rendering it inaccessible to cleavage by PIPLC. Its intracellular localization in the Golgi apparatus and in the endo-lysosomal compartment is similar to the localization of PrP^{C} (discussed above). Possible differences in localization appear to be more quantitative than qualitative by nature but

need further investigation and better tools need to be developed to investigate these issues.

III.14.2.2: Cellular compartments where PrP^{C} to PrP^{Sc} conversion could occur

III.14.2.2.1: The endoplasmatic reticulum (ER)

One of the "hot-topics" in prion-research is the quest for the site or organelle where the process of conversion from PrP^{C} to PrP^{Sc} occurs. This event might occur on the plasma membrane, the first site where endogenous PrP^{C} and exogenous PrP^{Sc} are presumed to interact, or immediately after internalization, in the endolysosomal compartment (Borchelt et al., 1992; Prado et al., 2004) (Fig. 28).
One intriguing possibility could be that once internalized, PrP^{Sc} undergoes retrograde transport to the Golgi apparatus and/or the ER, where it could perturb the biosynthesis of PrP^{C} and imprint its malconformation directly on the nascent wild-type protein (Fig. 28).
Rab6 is a GTPase involved in the retro-transport from Golgi apparatus to ER (rev. (Darchen and Goud, 2000)). The idea of possible PrP^{C}-PrP^{Sc} interaction in the ER is supported by a report in which increased retrograde transport of PrP^{C} was achieved by expression of constitutively active Rab6-GTPase (Fig. 28), which also led to augmented amounts of PrP^{Sc} in infected cells (Beranger et al., 2002). This suggests that the ER could have an important role in the conversion-process. One could also argue that a high amount of potential substrate for the conversion-process transits through the ER. It is also possible that nascent PrP^{C} is more prone to malconformation than mature protein (Campana et al., 2005). The role of the ER in pathological conversion is also supported by data indicating that some hereditary mutant forms of PrP^{C} produce biochemically identifiable intermediates, the first of which is generated in the ER (Harris, 1999b; Harris, 2003) (Fig. 28). To this aim, CHO cells (Chinese hamster ovary) overexpressing pathological mutants were metabolically labelled so that it was possible to monitor the kinetics of the acquisition of three characteristic traits of PrP^{Sc} (resistance to PIPLC, detergent-insolubility and resistance to protease-digestion). This enabled a kinetic description of the conversion process: within minutes after synthesis (with the protein

still in the ER) PrP acquires PIPLC-resistance, typical of PrP^{Sc} as discussed above. After one hour, it acquires the second PrP^{Sc}-trait, insolubility in detergents, which reflects the aggregation state of the proteins. This was confirmed with sucrose gradients, which showed a gradual decrease in signal sedimenting at 4S (i.e. monomeric PrP) within time, as well as a gradual accumulation of PrP-species sedimenting at more than 20S (i.e. aggregates of more than 30 molecules of PrP). The third and final phase-characteristic, that these PrP-molecules acquire, is the notorious resistance to proteases, which reaches a maximum only after hours of labelling (Harris, 1999b; Harris, 2003). This lead to the hypothesis that the PIPLC-resistance, which occurs while the protein is still present in the ER, might be the first characteristic for the conformational change while detergent insolubility and protease-resistance are only second and third trait to change and happen only later.

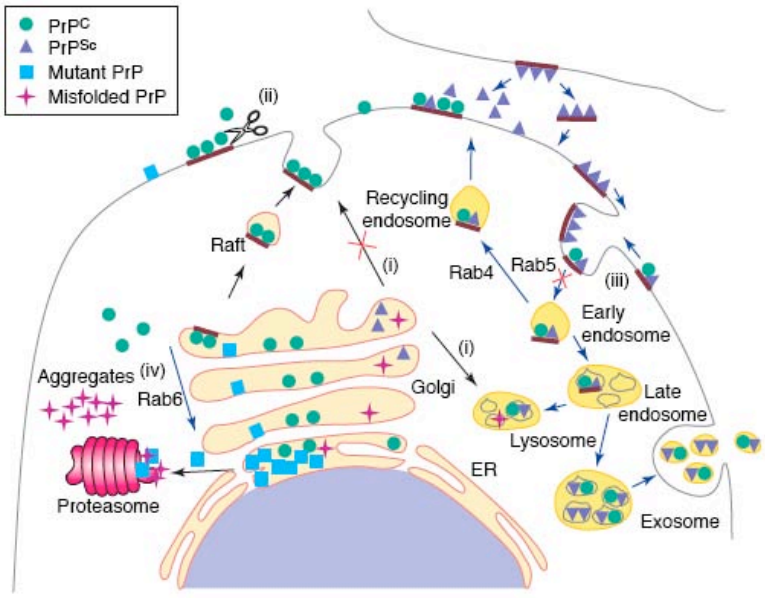

Fig. 28. Possible sites for the production of PrP^{Sc}. PrP^{C} (green circles) is synthesized in the ER. Possible interacting proteins like misfolded PrP and pathological mutants (blue rectangles) are also found here and are retrotranslocated to the proteasome. PrP^{C} passes through the Golgi apparatus to the surface where it might interact with exogenous PrP^{Sc} (blue triangles) either inside or outside of lipid rafts. During its lifetime PrP^{C} recycles between membrane and intracellular compartments and might therefore interact with its malconformed counterpart in the endolysosomal pathway. Abrogation of PrP^{C}-transport to the surface (i) abolishes infection of cells, the same is achieved by induction of shedding of PrP^{C} from the cell surface (ii). Reduction of internalization of PrP^{C} (iii) also decreases the amount of produced PrP^{Sc} (adapted from (Campana et al., 2005)).

Altogether these data suggest that the ER may have two roles in the conversion process. Firstly the ER could be directly involved in the case of hereditary TSEs, where malconformed PrP-forms may intimately interact with nascent proteins in the ER. Secondly, in the case of infectious TSEs, the ER could be the site of amplification for PrP^{Sc}, previously produced at other sites in the cell (reviewed in (Campana et al., 2005)).

III.14.2.2.2: On the way to the plasma membrane

Several studies indicate that conversion of PrP^{C} can only occur once the protein has reached the plasma membrane (Caughey and Raymond, 1991; Taraboulos et al., 1992). Consistent with this, it has been shown that infection of cells can occur by bringing them into contact with a simple metal rod on which PrP^{Sc} had been previously adsorbed (Weissmann et al., 2002), suggesting that extracellular contact on the plasma membrane suffices. Reports supporting this notion showed that removal of PrP^{C} from the plasma membrane (either by inducing endocytosis by antibody-crosslinking or by enzyme-induced shedding) (Fig. 28) cured chronically infected cells and prevented *de novo* infection (Caughey and Raymond, 1991; Enari et al., 2001; Taraboulos et al., 1992). In addition treatment with suramin, a compound, which induces PrP-accumulation in a post ER/Golgi compartment and therefore blocks its expression on the plasma membrane, was able to cure chronically infected cells (Gilch et al., 2001; Taraboulos et al., 1992).

Why the presence of PrP^{C} on the plasma membrane is a prerequisite for the conversion process is not clear, but a few theories have been proposed: i) Post-translational modifications (such as glycosylation-maturation in Golgi apparatus) could be required for the conversion process; ii) the lipid environment of the plasma membrane could be relevant for the refolding process itself or for enabling the interaction between PrP^{C} and PrP^{Sc}; iii) finally one could envision the involvement of a yet to be discovered local factor supporting the conversion process (Campana et al., 2005; Gilch et al., 2001; Prusiner, 1998).

III.14.2.2.3: The endocytic compartment

Some experimental results are compatible with the notion that the endolysosomal pathway is the site of scrapie conversion. Blocking the internalization of PrP^{C} by low temperatures also eliminates PrP^{Sc} from the membrane of infected cells in culture, suggesting that internalization is required (Borchelt et al., 1992; Caughey and Raymond, 1991; Horonchik et al., 2005; Taraboulos et al., 1992). Additionally, it was reported that PrP^{Sc} is proteolytically trimmed at its N-terminus in an acidic compartment immediately after its synthesis and in some cases accumulates in late endosomes (Arnold et al., 1995; McKinley et al., 1991; Taraboulos et al., 1992), which supports the role of endocytic compartments in conversion. Another finding is that expression of the dominant negative version of the GTPase Rab4, which inhibits recycling to the plasma membrane (Fig. 27) (thereby leading to an increase of PrP-protein in the endolysosomal compartment), resulted in increased production of PrP^{Sc} (Beranger et al., 2002). It could be that the conversion process requires partially denaturing conditions such as those found in compartments with acidic pH (i.e. lysosomes or late endosomes) (Prusiner, 1996).

Involvement of clathrin-coated vesicles in the conversion process was also suggested by some reports (Kaneko et al., 1997; Peters et al., 2003; Sunyach et al., 2003; Vey et al., 1996). Since the endocytosis mechanism of PrP^{C} is still unclear (discussed above) this possibility needs to be further investigated.

III.14.2.2.4: The role of lipid rafts in the conversion process

Evidence that lipid rafts are involved in human pathologies abounds (reviewed in (Fantini et al., 2002; Simons and Ehehalt, 2002)). As shown for other pathogens it has been hypothesized that prions exploit lipid rafts for entering cells (Simons and Ehehalt, 2002; van der Goot and Harder, 2001) (Fig. 28 and Fig. 29).

It seems that lipid rafts are a key element for the formation of PrP^{Sc}. Although the exact mechanisms remain obscure and are debated by researchers, some models could be envisioned.

1) Lipid rafts could act as transport platforms for taking PrP^{C} to specific intracellular compartments where it could encounter PrP^{Sc} (Fig. 29A). Data from our own laboratory show that in transfected cells, treatments that destabilize the association of PrP^{C} with lipid rafts does not alter its exocytic transport to the plasma membrane (Sarnataro et al., 2002), but slows down its endocytosis (Sarnataro et al., in preparation). These data support the hypothesis that rafts could be involved in regulating PrP^{Sc} conversion during endocytosis.
2) Lipid rafts might contain machinery such as proteins or lipids that are indispensable for the formation of PrP^{Sc} (Fig. 29B). This would be supported by the factor X-hypothesis (see paragraph III.12).
3) Lipid rafts may facilitate the encounters between PrP^{C} and PrP^{Sc} molecules (e.g. by clustering the molecules) and therefore favour conversion (Fig. 29C). In this hypothesis in which lipid rafts serve as a meeting place, is indirectly supported by work from Baron et al., which showed that PrP^{C} could be converted into PrP^{Sc} only upon insertion into contiguous membranes (Baron et al., 2002).
4) Another alternative could be that the interaction of PrP with different lipids produces different kinds of conformations (Fig. 29D). In this model, the different lipids could act as lipochaperones and facilitate/preserve the unfolding of α-helical or the refolding into β-helical conformations.

The notion that lipid domains somehow participate in the conversion process came at first from reports showing that both PrP^{C} and PrP^{Sc} are associated with DRMs (Baron and Caughey, 2003; Baron et al., 2002; Botto et al., 2004; Naslavsky et al., 1997; Taraboulos et al., 1992; Taraboulos et al., 1995). Additionally it was shown that prion-aggregates contained low amounts of two sphingolipids (galactosylceramide and sphingomyelin) usually found in lipid rafts (Klein et al., 1998b). It is important to stress that in some cases different lipid rafts appear to be hosting PrP^{C} versus PrP^{Sc} and that these can be separated by solubilization and density floatation techniques (Naslavsky et al., 1997). This suggests that the conversion process is not just a matter of refolding the participating PrP^{C}-protein but that PrP^{Sc} actively influences the surrounding lipid raft-

compartment (Fig. 29A) or that the two proteins have distinguishable affinities for different DRMs (maybe due to their folding differences) (Fig. 29B).

One interesting piece of evidence hinting at the role of rafts in conversion was a report showing that a transmembrane form of PrP^{C} does not localize to lipid rafts anymore. This form was also resistant to the conversion process while co-expressed normal PrP^{C} was not (Kaneko et al., 1997; Taraboulos et al., 1995). The destabilization of lipid rafts by depleting cholesterol in living cells also resulted in inhibition of PrP^{Sc}-production (Mange et al., 2000; Taraboulos et al., 1995); however depletion of sphingolipids resulted in an increase of PrP^{Sc}-replication (Naslavsky et al., 1997). This suggests that lipid rafts might support the transconformation process.

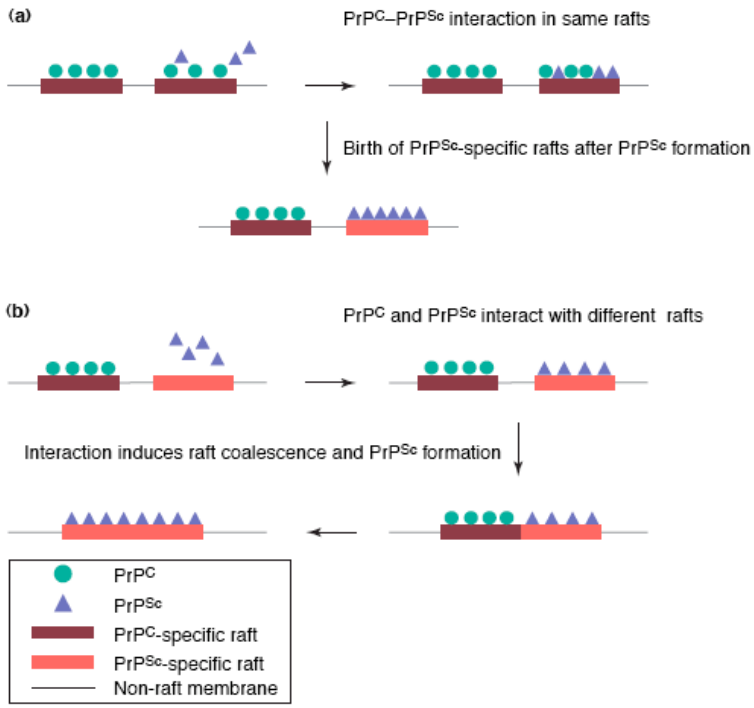

Fig. 29: Two proposed models of how PrP^{C} and PrP^{Sc} could interact with lipid rafts. Depending on their conformation, PrP-proteins could interact with different lipid rafts. **A)** PrP^{C} is normally sequestered in lipid rafts. Exogenous PrP^{Sc} interacts with PrP^{C} inside of lipid rafts and converts it; this leads to a change of PrP-interaction with the lipids of the rafts and therefore gives rise to the formation of distinct lipid rafts. **B)** Exogenous PrP^{Sc} has an affinity for lipid rafts that differs from PrP^{C} and therefore gives rise to different lipid rafts. Coalescence of these different lipid rafts leads to transfer of PrP^{Sc}, conversion and formation of a bigger PrP^{Sc}-specific lipid raft (adapted from (Campana et al., 2005)).

On the other hand, other reports speak against this hypothesis and for a protective role of DRMs. Utilizing a cell free conversion system, Baron et al., showed that GPI-

anchored PrP^{C} is present in DRMs and resistant to conversion by PrP^{Sc}, unless its GPI-anchor is cleaved by PIPLC-treatment, which results in the release of PrP^{C} from DRMs into the medium. Conversion of PrP^{C} could also be achieved by promoting the fusion of different membranes containing PrP^{C} or PrP^{Sc} by high levels of polyethylene glycol. Interestingly this treatment also disrupts raft structures (Baron et al., 2002). Results obtained in our laboratory showed that cholesterol-depletion increased the amounts of misfolded PrP^{C} (PK-resistant PrP) in the endoplasmatic reticulum and would therefore argue for a role of DRMs in stabilizing the tertiary structure of the cellular protein (Campana et al., 2006; Sarnataro et al., 2004).

Two independent reports suggested that lipids could act as lipochaperones and participate in the folding/misfolding of proteins (Bogdanov and Dowhan, 1999; Sanders and Nagy, 2000). Others reported that binding to membranes containing the monoganglioside GM1 supported the refolding of Alzheimer amyloid peptide A beta (1-40) towards a β-sheet enriched structure (Choo-Smith and Surewicz, 1997). The influence of lipids on conversion was also the subject of a recent report from Wang et al., who found that lipid interaction with recombinant PrP-protein initiated conversion of full-length α-helix-enriched protein to different forms, as well as to a β-sheet enriched form which was resistant to proteinase K digestion (Wang et al., 2007).

Recombinant PrP-protein was also utilized for studies on differences in lipid-affinity. Critchley et al., showed that PrP^{C}-resembling, α-helix-enriched PrP-forms (α-PrP) binds in decreasing order of affinity to membranes enriched in palmitoyl-phosphatidyl-glycerol (POPG), di-palmitoyl-phosphatidyl-choline (DPPC) and with lowest affinity lipids from lipid rafts (POPG > DPPC > lipids from lipid rafts), suggesting that at steady-state PrP^{C} might be situated outside of rafts (Critchley et al., 2004). Other studies reported that binding of PrP^{C} to lipid rafts stabilizes the α-helical structure of PrP, while interaction with negatively charged lipids (as found in higher amounts outside of lipid rafts) increases the content of β-sheets and even produces a disruptive effect on membranes (Sanghera and Pinheiro, 2002). The same group also reported that β-sheets enriched PrP is unfolded upon insertion into "lipid raft-like membranes" and is converted into fibrils (Kazlauskaite et al., 2003) arguing for a chaperone-like activity for lipids and a potentially protective role for lipid rafts in the conversion of PrP^{C}-protein (discussed above).

It appears that the binding of PrP^C to rafts could induce the folding of the unstructured N-terminal part of the protein and thereby stabilize the wild-type conformation of the protein. This notion is strengthened by findings that PrP^C situated inside lipid rafts is more resistant to conversion to PrP^{Sc} (Baron et al., 2002) and also that the inhibition of sphingolipid-synthesis, and thereby perturbation of lipid raft composition, increases the production of PrP^{Sc} (Naslavsky et al., 1997). The appearance of exogenous PrP^{Sc} might negatively affect the stabilizing effect of lipid rafts or might be not available when PrP^C exits lipid rafts during its endocytosis (Sunyach et al., 2003).

Data from our own laboratory in which lipid rafts were perturbed by cholesterol depletion, argue for a model in which lipid rafts act as a protective surrounding for PrP^C and agrees well with the aforementioned reports that lipid rafts confer protection by stabilizing the α-helical folded form of the protein both in the case of PrP^C and of some mutants (Campana et al., 2006; Sarnataro et al., 2004).

In contrast recombinant β-sheet-enriched protein has an altered affinity for lipid rafts and tends to produce fibrils therein (Kazlauskaite et al., 2003), showing that the role of lipid rafts in the pathogenesis is still unclear.

III.15: Exogenous prion-invasion and their dissemination in organisms

III.15.1: Dissemination in organisms: a short overview

In experimental conditions, prions can be introduced exogenously into animals from the periphery (intraperitoneal or oral inoculation) or directly into the central nervous system (CNS) (intracerebral inoculation). Medical intervention represents an accidental way of infection of patients (e.g. blood transfusion, intracranial surgery) and is therefore called "iatrogenic", deriving from ancient Greek, meaning "caused by a medical doctor" (see paragraph III.C.5.c). In the case of naturally occurring infections it is widely believed that prions are most commonly contracted by the oral route ingesting contaminated foodstuffs. (rev. in (Mabbott and MacPherson, 2006)). The exogenously derived TSE-forms are summarized in Table 5.

Following oral challenge, prions radiate away from the entry site in a characteristic way, concentrating in the adjacent secondary lymphoid organs and ending in the spleen (Fig.

30) within days and reaching a plateau after a few weeks (Bruce, 1985; Bueler et al., 1993; Rubenstein et al., 1991). This strongly suggests the involvement of the lymphoid system (discussed below) in the initial steps of invasion. The peripheral sympathetic neuronal system (SNS in Fig. 30) is believed to play a decisive role in the "switch" from the initially involved immune cells to the neuronal system. This is underlined by findings that sympathectomy in animals right before or after oral challenge significantly delays the onset of disease, while sympathetic hyperinnervation of the spleen and other lymphoid organs leads to a significant shortening of the incubation period (Clarke and Haig, 1971; Glatzel et al., 2001). Additionally, intraperitoneal challenge with prions leads to their first appearance in neurons of the thoracic spinal cord in regions corresponding to the entry site of the splanchnic nerves of the sympathetic nervous system (Beekes et al., 1996; Cole and Kimberlin, 1985). Prions were also shown to accumulate in sympathetic ganglia (McBride and Beekes, 1999), emphasizing once more the probable involvement of the peripheral sympathetic neuronal system.

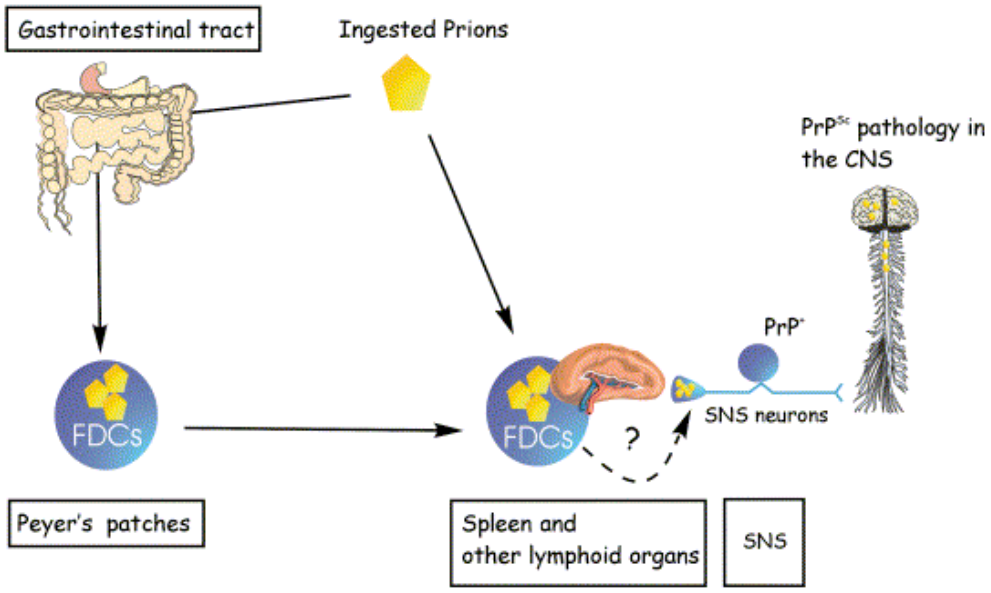

Fig. 30: Overview of chronological steps following oral challenge with PrP^{Sc}. Shortly after transferring the intestinal epithelium, prions are found in secondary lymphoid structures emanating away from the entry site, (i.e. first in adjacent Peyer's Patches, later in the spleen and on follicular dendritic cells (FDCs)). The sympathetic nervous system (SNS neurons) plays a relevant role in the transfer to the peripheral neuronal system. Once prions arrive in the central nervous system (CNS) the progression of disease is independent of the immune system (adapted from (Nicotera, 2001)).

Nevertheless it is worthwhile to mention that even sympathectomized mice succumb to the disease, suggesting either incomplete sympathectomy or the existence of another, less efficient entry route, as proposed for the vagal nerve (Baldauf et al., 1997; Beekes et al., 1998). Additionally it must be stressed that the enteric nervous system was also assigned an important role in different forms of TSE-diseases, as suggested for scrapie in sheep (Heggebo et al., 2003; van Keulen et al., 2000) and vCJD in humans (Haik et al., 2003) making prions quite "promiscuous" (i.e. without any apparent tropism, when invading the CNS of organisms from the periphery.

TSE disease	Natural host species affected	Route of transmission
Variant CJD	Humans	Ingestion of BSE-contaminated food.
Iatrogenic CJD	Humans	Usage of CJD-contaminated surgical instruments, vCJD-infected blood donors
Kuru	Humans	Ritualistic cannibalism
Scrapie	Sheep, goats and mouflon	Ingestion, horizontal transmission
BSE	Cattle	Ingestion of BSE-contaminated meat and bone meal
Chronic wasting disease (CWD)	Mule deer, elk and moose (and others)	Ingestion, horizontal transmission
Transmissible mink enceph.	Farmed mink	Ingestion, source unknown
Exotic ungulate enceph.	Zoological greater kudu, nyala and oryx	Ingestion of BSE-contaminated food

Table 5: Overview of exogenously caused TSE-diseases (adapted from (Mabbott and MacPherson, 2006)).

III.15.2: Cells (possibly) involved in dissemination of prions prior to neuroinvasion

III.15.2.1: M cells and gut epithelium

The acidic environment of the stomach cannot fully protect against ingested prions (Martinsen et al., 2002). It is widely accepted that entry into the affected organism occurs over the gut epithelium, probably involving microfold cells (M cells), a cell type specialized in transepithelial transport of particles and macromolecules from the lumen

of the intestine. The involvement of M cells has been shown in *in vitro* experiments (Heppner et al., 2001) (Fig. 31, A), yet still awaits confirmation from *in vivo* studies. Nevertheless, other mechanisms of passing the gut epithelium could be envisioned: Ferritin, a molecule abundantly found in meat and absorbed by the intestine was shown to complex with fragments of PrP^{Sc}. In vitro, these complexes were readily transported in vesicles through an epithelial monolayer of Caco-2 cells, a model, which simulates an intestinal epithelium (Mishra et al., 2004) (Fig. 31, B).

Dendritic cells might also acquire antigens directly from the intestinal lumen by "wiggling" dendrites through the epithelial barrier into the lumen and sample PrP^{Sc}, as has been shown with lumenal bacteria (Rescigno et al., 2001) (Fig. 31, C).

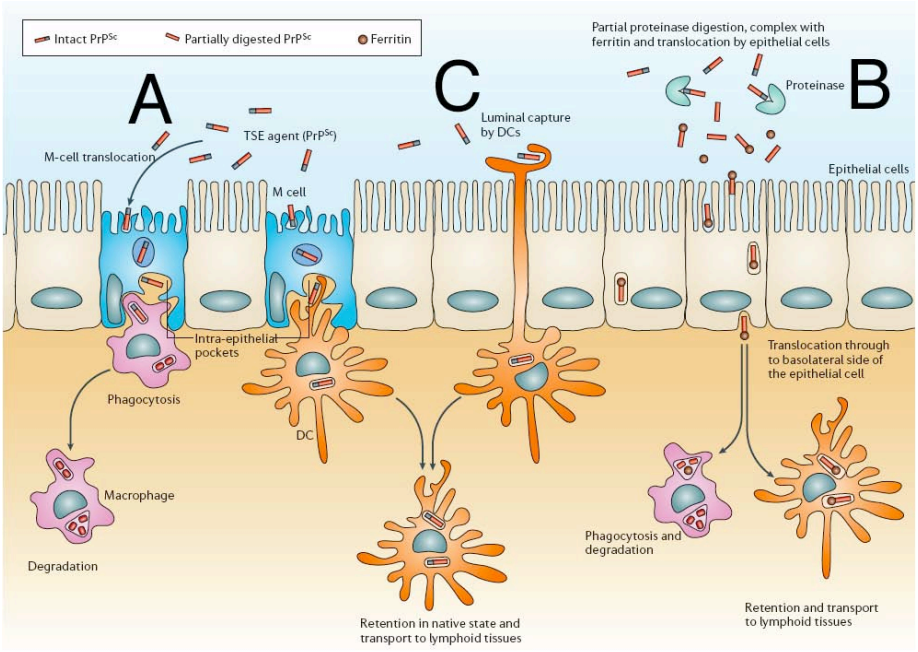

Fig. 31: Proposed mechanisms of how PrP^{Sc} could cross the intestinal epithelium.

Orally ingested PrP^{Sc} could traverse the intestinal epithelium by passage through M cells, a cell type specialized for transcytosis (**A**). PrP^{Sc}-fragments could cross the intestinal epithelium complexed to ferritin molecules (**B**). Dendritic cells (DCs) could insert dendrites through the epithelial barrier and sample PrP^{Sc} directly from the lumen (**C**). Macrophages (pink) or dendritic cells (orange), or other cells as discussed below, could sample prions once these have passed through M cells (adapted from (Mabbott and MacPherson, 2006)).

Material, which is transcytosed by M cells, exits on the non-lumenal side into the so-called intra-epithelial pocket of M cells (Fig. 31, A). It is believed that passage through M cells is the main mechanism by which PrP^{Sc} crosses the intestinal epithelium. If this

is the case, then data suggest that the cell-type sampling the molecules from the intra-epithelial pocket decides on the fate of PrP^{Sc} (i.e. degradation or preservation and spreading) (Fig. 31, A). The other cells possibly involved in the steps following transcytosis are examined in the paragraphs below:

III.15.2.2: Lymphocytes

Although they sample proteins from underneath M cells, T-cells and B-cells do not acquire measurable levels of PrP^{Sc}-protein upon intra-intestinal challenge (Huang et al., 2002). Furthermore T-cells seem to not be needed for prion conversion and do not play a role in TSE-diseases, since PrP-expression directed by the T-cell specific Lck-promoter in PrP knockout mice did not allow prion replication in the thymus, spleen or brain following intraperitoneal inoculation (Raeber et al., 1999).

B-cells, on the other hand, do not require endogenous PrP^{C}-expression for allowing prion-invasion (Klein et al., 1998a) and ablation of B-cells slows down prion infections. This seems to be due to their contribution to the organization of secondary lymphoid tissue architecture (Montrasio et al., 2000; Tumanov et al., 2002). Thus, lymphocytes appear to play only an indirect role in prion invasion as shown for B-cells.

III.15.2.3: Macrophages

Macrophages were among the first cells to be studied in the context of prion-related diseases (Lavelle et al., 1972; Narang et al., 1972). However, despite of many studies, their role remains uncertain (see below). Upon oral challenge, deposition of PrP^{Sc} in lysosomes of macrophages was reported in several organs such as in Peyer's Patches from hamsters (Beekes and McBride, 2000), in splenic B-cell follicles from sheep (Herrmann et al., 2003) as well as from murine spleens (Jeffrey et al., 2000) or mule deer's tonsils (Sigurdson et al., 2002). Due to the degradative role of lysosomes, this finding suggests that macrophages are involved in the degradation of sampled prions, rather than in their spreading. This was also confirmed by older reports that utilized peritoneal macrophages and challenged them with prions *in vitro*, showing that these cells readily sampled prions (Carp and Callahan, 1981) and that infectivity decreased

with extended incubation of scrapie-charged macrophages (Carp and Callahan, 1982). More recently, two *in vivo* studies supported the role of macrophages in clearing prion infection. These studies utilized dichloromethylene biphosphonate, a drug known to deplete macrophages transiently, and could show that depletion of these cells right before oral or intraperitoneal challenge with prions lead to a precocious increase of PrP^{Sc} in lymphoid tissues, thus arguing in favour of a protective role for macrophages (Beringue et al., 2000; Maignien et al., 2005).

III.15.2.4: Follicular dendritic cells (FDCs)

This somewhat exotic cell-type seems to play a very relevant role for TSE-diseases and therefore requires a few introductory phrases. FDCs are believed to derive from non-haematopoietic stromal precursor cells, are non-phagocytic and non-migratory and therefore by origin and phenotype not related to and not to be confused with dendritic cells (DCs) (rev. in (Imazeki et al., 1992; Shortman and Liu, 2002)). Highly immobile and long-lived, these cells reside in primary B-cell follicles and produce numerous fine dendrites used for trapping antigens on their surface, without internalizing them (Mandel et al., 1981). On these dendrites are located receptors, recognizing the Fc-region of antibodies (Fc-receptors) and also complement receptors CR1 and CR2, which are responsible for surface-retention of opsonized antigen, generation of high-affinity antibodies and maintenance of immunological memory (Kosco-Vilbois, 2003; Yoshida et al., 1993).

In the context of TSE-diseases, these cells were shown to express high amounts of PrP^{C} (Brown et al., 1999) and it was described that they accumulate PrP^{Sc}-protein in the initial steps of infection on the plasmalemma and within the extracellular spaces around their dendrites. However, it is not clear whether these cells produce new PrP^{Sc}-molecules (Jeffrey et al., 2000; Kitamoto et al., 1991; van Keulen et al., 2000). Additionally it was reported, that depletion of FDCs caused by administration of a soluble Lymphotoxin-β-Receptor-construct led to decreased prion-susceptibility (i.e. lower penetrance) and delayed neuroinvasion (Mabbott et al., 2000; Mabbott et al., 2003; Montrasio et al., 2000). For these reasons it is uncontested that FDCs play a relevant, yet unclear role in TSE-diseases. However, since FDCs are highly immobile,

they cannot be involved in transporting prions from the intestinal entry-site to secondary lymphoid structures. Additionally they were not found to be closely juxtaposed to the peripheral sympathetic neuronal system; rather they occupy anatomical sites different from sympathetic nerve endings in spleen (Fig. 32) (Defaweux et al., 2005; Dorban et al., 2007; Lorton et al., 1991). Therefore they are probably not involved in the direct process of peripheral neuroinvasion but may act as a PrP^{Sc}-reservoir, as suggested by others (Aguzzi et al., 2003; Glatzel and Aguzzi, 2001).

III.15.2.5: Dendritic cells (DCs)

DCs are one of the cell types found in intraepithelial pockets of M cells (Fig. 31) (Liu and MacPherson, 1993) and are specialized to sample antigens in the periphery and delivering them to lymphoid tissues for starting an immune response (rev. in (Shortman and Liu, 2002)). As mentioned before (Fig. 31, C) DCs can acquire pathogens and antigens without the assistance of M cell by a fascinating process in which they insert their dendrites between the cells of the intestinal layer (Niess et al., 2005; Rescigno et al., 2001). Antigens transcytosed by M cells and surrounding DCs are continuously endocytosed by their membrane veils but are not necessarily degraded in the lysosomal department (see below). Firstly, DCs can reduce the acidification of their early phagosomes by sustained activity of a NADPH-oxidase therein, called NOX2 (Savina et al., 2006), in a Rab27a-dependent manner (Jancic et al., 2007), resulting in an increased alkalinization of these organelles (Delamarre et al., 2005). Secondly, DCs were reported to possess receptors of the FcγRIIB-class, which are utilized for binding and uptake of antigens. Interestingly it was reported that antigens bound by this type of receptors are not directed to the lysosomal compartment but are rerouted into a nondegradative intracellular compartment and recycled to the cell surface for presentation to the B-cell receptor (BCR) on B cells (Bergtold et al., 2005). Thus, DCs with both degradative and nondegradative pathways are ideally suited for antigen uptake; furthermore they are mobile and are also found in the intraepithelial pockets of M cells. Nevertheless, their role remains unclear with respect to their interaction with prions due to partially contradicting reports. Indeed, it has been shown that myeloid DCs and skin-derived DCs sample readily PrP^{Sc} *in vitro* and also quickly degrade it (Luhr et al., 2002; Mohan

et al., 2005) in a cysteine protease-dependent manner (Luhr et al., 2004) thus questioning the role of DCs in prion-diseases.

In a different approach, Lymphocytic Choriomeningitis Virus (LCV), a virus, which has a tropism for and is toxic for $CD11^c$-positive DCs, was utilized to deplete DCs *in vivo*. Upon oral or intraperitoneal challenge with prions, LCV-treated mice succumbed to the disease as quickly as did the control-mice. Since the DC-specific viral ablation did not alter the kinetics of scrapie-neuroninvasion, the group contested the role for DCs in TSEs (Oldstone et al., 2002)**.** For the sake of completeness it has to be stressed that in this study a particularly high amount of prions was used, probably flooding the organism and overcoming all possible cell-based incubation differences.

In contrast with these reports, it was shown that DCs capture and retain PrP^{Sc} in its native state without degrading it (Huang et al., 2002) and that DCs from infected mice were sufficient for *de novo* infection when adoptively transferred into healthy animals (Aucouturier et al., 2001). Additionally prion-protein fragments were shown to act as chemoattractants for dendritic cells (Kaneider et al., 2005; Kaneider et al., 2003) and the involvement of dendritic cells in transporting PrP^{Sc} from the intestinal entry site to the secondary lymphoid structures was reported (Huang et al., 2002).

Intriguingly, in contrast to FDCs, dendritic cells have been frequently reported to physically interact with peripheral nerve endings (Goehler et al., 2000; Goehler et al., 1999; Hosoi et al., 1993) and have often been proposed to be involved in transferring TSE-agents to the peripheral nervous system (Aucouturier et al., 2001; Defaweux et al., 2005; Dorban et al., 2007). Additionally, these cells are in close contact with sympathetic neurons, when extravasating from the blood circulation and homing to the spleen. The spleen is a lymphoid organ in which sympathetic nerve endings ensheath the splenic artery with highest density at the splenic central arteriole (Fig. 32).

Once DCs have exited the central arteriole, they come in close contact with T-cells in the white pulp for initiating an immune response (Shortman and Liu, 2002) (Fig. 32); however a minor group has been shown to also enter B-cell follicles (Berney et al., 1999; Yu et al., 2002).

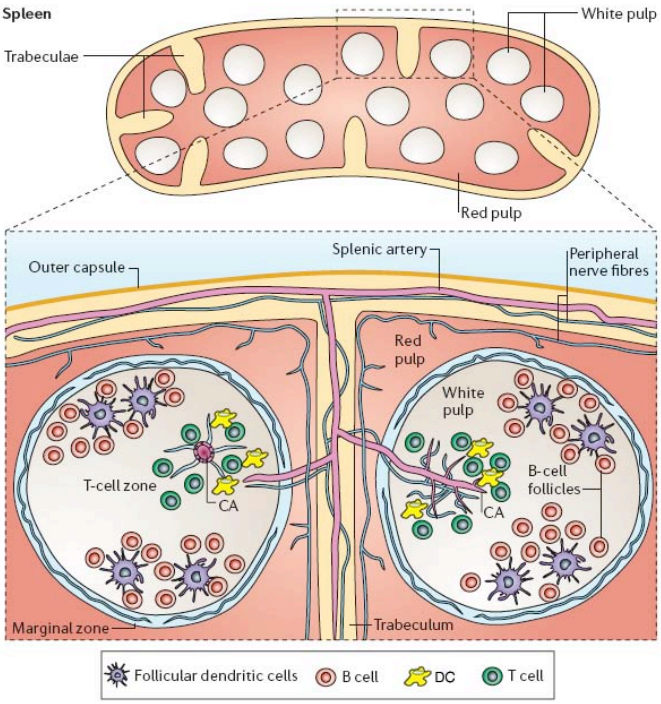

Fig. 32: Innervation of the spleen and positioning of immune system cells therein. The upper part depicts a cross section of spleen with the relevant regions of the trabeculae, by which arterioles enter the spleen, which are closely ensheathed by a web of sympathetic neurons. Sympathetic neurites terminate at central arterioles (CA) in the white pulp region (lower part). Close to the central arteriole and to the sympathetic nerve endings are T cells (green) and dendritic cells (yellow) found. B cells (red) and Follicular dendritic cells (FDCs) (violet) are situated in the so-called B-cell follicles at a distance from the central arteriole and sympathetic nerve endings. A very minor group of DCs were reported to localize also to B-cell follicles but are not shown here (adapted from (Mabbott and MacPherson, 2006)).

III.15.3: Intercellular transfer of PrP^{Sc}

Once prions are transported into close proximity of nervous endings (be it the enteric nervous system, parasympathetic system or the sympathetic system as described above), they must be taken up by neurons. If prions are to be actively delivered to neurons by cells such as those of the immune system a mechanism for intercellular transfer of proteins is required. The mechanism by which this occurs is still not understood but some possibilities have been proposed, as outlined below.

III.15.3.1: Transfer by exosomes

Quite a number of cell-types, such as DCs, lymphocytes, mast cells, platelets and epithelial cells, have been shown to actively exocytose small vesicles of 30 to 100 nm in diameter. These vesicles, termed exosomes, derive from multivesicular endosomes and appear to be enriched in cell-specific proteins (rev. in (Thery et al., 2002)). Although their function is unclear, a number of recent reports show that exosomes might be used for disposal of obsolete proteins and antigen presentation and induction of immune responses (Segura et al., 2005; Segura et al., 2007). Additionally researchers showed, albeit indirectly, that human immunodeficiency virus (HIV) might utilize exosomes as a "Trojan horse" (Gould et al., 2003; Nguyen et al., 2003): This was achieved by comparing the protein profile deriving from purified exosomes-membranes with that of HIV-membranes. Intriguingly, cells infected with PrP^{Sc} secreted exosomes containing prions and purified exosomes from these cells have been shown to be infectious for healthy animals when inoculated intracerebrally (Fevrier and Raposo, 2004). Still, one has to mention that in this report proof for the exosome-hypothesis as a means to spread infection was administration of highly enriched exosome-fractions intracerebrally (and not peripherally) in animals and therefore did not address directly the question of intercellular transfer.

III.15.3.2: Transfer by coated viruses

Membrane-coated viruses were described to contain pieces of host-membrane and host-proteins therein upon exiting an infected cell (Ott, 1997; Pelchen-Matthews et al., 2003; Raposo et al., 2002). Recently it was shown that the supernatant of cells, coinfected with prions and moloney murine leukemia virus (MoMuLV), contained prions in the excreted viral particles and that viral infection lead concomitantly to an increased exocytosis of prions in exosomes (Leblanc et al., 2006). This lead to the proposal that membrane-coated retroviruses, upon exiting cells, co-infected by the virus and with prions, could carry PrP^{Sc} over into a new host cell. Therefore it was proposed that membrane-coated viruses could serve as a vector for intercellular prion transfer.

III.15.3.3: Transfer by GPI-painting

GPI-painting describes the process of post-translational transfer of glycophosphoinositol-anchored (GPI-anchored) membrane proteins inserted into the outer membrane layer from one cell to another when closely juxtaposed. This somewhat ill defined and even less understood intermembrane transfer of GPI-anchored proteins was first described between murine red blood cells and endothelial cells *in vivo* (Kooyman et al., 1995) where it occured with a surprisingly high efficiency. The process of GPI-painting has also been specifically described for the GPI-anchored PrP^{C}-protein (Liu et al., 2002). Although this work limited itself to the study of PrP^{C} only, one can envision that PrP^{Sc} could also transfer intercellularly utilizing the same mechanism, since PrP^{Sc} retains its GPI-anchor and is also partially found on the outer plasma membrane layer (Jeffrey et al., 1992; Stahl et al., 1990; Turner, 1990).

III.15.3.4: Uptake of infectious apoptotic remnants

Two papers described the intercellular transfer of PrP^{Sc} without offering an obvious mechanism for this process (Kanu et al., 2002; Paquet et al., 2007). Kanu et al. showed that the medium of infected cells by itself was not infectious to healthy cells (therefore excluding exosomes) and that physical separation of healthy and infected cells by porous filters also abrogated transfer. Additionally the group could show that dead infected cells are still able to infect acceptor cells when in close contact to each other (Kanu et al., 2002). Paquet et al. also showed that closely positioned cells are more easily infected than cells further away from the infective donor cells (Paquet et al., 2007). The findings from these two articles suggests that a cell-to-cell contact is needed for the prion transfer and could be explained as being due to the uptake of infectious apoptotic remnants by living healthy cells.

III.16: Tunneling nanotubes (TNTs)

Recently a novel way of cell-to-cell communication has been described (rev. in (Gerdes et al., 2007)). Immortalized cell lines as well as primary cells of epithelial, neuronal and immunologic origin were found to produce very fine membrane channels between them in *in vitro* cultures (rev. in (Gerdes et al., 2007)). Depending on the cell-type used in the studies, the diameter and the length of these connections, termed tunneling nanotubes (TNTs), were very heterogeneous. The diameter of TNTs in the case of PC12 cells (rat pheochromocytoma cells) varied between 50 to 200 nm (Rustom et al., 2004), while those of macrophages were thicker in diameter (≥ 700 nm) (Onfelt et al., 2004); human endothelial progenitor cells and neonatal rat cardiac myocytes produced connections which ranged between 50 to 800 nm in diameter (Koyanagi et al., 2005) and DU 145 human prostate cancer cells produced the thickest described TNTs at more than 1000 nm in diameter (Vidulescu et al., 2004). The length of these structures ranged from a few microns up to 800 μm (Onfelt et al., 2004; Vidulescu et al., 2004).

One hallmark of these connections is that they are not attached to the substratum but are suspended freely above the substratum and connecting cells at their nearest distance (Fig. 33) (Hodneland et al., 2006; Rustom et al., 2004).

Strikingly, TNTs permitted the active intercellular exchange of plasma membrane components like surface receptors (HLA-A, B and C as well as MHC I) as well as a variety of vesicular structures, such as lysosomes, and mitochondria (Fig. 33) (rev. in (Gerdes et al., 2007)).

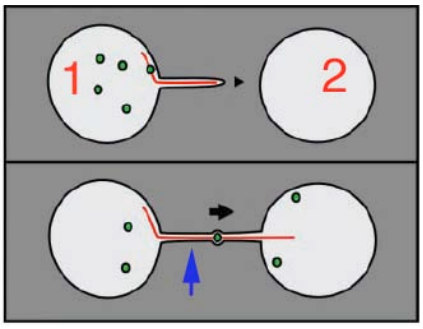

Fig. 33: Proposed model for organelle transfer by TNTs. A cell initiates the build-up of a TNT (termed donor, cell 1 in upper panel) and connects to another cell (termed receptor, cell 2 in upper panel). Once established, the TNT-connection can transfer organelles (green circles) intercellularly. A hallmark of TNTs is that they are not attached to the substratum but are suspended freely in the cell culture medium (blue arrow in lower panel) (adapted from (Rustom et al., 2004)).

The mechanism of TNT-formation and their functional role are not yet clear, however it appears that they contain cytoskeletal filaments involved in stabilizing the connections between cells. In most of the reported cases actin-filaments seem to span the entire length of TNTs; however heterogeneity in the cytoskeletal content, depending on the diameter of TNTs, has been reported (Onfelt et al., 2006).

In particular in the case of monocyte-derived macrophages TNTs with a diameter larger than 700 nm contained both actin-filaments (F-actin) and microtubuli, while TNTs with a diameter inferior to 700 nm contained only actin-filaments. The importance of F-actin for TNT-formation and –stability was further supported by the fact that usage of H_2O_2 promoted actin polymerization, as well as the formation of TNTs in primary cultured rat astrocytes (Zhu et al., 2005), a result further corroborated by the absence of TNTs upon treatment of cell cultures with the actin-depolymerizing drug latrunculin B (Rustom et al., 2004).

Since connections between cells were frequently observed between parting cells, (i.e. cells previously with membrane contact moving away from each other), it was proposed that TNTs could also exist between cells with abutting membranes (Onfelt et al., 2004).

In light of these results, TNTs could be a fascinating possibility for intercellular prion for several reasons:

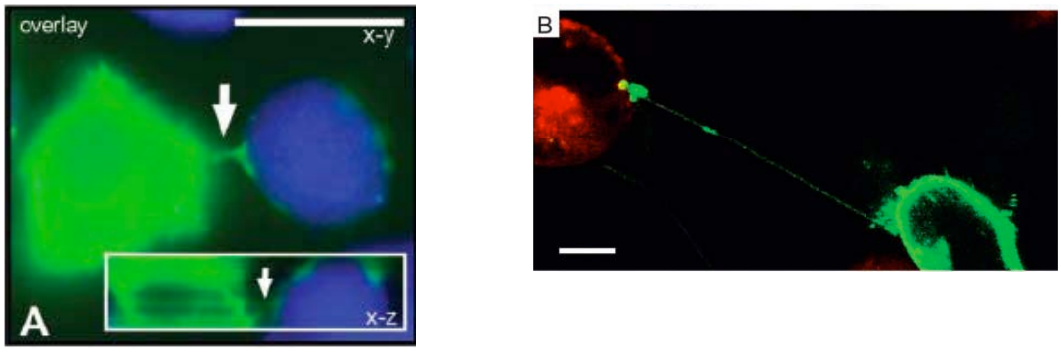

Fig. 34: Intercellular transfer of membrane content between differentially labelled co-cultured cell populations. Left picture depicts the transfer of membrane-attached farnesylated enhanced Green Fluorescent Protein (fEGFP) to another PC12 cell-population, previously stained in blue (size bar 20 μm) (from (Rustom et al., 2004)). The box inset is a X-Z reconstruction, showing that the connection is not attached to the substrate (white arrow). **Right picture** shows a previously transfected GFP-GPI chimeric protein transferring from a immortalized B-cell to another B cell-population, previously stained in red (size bar 10 μm) (from (Onfelt et al., 2004)). Note the heterogeneity in length and diameter between the two presented examples.

1) TNTs were shown to enable the transfer of plasma membrane components (Rustom et al., 2004) including a GPI-anchored Green Fluorescent Protein (GFP-GPI) (Onfelt et al., 2004) (Fig. 34). Since PrP^{C} has a GPI-anchor and its malconformed counterpart, PrP^{Sc}, retains its GPI-anchor (Stahl et al., 1987) one could easily envision their intercellular transfer by TNTs along the plasma membrane derived surface.

2) TNTs were reported to transfer lysosomes and other endosome-related vesicles between cells (for overview refer to (Gerdes et al., 2007)). This is very interesting for the prion-field, since PrP^{Sc} has been reported in endosomal structures, mainly late endosomes and lysosomes (Peters et al., 2003; Pimpinelli et al., 2005; Shyng et al., 1994) but also in the Golgi apparatus (Barmada and Harris, 2005; Taraboulos et al., 1990).

3) TNTs can be produced between different cell types and dendritic cells (DCs) are particularly apt at producing intercellular connections (Watkins and Salter, 2005). Önfelt and colleagues could show that TNTs were able to connect B-cells and Natural Killer cells (NK-cells), proving that TNTs are not restricted to cells of the same kind (Onfelt et al., 2004). Additionally, another publication reported that DCs derived from CD14+ peripheral blood monocytes from human donors are particularly apt at producing TNTs between cells and elegantly demonstrated rapid intercellular communication and exchange between these cells (Watkins and Salter, 2005). Interestingly, CD14+ cells, from which these abovementioned DCs derived, have been recently reported to be the population with highest prion infectivity in blood (Andreoletti, Congress Prion2007). This makes TNTs an interesting candidate-mechanism by which prions could "switch" from the immune system cells to the peripheral nervous system in secondary lymphoid structures, possibly involving dendritic cells passing prions directly to peripheral nervous endings.

4) The *in vitro* described TNTs could potentially have an *in vivo* counterpart as shown for the wing imaginal discs from *Drosophila melanogaster* (Hsiung et al., 2005; Ramirez-Weber and Kornberg, 1999) and could therefore also occur in whole organisms. Cautious *ex vivo* preparations of wing imaginal discs enabled these researchers to describe thin cytoplasmic extensions (length 700 µm, diameter 200 nm), termed cytonemes, radiating into the center of the imaginal disc, connecting different cells and enabling these to transfer vesicular structures. Provided that TNTs described *in vitro* and cytonemes *ex vivo* in *Drosophila melanogaster* are closely related structures

and that these structures occur also in tissues and intact organs of higher organisms, these connections represent a novel means of cell-to-cell communication and might have a major role in intercellular transfer of prions. Indeed, DCs were reported to be very apt at producing TNTs and because they can be in intimate contact with neurons of the peripheral nervous system (see III.15.2.5), this cell type could possibly produce TNTs and allow prions the “switch” from the immune system to the peripheral nervous system.

IV. Working hypothesis

In spite of intensive research on prion diseases for almost a century, a number of questions are still unanswered or not sufficiently covered in this field of biological/medical research.

As mentioned in the introduction, Transmissible Spongiform Encephalopathies (TSEs) can be subdivided into different forms based on their different clinical manifestations and/or aetiologies. Specifically Creutzfeldt-Jakob disease (CJD) is arborized into four subgroups:

1) Familial CJDs (fCJD) are the group of diseases where patients host a mutation in the *prnp*-gene and therefore express a faulty PrP^{C}-protein. As with all diploid organisms, humans possess two alleles for each gene and the vast majority of patients affected by fCJD express one healthy and one mutated allele of the *prnp*-gene in the same cell.

2) Variant or new variant CJD (vCJD or nvCJD) is believed to derive from ingestion of contaminated foodstuffs. Since BSE and vCJD share similar characteristics in experimentally infected animals, ingestion of BSE-contaminated beef could be a probable explanation as a cause for vCJD. Upon presumed entry by the intestine of the host organism, prions invade the peripheral nervous system and are retrogradely transported to the central nervous system (CNS). Cells involved in this process and the mechanism by which prions are transported from the site of entry to the CNS is not understood.

3) Iatrogenic CJD (iCJD) derives from accidental exposure to contaminated materials during medical interventions. This occurs for instance when dura mater, blood or cadaver-derived growth hormones originating from asymptomatic CJD-carriers are administered to recipients. Surgical instruments previously utilized on CJD-patients are also implicated in infection of new patients.

4) Sporadic CJD (sCJD) is a form of the disease believed to develop upon spontaneous somatic DNA-mutation or spontaneous misfolding on protein-level and is neither linked to an inherited mutation nor to ingestion of contaminated food nor the medical exposure.

In the course of my work on the prion protein I focused on the intracellular and intercellular trafficking of mutant and pathological forms of PrP as found in fCJD and vCJD and wanted to address specific aspects of these diseases:

- The intracellular trafficking and reciprocal influence of co-expressed wild-type and mutant prion proteins as found in fCJD. This part of my studies is based on the hypothesis that co-expressed wild-type and mutant proteins interact with each other and possibly influence the glycosylation, the cellular localization or the subcellular localization of one or the other form. This hypothesis was to be tested by various biochemical and microscopical approaches. Additionally, we were interested in understanding whether co-expressed wild-type and mutant proteins were in close enough proximity that the mutant could imprint its malconformation on the wild-type form. We planned to address this question by FRET (fluorescence resonance energy transfer). Revealing anomalies linked to the presence of both forms in the same cell could further explain the pathogenesis of TSE-diseases and therefore might provide information on how to counter these effects in afflicted individuals in the future.
- The intercellular trafficking of prion proteins from the intestinal entry site to the CNS, as found in vCJD. This part of my thesis is based on the hypothesis that intercellular spreading of exogenously derived prions starts with the sampling of prions by dendritic cells (DCs). These could interact closely with the peripheral nervous system (PNS) in secondary lymphoid organs (e.g. lymph nodes, spleen) and transfer prions to the PNS. The mechanism for transfer of prions is also not known. My hypothesis was to test the involvement of tunneling nanotubes (TNTs) in this process. I planned to address this hypothesis by a microscopy-based approach, imaging fixed and living cells and their interaction with each other. With promising preliminary results obtained from studying immortalized epithelial and neuronal cell lines, we decided to further test our hypothesis by characterizing the interaction of primary cells, such as bone-marrow derived dendritic cells (BMDCs) and primary hippocampal neurons. By setting up this *in vitro* model of neuro-immune interaction, I tried to mimic the close interaction of DCs and peripheral neurons I had previously found by immunohistofluorescence experiment done with murine splenic tissue. Understanding the basics of the spreading of prions and their mode of intercellular transfer, would not only

answer a highly interesting scientific question but might provide information on how to block their spreading in a clinical context (e.g. upon acute prion exposure).

V. Results

V.1: Manuscript 1

This manuscript has been submitted to the journal Traffic and we are currently addressing the comments of the referees.

V.1.1: General Introduction

One of the large TSE-subgroups consists of the familial forms of TSEs, which are all caused by insertions, deletions or exchanges in the *prnp*-gene. This newly produced protein is generally termed PrPmut and sometimes named after the aminoacid exchange it encodes for. Since the mutations are encoded on the nucleic acid level, they are passed on to the offspring of the carrier following Mendelian rules.

In the first part of my thesis I concentrated on these hereditary forms of prion-diseases, specifically on characterizing the interaction of co-expressed PrP^C and inheritable mutant forms of the prion protein in the same cell. With the vast majority of patients affected by inheritable TSE-diseases being heterozygous, we were interested to simulate this situation *in vitro* by co-expressing wild-type and mutant protein forms in the same cells. Heterozygosity and its consequences for prion pathogenesis are a neglected topic in prion research. In fact only one publication focused specifically on this aspect of prion pathogenesis but this study was limited to the biochemical aspects of the interaction and no reciprocal biochemical influence between the co-expressed proteins was found (Lehmann et al., 1997).

For this reason we decided to address the problem using a different approach: First we engineered a wild-type form of PrP^C tagged with yellow fluorescent protein (YFP-PrPwt) as well as several mutant forms of PrP-proteins tagged to cyan fluorescent protein (CFP-PrPmut) (see below). In order to distinguish between the two forms biochemically, we inserted into the mutant protein-forms a so-called 3F4-tag (aa 106-126). This tag is expressed naturally in hamster PrP^C and does not affect the characteristics of the prion-protein (Kascsak et al., 1987). This sequence has the

advantage to be specifically recognized by monoclonal antibodies and therefore allowed us to discern between the tagged mutant prion-forms and the untagged wild-type prion-form in our system (Bolton et al., 1991). These different plasmids were then stably co-transfected in cell cultures and allowed us to study the interaction of wild-type and mutant prion-protein forms in the same cells.

As cell-system for these experiments we chose a cell line called FRT (Fisher Rat Thyroid) cells, a polarized epithelial cell line derived from rat thyroids (Nitsch et al., 1985). We utilized these cells for two reasons: Firstly, these cells have been previously utilized in studies on the trafficking of proteins (Campana et al., 2006; Sarnataro et al., 2004; Sarnataro et al., 2002) and secondly, these cells do not express endogenous PrP^C, which might influence the study of the ectopically introduced co-expressed chimeric proteins.

Murine PrP^C was engineered to contain an N-terminally attached yellow fluorescent protein (YFP) and once transfected in FRT-cells gave rise to the cell line termed YFP-PrPwt.

The mutants we had chosen for this study were described as belonging to two different subtypes of inheritable TSE-diseases. The first mutation, found in fCJD, is an exchange from glutamic acid to lysine at amino acid (aa) position 200 (human numbering). This resulted in a mutant chimeric protein N-terminally tagged to cyan fluorescent protein (CFP). The FRT-cell line obtained by stable transfection was termed CFP-PrPE200K. The second mutation, found in GSS, is an exchange from alanine to valine at aa position 117, giving rise to a fluorescently tagged protein and a stable FRT-cell line termed CFP-PrPA117V. Additionally, by utilizing the FRT(YFP-PrPwt) cell line, we produced two cell lines stably expressing YFP-PrPwt and one of the mutants. This gave rise to the cell lines termed 2xEK (co-expressing YFP-PrPwt and CFP-PrPE200K) and 2xAV (co-expressing YFP-PrPwt and CFP-PrPA117V).

Populations such as the Libyan Jews and others have an increased incidence of fCJD-familial disorders, such as the E200K-mutation (100 times higher than the worldwide population, i.e. approximately 100/Million) (Gabizon et al., 1994a; Gabizon et al., 1994b; Kahana et al., 1974). Due to this, fCJD-diseases moved into scientific focus and allowed several pathological and epidemiological studies on the E200K mutation. However, considerable controversy on its cellular and subcellular characterization and localization exists, making additional studies necessary (see below in Introduction of Manuscript 1).

The A117V mutation on the other hand, has only been studied with respect to some very specific characteristics (see chapter III.6.2) and therefore required further studies on cellular and subcellular localization.
In the course of this work, we included a third mutant in our studies, which we had previously extensively characterized in our laboratory (Campana et al., 2006). This is a glycosylation mutant-protein, which hosts a threonine to alanine exchange at aa position 182 and is utilized here as a CFP-chimeric form, termed CFP-PrP182A. This mutant has been previously analyzed in the laboratory for subcellular localization, trafficking and biochemical characteristics (Campana et al., 2006).

V.1.2: Objectives

Because previous results on the biochemistry and localization of these mutants were not conclusive (Capellari et al., 2000; Goldfarb et al., 1992; Hegde et al., 1998; Hsiao et al., 1989; Kovacs et al., 2001; Negro et al., 2001; Piccardo et al., 1998; Rosenmann et al., 2001; Tateishi et al., 1990), we set out first to investigate the general traits (i.e. scrapie-like properties such as insolubility and resistance proteinase K-digestion, glycosylation pattern) of these chimeric proteins, comparing the chimeric proteins with their untagged counterparts. Next we characterized the cellular localization of all chimeric proteins, with our main emphasis being on the differences between the single-expressing cell lines (YFP-PrPwt or CFP-PrPmut) and the double-expressing cell lines (2xAV, 2xEK). Indeed, changes of localization of either the wild-type or the mutant protein caused by the presence of its homologue might shed light on the pathogenesis of these diseases. Next, we examined the subcellular compartmentalization (i.e. presence in detergent resistant membranes (DRMs)) of proteins in single- and double-expressing cells, in order to see whether the co-presence of wild-type and mutant proteins would somehow affect their presence in lipid membrane domains. Differences in membrane compartmentalization may not only provide information on possible mechanisms of pathogenesis but might also shed light on the debated role of lipid domains in the transconformation process of PrP^{C} to PrP^{Sc}. Finally by utilizing FRET (fluorescence resonance energy transfer), we addressed the question whether and where co-expressed YFP-PrPwt and CFP-PrPmut would interact closely in the cell. Since this energy transfer by FRET occurs only when fluorophores are in close proximity (3-10 nm)

(reviewed in (Kenworthy and Edidin, 1998)), information obtained by this technique may give important clues as to whether and where wild-type protein and its pathogenic homologue are close enough to allow malconformation of the former by the latter and possibly to reciprocally influence their function.

V.1.3: Summary of results

A plasmid encoding the murine form of PrP^C ($moPrP^C$) N-terminally linked to CFP and containing a 3F4-tag was used to generate the mutant PrP forms by site-directed mutagenesis. It is worthwhile to mention that $moPrP^C$ differs by one amino acid from the human PrP^C-form (see III.7.3), therefore mutations were shifted by one amino acid (A117V in humans becomes A116V in mouse, E200K in humans becomes E199K in the mouse system).

Following transfection we obtained several FRT-cell clones stably expressing either YFP-PrPwt, CFP-PrPE199K or CFP-PrPA116V. Utilizing different antibodies directed against either the N- or C-terminal region of the PrP-protein, we analysed both the size and the level of expression of the chimeric proteins by western blot. All three chimeric forms migrated at their expected molecular masses of approximately 58-60 kDa with distinct bands representing the di-, mono- and unglycosylated forms of the protein (see chapter III.8 and compare with Fig. 16). By utilizing an antibody directed against the 3F4-tag (expressed only in the CFP-PrPmut forms), we confirmed that we could biochemically distinguish the mutant chimeras from the wild-type chimeric protein. We also observed that the major band of CFP-PrPE199K protein migrates between the di-glycosylated and mono-glycosylated forms of YFP-PrPwt and CFP-PrPA116V. Since the second glycosylation site of the PrP^C-protein is at position aa 197 (in human PrP) (see chapter III.7.3 and Fig. 11 therein), this suggested an incomplete glycosylation of this mutant. To test this we treated protein samples with the enzyme N-glycosidase F (PNGase F), thereby removing attached N-glycans and analyzed these by SDS-PAGE and western blotting. Upon this treatment all three samples (YFP-PrPwt, CFP-PrPA116V and CFP-PrPE199K) revealed a sharp band at approximately 40-45 kDa confirming our hypothesis that the observed differences in size are due to differential glycosylation. Additionally we monitored the same difference in migration when YFP-

PrPwt and CFP-PrPE199K were co-expressed, suggesting that the differential glycosylation patterns are not affected by the simultaneous presence of both proteins.

Next we characterized the expressed proteins in the three single expressing cell lines for insolubility and sensitivity to Proteinase K (PK)-digestion. We found that the CFP-A116V-protein is soluble (10% sedimented) and sensitive to PK-digestion comparable to the wild-type chimera, YFP-PrPwt. This result is in agreement with data obtained for this mutant not fused to a fluorophore (Tateishi et al., 1990). However, CFP-E199K showed an increased insolubility (35-40%) and was weakly resistant to PK-digestion, as already reported by others for proteins not fused to fluorophores (Capellari et al., 2005; Lehmann and Harris, 1996a; Lehmann and Harris, 1996b). These data indicated that the chimeric proteins behaved as their wild-type counterparts, suggesting that the fusion with the GFP does not alter the intrinsic characteristics of the proteins. As a next step we sought to determine the cellular localization of the proteins in single- and double-expressing cells. For this we utilized fluorescent microscopy on fixed and permeabilized cells, assaying for colocalization of the fluorophore-attached chimeric PrP-proteins with antibodies against markers of different intracellular organelles. We found that all chimeric proteins (both the wild-type and the mutant forms) preferentially localized to the Golgi apparatus and the plasma membrane. This intracellular localization was maintained also in the case of cells co-expressing wild-type and mutant forms. Since wild-type and mutant proteins colocalized extensively at the Golgi apparatus and on the plasma membrane, we tested whether they occupied the same membrane microdomains. We addressed this by extraction at 4°C in the presence of detergents (Triton X-100) and subsequent centrifugation in a discontinuous sucrose gradient. The single-expressed chimeric proteins associated with detergent resistant membranes (DRMs) to very different degrees: 35%±11 of YFP-PrPwt, 18%±3 of CFP-PrPA116V and 50%±10 of CFP-PrPE199K associated with detergent resistant membranes (n=3). Surprisingly, the same assay performed on the double-expressing cells gave different results. Here, the amounts of both the wild-type and mutant proteins found in the DRMs increased dramatically. Cells co-expressing wild-type and the mutant A116V (2xAV) had 61%±13 of both forms in DRMs and when revealed with α3F4-antibody (recognizing only the mutant prion protein) we found 59±14 of CFP-A116V floating in the DRM-fraction. This increase was even more pronounced in the case of the cells co-expressing YFP-PrPwt and CFP-E199K. Here we found 95%±20 of both proteins in DRMs, while

the mutant itself floated with 86%±22. Co-expression of the CFP-PrPT182A mutant with the YFP-PrPwt protein lead also to a substantial increase of wild-type protein in DRMs (more than 80%), while the association with DRMs of the mutant protein remained maximal (<90%) as previously seen in cells expressing the T182A-mutant only.

DRMs and their protein composition did not appear to be generally disturbed in the double-expressing cell lines, since controls with GM-1 and flotillin-1, two proteins associated with DRMs, did not reveal any differences when single- and double-expressing DRM-preparations were compared. Additionally, we could not find the same increase in DRM-content when the wild-type protein was expressed as both YFP and CFP (+3F4-tag) linked chimeric forms. This suggests that the increased localization in DRMs is caused by the presence of the respective mutant protein forms and its interaction with wild-type protein. Additionally, these data indicate that PrPwt and PrPmut affect each other's presence in membranes and therefore might interact in cellular membrane subdomains. Analysis of the lipids, which co-immunoprecipitated with PrP^C and the mutant prion-proteins from single-expressing cells, showed no qualitative or quantitative differences supporting the notion that these proteins occupy the same membrane microdomains in cells and might interact with each other therein. Additionally, analysis by FRET showed no differences between cells co-expressing YFP-PrPwt and one of the CFP-linked mutants and control cells co-expressing YFP-PrPwt and CFP-PrPwt at the level of the Golgi apparatus and on the plasma membrane, arguing for a close interaction between the wild-type protein and the mutant forms both at the level of the Golgi apparatus and on the plasma membrane**.** This was further supported by colocalization experiments and confocal analysis in cells co-expressing YFP-PrPwt and CFP-T182A. We found that when co-expressed with the T182A-mutant, YFP-PrPwt, does not reach the surface but was retained intracellularly and colocalized extensively with the mutant in the Golgi-apparatus suggesting that an interaction with the mutant protein leads to its retention.

V.1.4: Discussion

In spite of intense studies for almost a century, the mechanisms of pathogenesis of TSE-diseases remain largely enigmatic. The goal of this work was to simulate in cell-culture

the heterozygous situation found in the majority of people affected by familial TSEs. The mutant proteins we had chosen for our study belonged to the best-studied forms in the prion field (Capellari et al., 2000; Goldfarb et al., 1992; Hegde et al., 1998; Hsiao et al., 1989; Kovacs et al., 2001; Negro et al., 2001; Piccardo et al., 1998; Rosenmann et al., 2001; Tateishi et al., 1990). However, differing results have been reported concerning their characteristics and cellular and subcellular localization. Additionally, the reciprocal influence of co-expressed wild-type and mutant forms in these systems had been largely neglected or was limited to biochemical characterization (Lehmann et al., 1997). The results we describe here show that the mutants A116V and E199K are mainly PK-sensitive and detergent soluble, as shown previously for the T182a mutant (Campana et al., 2006). This agrees with previous data published on the A116V mutation (Piccardo et al., 1998; Tatzelt and Schatzl, 2007). For the E199K mutation, our results agree with the data described for fibroblasts derived from human skin (Rosenmann et al., 2001) but contrasts with the report of others, who showed in CHO-cells, that this mutation conferred resistance to Proteinase K-digestion and insolubility in detergents (Daude et al., 1997; Lehmann and Harris, 1996a; Lehmann and Harris, 1996b). These differences could be explained by the differences in the cell systems used. On the other hand, our findings that the E199K mutant reaches the cell surface of FRT-cells, is supported by the results from Lehmann et al. (Lehmann and Harris, 1996a; Lehmann and Harris, 1996b) but is in conflict with the data presented by another group, who could not find the bovine homologue of the E200K mutation on the plasma membrane of several cell lines tested (Negro et al., 2001). The explanation for this discrepancy might be that we, as Lehmann and colleagues, used the mouse homologues of the human mutation (Lehmann and Harris, 1996a; Lehmann and Harris, 1996b), while Negro and colleagues utilized the bovine homologue (Negro et al., 2001). Alternatively, the differences at hand may be caused by different cell systems and constructs utilized in the different works as shown in the case of PrP^{C}. Indeed, our laboratory has described murine PrP^{C} as localized to the basolateral membrane of polarized FRT-cells (Sarnataro et al., 2002), while very recently another group reported the human protein's localization to be apical in polarized Caco-2 cells (De Keukeleire et al., 2007). This stresses the possibility that differences in localization and biochemical traits might be linked to the use of different cell-lines and expression systems.

Membrane microdomains have long been implicated in prion pathogenesis, since both PrP^{C} and PrP^{Sc} have been found to localize to DRMs (Baron and Caughey, 2003; Baron

et al., 2002; Botto et al., 2004; Naslavsky et al., 1997; Taraboulos et al., 1992; Taraboulos et al., 1995) although their role (in protection or promotion of transconformation) remains ambiguous (see III.14.2.2.4). In this work we show that the mutant proteins expressed in isolation associate with DRMs in different proportions, suggesting that these differences might be mutation-specific. Our finding that increased amounts of wild-type and mutant prion proteins are observed in DRMs in double-expressing cells underlines the notion that DRMs might have an important role in the conversion process.

We undertook several controls to rule out artefacts: Most importantly co-expressing two different wild-type forms, YFP-PrPwt and CFP-PrPwt (containing the 3F4 tag), did not increase the DRM-content of these proteins. This suggests that neither the interaction of the two fluorophores nor some ill-defined effect of the 3F4-tag is involved in the findings described above. Additionally we believe that general disturbance of the membrane can be ruled out, because two well-characterized markers for membrane microdomains, GM1 and flotillin-1, did not show any increased sequestration in DRMs of double-expressing cells. We believe that these controls suggest that the increased DRM-content is caused by the co-expression of the wild-type and the mutant protein and more importantly by reciprocal effects of one on the other. Another piece of information that indicates the co-existence of the proteins in common domains is that the lipid species co-immunoprecipitating with wild-type and mutant protein forms did not show any qualitative or quantitative difference. This argues for the hypothesis that these proteins occupy membrane rafts of the same kind and could therefore interact therein. This is further supported by our FRET-data, which shows that wild-type and mutant proteins are closely juxtaposed in the Golgi apparatus, as well as on the plasma membrane. In addition the data on the T182A mutation showed that coexpression with the wild type results in increased intracellular retention of the wild type, suggesting once more that both proteins interact with each other.

As mentioned previously, membrane microdomains play an important, albeit hotly debated role in prion pathologies. Some groups are ardent supporters of the hypothesis that transconformation occurs in rafts, while others speak in favour of the idea that rafts are an environment that protects against prion transconformation. As described in chapter III.14.2.2.4, numerous scientific publications arguing for one or the other can be found. Our finding that co-expressed proteins are enriched in DRMs unfortunately does not resolve this debate, since it could be in agreement with both hypotheses. Our

findings could nevertheless shed some light on the pathogenesis of TSE-diseases. As mentioned before, a publication utilizing the same experimental approach could not find a transfer of pathological characteristics from mutant to co-expressed wild-type protein by biochemical means (Lehmann et al., 1997). This suggested that some other aspect might cause pathology and led us therefore to look at other criteria. The increased sequestration of both mutant and wild type forms in DRMs lead us to hypothesise that PrP^{C} and PrPmut interaction promotes a PrP^{C}-disequilibrium in cell membranes (raft versus non-raft), which might lead to the development of pathologies in heterozygous carriers of these mutations. Unfortunately, with no clear function described for either PrP^{C} or PrP^{Sc}, this gives us no real basis for understanding the pathogenesis of these enigmatic diseases. However, a number of hypotheses could be envisioned with respect to how pathogenesis could occur:

- Gain of function of PrP^{C}. Provided that PrP^{C} serves a yet-to-be-defined function in membrane rafts, the presence and the interaction with its mutant homologue, could lead to its increased sequestration therein and thence to increased activity. In cells, susceptible to these disturbances (e.g. neurons), this might consequently lead to cell-toxicity.
- Loss of function of PrP^{C}. The opposite of the hypothesis presented above could also be envisioned. Assuming a function of PrP^{C} outside of rafts, its interaction with its mutant homologue leading to increased sequestration in membrane domains could by consequence lead to a drop of its functional activity and therefore to cell-toxicity in susceptible cells
- Indirect gain or loss of function. Other proteins or factors interacting with PrP^{C} or PrPmut (Protein X, see III.12), could be affected by the increased sequestration of PrP^{C}/PrPmut in DRMs and could lead to a Protein X-based gain/loss of function.
- Gain/loss of function for PrPmut. As described above for PrP^{C} the increased sequestration of PrPmut into DRMs could produce a toxic effect in heterozygous carriers of the mutation.

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Co-expression of wild-type and mutant prion proteins alters their partitioning into detergent resistant membranes

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Abstract:

Transmissible spongiform encephalopathies (TSEs) are a group of diseases of infectious, sporadic and genetic origin, found in higher organisms and caused by the pathological form of the prion protein. The inheritable subgroup of TSEs are linked to insertional or point mutations in the prion gene *prnp*, which favour its misfolding and are passed on to offspring in an autosomal-dominant fashion. The large majority of patients with these diseases are heterozygous for the *prnp* gene, leading to the co-expression of the wild-type form (PrP^C) and the mutant form (PrPmut) in the carriers of these mutations. In order to mimic this situation *in vitro*, we produced FRT cells coexpressing wild-type PrP alongside mutant versions of PrP including A117V, E200K and T183A relevant to the human TSE-diseases Gestmann-Sträussler-Scheinker Disease (GSS) and familial Creutzfeldt-Jakobs-Disease (fCJD). We found that co-expression of mutant PrP with wild-type proteins does not affect the glycosylation pattern nor the biochemical characteristics of either protein. However we provide evidence that the mutant form alters the subcellular localization and the membrane environment of the wild-type protein in co-transfected cells. Specifically, co-expression of the wild-type and mutant proteins leads to an increased sequestration of both proteins in detergent resistant membranes (DRMs), a site believed to be involved in the pathological conversion of the prion protein or protection thereof. Our data indicate that altered membrane environment of PrP may play a role in the development of these diseases.

Introduction:

Prions are infectious proteins found in organisms as far apart as animals and yeast. The group of diseases caused by prions in higher organisms are called transmissible spongiform encephalopathies (TSEs), lethal neurodegenerative diseases which in humans can have different aetiologies: genetic, infectious and sporadic (1, 2). The common denominator of all TSEs is believed to be the prion protein, in its healthy form termed PrP^C, a glycosylphosphatidylinositol (GPI)-anchored protein with unknown function, yet widely expressed in tissues, particularly in cells of the immune system and the central nervous system (3-5). The hallmark of most TSEs is the appearance of a new isoform of PrP, termed PrP^{Sc} which has undergone a refolding-process from a formerly α-helix rich form to a β-sheet enriched isoform which is partially protease-resistant, insoluble in detergents and is not released from the plasma membrane by treatment with phosphatidylinositol-specific phospholipase C (PIPLC) (6). Whereas in infectious transmissions the acquired PrP^{Sc} could originate from ingestion of contaminated food, all the inheritable forms (about 10% of human TSEs) are linked to mutations in the coding region of the prion protein gene (*prnp*), which is found on chromosome 20 in humans (7). Presently, more than 20 nonsense-, insertion- and point-mutations leading to disease have been described and due to their clinicopathological traits, these autosomal-dominant diseases have been divided into three subgroups, namely familial Creutzfeldt-Jakob-Disease (fCJD), Fatal Familial Insomnia (FFI) and Gerstmann-Sträussler-Scheinker Syndrome (GSS). Although it is believed that PrP^{Sc} acts as a catalyst for imprinting its malconformation on PrP^C (8), where in the cell and how this process of pathological conversion occurs and why this leads to pathology is yet to be resolved (rev. in (9)). Therefore, understanding the subcellular localization and the membrane environment in which PrP^C and PrP^{Sc} could physically interact --for the latter to induce malconformation of the former — is vital for understanding the pathogenesis of these disorders. An important role in the conformational conversion process has been attributed to specialized detergent resistant membrane domains (DRM or lipid rafts) enriched in cholesterol and sphingolipids. Indeed it has been shown that PrP^C and PrP^{Sc} associate to these domains (10, 11) and that DRM-associated PrP^C needs its malconformed counterpart PrP^{Sc} to be inserted into contiguous membranes in order to allow conversion (12). Furthermore, cholesterol depletion decreased the amount of PrP^{Sc} production in infected cells (13), while depletion of specific sphingolipids increased the amount of PrP^{Sc} (14). These data suggest that the major components of DRMs (cholesterol and sphingolipids) have an effect on prion conversion. In addition it has been shown that immature PrP^C is already associated with DRMs in the endoplasmatic reticulum (ER) and that misfolding of PrP^C is increased upon cholesterol depletion (15, 16), suggesting that DRM association in the ER might be required for the correct folding of PrP. This seems also to be the case for some PrP-mutants (16) although the site of misfolding in the infectious and inherited forms of the disease could be different (rev. in (9, 17)).

Alteration in the intracellular trafficking of the prion protein could also have a role in the pathology of the inherited disease as evidenced by the fact that some pathological mutants have a different intracellular localization compared to the wild-type protein. We and others have previously studied the biogenesis, intracellular pathway and subcellular localization of T182A, a pathological glycosylation mutant found in a familial form of CJD (16, 18, 19). Here we analyzed two different mutants, E200K (glutamate to lysine amino acid exchange at position 200) and A117V (alanine to valine exchange at position 117) associated with fCJD and GSS diseases respectively (20-23). Although these two mutants are among the best-characterized, controversial results have been published regarding their biochemical characterstics and subcellular localization. Specifically, while some groups reported that E200K, is detergent insoluble, PK-resistant and localized to the cell surface (24-26), others found that it was soluble in detergents, PK-sensitive and segregating into cholesterol-enriched microdomains in transgenic mice, as well as in primary fibroblasts from fCJD-patients but not in brain extract of the same patients (27, 28). In addition the bovine form of PrP harbouring the

bovine homolog of the human E200K mutation was reported to accumulate in the ER and in the Golgi but not on the plasma membrane and was shown to associate with cholesterol-enriched microdomains (27).

From the group of GSS-diseases, one of the best studied mutants in terms of membrane insertion and neuropathology, is the A117V mutant, exchanging alanine for valine (A117V) (23, 29, 30). This substitution belongs to a remarkable group of mutations that were shown to be responsible for the increased production of a transmembrane form of PrP^{C}, termed ^{Ctm}PrP (Ctm for transmembrane, with its COOH-terminus in the ER lumen and the NH_2-terminus in the cytosol) which possibly causes or at least contributes to the neurotoxic effect of PrP-A117V (21). However its biochemical characteristics and intracellular localization are largely unknown.

Another notable characteristic of the familial TSE-diseases that has been somewhat neglected is the fact that the majority of patients are heterozygous for the mutations of the *prnp*-gene (31). We were interested in characterizing this clinically relevant situation of heritable prion disease by mimicking heterozygosity in a cell culture model in order to examine the phenotypic effect of the heterozygous genotype at the subcellular and biochemical level.

To this aim we co-expressed each of the mouse analogs of these three PrP-mutants, E199K, A116V and T182A, together with mouse PrP^{C} in Fischer Rat Thyroid (FRT) cells, which have been extensively characterized for the trafficking of PrPwt and some PrP mutants (15, 16, 32). In these cells, we analyzed the trafficking and metabolic characteristics of both the wild-type and the mutant forms. We found that co-expression of wild-type and one of the mutant PrP-forms does not alter their respective glycosylation patterns nor their subcellular localization in the Golgi-apparatus or on the basolateral plasma membrane. However, upon co-expression, the amount of both forms was substantially increased in DRMs, suggesting that the presence of both forms in the same cell perturbs the membrane distribution of PrP^{C} and of its mutant counterpart. This indicates a possible interaction of the two forms in this domain, which could lead to the progress of the pathology in heterozygous patients.

Materials and Methods:

Reagents and antibodies

Cell culture and reagents were purchased from Gibco Laboratories (Grand Island, NY). The α-PrP antibodies SAF32 (recognizing the octa-repeat region (aa 59-88 in human numbering) located in the N-terminal part of PrP) and SAF61 (recognizing the amino acids 142-160 (human numbering) of the C-terminal part of PrP) were a kind gift from J. Grassi (CEA, Saclay, France). The α-GFP antibodies (A11120) used for immunoprecipitation were purchased from Molecular Probes. Protein-A-Sepharose was bought from Pharmacia Diagnostics AB (Uppsala, Sweden). Antibodies against calnexin and EEA1 were from StressGen Biotechnologies Corp. (Victoria, BC, Canada). The antibody against giantin was from BAbCO (Berkeley Antibody Company, Richmond, CA). The antibody against flotillin-1 was from Transduction Laboratories (Beckton Dickinson).

PrP constructs, transfection and cell culture

$MoPrP^{C}$ was previously engineered for expression in a pEYFP-C1-plasmid, containing a Zeocin-resistance (YFP-PrPwt). Plasmid encoding for $moPrP^{C}$, N-terminally linked to cyan fluorescent protein (CFP-PrPwt) in a pCFP-C1, containing a 3F4-tag and a G418-resistance was used to generate mutant PrP forms by site-directed mutagenesis using the QuickChange II XL site directed mutagenesis kit (Stratagene). The alanine to valine exchange at position 116 in the $moPrP^{C}$ was made by the oligonucleotide 5'-AGGGGCTGCGG**T**AGCTGGGGCAGT-3', for the lysine to glutamate change at position 199 in $moPrP^{C}$, the oligonucleotide 5'-GGGGAGAACTTCACC**A**AGACCGATGTGAAGAT GA-3' were obtained from Stratagene (Amsterdam, NL) was used. The constructs were stably transfected with Lipofectin Reagent from Invitrogen (Carlsbad, CA). Stable clones were selected with Zeocin for YFP-PrPwt and G418 for CFP-PrPA116V and CFP-PrPE199K. FRT cells stably expressing the different constructs were cultured in F12 Coon's modified medium from Euroclone (Milan, Italy) containing 5% FBS.

N-glycosidase F treatment

Samples were eluated in 50μl of 50mM PBS, 10mM EDTA, 0.5% TX-100, 0.1 SDS, 1% β-Mercapto-Ethanol, boiled for 5 minutes at 100°C and then incubated with 5U PGNase F at 37°C for 16 hours. Then samples were incubated with 5U PGNase F for 2 more hours, incubated with Laemmli-Buffer for 10 minutes at 100°C and run on 12% polyacrylamide gels and revealed by Western-Blot.

Assays for scrapie-like properties

Triton/Doc insolubility

Triton/DOC insolubility assay wasperformed as previously described (32). Briefly, cells were lysed in Triton/Doc buffer (0.5% Triton X-100, 0.5 Na deoxycolate, 150mM NaCl and 100 mM Tris, pH7.5) for 20 minutes and cleared lysates were centrifuged at 265000Xg for 40 minutes in a TLA 100.3 rotor of Beckman Optima TL ultracentrifuge. Supernatant was isolated from pellet and the proteins from both fractions was recovered by Trichloroacetic acid-precipitation (TCA). It was previously shown that in these conditions PrP-forms with PrP^{Sc}-characteristics are preferentially found in the pelleted fraction (26).

Proteinase K (PK) digestion

Assay was performed as previously described (32). Briefly, lysates were digested with PK (3,3 μg/mg of total protein) for 2, 5 and 10 minutes, TCA precipitated and then visualized by SDS/PAGE and western blot.

Fluorescence microscopy

FRT cells stably expressing the different constructs were grown either for 2 days on coverslips or 4-5 days on transwell filters, washed with PBS, fixed in 2% paraformaldehyde, permeabilized with 0.075%

saponin and processed for indirect immunofluorescence using specific antibodies. In some cases PrPwt and its mutated homologs were visualized with SAF32/SAF61 as primary antibodies and FITC- or TRITC-conjugated secondary antibodies, while calnexin, giantin and EEA1 were revealed with TRITC-conjugated secondary antibodies using a Zeiss laser scanning confocal microscope (LSM 510). For lysosome staining, cells were incubated for 1 hour with Lysotracker (1: 10000) in complete medium, washed and fixed.

Biotinylation assays
Confluent monolayers were achieved by culture for 4-5 days on transwells, biotinylated and processed for immunoprecipitation as described elsewhere (33). To recover the immunoprecipitated proteins, the samples were boiled in Laemmli buffer for 10 minutes at 100°C and then loaded on 12% polyacrylamide gels. After transfer on nitrocellulose by western blot, biotinylated PrP^C and PrPmut were revealed by horseradish-peroxidase (HRP)-conjugated streptavidin (Amersham).

Sucrose density gradients
Sucrose gradient analysis of TX-100-insoluble material was done after protocols published elsewhere (34, 35). Briefly, after lysis in TNEV/1% TX-100 buffer on ice, cells were scraped off, homogenized and brought to 40% sucrose and placed at the bottom of centrifuge tubes. On top, a discontinuous sucrose gradient (30% first, then 5% sucrose on top) covered the lysates. These were centrifuged at 39,000 rpm for 17 hours in an ultracentrifuge (SW41 from Beckman Coulter). Fractions of 1ml were cautiously taken off from the top to the bottom of the gradients, run on 12% polyacrylamide gels, blotted and revealed by western blot.
To reveal the distribution of GM1 in the gradient, 20μl of each fraction were spotted on nitrocellulose membrane and detected with HRP-conjugated cholera toxin B subunit (Sigma-Aldrich).

Results:

All three chimeric proteins have the predicted size but different patterns of glycosylation

Fisher Rat Thyroid cells (FRT) as well as other epithelial cells have previously been shown to be a good model for studying prion trafficking (15, 16, 25, 26, 32, 36-38). FRT cells have been used to study the exocytic pathway of murine PrP^C, of a hereditary mutant (PrPT182A) and of an anchorless form of PrP (PrPΔGPI (15, 16, 32, 38). In order to study the biochemical properties and subcellular localization of two inheritable pathological mutant forms found in familial CJD (E200K) and in GSS (A117V) and their interaction with PrPwt, FRT cells were stably transfected with cDNAs encoding for murine PrPwt or the two mutants fused at the N-terminus either to Yellow Fluorescent Protein (YFP-PrPwt) or to Cyan Fluorescent Protein (CFP-PrPA116V, CFP-PrPE199K). Furthermore, in order to biochemically distinguish wild-type from mutant PrP, a 3F4-tag was introduced into the mutant PrP variants (39-41). Different FRT clones, stably expressing either YFP-PrPwt, CFP-PrPA116V or CFP-PrPE199K were analysed for the size and the level of expression of the chimeric proteins by western blot, using different antibodies directed against either the N- or C-terminal region of PrP or against the 3F4-tag (where inserted). Compared to YFP-PrPwt the two mutants were expressed at lower levels and all three chimeric forms migrated at the expected molecular masses of approximately 58-60 kDa (Fig. 1A). However the ratios between di-, mono- and unglycosylated bands were different, among the chimeric proteins (Fig. 1A). In particular the major glycosylation form of CFP-PrPE199K migrates between the di-glycosylated and mono-glycosylated forms of YFP-PrPwt or CFP-PrPA116V, suggesting an incomplete glycosylation of this mutant (Fig. 1A, left and center) as shown before (24). When incubated with an anti-3F4-tag antibody, no reactivity was seen with YFP-PrPwt, while the mutant expressing cell lines showed the same differences between the different isoforms (Fig. 1A, right).
In order to understand whether the above mentioned differences in size were related to different glycosylation patterns for the different mutants, we immunoprecipitated cell lysates of FRT cells expressing either YFP-PrPwt, CFP-PrPA116V or CFP-PrPE199K and treated the immunoprecipitates with N-Glycosidase F (PNGase F) in order to remove N-Glycans (Fig. 1B). Upon deglycosylation all three samples revealed a sharp band at approximately 40-45 kDa, indicating that the observed differences in size are due to differential glycosylation (Fig. 1B).
It had been previously reported that during the normal cycling of PrP^C between the plasma membrane and intracellular compartments, C-terminal fragments of the protein with sizes of 27-30, 22 and 18 kDa are generated. These forms represent C-terminal fragments of the highly glycosylated (27-30 kDa), intermediate (22 kDa) and unglycosylated (18 kDa) forms (42-44). However this seems to be cell-specific and was never observed in large amounts in FRT cells (15). In order to rule out the possibility that the transfected chimeric proteins were subjected to proteolysis, which would result in the loss of the N-terminally linked fluorophore, we immunoprecipitated the cell lysates using an α-GFP antibody. The immunoprecipitates were analyzed by western blotting with α-PrP antibodies directed either against the N-terminus or the C-terminus of PrP^C or by an α-GFP antibody. In all three cases, the strongest band appeared at a height of 48-60 kDa, representing the intact chimeric proteins of interest (Fig. S1A). In addition to the main band, other minor bands of approximatively 30 to 37 kDa appeared in the western Blots revealed with the α-PrP antibodies. Most likely these bands represented fluorescent protein-linked degradation products. Because they constituted only a minor fraction of the signal, we did not investigate these bands further (Fig. S1A).
In parallel to the biochemical experiments described above, we approached the question of the possible loss of the GFP fluorophore by proteolysis of the PrP-fusion proteins by immunofluorescence

microscopy. For this purpose we performed immunofluorescence experiments and confocal analysis and compared the signals deriving from the YFP- or CFP-linked chimeric proteins with the signal derived from indirect immunofluorescence using antibodies directed against either the N- or C-terminal part of PrP or with an α-GFP antibody. We found that there was an almost complete colocalization between the YFP and CFP signals of the transfected chimeric proteins and the signal from each of the three antibodies used. These experiments showed that there was no detectable fluorescent signal coming from fluorescent molecules (YFP or CFP) not linked to PrP (Fig. S1B), confirming that in our conditions the majority of the chimeric proteins are intact.

CFP-PrPE199K is more insoluble and less sensitive to Proteinase K treatment than the YFP-PrPwt and CFP-PrPA116V chimeric proteins

Next we analyzed the PrP^{Sc}-like traits of the chimeric proteins in single expressing cells by centrifugation of Triton/DOC cell lysates at 265,000 Xg for 40 minutes, a treatment which had been reported to sediment detergent insoluble PrP^{Sc}-like aggregates but not PrP^{C} (45, 46). While about 10% of YFP-PrPwt and of CFP-PrPA116V sedimented, CFP-PrPE199K repeatedly showed a higher percentage of insolubility (35-40%) (Fig. 1C). CFP-PrPE199K also repeatedly proved to be more resistant to PK-digestion. After 2 minutes of treatment with 3,3, µg of PK/1mg of total protein, approximately 20% was still present and after 10 minutes approximately 5% was still detectable by western blot while YFP-PrPwt and CFP-PrPA116V were completely digested (not shown). Taken together these experiments suggest that neither YFP-PrPwt nor the CFP-PrPA116V chimeric proteins display PrP^{Sc}-like characteristics similar to what was shown for the same proteins not fused to fluorophores (23). On the other hand CFP-PrPE199K seems to be more insoluble in detergents and shows a slightly higher PK-resistance, supporting the notion that this mutation confers minor PrP^{Sc}-like traits as shown before for the same PrP-mutant form not linked to GFP (24-26). These experiments also indicate that the fusion of GFP to the different PrP-forms does not alter the intrinsic characteristics of these proteins.

Both PrP mutants show a preferential localization to the Golgi-apparatus and the plasma membrane in single transfected cells

In order to analyze the intracellular localization of the two chimeric proteins, we performed indirect immunofluorescence experiments on permeabilized cells expressing either each of the mutants or PrPwt, utilizing several antibodies directed against well-characterized markers of different intracellular organelles (Fig. 2).

YFP-PrPwt, as well as CFP-PrPA116V and CFP-PrPE199K colocalized extensively with Giantin, a marker of the cis- and medial Golgi apparatus, whereas we could not see any colocalization with calnexin, a marker for the endoplasmatic reticulum (ER), or with any markers of the endocytic pathway, such as EEA1 for early endosomes and LysoTracker for lysosomes (Fig. 2). In addition all three chimeric proteins were found on the plasma membrane of cells. This localization is consistent with the localization observed for PrPwt (15) and the original A116V and E199K mutants constructs not fused to GFP when transfected in FRT cells (not shown), indicating that the GFP-tag did not affect the appropiate trafficking/sorting of the proteins.

YFP-PrPwt and CFP-PrPmut chimeric proteins maintain their glycosylation pattern and their intracellular localization upon co-expression in the same cell

Because we were interested in directly comparing the localization and eventually following the interaction between PrPwt and PrPmut in the same cell, we produced FRT clones stably co-expressing either YFP-PrPwt and CFP-PrPA116V (2XAV) or YFP-PrPwt and CFP-PrPE199K (2XEK) (Fig. 3). When revealed with anti-PrP antibodies against the N- or C-terminus (Fig. 3, left and center), we observed the di-, mono- and unglycosylated forms of YFP-PrPwt, as previously described in cells expressing only YFP-PrPwt (compare Fig. 3 with Fig. 1). When the same samples were revealed by western blotting with an α3F4-antibody (Fig. 3, right), we could see that the cells expressing both YFP-PrPwt and CFP-PrPA116V (2XAV) produced a mutant form with a strong diglycosylated form and a minor monoglycosylated subform, while the cells co-expressing YFP-PrPwt and CFP-PrPE199K (2XEK) produced only one strong band at a lower molecular weight than the diglycosylated form of either YFP-PrPwt or CFP-PrPA116V. These results suggest that the glycosylation pattern of each PrP-form is maintained despite the co-existence of the wild-type and mutant PrP-forms in one cell (Fig. 3, compare with Fig. 1).

We then analyzed whether the co-expression of PrPwt and PrPmut in the same cell could produce a change in their respective intracellular localization. To this aim we analyzed the intracellular localization of wt and mutant PrP by confocal microscopy using specific organelle markers (Fig. 4A and B), as we did for the single expressing clones (Fig. 2). A clear colocalization on the plasma membrane and with Giantin in the cis- and medial Golgi, was found in the case of the double expressing cells YFP-PrPwt/CFP-PrPA116V as well as YFP-PrPwt/CFP-PrPE199K, while no colocalization could be seen with neither calnexin, nor EEA1 nor LysoTracker (Fig. 4A and B). These results indicate that when co-expressed both forms colocalize at the same sites (cis-, medial Golgi and plasma membrane) and that neither PrPwt nor PrPmut seem to interfere with the intracellular trafficking of the other.

Because our laboratoy among others (32, 37) had previously shown a preferential basolateral localization for PrP^{C} in polarized cells, we analyzed whether this was also the case for YFP-PrPwt and for the two CFP-linked mutants (Fig. S2). To this end, we performed a biotinylation assay on filter-grown polarized cells. We found a strong signal on the basolateral site for PrPwt and PrPmut in both

single- and double expressing cells (Fig. S2A). Quantification of these results showed that approximately 80% of the signal was localized to the basolateral site in all cases, suggesting that the co-expression of PrPwt together with PrPmut in the same cells does not alter their trafficking to the plasma membrane (Fig. S2B). These results were also confirmed by confocal microscopy of polarized cells grown on filters (not shown).

Co-expression of YFP-PrPwt and CFP-PrPmut strongly increases their association with DRMs

Because PrPwt and PrPmut colocalize extensively at the Golgi apparatus and on the plasma membrane, we analysed whether they occupied the same membrane microdomains. Since PrP^C had been found in DRMs in FRT and other cells (10, 32, 37, 47, 48), we analyzed the presence of the chimeric proteins in detergent resistant membranes (DRMs) in single and double expressing clones (Fig. 5). We found that each of the chimeric proteins associated with DRMs to different degrees. In single expressing cells, quantification showed 35%±11 of YFP-PrPwt, 18%±3 of CFP-PrPA116V and 50%±10 CFP-PrPE199K in the floating fraction (Fig. 5A upper panel for blots, 5C for quantification). Interestingly, when we performed the same assay on the double expressing cell lines, the amount of the proteins found in the DRMs increased dramatically. In the case of the cells co-expressing YFP-PrPwt/CFP-PrPA116V we found 61%±13 of both PrP-forms in DRMs and revealed with α3F4-antibody, 59%±14 of CFP-PrPA116V floating in the DRM-fraction. For the cells co-expressing YFP-PrPwt/CFP-PrPE199K the increase was even more pronounced, since we found 95%±20 of both proteins to be localized to the DRMs and 86±22 when assayed for the CFP-PrPE199K-protein by α3F4-antibody (Fig.5B upper, 5C for quantification). The highly increased amount of both PrP-forms in the co-expressing cells was not due to a general disturbance of DRMs, since GM-1 and flotillin-1, two proteins associated with DRMs, were equally present in the DRM-fractions of single and double-expressing cells (Fig. 5 and not shown). These data strongly suggest that PrPwt and PrPmut are restricted to the same membrane subdomains where they might interact with each other. Because the differences in the migration of the bands corresponding to the wt and mutants were too subtle to appreciate (see Fig. 1 and Fig. 3), we could not carry out convincing co-immunoprecipitation experiments to prove interaction of PrPwt with PrPmut. Nevertheless, the enhanced enrichment into DRMs indicated that the mutant proteins could be influencing the localization of the PrPwt. However, since both mutants as well as PrPwt exhibit similar subcellular distributions, a subtle redistribution within membranes due to this interaction might occur but could not be detected. Therefore, we utilized a previously characterized mutant, PrP-T182A, known to exhibit a distinctly different subcellular distribution as previously shown (16, 19).

Interestingly, we found by immunofluorescence that when co-expressed with the T182A-mutant, YFP-PrPwt, does not reach the surface but was retained intracellularly and colocalized extensively with the mutant (Fig. 6A and inset). Also in this case, we found increased amounts of both forms in the DRM fractions of sucrose gradients, when co-expressed (Fig. 6B, compare upper with lower blots). Quantification showed that while in single expressing cells YFP-PrPwt associated with approximately 40% to DRMs (as also shown in Fig. 5A), the CFP-PrPT182A mutant associated to DRMs for more than 90%. In contrast to this, double-expression of the wild-type and the mutant protein, revealed a substantial increase in DRM association for YFP-PrPwt (more than 80%), while the association with DRMs of the mutant protein remained maximal (<90%).

Taken together these data suggest that mutant forms of PrP are able to interact with their wild-type counterpart, increase their DRM association and influence their intracellular localization.

Discussion:

In spite of intense research the pathogenesis of TSE-diseases is still only poorly understood. For example, inheritable human prion diseases such as familial CJD and GSS have been studied now for almost a century (the first case being described by Hans-Gerhard Creutzfeldt in 1920) while the mechanisms of pathogenesis remain enigmatic. The aim of this work was to simulate in a cell-culture system the heterozygous situation found in the majority of patients with inheritable TSE-diseases (31, 49, 50). Thus both PrP^C and PrPmut were expressed in the same cell, in order to analyze whether and how each of the two proteins affected the biochemistry, as well as the cellular and subcellular localization of each other. Although these mutants belong to the best studied pathological mutations found in inheritable prion diseases (18-20, 22, 51-53) differing results have been published about their characteristics and subcellular localization. Furthermore the reciprocal influence of mutants and PrP^C have not been specifically addressed (23-30) or were only characterized by biochemical means (54). A previous study, in which PrPwt was coexpressed with a mutant called PG11 in an *in vitro* system, revealed no transfer of mutant characteristics onto the coexpressed PrPwt-protein but the scope of this report was limited to an examination of the biochemical characteristics of the proteins (54). Our results indicate that both mutant forms (A116V, E199K) are mainly PK-sensitive and detergent soluble (Fig. 1). For the A116V mutation these findings corroborate previous data (23, 30). In the case of the E199K mutation, our findings resemble the results described by Rosenmann et al. in fibroblasts derived from human skin (28) but are in contrast to earlier reports, where it was shown that the E200K mutation conferred PK-resistance and detergent insolubility (25, 26, 36). These discrepancies might be due to the different cells used in the different studies. On the other hand, our data, showing that the E199K mutant reaches the cell surface of FRT-cells, confirms the results described by Lehmann et al. (25, 26) but contrasts

the report of Negro et al. (27), who showed that the bovine homologue of the E200K mutation was not detectable on the plasma membrane of neither HeLa, CHO nor N2a cells. This could be explained by the fact that we, as Lehmann et al. (25, 26), used the mouse homologues of the human inheritable diseases, while Negro et al. (27) studied the bovine homologue of E200K. Therefore it is possible that the difference in localization is linked to the different strains used as recently reported for human PrPwt (55).

Cholesterol-enriched membrane microdomains or lipid rafts as well as the GPI anchor are believed to be essential for PrP^{C} to PrP^{Sc} conversion, since exchanging the GPI-anchor for a transmembrane domain which prevents PrP^{C}-raft association lead to the block of PrP^{Sc} formation in infected cells (56). In addition, reduction of the intracellular levels of cholesterol, an integral compound of DRMs, also reduces the amount of newly made PrP^{Sc} in infected cells (13). Here we show that both mutants, E199K and A116V, are correctly transported to the plasma membrane and associate in different amounts to DRMs, supporting the hypothesis that these differences could be mutation-specific. Interestingly, the percentage of the protein floating in the DRM-fraction of sucrose gradients changed significantly when each of the mutants was co-expressed with PrPwt. In both co-expressing cell lines the floatation of YFP-PrPwt as well as CFP-PrPmut was increased when compared to the single-expressing cell lines (Fig. 5C). The findings we describe here are not caused by an additive effect of PrPwt and PrPmut in the floating fraction because not only total PrPwt was increased but also the PrPmut fraction by itself, thus suggesting that co-expression of PrPwt and PrPmut leads to their increased sequestration in DRMs. Thus, although the wild-type and mutant protein do not seem to interfere with the metabolism and glycosylation of the other (Fig. 3 and 4), they appear to influence each others' sequestration in subdomains of the plasma membrane and might affect their reciprocal localization. This hypothesis was confirmed by using an additional mutant, PrPT182A, previously shown to be blocked in the ER and in the cis-Golgi apparatus (16, 19). Indeed, co-expression of this mutant with PrPwt, lead to a specific increase of PrPwt in DRMs but also to the intracellular retention of PrPwt (Fig.6). Thus we hypothesize that the increased sequestration in DRMs as well as the delocalization might be a consequence of the interaction between the wild-type and mutant forms of PrP. This could also occur in the case of heterozygous carriers of mutations and therefore be involved in the pathogenesis and development of the hereditary heterozygous form of the disease. The role of DRMs in the pathology of TSEs is still under debate, since it had been postulated that DRMs represent a site responsible for pathological conversion from PrP^{C} to PrP^{Sc} (11, 13, 56). On the other hand we have shown that DRMs could fulfil the exact opposite role, because impairment of DRM-association by cholesterol-depletion significantly increased the amount of misfolded PrP^{C} as well as that of the mutant form PrPT182A (15, 16). This protective role of rafts is also supported by the biochemical studies on membrane interaction of PrP^{C} and PrP^{Sc} (rev. in (57)). The finding that increased amounts of PrP^{C} and PrPmut in DRMs only in double-expressing cells underlines the notion that DRMs are an important site for pathological conversion or protection thereof and needs to be further investigated. Why the amounts of PrPwt and PrPmut in DRMs increased when the two proteins were co-expressed, remains unclear. However one could imagine that their possible interaction could trigger a signal of increased sequestration into DRMs and therefore produce a disequilibrium in the membrane. This anomaly in turn could lead to a toxic effect specifically in neurons where PrP^{C} exerts its function (rev. in (58)). Whether the recruitment in lipid rafts and delocalization of PrP^{C} leads either to an impairment of PrP^{C}-function or to the toxic accumulation of the misfolded form, and how this contributes to the pathology in heterozygous carriers of these mutations needs to be further investigated.

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Figure Legends:

Figure 1: Biochemical characterization of YFP-PrPwt, CFP-PrPA116V and CFP-PrPE199K mutant forms

(A) FRT cells stably expressing either YFP-PrPwt, CFP-PrPA116V or CFP-PrPE199K were lysed with Lysis Buffer and upon normalization to total protein content subjected to SDS/PAGE and western blot using an α-PrP antibody recognizing a N-terminal part of PrP (left panel) or an α-PrP antibody directed against the C-terminal part of PrP (center panel) or an α-3F4 antibody (right panel).

(B) FRT cells stably expressing either YFP-PrPwt, CFP-PrPA116V or CFP-PrPE199K were lysed in Triton/Doc Buffer and immunoprecipitated with an antibody directed against the N-terminus of PrP. Immunoprecipitates were incubated in the presence or absence of N-Glycosidase F at 37°C as described in Materials and Methods. Samples were subjected to SDS/PAGE and WB and revealed by α-PrP antibody directed against the N-terminal part of PrP.

(C) FRT cells expressing either YFP-PrPwt, CFP-PrPA116V or CFP-PrPE199K were grown in 100mm dishes to confluency, lysed with Triton/Doc lysis buffer for 20' on ice and centrifuged as described in Materials and Methods. The resulting supernatant was divided from the pellet, and the pellet was resuspended in Triton/Doc lysis buffer at 30°C. Both, supernatant and resuspended pellet were subjected to SDS/PAGE and revealed by

western blot with an anti-C terminal PrP antibody (Fig. 1C, left panel). Blots were quantified by ImageJ (n=3) (Fig. 1C, right panel).

Figure 2: Intracellular localization of YFP-PrPwt or CFP-PrPA116V or CFP-PrPE199K in stably transfected FRT cells

(A-C) FRT cells stably expressing YFP-PrPwt (Fig.2, left panel) or CFP-PrPA116V (Fig.2, center panel) or CFP-PrPE199K (Fig.2, right panel) were grown on cover slips, fixed with 2% PFA, permeabilized with PBS/Gelatine (0,2%)/Saponin (0,075%) and incubated with antibodies, directed against proteins well established as intracellular markers, or with LysoTracker, a dye staining lysosomes. Upon mounting the cover slips were assayed by Confocal Microscopy. Size bars are 10μm.

Figure 3: Biochemical characterization of FRT cells co-expressing YFP-PrPwt and either of the two mutants

FRT cells stably co-expressing either YFP-PrPwt and CFP-PrPA116V (here referred to as 2XAV) or YFP-PrPwt and CFP-PrPE199K (here referred to as 2XEK) were lysed with Lysis Buffer and upon normalization to total protein content subjected to SDS/PAGE and western blot with an α-PrP antibody recognizing a N-terminal part of PrP (left panel), with an α-PrP antibody directed against the C-terminal part of PrP (center panel) and with an α-3F4 antibody (right panel).

Figure 4: YFP-PrPwt/CFP-PrPA116V or YFP-PrPwt/CFP-PrPE199K colocalize in double expressing cells

FRT cells stably co-expressing either YFP-PrPwt and CFP-PrPA116V (Fig. 4A) or YFP-PrPwt and CFP-PrPE199K (Fig. 4B) were grown on cover slips fixed with 2% PFA permeabilized with PBS/Gelatine (0,2%)/Saponin (0,075%) and incubated with antibodies, directed against proteins well established as intracellular markers, or with LysoTracker, a dye staining lysosomes. Upon mounting the cover slips were assayed by Confocal Microscopy. Size bars are 10μm.

Figure 5: Characterization of DRM association of YFP-PrPwt, CFP-PrPA116V and CFP-PrPE199K in single and double expressing FRT cells.

(A) FRT cells expressing either YFP-PrPwt, CFP-PrPA116V or CFP-PrPE199K were grown on 150mm dishes. Upon confluency, cells were lysed in 1% Triton X-100 and the cell lysates were run on a two-step (5-30%) sucrose density gradient. After centrifugation to equilibrium, twelve fractions were collected from top to bottom. Upon SDS/PAGE and western blot, YFP-PrPwt, CFP-PrPA116V or CFP-PrPE199K were revealed by an antibody against the C-terminal part of PrP (Fig. 5A upper part). In order to assay the efficacy of the fractionation, a sample of each fraction was hybridized on a Dot Spot with a subunit of Cholera-Toxin linked to Peroxidase, a molecule interacting strongly with GM-1 which is a typical marker of DRMs. (Fig. 5A, bottom part).

(B) FRT cells co-expressing either YFP-PrPwt and CFP-PrPA116V (here 2XAV) or YFP-PrPwt and CFP-PrPE199K (here 2XEK) were grown on 150mm dishes. Upon confluency, cells were lysed in 1% Triton X-100 and the cell lysates were run on a two-step (5-30%) sucrose density gradient. After centrifugation to equilibrium, twelve fractions were collected from top to bottom. Upon SDS/PAGE and western blot, YFP-PrPwt, CFP-PrPA116V or CFP-PrPE199K were revealed by an antibody against the C-terminal part of PrP (termed PrP total), in parallel the same amounts of each fraction were run and revealed by the antibody 3F4, showing only the amounts of PrPmut in the respective fraction (termed PrP mutant only)

(B, lower panel) In order to assay the efficacy of the fractionation, a sample of each fraction was hybridized on a Dot Spot with a subunit of Cholera-Toxin linked to Peroxidase, a molecule interacting strongly with GM-1, which is a typical marker of DRMs (lower panel).

(C) Blots of Sucrose-Two Step gradients were quantified by ImageJ (n=3). Results of single expressing cell-lines are depicted in black. Results of double-expressing cell-lines are depicted in grey.

Figure 6: Co-expression of YFP-PrPwt and CFP-PrPT182A in FRT cells leads to partial retention of YFP-PrPwt in the ER and increases the amount of YFP-PrPwt in DRMs.

(A) (upper panel) Confocal image of FRT cells co-expressing YFP-PrPwt and CFP-PrPT182A. Shown is a clone, expressing YFP-PrPwt in 100% of cells but which had partially lost expression of CFP-PrPT182A (in the center of image). Note that while in the cells expressing only PrPwt, the majority of the protein is on the plasma membrane (central part of left panel), in the cells expressing both wild-type and mutant form, PrPwt is retained intracellularly and colocalizes extensively with the mutant (highlighted in lower set of panels) suggesting that the interaction with CFP-PrPT182A leads to a partial intracellular retention of YFP-PrPwt. Size bar is 10μm. A magnification of rectangle from upper row is shown in the lower panel. Complete colocalization between YFP-PrPwt and CFP-PrPT182A was observed.

(B, two upper blots) Sucrose Density Gradients of cells expressing either YFP-PrPwt or CFP-PrPT182A. Treatment of cells as described in Figure 5A.

(B, two lower blots) Sucrose Density Gradients of cells co-expressing YFP-PrPwt and CFP-PrPT182A (here 2XTA). PrPwt and PrPmut were revealed by an antibody against the C-terminal part of PrP (blot termed PrP total), in parallel the same amounts of each fraction were run and revealed by the antibody 3F4, showing only the amounts of PrPmut in the respective fraction (termed PrP mutant only).

Figure S1: The majority of PrPwt and PrPmut are expressed as fluorophore-linked, chimeric proteins

(A) FRT cells stably expressing either YFP-PrPwt or CFP-PrPA116V or CFP-PrPE199K were lysed in Triton/Doc Buffer and immunoprecipitated overnight (ON) with an α-GFP antibody. Immunoprecipitates were subjected to SDS/PAGE and revealed by western blot using an α-PrP antibody against the N-terminal part of PrP (left panel), the C-terminal part of PrP (center panel) or with an α-GFP antibody (right panel).
(B) FRT cells stably expressing either YFP-PrPwt (left panel) or CFP-PrPA116V (center panel) or CFP-PrPE199K (right panel) were grown on cover slips, fixed with 2% PFA, permeabilized with PBS/Gelatine (0,2%)/Saponin (0,075%) and incubated with antibodies against the N-terminal part of PrP (upper panel), the C-terminal part of PrP (center panel) and with an α-GFP antibody (lower panel). Size bars are 10μm.

Figure S2: YFP-PrPwt, CFP-PrPA116V and CFP-PrPE199K are strongly localizing to the basolateral membrane in single- and double expressing polarized FRT cells
(A) FRT cells stably expressing YFP-PrPwt, CFP-PrPA116V and CFP-PrPE199K or the combination of YFP-PrPwt with one of the mutants were grown on Transwell filters for 4 days. Upon measuring of the transepithelial resistance, filters were either treated with biotin from the apical or basolateral side for two times 25'. After extensive washings, cells were lysed and immunoprecipitated ON at 4°C with an antibody against the C-term part of PrP or in the case of the double-expressing cells, when assayed for PrPmut, with an α-3F4 antibody. Immunoprecipitates were subjected to SDS/PAGE, protein bands were revealed with Streptavidin-Peroxidase (Fig. S2A).
(B) Blots (n=3) were quantified by ImageJ. Results from single expressing cell-lines are depicted in black, double expressing cell-lines are depicted in grey (Fig. S2B).

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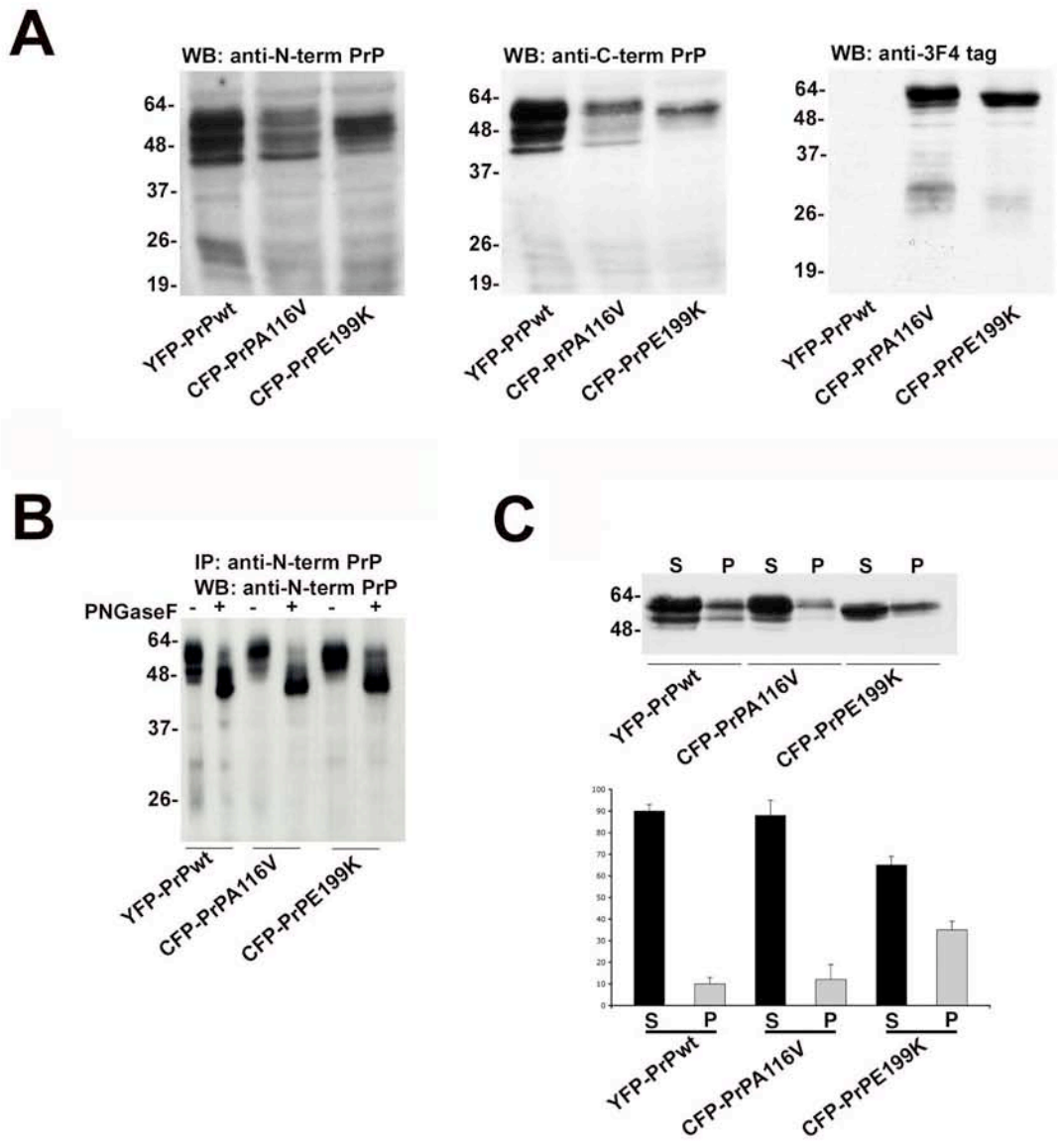
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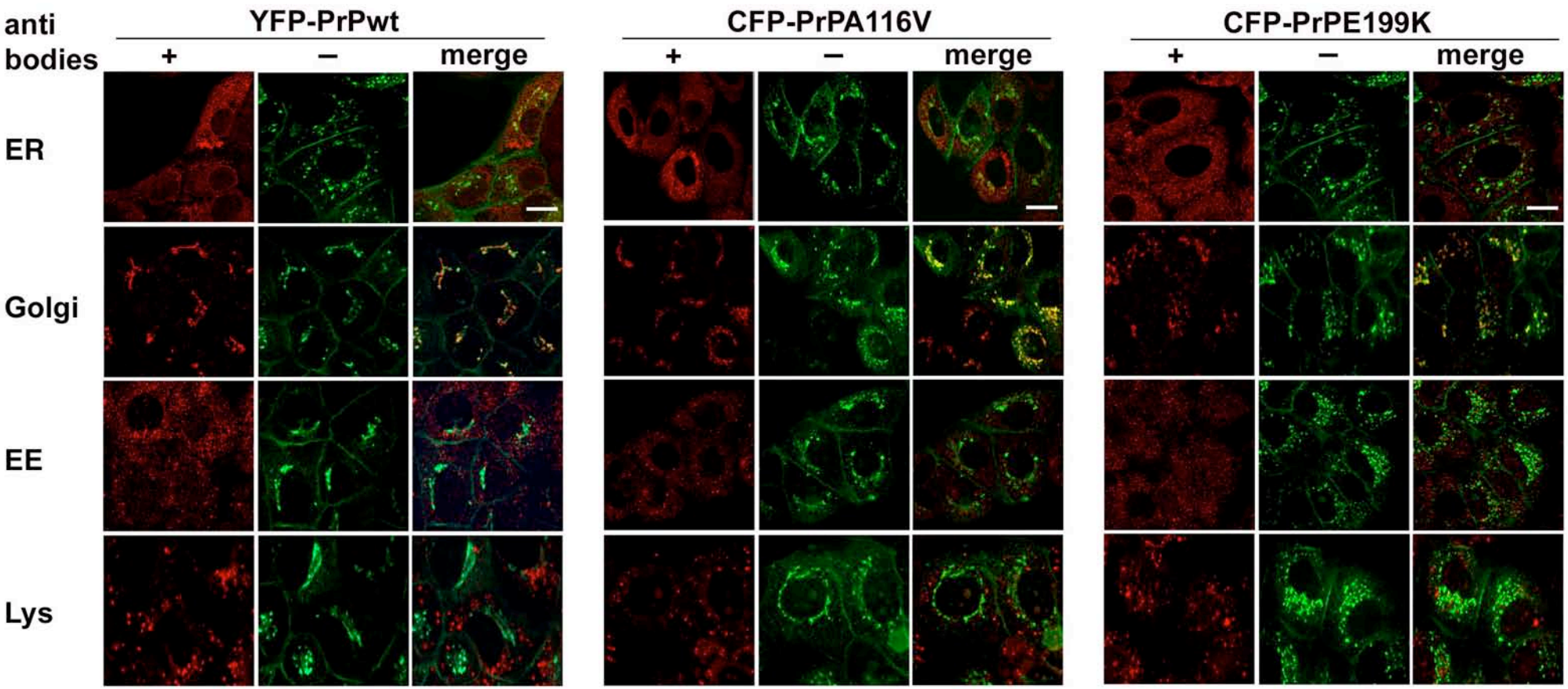
Figure 1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Lys

Figure 2

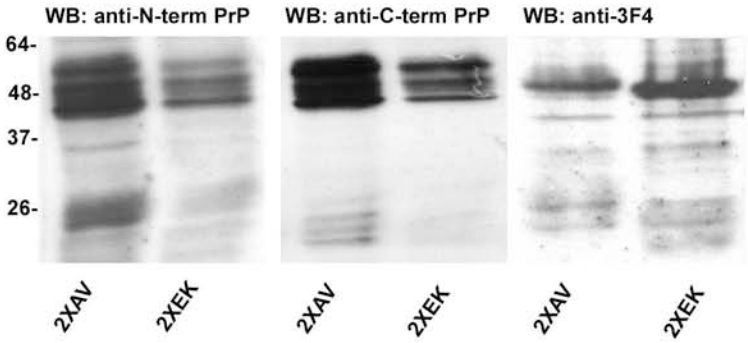

WB: anti-N-term PrP
WB: anti-C-term PrP
WB: anti-3F4
64-
48-
37-
26-
2XAV
2XEK
2XAV
2XEK
2XAV
2XEK

Figure 3

A

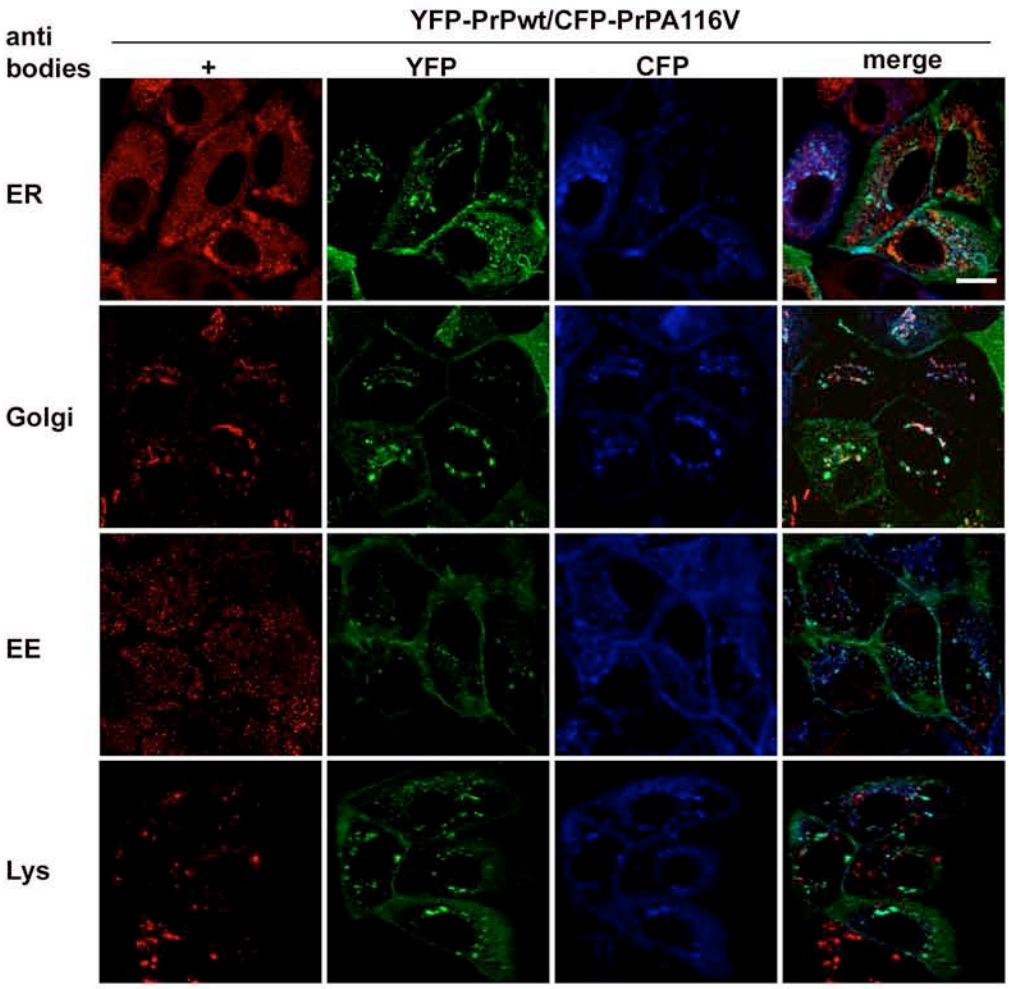

YFP-PrPwt/CFP-PrPA116V
anti bodies
+
YFP
CFP
merge
ER
Golgi
EE
Lys

Figure 4A

B

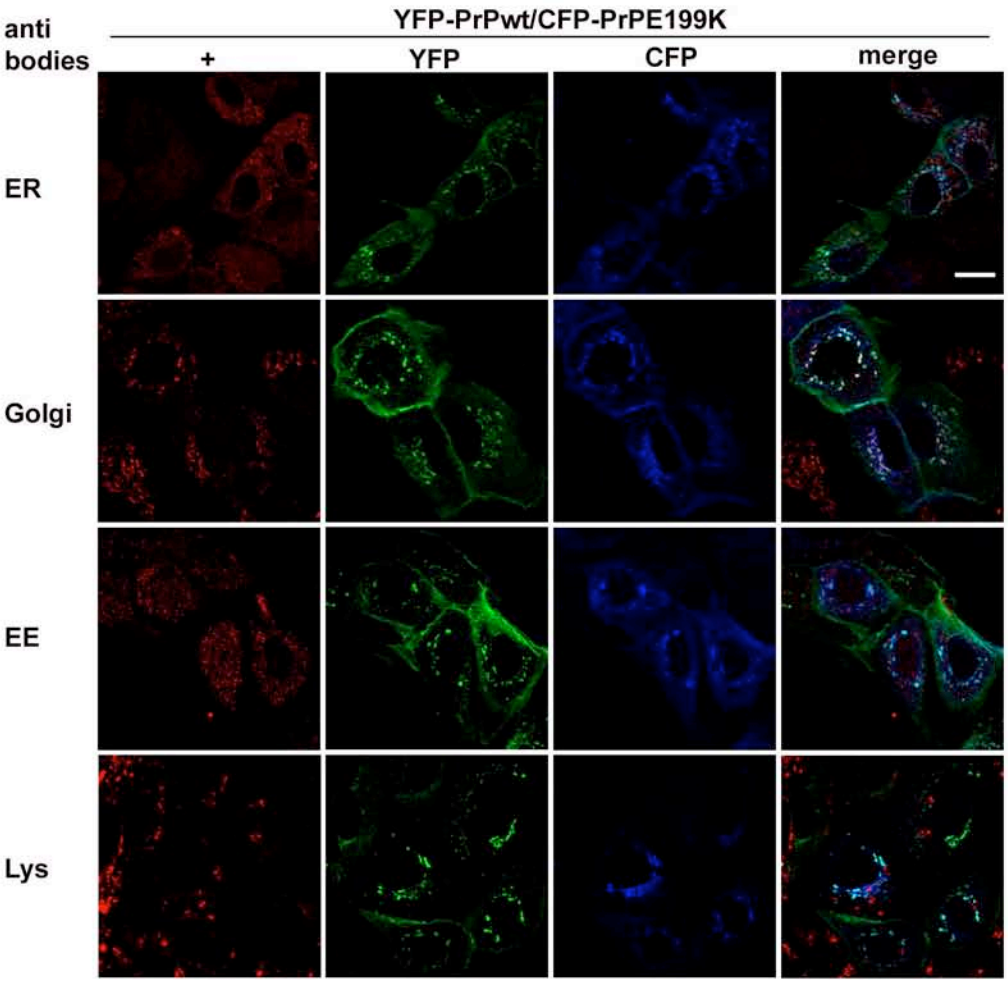

anti bodies
YFP-PrPwt/CFP-PrPE199K
+
YFP
CFP
merge
ER
Golgi
EE
Lys

Figure 4B

A

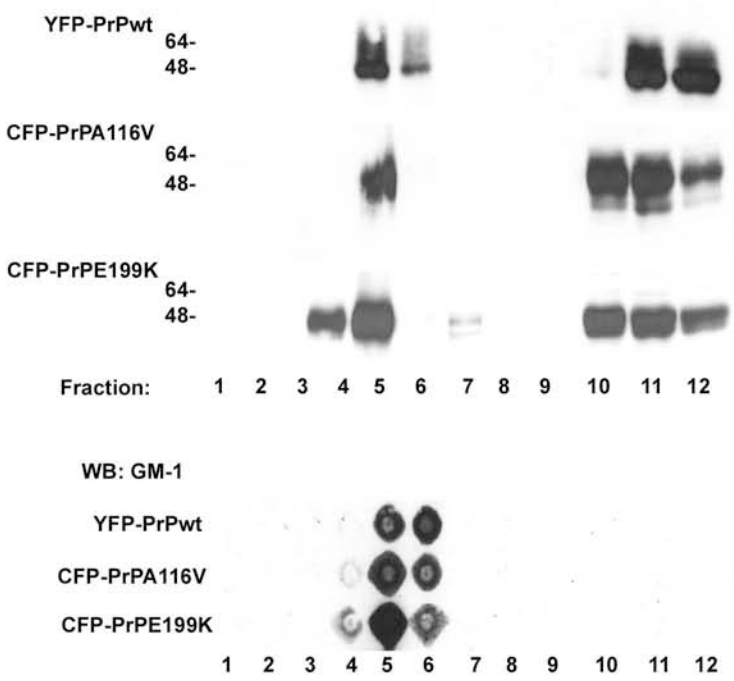
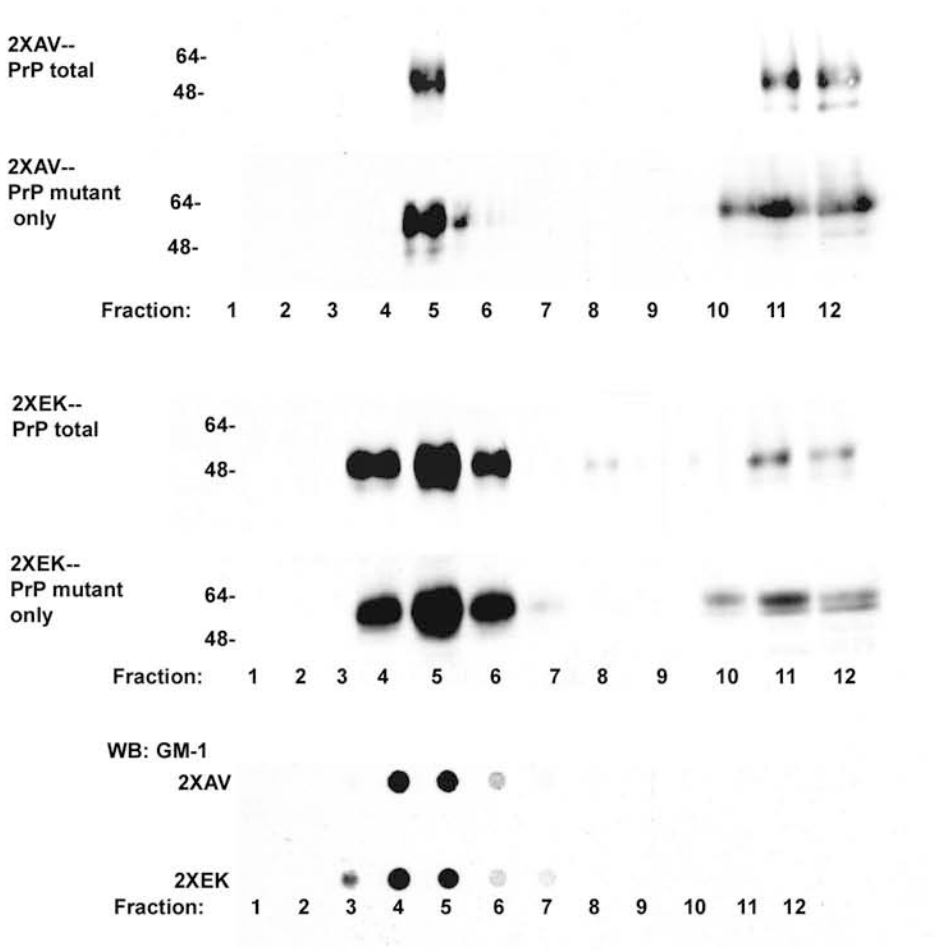

YFP-PrPwt
64-
48-
CFP-PrPA116V
64-
48-
CFP-PrPE199K
64-
48-
Fraction: 1 2 3 4 5 6 7 8 9 10 11 12
WB: GM-1
YFP-PrPwt
CFP-PrPA116V
CFP-PrPE199K
1 2 3 4 5 6 7 8 9 10 11 12

Figure 5A

B



C

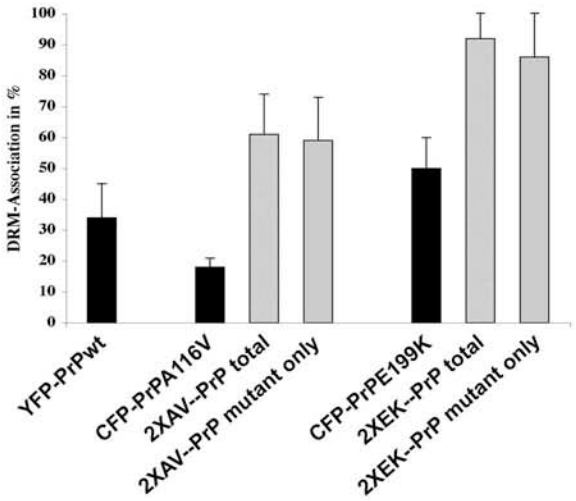


Figure 5 B and C

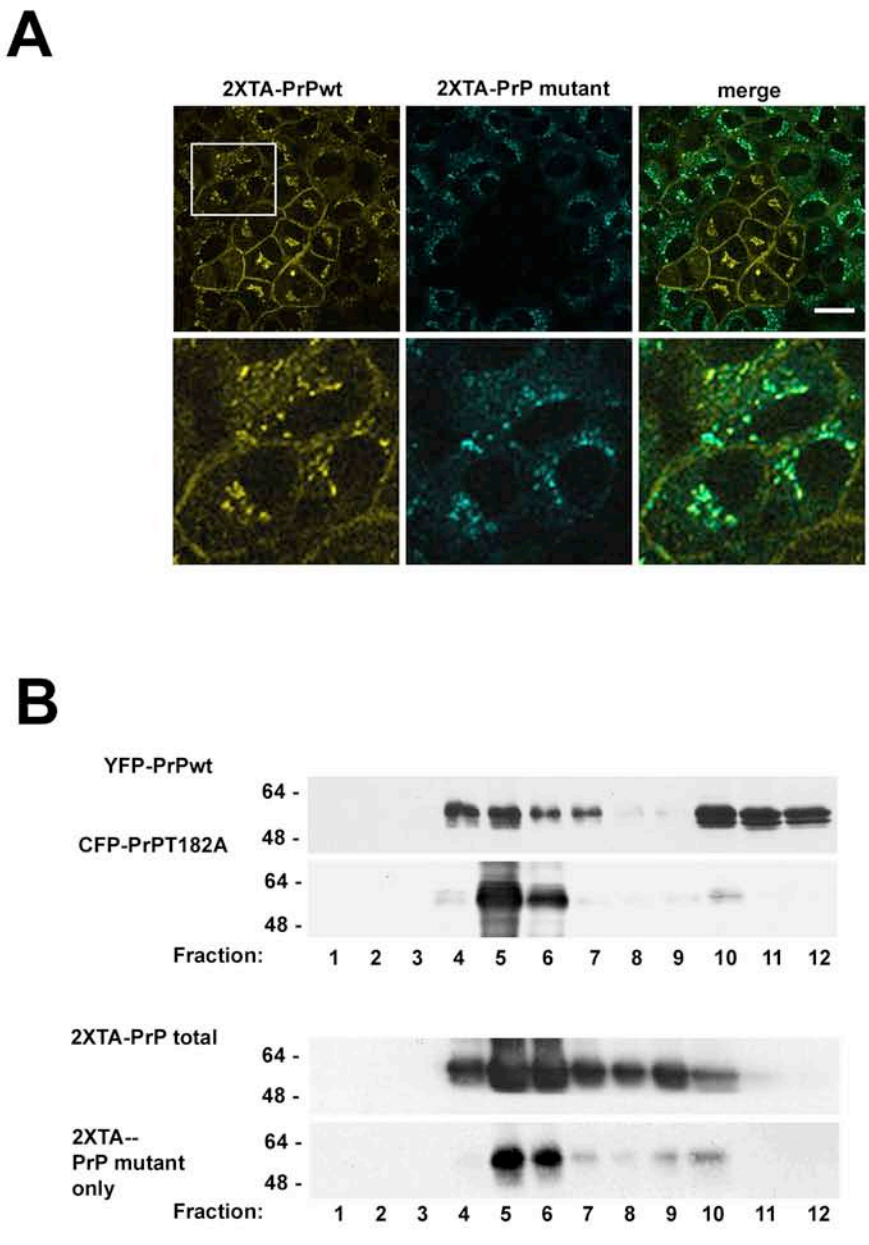
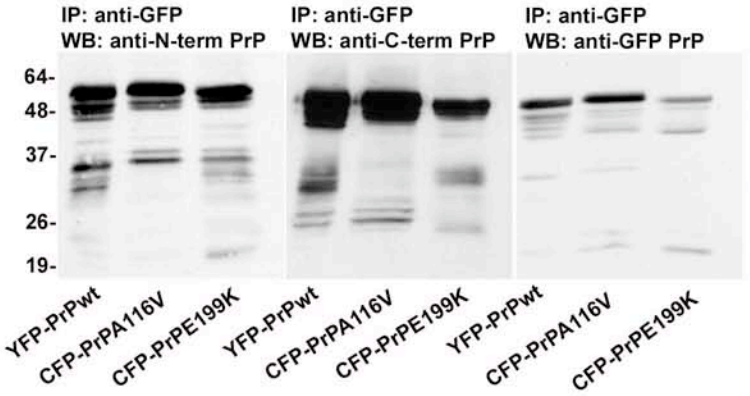
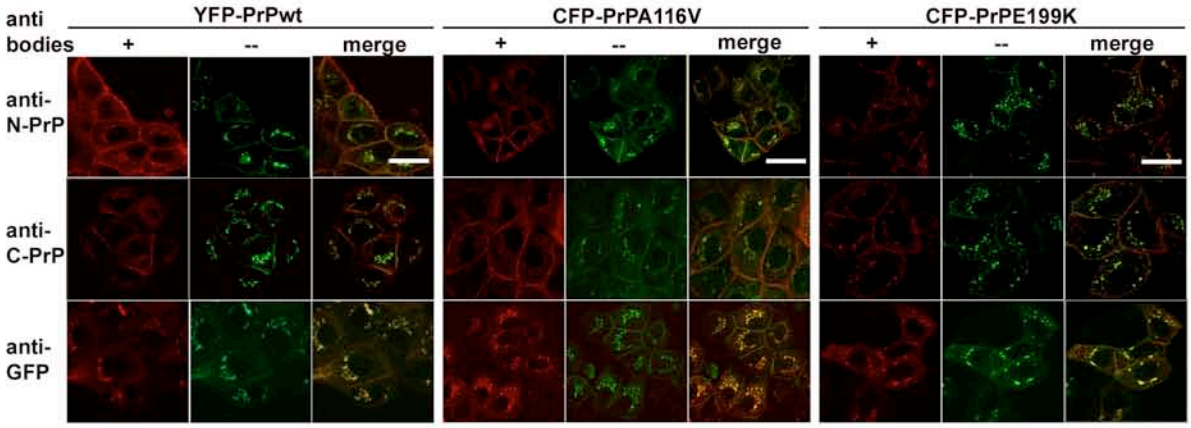

A
2XTA-PrPwt
2XTA-PrP mutant
merge
B
YFP-PrPwt
CFP-PrPT182A
64 -
48 -
64 -
48 -
Fraction:
1 2 3 4 5 6 7 8 9 10 11 12
2XTA-PrP total
64 -
48 -
2XTA--
PrP mutant
only
64 -
48 -
Fraction:
1 2 3 4 5 6 7 8 9 10 11 12

Figure 6

A

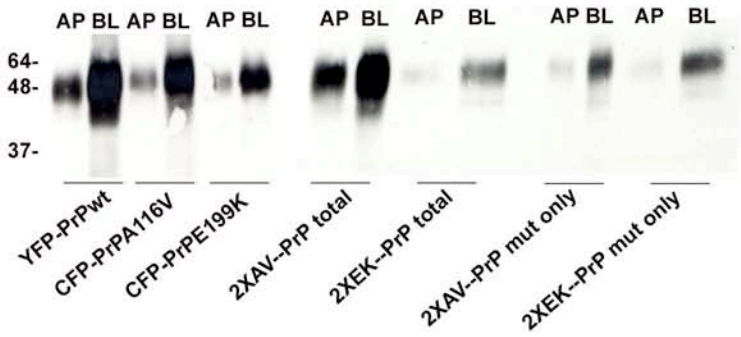


B

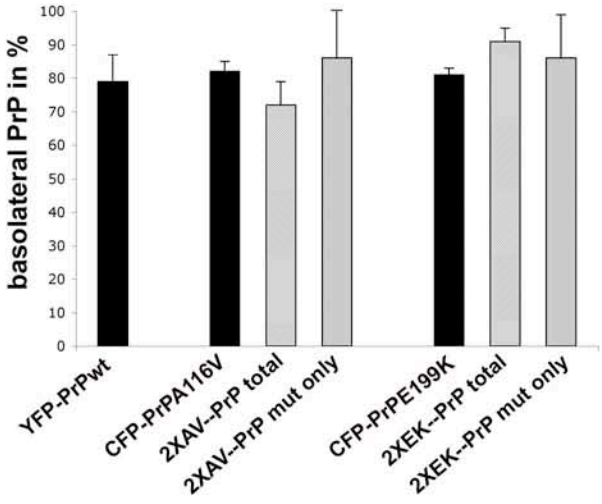


Supplementary Figure 1

A



B



Supplementary Figure 2

V.1.5: Additional data requested by the reviewers

The reviewers chosen by the journal "Traffic" reacted overall positively on the manuscript presented above. However, a few additional questions and controls were asked, which I was able to cover and are shown here:

1) DRM-sequestration in the case of FRT-cells co-expressing two wild-type proteins.
As described in detail above, co-expression of YFP-PrPwt and a mutant form linked to CFP (A116V, E199K, T183A) lead to a significant increase of PrPwt and PrPmut in DRMs. To rule out the possibility that this is as an unspecific effect of overexpression of two proteins and to show that this increase is mutant-specific we transfected the YFP-PrPwt single-expressing cells with a CFP-PrPwt-construct (containing the 3F4-tag) and performed a floatation assay (see Materials and Methods in Manuscript I). Quantification of DRM-association from Western blots utilizing antibodies recognizing either total PrP (black bar in quantification below, 40%±8) or only the 3F4-tag of CFP-PrPwt (grey bar in quantification below, 39±5) showed both to be associated to DRMs with similar amounts, as also seen for single-expressed YFP-PrPwt (35%±11). This let us conclude that there is no increase in DRM-association, when two wild-type forms are co-expressed and also suggests that the changes in DRM-sequestration, as observed in cells co-expressing YFP-PrPwt and CFP-PrPmut, are probably mutant-specific. This supports our hypothesis that the changes in DRM-association, as found with co-expressed wild-type and mutant, might be relevant for pathology in heterozygous carriers.

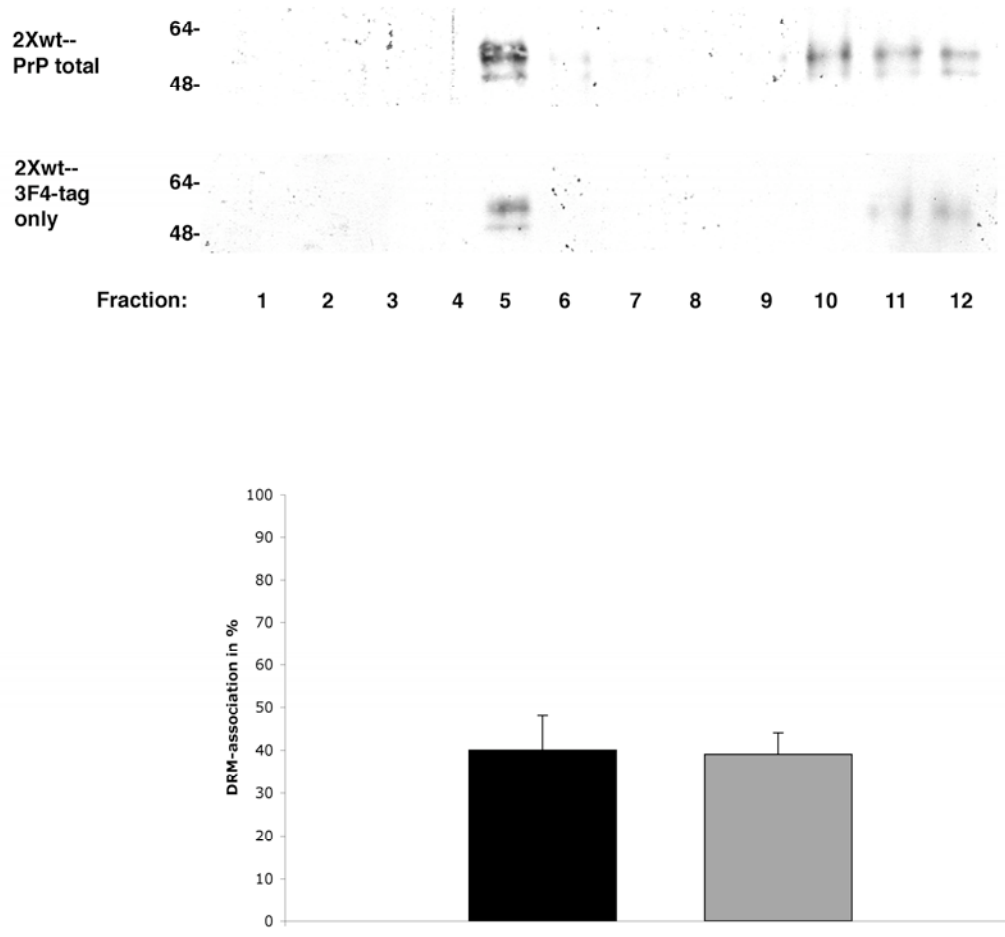


Fig. 35: Co-expression of two wild-type forms, YFP-PrPwt and CFP-PrPwt (containing a 3F4-tag) does not lead to an increased sequestration of PrP-proteins in DRMs. (above) Cells co-expressing YFP-PrPwt and CFP-PrPwt (with 3F4-tag) were run on a discontinuous sucrose gradient after extraction in cold detergents and revealed by Western Blot with antibodies recognizing both wild-type forms (termed 2Xwt-PrP total) or only the 3F4-tagged CFP-PrPwt (termed 2Xwt--3F4-tag only). **(below)** Quantification of amounts of protein associated to DRMs shows no difference between the total amount of PrPwt (black bar) and the co-expressed CFP-PrPwt (grey bar).

2) Analysis of lipids co-immunoprecipitating with PrP-proteins

Another comment of the reviewers was related to the nature of the DRMs in which PrPwt and PrPmut resided; specifically whether these lipid domains were the same or of different kinds. To answer this question we purified DRMs by sucrose density gradients from cells expressing different PrP-proteins (as specified in Fig. 36 below) from which the different PrP-isoforms were immunoprecipitated. The lipids, which co-immunoprecipitated with the different proteins of interest, were then subjected to analysis by high-performance thin layer chromatography (HPTLC) (for detailed

Materials and Methods see below). As shown in Fig. 36, we could not find any significant qualitative or quantitative difference in lipids co-immunoprecipitating with the proteins, suggesting that the proteins most probably occupy the same lipid domains in cells.

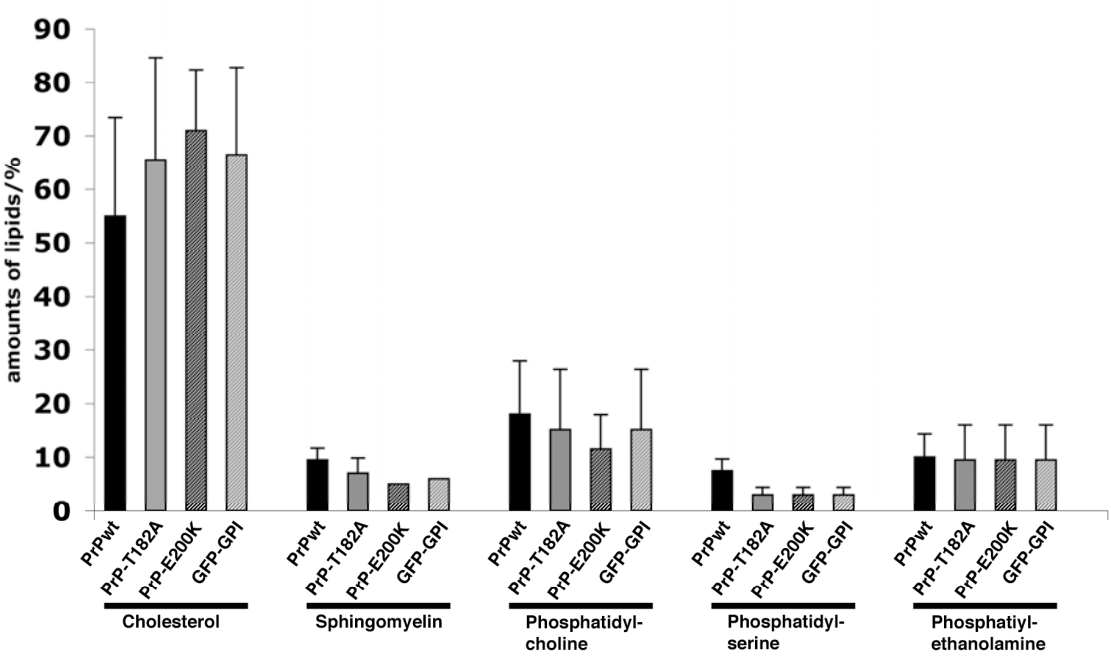


Fig. 36: Analysis of lipds associated with different PrP-forms in detergent resistant membranes (DRMs). Cells expressing the different PrP-proteins (see graph) were subjected to sucrose density gradients. Proteins of interest were immunoprecipitated from the DRMs-fraction and co-immunoprecipitated lipids were analyzed by high-performance thin layer chromatography (HPTLC). Quantification of lipids associated to different proteins shows no significant quantitative or qualitative difference.

The following is the detailed description of Material and Methods, as used for this experiment (see also (Tivodar et al., 2006).

Sucrose density gradients

Cells that had just reached confluency in 150-mm dishes were subject to ultracentrifugation on discontinuous sucrose gradients as previously described (Prinetti et al., 2000). Briefly, cells harvested in PBS containing 0.4 mM Na_3VO_4 were suspended in 1 ml lysis buffer (1% TX-100, 10 mM Tris buffer, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM PMSF and 75 mU/ml aprotinin), allowed to stand on ice for 20 min and homogenized. Post-nuclear supernatants (~ 8 mg proteins) were mixed with an equal volume of 85% sucrose (w/vol) in 10 mM Tris buffer pH 7.5, 150

mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, placed at the bottom of a discontinuous sucrose gradient (30–5%) in the same buffer and centrifuged at 200,000 g for 17 hrs at 4°C. After ultracentrifugation, eleven 1 ml fractions were collected starting from the top of the tube. Fraction 5 located at the interface between 5 and 30% sucrose was regarded as the sphingolipid-enriched membrane fraction. The bottom fraction (fraction 11) contained a pellet, which was carefully homogenized before analysis.

Immunoprecipitation

Aliquots of fraction 5 (800 ml) diluted with 200 µl lysis buffer (5% TX-100, 10 mM Tris buffer pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM PMSF and 75 mU/ml aprotinin) were precleared twice with dynabeads for 2 hrs and incubated overnight at 4°C with anti-PrP (Saf32) antibodies. Immunoprecipitates were recovered using protein G-coupled magnetic beads

After washing 3 times with lysis buffer, lipids were extracted from the immunoprecipitates and analyzed as described above and SDS-sample buffer was added to the beads. 1/5 of the samples were analyzed by SDS-PAGE (not shown).

Analysis of lipids

The sucrose gradient fractions obtained were analyzed for content of lipids. Samples were dialyzed and lyophilized, and lipids were extracted twice with 0.4 ml chloroform/methanol 2:1 (v/v) (Riboni et al., 1992). Cholesterol was separated by monodimensional high-performance thin layer chromatography (HPTLC) using the solvent system hexane/diethylether/acetic acid, 80:20:1 (v/v/v) and quantified after separation on HPTLC followed by visualization with 15% concentrated sulphuric acid in 1-butanol. Phosphatidylcholine was separated by a two-run monodimensional HPTLC using the solvent system chloroform/methanol 9:1 (v/v), followed by the solvent system chloroform/methanol/acetic acid/water 30:20:2:1 (v/v/v/v) and quantified after separation on HPTLC followed by specific detection with a molybdate reagent. The quantity of cholesterol and phosphatidylcholine were determined by densitometry and comparison with known amounts of standard lipids using the Molecular Analyst program (Bio-Rad Laboratories).

3) Interaction of co-expressed PrP-isoforms tested by Fluorescence resonance energy transfer (FRET)

As a next question the reviewers asked whether co-expressed wild-type and mutant proteins were in close enough proximity so that the mutant could modifiy the conformation of the wild-type form. They proposed to test this by performing Fluorescence resonance energy transfer (FRET)-experiments. Energy transfer by FRET occurs only when fluorophores are in close proximity (3-10 nm) (reviewed in (Kenworthy and Edidin, 1998)). Positive information obtained by this technique may give important clues as to whether and where wild-type protein and its pathogenic homologue are close enough to allow malconformation of the former by the latter and possibly to reciprocally influence their function. To answer this question we cultured FRT-cells expressing the proteins of interest on coverslips. After fixation in paraformaldehyde (2%) cells were mounted in PBS/Glycerol (1:1) and assessed for FRET by photobleaching of the acceptor (for description of the technique see (Kenworthy and Edidin, 1998)) on a SP 5 Leica-confocal microscope (Leica, Germany) utilizing the supplied software. The results of the measurements ($n \geq 15$ for each) are shown in Fig. 37 below. Cell lines co-expressing YFP-PrPwt and each of the CFP-linked mutants (A116V (termed 2XAV), E199K (termed 2XEK), T182A (termed 2XAT)) presented intracellularly the same FRET-efficiency as the positive control co-expressing two wild-type forms (YFP-PrPwt and CFP-PrPwt, termed 2Xwt). This suggests that wild-type and mutant molecules are in close proximity at the level of the Golgi-apparatus. When FRET-efficiency was measured on the plasma membrane, cells co-expressing YFP-PrPwt and CFP-PrPE199K (2XEK) showed comparable efficiencies as the positive control (2Xwt). Interestingly, the cells co-expressing YFP-PrPwt and CFP-PrPA116V (2XAV) showed a somewhat diminished FRET-efficiency, which suggests that these two molecules interact more closely intracellularly and less once arrived on the plasma membrane. As expected, cells expressing CFP-PrPA116V only, serving here as a negative control, showed only a minimal amount of FRET-efficiency. We are currently reproducing these data to have better statistical validity. However it seems to be clear that for all analyzed mutants there is a close interaction with the wild-type form on the plasma membrane as well as intracellularly, thus supporting our hypothesis.

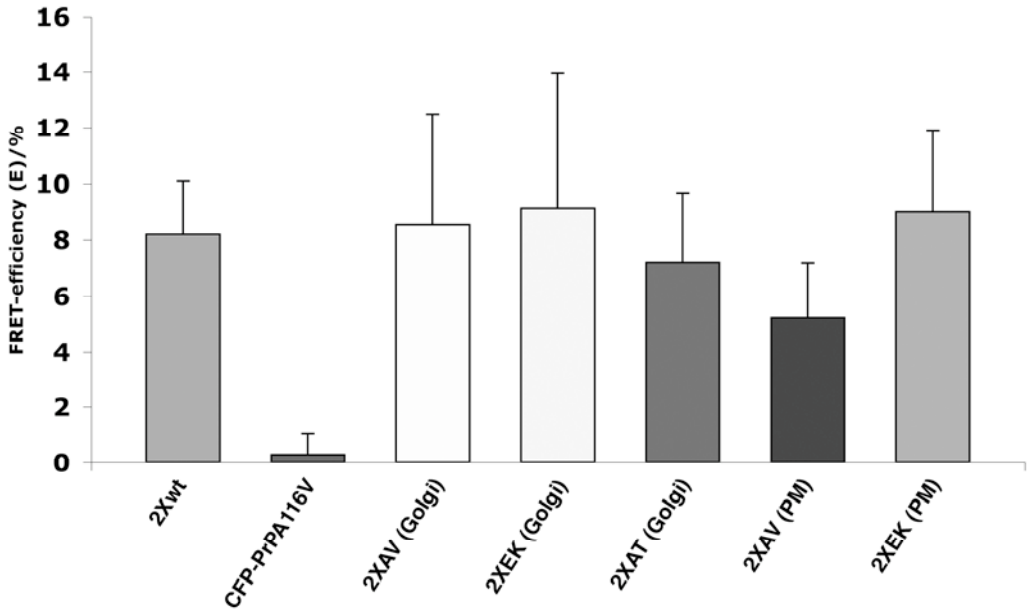


Fig. 37: FRET-efficiency measurements shows that wild-type and mutant PrP-forms are in close proximity. FRT-cells expressing different constructs were grown on coverslips, fixed and assayed for FRET-efficiency. FRT-cells co-expressing two wild-type forms (YFP-PrPwt, CFP-PrPwt, termed 2Xwt) served as positive control, while cells expressing CFP-PrPA116V only were utilized as negative control. Quantification shows that cells co-expressing YFP-PrPwt and either of the CFP-linked mutants (A116V (termed 2XAV), E199K (termed 2XEK), T182A (termed 2XAT)) presented FRET-efficiencies as high as the positive control in the Golgi-apparatus. On the plasma membrane, 2XAV presented somewhat diminished FRET-efficiencies when compared to 2Xwt.

4) Colocalization studies of mutants not linked to CFP-fluorophores in FRT-cells.

As a last question the reviewers wanted us to test whether the attachment of fluorophores affects the trafficking of the mutant proteins. To answer this question we transfected FRT-cells with the plasmids encoding only for murine PrP, hosting the alanine to valine change at position 116 and the glutamate to lysine change at position 199. Cells expressing either PrP-A116V or PrP-E199K were grown on coverslips and fixed with PFA (2%), then incubated with an antibody directed against the N-terminal part of the PrP-protein and antibodies directed against various organelle markers (calnexin in the case of the endoplasmatic reticulum, Giantin for cis- and medial Golgi and LysoTracker for lysosomes) (see Materials and Methods of Manuscript I). Image acquisition and analysis was performed by confocal microscopy (Zeiss LSM 510). As shown in Fig. 38, the PrPmut-proteins behaved just as their fluorophore-attached counterparts (see Manuscript I), colocalizing with Giantin and also localized to the plasma membrane. This was also consistent with the previously published data for the

PrP-T182A mutant (Campana et al., 2006; Sarnataro et al., 2004). This suggests that the attachment of the CFP-fluorophore does not affect their intracellular trafficking.

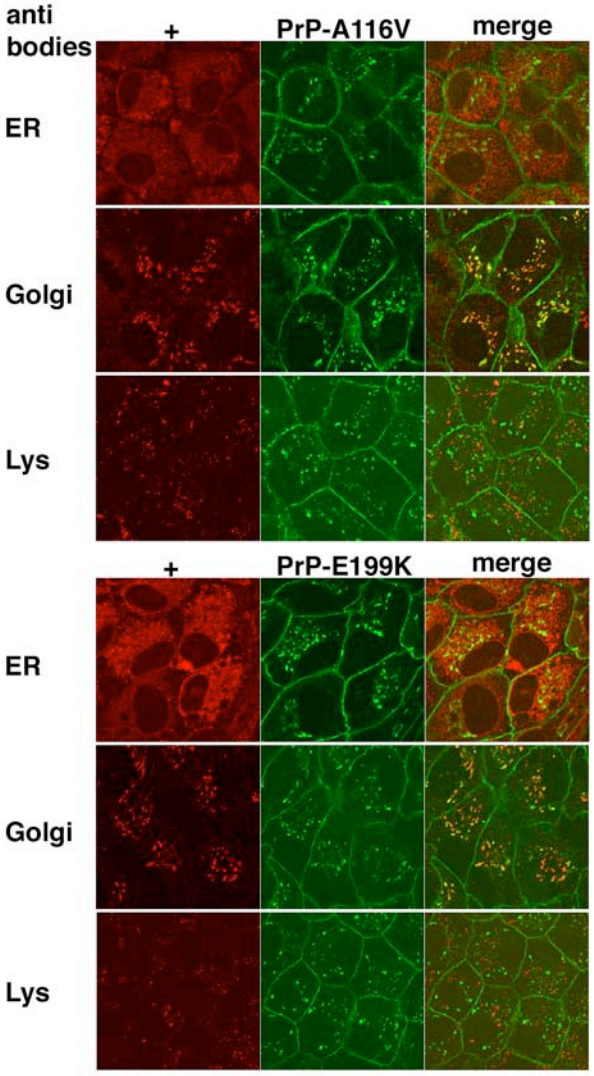


Fig. 38: Colococalization studies for the mutants PrP-A116V and PrP-E199K. Immunofluorescence and confocal microscopy on fixed cells, expressing the non-CFP-attached forms of the PrP-mutants A116V and E199K, shows colocalization with the utilized Golgi-marker (Giantin) and deposition on the plasma membrane.

V.2: Manuscript 2

This manuscript has been submitted to the journal Science and is currently under revision.

V.2.1: General introduction

In the second part of my thesis I focused on the acquired forms of prion-diseases, specifically on the question of how prions are transmitted from cell to cell and which cells could be involved in the process of transmission of the disease from the periphery to the central nervous system. As described in chapter III.15, the most common route of infection with exogenous prions is by oral uptake of contaminated foodstuffs. Based on results from *in vivo* and *in vitro* studies, the common belief is that the entrance into the affected organisms occurs by a transcytotic mechanism by microfold cells (M cells) of the intestinal system (Heppner et al., 2001). By inspecting samples from animals, sacrificed at various times post oral exposure, researchers found that prions radiated away from the intestinal entry site and infected adjacent secondary lymphoid organs within days to weeks prior to neuronal invasion (Bruce, 1985; Bueler et al., 1993; Rubenstein et al., 1991). Due to their first appearance in lymphoid organs, it was hypothesized that cells of the immune system might play a role in the spread of prions throughout the organism and as of late numerous immune cell types, such as macrophages, B cells and dendritic cells (DCs) have been implicated in this process (see III.15.2 and references therein). Follicular dendritic cells (FDCs) have also been proposed to be involved in prion diseases, however their role remains enigmatic (III.15.2.4). Specifically, the spleen received much attention in prion diseases and early works have already emphasized its role in acquired prion diseases (Clarke and Haig, 1971). Lately, the gut associated lymphatic tissue (GALT) has received increased attention from researchers and is now considered to be the first site of prion accumulation following oral inoculation (Glaysher and Mabbott, 2007). A common trait of the spleen and GALT is their strong innervation by the peripheral nervous system (III.15.1); their role as access points for prions to the central nervous system (CNS) is uncontested. The sympathetic nervous system (SNS) is believed to play a decisive role in handing prions from an unspecified immune system cell to the CNS, since it was shown that sympathectomy prior to or just after oral prion challenge significantly

delayed the progression of the disease, while splenic hyperinnervation significantly reduced the peripheral incubation period and lead to a more rapid invasion of the CNS (Clarke and Haig, 1971; Glatzel et al., 2001). As described in detail in III.15.2 evidence suggests that the immune cells transferring prions from their entry site to the secondary lymphoid organ must be mobile and should not destroy sampled antigens. This would explain the radiation of prions from the intestinal entry site and their still active infectivity after transportation to the secondary lymphoid organs. Additionally these immune system cells should interact closely with the peripheral nervous system in order to allow the transfer of prions from the immune system cell to the peripheral nervous system (Beekes and McBride, 2007).

These prerequisites make dendritic cells (DCs) a suitable candidate, because they are highly mobile and because they do not necessarily destroy sampled antigens, as shown for PrP^{Sc} (Huang et al., 2002). In addition these cells are in close interaction with the peripheral nervous system (Aucouturier et al., 2001; Defaweux et al., 2005; Dorban et al., 2007) and could therefore allow intercellular transfer by an unknown mechanism (see paragraph III.15.2.5).

Until now a variety of mechanisms for intercellular transfer have been suggested:

GPI-painting, an ill-understood process, in which GPI-anchored proteins transfer from the plasma membrane of one cell to another cell has been shown for PrP^{C} (Liu et al., 2002). Since PrP^{Sc}, like PrP^{C}, contains a GPI-anchor (Stahl et al., 1987), one could envision that this also occurs for the pathogenic form.

Another possibility for intercellular prion transfer is *via* exosomes (see paragraph III.15.3.1). These vesicular structures with a diameter of 30 to 100 nm are derived from multivesicular endosomes, contain cell-derived proteins and are excreted by a number of cell-types such as DCs, lymphocytes, mast cells, platelets and epithelial cells (Thery et al., 2002). Additionally, they have been shown to contain PrP^{Sc} and are able to infect animals when inoculated intracerebrally in enriched amounts (Fevrier and Raposo, 2004). As mentioned above, DCs were shown to excrete high amounts of exosomes and emphasizes once more the potential role of these cells in transferring prions to the peripheral nervous system. However it must also be stressed that although Fevrier et al. proposed exosomes to be a means of intercellular transfer of prions, they only showed this in an indirect way by inoculating animals intracerbrally with enriched preparations of exosomes.

Another intriguing possibility for how prions might transfer intercellularly is *via* the recently described tunneling nanotubes (TNTs) (Onfelt et al., 2006; Onfelt et al., 2004; Rustom et al., 2004). These structures are described in depth in paragraph III.16, where I also discuss why TNTs could be another (not necessarily exclusive) means by which prions could transfer intercellularly. Here as an overview the arguments that speak in favour of TNTs as a means for intercellular transfer of prions:

i) GPI-anchored proteins and endosomal-derived organelles can transfer intercellularly *via* TNTs, which makes these structures very interesting for the prion-field, since PrP^{Sc} retains its GPI-anchor (Stahl et al., 1987) and is also found in endosomal structures, such as lysosomes and endosomes (Peters et al., 2003; Shyng et al., 1993).

ii) TNTs connect cells of different origin and DCs are particularly apt at producing these structures, allowing quick intercellular transfer (Watkins and Salter, 2005).

iii) TNTs may also be produced *in vivo*, since structures resembling TNTs, termed cytonemes, were described in *ex vivo* preparations from *Drosophila melanogaster* (Hsiung et al., 2005; Ramirez-Weber and Kornberg, 1999).

V.2.2: Objectives

Due to the reasons stated above I believe that TNTs, possibly involving dendritic cells as one of the participating cell-types, could be a means by which prions could transfer from cell to cell. TNTs, supposedly used by cells as a novel means for cell-cell communication, are very fine and dynamic structures and therefore require a microscopy-based approach in fixed, ideally in living cells for their study. We decided to first work with HEK 293 cells, since these were reported to produce TNTs (Rustom et al., 2004). We reasoned that this robust immortalized cell line would allow us to set up the appropiate conditions for studying these structures, since the study of living cells is a non-trivial approach and requires fine-tuning. Once protocols and techniques were established we switched to an immortalized neuronal cell-line more relevant to prion pathology. For this part of my studies we decided to work with CAD-cells, a murine cell line of catecholaminergic origin (Qi et al., 1997). These cells were reported to produce structures reminiscent of TNTs (Li et al., 2007b). Additionally this cell-line is easily transfectable with plasmids and infectable with prions (personal communication from H. Laude). Thus we used a CAD-cell line, chronically infected with the scrapie

strain 139A (scCAD) (kind gift from H. Laude), which allowed us to advance our studies more rapidly. The undoubtedly most ambitious part in this work was to understand the *in vivo* relevance of the data we had obtained *in vitro*. For this we set up the coculture of primary cell lines of different origins and tested whether the cells would produce TNTs and whether PrP^{Sc} would transfer between them *via* these structures. For the reasons stated in the introduction and in paragraph III.15.2.5, we considered the interaction of dendritic cells with neurons to be the most interesting scenario. For these experiments several techniques and protocols had to be established in the laboratory. The culture of murine bone-marrow derived dendritic cells (BMDCs) and the culture of murine hippocampal neurons was established. These cells were then cocultured in order to simulate the neuro-immune interaction *in vitro*. In order to be able to distinguish the neurons by fluorescent microscopy we transduced them with a retroviral system coding for green fluorescent protein (GFP) linked to murine wild-type PrP (GFP-PrPwt) that was engineered in the laboratory. Traditional microscopic studies of PrP^{Sc} required up to today fixation of cells and treatment with harsh chaotropic agents to reveal PrP^{Sc}-specific epitopes (Taraboulos et al., 1990). In order to study PrP^{Sc}-transfer in living cells, we decided therefore to purify PrP^{Sc} from the brains of terminally ill mice and directly link this to Alexa-fluorophores by adapting a previously published protocol (Magalhaes et al., 2005).
Utilizing the cell-systems and protocols described above we then were able to test the hypothesis that TNTs are responsible for intercellular PrP^{Sc}-transfer.

V.2.3: Results

In order to determine whether we could observe tunneling nanotubes (TNTs) between different cells, we transfected HEK 293 cells with GFP-PrPwt and cocultured these with an unlabelled cell population. After fixation and immunofluorescent staining for various cytoskeletal proteins (see below), we proceeded to confocal microscopy. Indeed, we found tubes containing GFP-PrPwt connecting cells of the two populations, thereby excluding incomplete cytokinesis of daughter cells. A hallmark of TNTs is that they are not attached to the substratum (Rustom et al., 2004). X-Z reconstructions of cells showed in our case that connections hovered freely in the cell medium without contact to the substratum (see also movie S1 on CD). Additionally, as reported previously

(Onfelt et al., 2006), we also observed cytoskeletal heterogeneity based on the diameter of TNTs. In thicker TNTs (diameter 500-700nm) we observed positive staining for filamentous actin (F-actin) and tubulin, while thinner connections (diameter approximately 300nm) contained F-actin but little or no tubulin. These initial findings lead us to conclude that we were indeed looking at the phenomena previously termed tunneling nanotubes.

When TNTs were first described, the authors hypothesized based on their observations, that cells interacting by TNTs could be subdivided into a donor and a recipient (the first providing the tube and connecting to the latter) (Rustom et al., 2004). Studying cocultures of differentially labelled cell populations, we monitored tubes deriving exclusively from one of the labelled subgroups. This supported the initial hypothesis of a donor and a receptor. In spite of this, when differently labelled cells were cocultured, we repeatedly observed TNTs, which harboured GFP-PrPwt in one half of the tube and the other fluorophore in the second half, thus suggesting that both cells might contribute to the build-up of the TNT. For testing the dynamics of the formation of TNTs, we utilized live cell microscopy, specifically a spinning disc confocal microscope. For this experiment HEK 293 cells were labelled with TAMRA, a dye labelling lipids in membranes, and then processed these cells for imaging (for example see also movie S4 on CD). Already at the beginning of the recording we found cells connected by TNTs. Furthermore, we monitored the active build-up of a TNT, which lasted in the shown example approximately 5 minutes and which connected two cells for about 13 minutes (total recording time of this movie was 30 minutes). Interestingly, we repeatedly could see that both cells participated in this process, thereby excluding incomplete cytokinesis of daughter cells and demonstrating that both cells could participate in the formation of TNTs.

Next we analyzed whether TNTs could transfer PrP^C intercellularly. For this we cocultured one cell-population transfected with GFP-PrPwt and labelled with LysoTracker with another population labelled only with LysoTracker and imaged these by live microscopy. The presented example shows that, during the course of 30 minutes lysosomes were observed to exit from the GFP-PrPwt cells and move *via* a TNT to another cell. These vesicles were measured at a speed of 40-60 nm/s, in agreement with vesicular transport kinetics in TNTs as previously reported (Rustom et al., 2004). Additionally, we observed the movement of GFP-PrPwt labelled membrane from one cell to another, suggesting that GFP-PrPwt could spread intercellularly by plasma

membrane transfer on TNTs (see also movie S5 on CD). Using cells labelled the same way, we could also monitor transfer of vesicles containing GFP-PrPwt transferring intercellularly *via* TNTs with a speed of 40-60 nm/s (for example see also movie S6 on CD). Interestingly, we observed that GFP-PrPwt was transferred in vesicles of different kinds, some of which were also positive for LysoTracker. This suggests that PrP^C-proteins can be transferred intercellularly contained in acidic vesicles as well as in other vesicles. Based on colocalization data, Rustom and colleagues proposed the involvement of molecular motors (e.g. myosin Va) in this process (Rustom et al., 2004). We were therefore interested to analyze the characteristics of vesicular movement in TNTs and did this by studying their so-called mean square displacement (MSD) (see manuscript 2 Fig.S4 and Materials and Methods). We found that they moved with a directed, non-brownian character, suggesting the use of molecular motors in the transfer-process. Additionally, when fixed cells were analyzed for colocalization of GFP-PrPwt-positive vesicles with different molecular motors, we found actin-based motors such as myosin Va and myosin VI to colocalize with these vesicles (approximately 50% each). On the other hand we found little colocalization with a microtubule associated motor of the kinesin superfamily, Kif 3a. These data taken together confirmed the involvement of actin based molecular motors in the transfer of vesicles by TNTs.

We could also observe transfer of GFP-PrPwt in tubulovesicular structures, which filled the length of the TNT between cells. To examine this further we utilized X, Y and Z information and reconstructed one of these examples while applying a threshold for GFP-PrPwt content (blue stands for high GFP-PrPwt content, grey for lower amounts) (see movie S8 on CD). Here, GFP-PrPwt appears to be transferred in tubulo-vesicular structures reminiscent of the trans-Golgi-network (TGN). Nascent PrP^C is known to pass through the Golgi-apparatus before transferring to the plasma membrane (see paragraph III.14.1.1) and PrPSc was described to localize partially in the Golgi-apparatus . Others have hypothesized that TNTs could be involved in the intercellular transfer of the TGN (Galkina et al., 2001). This prompted us to test whether GFP-PrPwt could be transferred intercellularly while in the Golgi-apparatus (Taraboulos et al., 1990). For this we transfected HEK 293 cells with GFP-PrPwt and cocultured these with unlabelled cells for 24 hours. Then, we fixed and labelled them with antibodies directed against Golgi-markers, such as Giantin (a marker for cis- and medial Golgi). We observed TNTs connecting differentially labelled cells, which contained both the

Golgi-marker and GFP-PrPwt. These data taken together suggest that Golgi-derived material containing GFP-PrPwt can be transferred intercellularly *via* TNTs.

With these encouraging results, I decided to switch to the aforementioned immortalized neuronal CAD-cell line (Qi et al., 1997).

We first analyzed whether we could repeat the coculture experiments with two differently labelled populations as described above. Coculture of GFP-PrPwt-transfected cells with cherry-rab 6 (a Golgi marker) transfected cells for 48 hours, produced typical TNTs not attached to the substratum (as shown by X-Z reconstructions). Additionally we observed GFP-PrPwt-transfer *via* both membrane "surfing" and vesicular structures. We could as well repeat the experiments showing transfer of GFP-PrPwt contained in Golgi-derived vesicles in both fixed cells and in living cells (see also movie S9 on CD). Altogether these data indicate that GFP-PrPwt can be transferred between cells of neuronal origin by transfer on the PM of TNTs or by vesicular transport inside of TNTs.

Next step was to study whether endogenous PrP^{C} and PrP^{Sc} can also transfer intercellularly *via* TNTs. In fixed samples we could indeed observe vesicles inside of TNTs containing endogenous PrP^{C} in the case of non-infected CAD-cells and PrP^{Sc} in CAD-cells chronically infected with the prion strain 139A (scCAD). Some of the transferring vesicles also contained lyso-bisphosphatidic acid (LBPA), a marker of late endosomes (Kobayashi et al., 1998). Because discerning between PrP^{C} and PrP^{Sc} required fixation of cells and a pre-treatment with guanidium hydrochloride, this approach was limited to studying fixed cells. Thus, in order to be able to observe the transfer of PrP^{Sc} in living cells, we decided to produce fluorescently-labelled PrP^{Sc} (Alexa-PrP^{Sc}) (labelled with Alexa-568nm) by adapting a previously published protocol (Magalhaes et al., 2005). scCAD-cells were charged with Alexa-PrP^{Sc} for 6 days, extensively washed and cocultured for 24 hrs with non-infected CAD-cells, which were previously transfected with a GFP-GPI construct. We could monitor TNTs, unattached to the substratum, between the two populations, which also contained Alexa-PrP^{Sc} particles. Upon close inspection we could also observe fluorescent particles, which had already been transferred into the lumen of the recipient cell. This demonstrates that fluorescently labelled PrP^{Sc} can transfer by TNTs to other cells, showing that TNTs could represent a means to spread prion infection between cells of neuronal origin.

Finally, we wanted to understand whether TNTs could support the transfer of Alexa-PrP^{Sc} between primary cells of different origins. By immunohistofluorescence we could

observe dendritic cells closely juxtaposed to sympathetic neurons in murine spleen-cuts, supporting the hypothesis that these two cell-types were in close enough proximity to support the intercellular transfer of PrP^{Sc} *via* TNTs. Because the resolution of current imaging systems does not allow the analysis of TNTs in living tissues, we simulated the interaction of DCs with neurons by coculturing murine bone marrow derived dendritic cells (BMDCs) with primary murine hippocampal neurons *in vitro*. After 24 hours of coculture we found TNTs between neurons and BMDCs, which were not attached to the substratum (see movie S10 on CD). Furthermore, using cocultures of BMDCs, previously stained with LysoTracker, and neurons, previously transduced with GFP-PrPwt, we observed LysoTracker-positive vesicles within neurons in close vicinity to TNTs beween the two cell-types. This suggests that lysosomal vesicles can transfer *via* TNTs from BMDCs to neurons.

When BMDCs were charged with Alexa-PrP^{Sc} and were cocultured with GFP-PrPtwt-transduced hippocampal neurons, we found TNTs connecting the two different cell types as well as the presence of Alexa-PrP^{Sc} in the neuronal cell bodies and neurites close to the intercellular connection. These data taken altogether show that DCs can interact with neurons *via* TNTs and that intercellular transfer of prions between these two cell types can occur *via* these connections.

V.2.4: Discussion

In diseases such as variant CJD (vCJD) exogenous PrP^{Sc} is believed to enter the organism by contaminated foodstuffs. Comparison of mice experimentally infected with prions from BSE-cattle and patients who succumbed to vCJD, led researchers to conclude that the most likely cause for vCJD in humans was the consumption of BSE-contaminated beef (Bruce et al., 1997; Hill et al., 1997). It is generally assumed that prions from contaminated foodstuffs enter the body *via* M-cells in the intestine (Heppner et al., 2001). As discussed in depth in paragraph III.15.1, following oral challenge, prions spread from the entry site, concentrate in the adjacent secondary lymphoid organs and are found in the spleen within days and reach a plateau after a few weeks (Bruce, 1985; Bueler et al., 1993; Rubenstein et al., 1991) (rev. in (Daude, 2004)). Due to the fact that secondary lymphoid organs close to the site of entry were found to concentrate prions in the organism, researchers concluded that the immune

system plays a decisive role in the peripheral progression of the disease. These findings were substantiated by the fact that splenectomy shortly before or during oral challenge of animals with prions lead to a significant prolongation of the incubation, thus suggesting that the spleen might play an important role in the peripheral invasion of the disease (Fraser and Dickinson, 1970; Kimberlin and Walker, 1989).

As the disease progresses, it is believed that the peripheral sympathetic neuronal system (SNS) serves as a portal for prions to enter the central nervous system. This is emphasized by findings that sympathectomy in animals immediately before or after oral challenge significantly delays the onset of disease, while sympathetic hyperinnervation of the spleen and other lymphoid organs leads to a significant shortening of the incubation period (Clarke and Haig, 1971; Glatzel et al., 2001). Upon intraperitoneal challenge, prions manifest themselves first in neurons of the thoracic spinal cord in regions corresponding to the entry site of the splanchnic nerves of the sympathetic nervous system (Beekes et al., 1996; Cole and Kimberlin, 1985). Prions were also shown to accumulate in sympathetic ganglia (McBride and Beekes, 1999), underlining the probable involvement of the peripheral sympathetic neuronal system. Nevertheless it is worthwhile to mention that sympathectomized mice succumb to the disease, although with some delay compared to control animals, indicating the existence of other less efficient entry routes, possibly *via* the vagal nerve (Baldauf et al., 1997; Beekes et al., 1998).

Dendritic cells, a highly mobile cell-population of the immune system, have frequently been reported to be in close physical contact with peripheral nerve terminations (Goehler et al., 2000; Goehler et al., 1999; Hosoi et al., 1993). Due to this and their ability to preserve antigens undegraded, dendritic cells have been proposed to play a role in the "switch" of prions from the immune system to the peripheral nervous system (Aucouturier et al., 2001; Defaweux et al., 2005; Dorban et al., 2007).

However, the mechanism as to how this presumed transfer from immune system cells to peripheral neurons occurs remains enigmatic. Proposed mechanisms include transfer *via* exosomes, membrane-coated viruses or a process termed GPI-painting (see paragraph III.15.3).

Tunneling nanotubes (TNTs) are structures, which have been recently described *in vitro* (Rustom et al., 2004) and presumably serve as a means for cell-cell communication. Specialists from the field suggest that TNTs might have a counterpart *in vivo*, termed cytonemes, which were described in *ex vivo* preparations of the wing imaginal disc from

Drosophila melanogaster and are presumably involved in decapentaplegic-signalling (Hsiung et al., 2005; Ramirez-Weber and Kornberg, 1999).

In this part of my thesis I wanted to analyze whether prions could exploit tunneling nanotubes for intercellular spreading *in vitro* and whether this might occur *in vivo* between dendritic cells and peripheral neurons in secondary lymphoid organs such as the spleen.

In the work presented here we demonstrate that TNTs are built between cells of different populations, therefore excluding incomplete cytokinesis of daughter cells. Additionally, we have contributed to the understanding of the mechanism how TNTs are built up between cells. By utilizing differentially labelled cell population, we found TNTs labelled for one half with membrane from one cell and for the other half with membrane derived from the other cell population. This suggests that both cells can contribute to the building of the TNT, in contrast to the previous hypothesis of a donor and an acceptor cell (Rustom et al., 2004). Additionally in living cells, we observed that both cells could initiate the TNT-build-up in a spatio-temporal controlled manner, strongly suggesting either the existence of even finer physical connections escaping the detection by the microscope or the existence of secreted messengers. The mechanism as to how cells initiate the construction of TNTs and as to how cells utilize cytoskeletal elements for stabilizing these tubes remains largely unclear and represents an interesting avenue for future research. We could find cytoskeletal heterogeneity depending on the diameter of TNTs in agreement with previous work (Onfelt et al., 2006). Utilizing sophisticated measurement methods (see Material and Methods of Manuscript 2) we could establish a cut-off number for diameter thickness (approximately 500nm) and cytoskeleton differences deriving from there: TNTs with a diameter of 300nm contained filamentous actin (F-actin) but little or no tubulin. In contrast, TNTs with thicker diameters always contained F-actin and microtubules. Why difference in diameter leads to this cytoskeletal heterogeneity remains unanswered, but it could be envisioned that thicker connections need additional stabilizing cytoskeletal elements such as microtubules. Also this question needs to be further examined.

In the course of this work, I believe to have demonstrated that prions utilize TNTs for intercellular transfer both by plasma membrane and by vesicular transfer:

1) Plasma membrane transfer. We could show in fixed and living cells that a GFP-linked, ectopically expressed protein (GFP-PrPwt) can transfer by “surfing” on the plasma membrane of TNTs from one cell to another. This is consistent with previous

findings as shown for proteins attached to the inner side of the plasma membrane (farnesylated EGFP) (Rustom et al., 2004), to the external side of the plasma membrane (GFP-GPI, a GPI-anchored GFP-molecule) (Onfelt et al., 2004) and also for pathogens such as Mycobacterium bovis and Murine Leukemia Virus (MLV) (Onfelt et al., 2006; Sherer et al., 2007). These findings suggest that plasma membrane and associated elements can exchange between cells by TNTs. The mechanism underlying this exchange of plasma membrane content remains to be further investigated.

2) Vesicular transfer. We could show that GFP-PrPwt is transferred by TNTs in vesicles of lysosomal origin, in Golgi-derived structures as well as other vesicular carriers. By analyzing the mean square displacement (MSD) of vesicles contained in TNTs (see Manuscript 2 and Materials and Methods therein), we concluded that their motion is of a non-brownian, directed nature. This suggested the participation of molecular motors, which was further substantiated when we found that several actin-based molecular motors such as myosin Va and myosin VI colocalized with GFP-PrPwt-positive vesicles inside TNTs. On the other hand, we could not find colocalization between a microtubule-based motor (Kif 3a) and PrP-containing vesicles inside TNTs, thus suggesting no involvement of this motor in the transfer of GFP-PrPwt-positive vesicles *via* TNTs. These data suggest that actin-based motors but not microtubules motors might be involved in the process of intercellular transfer *via* TNTs. Importantly, in cells of neuronal origin we found that not only ectopically expressed GFP-PrPwt is transferred *via* TNTs but also endogenous PrP^{C} and PrP^{Sc}, as well as Alexa-labelled PrP^{Sc}. This strongly suggests that prions can transfer *in vitro* between neurons *via* TNTs. Based on our coculture experiments with primary neurons and bone marrow derived dendritic cells in which lysosomes and Alexa-PrP^{Sc} efficiently transferred to neurons *via* TNTs, we propose that this transfer occurs also *in vivo*. The fact that we observed dendritic cells in close proximity to peripheral sympathetic neurons in spleen by immunohistofluorescence, underlines the plausability of our hypothesis. In conclusion we propose that TNTs may represent one of the possible means of transfer of infectious PrP from the periphery to the central nervous system (CNS) and within cells of the CNS. If this holds true, it will be important to develop methods that temporarily inhibit production of TNTs and/or knock-down of dendritic cells as a treatment against acute prion exposure.

Prions hi-jack tunneling nanotubes for intercellular spread

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Abstract:
In the infectious variant of Creutzfeldt-Jacobs disease (vCJD), prions (PrP^{Sc}) enter the body by oral exposure with contaminated foodstuffs. Prions may then spread from the intestinal entry site to the central nervous system (CNS) by intercellular transfer from the lymphoid system to the peripheral nervous system. However, the mechanism of cell-to-cell spread remains elusive. Tunneling nanotubes (TNTs) have recently been identified as a novel means of cell-cell communication. Here we show that TNTs transfer cellular PrP and PrP^{Sc} between cells of the same and different origin. Significantly, we observed fluorescently-labelled PrP^{Sc} transferring *via* TNTs from dendritic cells to primary neurons. Thus, TNTs sustain intercellular prion transfer and may be involved in its spread from the periphery to the CNS by neuro-immune interactions.

Transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative diseases of either genetic or acquired origin. The suspected cause of these diseases is a malconformed and infectious form of the naturally expressed PrP^{C}-protein, termed PrP^{Sc} or prion (*1*). In the case of one of the TSEs, variant Creutzfeldt-Jacobs disease (vCJD), prions enter organisms by oral exposure with contaminated foodstuffs. The mechanism by which exogenously acquired PrP^{Sc} invades the CNS from peripheral sites of infection remains enigmatic. It is hypothesized that PrP^{Sc} passes from the gut through the lymphoreticular system (LRS) and lymphoid organs to the peripheral nervous system (PNS) and finally to the central nervous system (CNS) (*2-4*). It is generally believed that the initial phase of prion replication occurs in the lymphoid organs and that transfer from LRS to PNS occurs by cellular interactions therein (*5, 6*). Several mechanisms have been proposed for these intercellular transfer events, including hitch-hiking on membrane-coated viruses, transfer *via* exosomes or by GPI-painting (*7-9*) and may involve diverse cell types such as dendritic cells, follicular dendritic cells or macrophages (*10-12*).

Recently, a novel mode of cell-cell communication, consisting of thin membrane tubes interconnecting cells, termed tunneling nanotubes (TNTs) or cytonemes, has been described both *in vitro and in vivo*. (13-17). Proteins attached to the plasma membrane by lipid-based moieties (such as farnesyl groups or GPI-anchors) can be transferred from cell to cell *via* TNTs (*14, 16*). This is particularly interesting regarding the mechanism of intercellular spread of prions, because both PrP^{C} and PrP^{Sc} contain a GPI-anchor (*18*). To determine whether cellular PrP (PrP^{C}) could be transferred between cells by TNTs, we transfected HEK 293 cells (previously shown to form TNTs (16)) with a cDNA encoding mouse PrP^{C} fused to GFP (GFP-PrPwt) (20) . Tubular connections containing GFP-PrPwt were observed between two different cell populations (Fig. 1A). We could exclude that this was due to incomplete cytokinesis of daughter cells because we found connections between GFP-PrPwt expressing cells and non-expressing cells (Fig. 1A). In order to characterize the cytoskeletal content of these connections we stained cells for F-actin and alpha-tubulin and measured their thickness and length (for measurements see (20)). We found heterogeneity with respect to thickness and cytoskeletal content. While thicker connections (diameter 500-700nm,), contained both F-actin and tubulin (Fig. 1A), thinner connections (diameter 300 ± 50nm,) contained F-actin but little or no tubulin (Fig. S1). Similar differences in thickness and length have also been observed in human macrophages (19). Connections between cells were also very diverse in length, varying between approximately 5 μm and more than 50 μm (not shown), and also

form networks of tubes (Fig. S2A) as previously reported (14, 16, 21). Furthermore, X-Z reconstructions revealed that these connections were not attached to the substratum**,** a characteristic of TNTs (Fig. 1B, Fig. S1, Movie S1) (16).

When TNTs were first described, it was hypothesised that cells interacting by TNTs could be subdivided into a donor and a recipient, (i.e., a cell providing the tube and a cell receiving the tube (16, 22). Coculturing differentially labelled cell populations, we observed tubes deriving exclusively from cells of either one or the other population, supporting this hypothesis (Fig. 1C, compare tubes connecting cells 1 and 2). However, TNTs, which harboured both colours, were also observed, suggesting that both cells can participate in their formation (Fig. 1C, compare tube connecting cell 1 and 3). To test this hypothesis, we examined the dynamics of TNT-formation by live cell microscopy. HEK 293 cells in suspension culture were stained with a membrane dye to visualize the plasma membranes and imaged over the course of 30 minutes. At the beginning of the recording a number of cells were generally found connected by TNTs (see Fig. 1D, cells 1 and 2 in panel 0s, and Movie S4). In addition, we observed the active build-up of TNTs, which could involve either one or both cells and connected them for several minutes (for a representative example see Fig. 1D, cells 1 and 3 in panels 112s to panel 896s). In the case shown here, both cells participated in establishing a connection and after approximately 5 minutes one full TNT had formed and lasted for 13 minutes (see Fig. 1D, panel 112s to 448s). Therefore both cells can participate in establishing a TNT, suggesting that the current model of one donor and one recipient might be incomplete (22). We also show that these TNTs were functional because they could transfer lysosomes (Movie S2 and S3), as previously reported (22).

Our next objective was to analyze whether these structures could transfer PrP^C. We therefore co-cultured one HEK 293 cell-population transfected with GFP-PrPwt (22) and labelled with LysoTracker with another population of cells labelled only with LysoTracker and imaged these by live microscopy (20). During the course of 30 minutes LysoTracker-positive vesicles were observed to exit from the GFP-PrPwt positive cell (Fig. 2A, cell 1) towards a recipient cell with a speed of 40-60 nm/sec (for speed measurements see (20)) (see Fig. 2A, thin arrows in all panels), in agreement with lysosomal vesicles transport kinetics previously described in TNTs (16). Additionally, movement of GFP-PrPwt labelled membrane from the donor cell towards the recipient cell was observed (Fig. 2A, cell 2), suggesting that PrP^C could spread intercellularly by plasma membrane-transfer along the surface of the tube (Movie S5).

In addition to membrane “surfing”, vesicular structures have been reported to transfer intercellularly inside TNTs, including lysosomes, synaptophysin-positive vesicles and

mitochondria (16, 19, 22). PrP^{C} and its pathogenic conformer, PrP^{Sc}, can be found in endosomal structures, such as lysosomes and late endosomes (23-25) as well as in the Golgi-apparatus (26, 27). Thus, we analyzed whether PrP^{C} could also be transferred intercellularly through TNTs in cytosolic vesicles. To this end we monitored HEK 293 cells, transfected with GFP-PrPwt and stained with LysoTracker (for example see Fig. 2B). We repeatedly observed transfer of GFP-PrPwt-positive vesicles *via* TNTs. As shown in figure 2B, a vesicle double-positive for GFP-PrPwt and LysoTracker (thin arrow in all panels, Fig. 2D and Fig. S3 for quantifications of fluorescence) followed by a single positive GFP-PrPwt vesicle (Fig. 2B, thick arrow in all panels and Fig. 2D) transferred from one cell to another *via* a TNT over a time of approximately eighteen minutes (movie S6). 3-D reconstruction of the images confirmed that the vesicles moved inside the tube (Fig. 2C and movie S7) with a mean speed of 40-60 nm/s (20). Altogether these data demonstrate that GFP-PrPwt can be transferred *via* TNTs in cytosolic vesicles of both lysosomal and non-lysosomal origin (Fig. S2B). Measurements of the mean square displacement (MSD) (20) of vesicles suggested the involvement of molecular motors in vesicular transfer (Fig. 2E, details on MSD calculation are described in Fig. S4). Indeed, approximately 50% of GFP-PrPwt containing vesicles colocalized in TNTs with the actin-based molecular motor myosin Va (Fig. S5), as previously found for vesicular transfer in TNTs (16, 22). In addition we found that approximately 45% of GFP-PrPwt positive vesicles colocalized with Myosin VI, another motor involved in organelle transport (28), while there was no colocalization of vesicles with Kif 3a, a microtubule-associated motor belonging to the kinesin superfamily (Fig. S5) (29).

GFP-PrPwt was also observed inside TNTs in tubulovesicular structures, which filled the length of the nanotube (diameter 600nm) from donor to recipient cell (Fig. 2F and movie S8). One example is shown as a three dimensional reconstruction of Fig. 2F, in which we applied a colour threshold to distinguish differences in GFP-PrPwt content. Here, GFP-PrPwt appears to be inside the TNT in tubulo-vesicular structures reminiscent of the TGN (Trans Golgi Network) (movie S8), which had been previously suggested to transfer by TNTs (30). Nascent PrP^{C} passes through the Golgi-apparatus before reaching the plasma membrane (31) and, as expected, in HEK 293 cells intracellular GFP-PrPwt extensively colocalizes with Giantin, a well-defined marker of the cis and medial Golgi-apparatus (Fig. S6). To analyze whether GFP-PrPwt could be transferred by TNTs when in the Golgi apparatus, HEK 293 cells expressing GFP-PrPwt were cocultured with untransfected cells for 24 hrs, fixed and labelled with antibodies directed against Giantin. TNTs connecting the two cell populations and containing both the Golgi-marker and GFP-PrPwt were observed (Fig. 2G). Thus, Golgi

derived vesicular and tubulovesicular structures containing GFP-PrPwt can be found in TNTs, suggesting that GFP-PrPwt may also be transferred intercellularly within these structures.

To examine whether TNTs represent a possible means for prions transfer in neuronal cells, we utilized CAD-cells, a mouse neuronal cell line of catecholaminergic origin. These cells have the advantage of being infectable with prions (Fig. S7) and have been recently shown to produce cellular extensions reminiscent of TNTs (reviewed in (32)).

Co-culture for 48-72 hours of GFP-PrPwt-transfected CAD-cells (CAD(GFP-PrPwt)) with another population of CAD-cells transfected with either cherry-rab 6 or labelled with LysoTracker produced typical TNT-connections containing GFP-PrPwt on their surface and inside vesicular structures of lysosomal and Golgi origin (Fig. S8 A-C). TNT-based transfer between CAD cells was also observed in live cells by labelling cells with GolgiTracker (Fig. S9 and Movie S9). Like for HEK 293 cells, these data indicate that exogenously expressed GFP-PrPwt can be transferred between cells of neuronal origin by "surfing" on the plasma membrane of TNTs and by vesicular transport inside of TNTs.

Next, we wanted to evaluate whether endogenous PrP can transfer *via* TNTs, and more importantly whether this represents a means of intercellular spread of PrP^{Sc} from infected to non-infected cells. In order to address these questions, we first analyzed the transfer of PrP between either non-infected CAD-cells or CAD-cells infected with the 139A-prion strain (scCAD) (33) in fixed samples (Fig. 3 A, B and Fig. S10). In both cases we found PrP^{C} and PrP^{Sc} within vesicular structures inside TNTs, indicating transfer ocurring between cells. Some of the vesicles inside TNTs were also positively stained for lyso-bisphosphatidic acid (LBPA) (Fig. 3 A-D and Fig. S10), a marker of late endosomes (34).

The experiment described above with infected CAD cells had to be performed in fixed conditions because of lack of antibodies specifically recognizing PrP^{Sc} in live cells. To follow PrP^{Sc} transfer in live conditions we produced fluorescently-labelled PrP^{Sc} (Alexa-PrP^{Sc}) by adapting (20) a previously published protocol (35) (Fig. S11). ScCAD-cells were loaded with Alexa-PrP^{Sc} (Fig. 3E, cell 1), washed extensively, co-cultured for 24 hrs with CAD cells previously transfected with GFP-GPI (Fig. 3E, cell 2) and imaged by live microscopy. We could detect TNTs (Fig. S12) containing Alexa-PrP^{Sc} between cells of the two populations (Fig. 3E, arrowheads) and found Alexa-labelled particles in the lumen of a non-loaded cell (Fig. 3E arrows and inset E1). Importantly we could not detect any fluorescence, when adding the supernatant from the Alexa-PrP^{Sc}-loaded cells to another unlabelled cell population (data not shown). This excludes transfer of fluorescently labelled PrP^{Sc} *via* the supernatant and

shows that PrP^{Sc} transferred from a loaded cell to recipient cells *via* TNTs, suggesting that this could be a means to spread the infection between neurons.

We next addressed whether TNTs could support the transfer of PrP^{Sc} between cells of different origin. TNTs may also be produced *in vivo*, as shown for the closely related structures termed "cytonemes" in *ex vivo* preparations of the wing imaginal discs of *Drosophila*, (13, 15), and may therefore represent an efficient mechanism for the transfer of PrP^{Sc}, sampled by cells at the peripheral entry-site, to neurons *in vivo*. Dendritic cells (DCs) have been shown to sample and transport PrP^{Sc} from the gut to peripheral lymphatic organs and also to be sufficient for *de novo* infection when adoptively transferred from infected animals to healthy animals (10, 36). It has also been reported that DCs are particularly apt at producing TNTs (21). Lymphatic organs like the spleen are innervated by the sympathetic nervous system (SNS), mainly ensheathing local blood vessels, called central arterioles; in infected animals the SNS has been shown to contain PrP^{Sc} in abundance (37, 38). Intriguingly, DCs were repeatedly found in close proximity to peripheral nerve endings and so it has been hypothesized that DCs could be involved in transferring prions to the peripheral nervous system (10, 39, 40).

By performing immunohistofluorescence-experiments on splenic sections we found DCs closely juxtaposed to neurites (Fig. 4A, arrows) of the SNS emanating from the central arteriole (Fig. 4A asterisk). Since current imaging systems do not have sufficient resolution to analyze TNTs in spleen tissue, we simulated the interaction between DCs and neurons by examining the interaction between bone-marrow-derived dendritic cells (BMDCs) and murine hippocampal neurons (20) *in vitro* (Fig. S13). After 24 hours of coculture, typical TNTs not attached to the substratum (Movie S10) were observed to connect neurons labelled in green (transduced with GFP-PrPwt (for details see (20))) with BMDCs (stained in red with LysoTracker) (Fig. 4B, arrowhead). Furthermore, we found LysoTracker-stained vesicles inside neurites connected to BMDCs by TNTs, suggesting transfer of LysoTracker-positive vesicles from BMDCs to neurons (Fig. 4B, arrows). In order to follow PrP^{Sc}-transfer, BMDCs were loaded with Alexa-PrP^{Sc} and cocultured with GFP-PrPwt-transduced hippocampal neurons. Also in these conditions we found TNTs connecting the two different cell types (Fig. 4C, arrowheads). Importantly we observed Alexa-PrP^{Sc} in the TNT itself, in the neuronal cell bodies and in the neurites close to the intercellular connection (Fig. 4C, arrows, Fig. S14). These data demonstrate that BMDCs interact with neurons *via* TNTs and suggests that transfer of prions between these two cell types occurs *via* these structures.

We demonstrate here that the cell-to-cell communication mechanism of TNTs is exploited by prions for intercellular transfer *in vitro* and may serve as a means of cross-infection *in vivo*. This is substantiated by several findings: i) exogenously expressed GFP-PrPwt can be actively transferred *via* TNTs in immortalized cells of epithelial and neuronal origin; ii) endogenous PrP^{C} and PrP^{Sc} can be transferred *via* TNTs between infected and non-infected cells of neuronal origin and iii) fluorescently labelled PrP^{Sc} can be transferred *via* TNTs from primary BMDCs to non-infected primary neurons. We therefore propose that TNTs mediate intercellular transfer of PrP^{Sc} *in vivo*. Similar structures, termed cytonemes, have been described in Drosophila and may represent an evolutionarily conserved mechanism of cell-cell communication (13, 15). Since DCs may interact with peripheral neurons in lymphoid organs, intercellular transfer would allow neurons to retrogradely transport prions to the CNS (41). Interestingly, it was recently shown that in blood from orally infected sheep, CD14+ cells (which include a subpopulation of DCs) contain the major part of infectivity (42), thus adding another layer of complexity for the involvement of DCs in spreading of prions from the blood flow. The finding that pathogens as diverse as viruses (*17*), bacteria (*19*) and prions can exploit TNTs for invading eukaryotic cells, implies an ancient evolutionary origin for these structures.

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Figure legends:

Figure 1:

HEK cells produce thick TNTs containing GFP-PrPwt.

A) Actin and tubulin content. HEK 293 cells were transiently transfected with a GFP-PrPwt construct and cocultured for 48-72 hrs with unlabelled cells; samples were fixed and labelled with phalloidin for staining actin filaments and an antibody against α-tubulin and imaged by confocal microscopy (Zeiss). Z-reconstruction of several planes is shown. Except where differently indicated, all scale bars represent 10 μm.

B) Inset representing an X-Z reconstruction of A), showing that the tube is not attached to the substratum (see arrow and also also Movie S1).

C) Both cells can contribute to TNT-formation. HEK 293 cells were either transiently transfected with a GFP-PrPwt-construct or stained with LysoTracker. After 24 hrs of coculture, cells were fixed and processed for imaging (Zeiss). Note that some tubes contain both GFP-PrPwt and LysoTracker.

D) Live analysis of TNT-formation. HEK 293 cells were mildly trypsinized, in order to detach them from the culture dish and labelled with TAMRA, a lipid dye, to visualize the plasma membrane. After extensive washing, cells were processed for live imaging on a Perkin-Elmer spinning disc confocal. Selected frames of a video sequence are shown, demonstrating that TNT-formation is an active process in which two cells can participate (build-up 5 minutes, duration of connection approximately 13 minutes) (see also Movie S4, 2 frames/s over 30 minutes). Shown is a Z-reconstruction of several planes.

Figure 2:

GFP-PrPwt transfers *via* TNTs between HEK 293 cells.

A) Transfer by membrane surfing. Two populations of HEK 293 cells were either double-labelled by staining with LysoTracker and transient transfection with a GFP-PrPwt-construct or stained with LysoTracker only. After 24 hrs of coculture, cells were processed for live imaging on a Perkin-Elmer spinning disc confocal. Selected frames of a video sequence are shown (see also corresponding Movie S5, 2 frames/s over 30 minutes). Thin arrows indicate LysoTracker-positive vesicles, while the thick arrow marks the front edge of the GFP-PrPwt-containing membrane. Shown is a Z-reconstruction of several planes.

B) Transfer of vesicles of both lysosomal and non-lysosomal origin. HEK 293 cells were transiently transfected with GFP-PrPwt, cultured for 24 hrs and then incubated with LysoTracker. After extensive washing, cells were processed for live imaging on a Perkin-Elmer spinning disc confocal. Selected frames of a video sequence are shown (see also corresponding Movie S6, 2 frames/s over 30 minutes). The thin arrow marks a GFP-PrPwt- and LysoTracker-positive vesicle; the thick arrow marks a GFP-PrPwt positive vesicle, which both, in a course of 18 minutes transferred from one cell to another with a speed of 40-60 nm/s. Z-reconstruction of several planes is shown.

C) 3D reconstruction of Fig. 2B and Movie S6. The background is displayedin grey in order to better depict the TNT and the contained vesicles. Selected frames of the second part of Movie S7 are shown (the first part of Movie S7 is a 360° spin with the tube as the longitudinal axis).

D) Analysis of fluorescence contained in the vesicles shown in Fig. 2B and 2C. Concurrent increase of signals at position of vesicle 1 (thick arrow) shows colocalization of GFP-PrPwt and LysoTracker therein, while the second vesicle (thin arrow) contains GFP-PrPwt only, suggesting a non-lysosomal origin (see also Fig. S3 and (*20*)).

E) Analysis of vesicle-movement by mean square displacement (MSD) suggests involvement of molecular motors in vesicle transfer. The data obtained from Movie S6 were analyzed by MSD plot. Slope of MSD-graphs describes the nature of the motion (slope inferior to 1 corresponds to diffuse undirected movement of objects while superior to 1 indicates actively transported objects). At beginning the green vesicle presents a slope inferior to one (closed

triangles) but changes later to slope higher than one. The red vesicle showed a slope higher than one. Symbols utilized: closed triangles (green vesicle in part 1 of movie (0'-10')), open triangles (green vesicle in part 2 of movie (10'-20')), circles (green vesicle in part 3 of movie (20'-30')), rectangles (double-positive vesicle in part 1 of movie (0'-10')) (see Fig. S4 and (*20*) for further detail).

F) TNT contain tubulovesicular structures. HEK 293 cells, transfected with GFP-PrPwt, can form TNTs that are not attached to the substrate (see X-Z, upper part) and transfer GFP-PrPwt containing material in long tubulovesicular structures reminiscient of TGN (see movie M8). Images were acquired with spinning disc confocal (Andor).

G) TNTs contain Golgi-structures. HEK 293 cells were transfected with GFP-PrPwt, cocultured with an unlabelled population, fixed after 24 hrs and immunostained for Giantin (a marker for cis- and medial-Golgi) and imaged by confocal microscopy on a Zeiss LSM 510. Shown is a TNT which contains GFP-PrPwt as well as the Giantin, suggesting transfer of Golgi-content by TNTs. A Z-reconstruction of several planes is shown.

Figure 3: Endogenous PrP^{C} and PrP^{Sc} transfer by CAD-cells and scCAD-cells *via* TNTs.
A) Transfer of endogenous PrP^{C} in late endosomes. PrP^{C} and late endosomes were detected by immunofluorescence with anti-prion antibodies and an antibody against a marker of late endosomes (LBPA) in CAD-cells. Cells connected by a TNT transferring PrP^{C} in late endosomes (arrows) are shown. Images were acquired using epifluorescent microscope (Marianis).

B) Transfer of endogenous PrP^{Sc} in late endosomes. Cultured scCAD-cells were fixed and treated with GndHCl to reveal PrP^{Sc}-epitopes and then immunostained anti-prion and anti-LBPA antibodies. Cells connected by a TNT transferring PrP^{Sc} in late endosomes (arrows) are shown.

C) Fluorescence values (ordinate/arbitrary units) of PrP^{C} (green rectangles) and LBPA (red triangles) from Fig. 3A were measured along the tube in a single confocal plane using ImageJ and plotted against the distance of the tube (abscissa/μm). Concurrent increases of the signals

at the same points along the tube (arrows depict vesicle 1, 2 and 3 from Fig. 3A) shows localization of PrP^{C} within late endosomal vesicles (see also Fig. S10).

D) Fluorescence values (ordinate/arbitrary units) of PrP^{Sc} (green rectangles) and LBPA (red triangles) from Fig. 3B were measured along the tube in a single confocal plane and plotted against the distance of the tube (abscissa/μm). Concurrent increase of the signals at the same points along the tube (arrows depict vesicle 1 and 2 from Fig. 3B) shows localization of PrP^{Sc} within late endosomal vesicles (see also Fig. S10).

E) Alexa-labelled PrP^{Sc} is transferred from scCAD-cells to CAD-cells. CAD-cells were infected with Alexa-PrP^{Sc} and kept in culture for 6 days for efficient uptake and breakdown of absorbed Alexa-PrP^{Sc} aggregates. Cells in suspension were washed several times to ensure loss of possible extracellular Alexa-PrP^{Sc} and then cocultured for 24 hrs with CAD-cells, previously transfected with GFP-GPI,. A TNT, connecting cells of the two populations with Alexa-PrP^{Sc}-particles contained within the tube (arrowheads) and the recipient cell (arrows and inset E1). Inset E1 depicts a slightly magnified zone of cell 2 with enhanced intensity in red channel for better visualization of Alexa-PrP^{Sc}-particles. Note that in order to visualize all Alexa-PrP^{Sc} molecules, high laser power was utilized, therefore highly fluorescent Alexa-PrP^{Sc}-particles are also weakly detected in the green channel. Cells were imaged by spinning disc confocal microscopy (Andor Revolutions).

Figure 4: Purified and fluorescently labelled PrP^{Sc} transfers *via* TNTs from bone-marrow derived dendritic cells to primary neurons.

A) Immunohistofluorescence of murine spleen. Antibodies recognizing specific markers of DCs ($CD11^{c}$, red) and sympathetic neurons (Tyrosine-Hydroxylase, green) were utilized on cryo-cuts of spleen tissue from C57/BL6 mice. Arrows mark DCs in close interaction with neurites extending away from central arteriole (asterisk). Images were obtained by confocal microscopy on a Zeiss LSM 510. The scale bar represents 5 μm.

B) LysoTracker-positive vesicles transfer from DCs to primary hippocampal neurons. Bone-marrow derived dendritic cells (BMDCs) were stained with LysoTracker. After extensive washing, BMDCs were cocultured for 24 hours with primary hippocampal neurons, previously transduced with a GFP-PrPwt-encoding viral vector and imaged by confocal

microscopy. A BMDC interacting with a neurite *via* a TNT (arrowhead) is shown; the presence of several LysoTracker-positive vesicles inside the neurite (arrows), suggests intercellular transfer. Images were obtained by confocal microscopy on a Zeiss LSM 510.

C) PrP^{Sc} transfers from DCs to primary hippocampal neurons. Bone-marrow derived dendritic cells (BMDCs) were infected with Alexa-PrP^{Sc} for 4 days. After extensive washing BMDCs were cocultured for 24 hours with hippocampal neurons, previously transduced with a GFP-PrPwt-encoding viral vector, and imaged by confocal microscopy. A BMDC charged with Alexa-PrP^{Sc} is shown interacting with a neurite *via* TNTs (arrowheads). Several Alexa-PrP^{Sc}-particles are observed: some at the base of the TNTs and inside of the neurite and several in the cell body of the neuron (arrows and Fig. S14). Images were obtained by confocal microscopy on a Zeiss LSM 510.

Supplementary Figures:

S1) HEK 293 cells produce thin TNTs, which contain actin but no tubulin.

As shown by others for human macrophages (*1*), TNTs are structurally distinct and distinguishable by their diameter and cytoskeletal content. Önfelt et al. (*1*) reported that a TNT-diameter less than 700 nm contained only actin filaments as cytoskeletal elements but no microtubulin, in contrast to the TNTs with a diameter superior to 700 nm. When HEK 293 cells were transiently transfected with a GFP-PrPwt construct, cocultured for 48-72 hrs with unlabelled cells, fixed and labelled with Alexa-546-Phalloidin for actin filaments and an antibody against α-tubulin and imaged by confocal microscopy, tubes with a diameter inferior to 500 nm (here 300 ± 30nm) were found with little to no microtubule content (*2*). Inset represents an X-Z reconstruction, showing that the tubes are not attached to substratum. Shown is a Z-projection of several confocal planes. Except where differently indicated, all scale bars represent 10 μm.

S2) HEK 293 cells produce TNT-networks containing both actin and tubulin.

A) Shown is a network of thick TNTs connecting several cells of different populations. HEK 293 cells were transiently transfected with GFP-PrPwt and cocultured with an unlabelled population for 24 hrs. After fixation, cells were immunostained with Alexa-546-phalloidin (for actin-cytoskeleton) and anti-tubulin antibodies and imaged by confocal microscopy.

B) Cells were treated as in A). Note the arrow which marks a GFP-PrPwt-vesicle inside of a thick, microtubule-positive TNT, suggesting its transfer therein.

S3) Quantification of fluorescence of GFP-PrPwt and LysoTracker in vesicles inside of TNTs.

Fluorescence values (ordinate/arbitrary units) of GFP-PrPwt (green curve) and LysoTracker (red curve) from Fig. 2B were measured along the tube in a single confocal plane and plotted against the distance of the tube (abscissa/nm) (see lower images for numbering of vesicles (left) and measuring distance (right)). Concurrent increase of the signals at the position of vesicle 1 shows colocalization of GFP-PrPwt and LysoTracker therein, while the second vesicle contains GFP-PrPwt only, suggesting its non-lysosomal origin. Measurements of fluorescence values were obtained using ImageJ software.

S4) Mean square displacement (MSD) measurements suggests the involvement of molecular motors in vesicular transfer by TNTs. The definition of MSD is given by $MSD(\Delta t) := \langle |\mathbf{x}(t+\Delta t) - \mathbf{x}(t)|^2 \rangle$. Here, $|\mathbf{x}(t+\Delta t) - \mathbf{x}(t)|$ is the distance travelled by the object over a time interval of duration Δt, and the squared magnitude is averaged (as indicated by the angle brackets) over many such time intervals. The MSD is usually fitted by a power law, i.e., $f(\Delta t) := C\ \Delta t^a$, where the exponent *a* typically characterizes the movement. If an object is undergoing a free Brownian motion, its MSD will exhibit a linear relation, (i.e., a = 1 with C proportional to the diffusion constant). A super-linear relation (a > 1) indicates actively transported objects showing directional movements; a sub-linear growth (a < 1) indicates confined diffusive movements. In order to calculate the parameters (C, a) we computed a fitted MSD-data set (shown in B) from the raw MSD-data set (shown in A).

A. the MSDs computed from the trajectories of the vesicles;
B. the fitted MSDs with a power law $f(\Delta t) := C\ \Delta t^a$.

We found that the green vesicle in the first recorded movie (out of three) exhibited a sub-linear MSD (a = 0.50), and super-linear relations for the remainder (a = 1.34 for the second part and a = 1.49 for the third part), suggesting confined diffusive movement at the beginning and switch to motor-based directed movement afterwards; the double-positive vesicle displayed a super-linear MSD with the highest slope among all the vesicles (a = 1.50).

Altogether this suggests the involvement of molecular motors for transferring vesicles by TNTs.

S5) Myosin Va and Myosin VI but not Kif3a colocalize with GFP-PrPwt containing vesicles inside of TNTs. HEK 293 cells were transiently transfected with GFP-PrPwt and cocultured with an unlabelled population for 24 hrs. After fixation, cells were immunostained with antibodes directed against either Myosin Va, Myosin VI or Kif3a. TNTs connecting GFP-PrPwt-expressing cells with unlabelled cells (thereby excluding incomplete cytokinesis) were chosen (n>10) and GFP-PrPwt positive vesicles colocalizing with the respective molecular motors MyoVa, MyoVI or Kif3a were quantified (total number of vesicles>20). Quantitative analysis shows that GFP-PrPwt-positive vesicles colocalized with MyosinVa in approximately 55% of cases and in approximately 50% of cases with Myosin VI. In contrast to this GFP-PrPwt-positive vesicles colocalized in only ca. 5% of cases with Kif3a. These data suggest the involvement of actin-bound MyoVa and MyoVI and no involvement of microtubuli-bound Kif3a as molecular motors for the transport of GFP-PrPwt-positive vesicles by TNTs.

S6) GFP-PrPwt is localized in the Golgi-apparatus in HEK 293 cells

HEK 293 cells were transiently transfected with GFP-PrPwt. After fixation, cells were immunostained with Giantin, a cis- and medial Golgi-marker, and imaged by confocal microscopy (Zeiss). Note the high percentage of colocalization between GFP-PrPwt and Giantin intracellularly.

S7) scCAD, murine catecholaminergic cells, are chronically infected with PrP^{Sc} which is resistant to Proteinase K-digestion. CAD cells and scCAD cells (infected with prion strain 139A) were lysed and incubated with Proteinase K (PK) where indicated. Samples were run on SDS/PAGE-Gel and analyzed by Western Blot utilizing anti-PrP-antibodies for detection of PrP^{C}/PrP^{Sc}-proteins. Note the complete digestion of PrP^{C} from CAD cell lysates treated with proteinase K-enzyme (PK) and the appearance of PK-resistant products at 25, 20 and 15 kDa in the case of scCAD cells.

S8) CAD cells produce TNTs and are able to transfer GFP-PrPwt intercellularly on the plasma membrane as well as in vesicles.

A) (upper) CAD-cells transfer GFP-PrPwt by the plasma membrane in long TNTs. CAD-cells were transfected with either GFP-PrPwt or cherry-rab 6 (a Golgi marker), cocultured for 24-48 hrs and imaged by confocal microscopy. A particularly long TNT (length 48 μm, diameter 1 μm) is shown, connecting cells from the two populations. Note that plasma membrane-bound GFP-PrPwt is apparently transferred to the recipient cell along the TNT as has been shown for GPI-anchored GFP by others (3, *4*). Images acquired by spinning disc confocal microscopy (Andor).

(lower) TNTs in CAD-cells are not attached to the substratum and transfer plasma membrane-bound GFP-PrPwt to other cells. An X-Z reconstruction of cells from Fig. S8 A is shown. The TNT in green is not in contact with the substratum, a characteristic feature of TNTs (*3*).

B) TNTs in CAD-cells enable transfer of LysoTracker-positive vesicles and GFP-PrPwt-containing vesicles. CAD-cells transfected with GFP-PrPwt and additionally stained with LysoTracker connected by TNTs can transfer LysoTracker-positive vesicles and GFP-PrPwt-containing (arrow) vesicles therein.

C) Transfer of Golgi-positive material. CAD-cells were transfected with GFP-PrPwt, cocultured with an unlabelled population of cells, fixed after 24 hrs and immuno-stained for Giantin and imaged by confocal microscopy (Zeiss). Shown is a Z-reconstruction of several planes in which cells are connected by a GFP-PrPwt-positive TNT that also contains numerous giantin-positive vesicles (arrow).

S9) Transfer of Golgi-particles by TNTs shown in living cells by staining with GolgiTracker.

A) Golgi-Tracker-positive material also containing GFP-PrPwt transfers intercellularly. CAD-cells were transiently transfected with GFP-PrPwt, cocultured with an unlabelled population for 24 hrs and then incubated with GolgiTracker. After extensive washing, cells were processed for live imaging. The arrow marks a particle positive for GFP-PrPwt and GolgiTracker, transferring from cell 1 to cell 2. Selected frames of a video sequence are shown (see also Movie S9, 2 frames/s over 20 minutes).

B) Fluorescence values of vesicles from Fig. S11A. Fluorescence values (ordinate/arbitrary units) of GFP-PrPwt (green curve) and GolgiTracker (red curve) were measured along the tube in a single confocal plane and plotted against the distance of the tube (abscissa/μm). Concurrent increase of the signals at position of the white arrow (image lower left) shows colocalization of GFP-PrPwt and GolgiTracker. Measurements of fluorescence values were obtained using ImageJ software.

S10) Analysis of fluorescence values of vesicles from Fig. 3A and Fig. 3B.

A) Fluorescence values (ordinate/arbitrary units) of PrP^{C} (green rectangles) and LBPA (red triangles) from Fig. 3A were measured along the tube (ImageJ) in a single confocal plane and plotted against the distance of the tube (abscissa/μm). Concurrent increase of the signals at the same points along the tube (arrows depict vesicle 1, 2 and 3) shows colocalization of PrP^{C} and LBPA.

B) Fluorescence values (ordinate/arbitrary units) of PrP^{Sc} (green rectangles) and LBPA (red triangles) from Fig. 3B were measured along the tube (ImageJ) in a single confocal plane and plotted against the distance of the tube (abscissa/μm). Concurrent increase of the signals at same points along the tube (arrows depict vesicle 1 and 2) shows colocalization of PrP^{Sc} and LBPA.

S11) Production and analysis of fluorescently labeled PrP^{Sc}.

A) Scheme of PrP^{Sc} purification and its labelling with Alexa-568-succinimidyl esters. PrP^{Sc}-containing brain homogenate from terminally ill mice was PTA-precipitated in order to enrich the PrP^{Sc} content. Proteinase K (PK) digestion removed proteinaceous contaminants. The remaining protein, highly enriched in PrP^{Sc}, was labelled with Alexa-Fluor-568-succinimidyl esters according to manufacturers protocol (*2*).

B) Fischer Rat Thyroid cells, which do not express PrP^{C}, were infected with proteinase K-digested, fluorescently-labelled, PTA-precipitated material from PrP^{Sc}-infected mouse brain (red). Cells were fixed, permeabilized and incubated in PBS (top panels) or guanidium hydrochloride (lower panels). Subsequently, the cells were stained with anti-PrP antibody SAF32 (green) and visualized by fluorescent microscopy. Images were acquired using epifluorescent microscope (Marianis).

C) Fluorescently-labelled PTA precipitate was subjected to SDS-PAGE and analyzed by fluorescent scanning or western blotting with anti-PrP antibody SAF32 as indicated. Arrows indicate the three glycosylated isoforms of proteinase K-resistant PrP^{Sc}. Arrowhead indicates undissociated PrP^{Sc} higher molecular weight complex.

S12) Z-reconstruction of Fig. 3E showing that the TNT is not attached to substrate. The background behind the tube has been artificially coloured in grey in order to better depict the TNT and the contained structures. Volocity and Osirix software were utilized.

S13) Characterization of bone-marrow derived cells.

Cells derived from bone-marrow (BM) of C57/Bl6 mice were characterized for dendritic cell-markers by fluorescence activated cell sorting (FACS). The white histograms represent the fluorescent intensities of the BM-derived cells labelled with antibodies against CD86 (left), MHC II (centre) and $CD11^{c}$ (right). The black histogram represents signal from isotype matched control antibodies.

S14) Intercellular transfer of Alexa-PrPSc between DCs and neurons *via* TNTs.

Bone marrow derived dendritic cells, charged with Alexa-PrP^{Sc} (red), are connected to neurons, transduced with GFP-PrPwt-virus (green), *via* TNTs (white arrowheads). Different confocal layers (Zeiss) (specified upper left) depict Alexa-PrP^{Sc}-molecules (yellow rectangles) inside of TNT connecting the two cells (left lane), inside of the neurite (centre) and at the distal end of another TNT (right lane). This suggests the intercellular transfer of Alexa-PrP^{Sc} from DCs to neurons *via* TNTs.

<u>Supplementary movies:</u>

M1) X-Z reconstruction of HEK 293 cells, connected by a thick TNT. Several Z-planes from Fig. 1A acquired by confocal microscopy were utilized. The movie shows a 360°-spin around the tube, demonstrating that the tube is not attached to the substratum. The movie was generated utilizing the LSM 510 software.

M2) Detached HEK 293 cells produce TNTs and can transfer LysoTracker-positive vesicles therein.

HEK 293 cells were stained with LysoTracker and then mildly trypsinized in order to detach them from the substratum. After extensive washing, the cells were allowed to recover for 30

minutes and then processed for imaging on a spinning disc confocal microscope (Perkin Elmer). The speed of the movie shows 6 frames/s. The recording time was 10 minutes. Note LysoTracker-positive vesicles entering the tube from the lower cell and exiting the TNT on the other side.

M3) X-Z reconstruction of the data from movie M2, showing a TNT between cells and transfer of LysoTracker-positive vesicles therein.
LysoTracker-positive vesicles are shown in white, while membrane is coloured in red (for better visualization).

M4) De novo TNT-formation can involve both cells.
HEK 293 cells were treated as described for Fig. 1D. The movie shows that TNT-formation is an active process in which both cells can participate, demonstrating that the donor-receptor subdivision as suggested by others (*3*) may not always apply. Live imaging was done with a spinning disc confocal microscope (Perkin Elmer). Shown are 2 frames/s over 30 minutes.

M5) LysoTracker-positive vesicles and plasma membrane-bound GFP-PrPwt can transfer intercellularly by TNTs. HEK 293 cells pretreated as described in Fig. 2A. Note that LysoTracker-positive vesicles from the GFP-PrPwt expressing cell enter the tube readily and transfer to the recipient cell with a measured speed of 40-60nm/s (see Materials and Methods). Note also the movement of the plasma membrane containing GFP-PrPwt along the TNT. This suggests transfer of plasma membrane and GFP-PrPwt therein by TNTs. Time of recording is 30 minutes shown with 2 frames/s.

M6) Vesicles double-positive for LysoTracker and GFP-PrPwt or positive for GFP-PrPwt alone transfer by TNTs intercellularly in HEK 293 cells. Cells were treated as described in Fig. 2B. Pictures were acquired by spinning disc confocal microscopy (Andor Revolution). Measured speed of vesicles were 40-60 nm/s. Time of recording is 3x10 minutes shown with 2 frames/s.

M7) Reconstruction of movie M6. Computer-aided simulation of an excerpt from movie M6. Shown first is a 360° spin utilizing the tube as the longitudinal spin-axis; thereafter the view changes to the original angle and motion of vesicles is followed in time. The

background has been coloured grey for better visualization. Computer-aided reconstructions were accomplished with the ImageJ, Volocity and Osirix software programmes.

M8) Transfer of tubulovesicular structure by a TNT. Computer-aided highly detailed 3D-reconstruction of images from Fig. 2F. A colour code for GFP-PrPwt content (blue for high content, grey for low content) was applied, showing a clearly tubulovesicular structure with high GFP-PrPwt-content transferring through the tube intercellularly. Computer-aided processing of data was done with ImageJ, Volocity and Osirix software programmes.

M9) Intercellular transfer of a GFP-PrPwt and GolgiTracker-positive structure in CAD cells.

Cells were treated as described in Materials and Methods. Pictures were acquired by spinning disc confocal microscopy (Andor Revolution). Measured speed of particle was ca. 10 nm/s. Time of recording was 20 minutes shown at 2 frames/s.

M10) Connections between primary hippocampal neuron and bone marrow derived dendritic cell are not attached to the substratum.

Coculture of neurons with dendritic cells, labelled with TAMRA, a lipid intercalating dye, as imaged by confocal microscopy. Shown is a whole Z-scan, starting at the bottom of the substratum and ending with the top of the DC. Note that the fluorescence inside of the TNT moves with each confocal layer (thickness 300nm). This demonstrates that the connection is not attached to the substratum. The movie was done with LSM 510 software and Image J. The scale bar represents 5 μm.

Materials and Methods:

Immortalized cell lines, transfections and transductions:

HEK 293 cells were cultured in DMEM (Sigma, St. Louis, USA) supplemented with 10% fetal bovine serum in a humidified atmosphere with 5% CO_2. CAD-cells (CAD wild-type and CAD infected with prion strain 139A (scCAD)) were cultured in DMEM/F12 (Invitrogen) supplemented with pyruvate (100 mM), HEPES (15 mM) (Invitrogen) and 10% fetal bovine serum. Transient transfections were performed with Lipofectamine (Invitrogen) according to the manufacter's instructions. pEGFP-C1 (Clontech), was engineered to express mouse PrP^C

between the restriction sites Nhe I and EcoR I, while the signal peptide of mouse PrP^C was inserted 5' of EGFP between the restriction sites NheI and PinaA I (kind gift from M. Sorgato, University of Padua, Italy). Cherry-rab 6, expressing rab 6 linked to the fluorescent protein "cherry", was a kind gift from the laboratory of B. Goud (Institut Curie, Paris, France). Murine wild-type PrP, was inserted in the vector pRRLsin.PPT.hPGK.GFPpre (kind gift from J.-M. Heard, Institut Pasteur, Paris) utilizing the restriction sites Sal I and BamH I, at a position 3' of GFP. The lentiviral vector was produced and collected as described elsewhere (*5*).

Primary cell lines:

Bone-marrow derived dendritic cells:

DCs were differentiated from bone marrow cells from 6- to 8-week-old C57 BL6 mice according to a method adapted from Méderlé et al. (*5*). Briefly, bone marrow cells were seeded at $2x10^6$ cells per 100 mm diameter Petri dish (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) in 10 ml of Iscove's modified Dulbecco's medium (IMDM; BioWhittaker Europe, Verviers, Belgium) supplemented with 10% heat-inactivated foetal calf serum (FCS; Dutscher, Brumath, France), 1.5% supernatant from a J558 cell line producing murine GM-CSF (*6*), 50 U/ml penicillin, 50 μg/ml streptomycin, 50 μM 2-mercaptoethanol and 2 mM glutamine (complete IMDM). Cultures were incubated at 37°C in a humidified atmosphere with 7% CO_2. On day 3, 10 ml of complete IMDM was added. On day 6, suspended cells and loosely adherent cells were harvested using prewarmed 1% EDTA in Dulbecco's PBS without Ca2+ and Mg2+ (Biochrom AG, Berlin, Germany). The recovered cells were further cultured under the same conditions as above. On day 10, cells were harvested with EDTA as above and distributed in hydrophobic 6-well plates (Evergreen Scientific, Los Angeles, CA) at a concentration of $9x10^5$ cells/well in 3 ml complete IMDM.

Embryonic hippocampal neurons were produced from C57/BL6 mice as published elsewhere (*7*) and were grown on glass bottom culture dishes (Mattek, USA) coated with poly-L-lysine (Sigma) according to the manufacturer's instructions for 2 days. Then, neurons were transduced with virus encoding GFP-PrPwt and cultured for another 3 days. Subsequently, cells were utilized for coculture experiments.

Antibodies

Anti-PrP-antibodies, recognizing the C-terminal part of PrP-proteins, termed SAF61 (amino acids 142-160, human numbering) were a kind gift from J. Grassi (CEA, Saclay, France).

Anti-Myosin Va antibody (LF 18) was obtained from Sigma, the anti-Myosin VI antibody was purchased from Sigma-Aldrich. The antibody against α-tubulin was obtained from Chemicon (USA), phalloidin-Alexa 546 was purchased from Molecular Probes (Eugene, USA). Antibodies against Giantin was purchased from Babco (Berkeley Antibody Company, USA). Anti-LBPA antibody was a kind gift from J. Gruenberg (University of Geneva, Geneva, Switzerland). The antibody against Tyrosine-Hydroxylase was obtained from Chemicon; the anti-CD11c-antibody was purchased from BD Biosciences/Pharmingen; biotin-conjugated anti-hamster IgG was purchased from Jackson ImmunoResearch/Dianova and streptavidin-FITC was purchased from BD Biosciences/Pharmingen. All other secondary antibodies were purchased from Molecular Probes. The Kif 3a antibody was a kind gift from C. Petit (Institut Pasteur, Paris, France).

Staining of living cells with organelle-specific dyes

Lysosomal staining with LysoTracker:

Living cells were seeded out on Ibidi plastic bottom dishes (Ibidi, Germany) and incubated with LysoTracker (Molecular Probes) (1:10000) for 1h at 37°C. After extensive washing cells were utilized as specified in each experiment.

Staining of Golgi-apparatus with GolgiTracker:

Cells were cultured on Glass bottom culture dishes (Mattek, USA) coated with poly-L-lysine (Sigma) according to the manufacturers instructions. Cultured cells were washed carefully three times with HMEM (HEPES 13. 8 mM, NaCl 137 mM, KCl 5. 4 mM, Glucose 5.5 mM, Glutamine 2.0 mM, KH_2PO_4 0.4 mM, Na_2HPO_4 0.18 mM, $CaCl_2 \bullet 2H_20$ 1.25 mM, $MgSO_4 \bullet 7H_2O$). Bodipy TR Ceramide (Molecular Probes) was complexed to BSA according to the manufacturer's protocol. Living cells were incubated with Bodipy TR Ceramide/BSA solution (5 μM) for 1h at 37°C. Cells were carefully washed once more with HMEM and then incubated for 1 h at 37°C in HMEM/1%FBS. Cells were washed three times with HMEM. To remove residual surface staining, cells were incubated for 15 minutes at 10°C with 5% fatty acid free BSA (Sigma) dissolved in HMEM and then washed three times with HMEM. This step was repeated three times. Cells were subsequently processed for live imaging.

Staining of fixed cells with antibodies

Cells cultured on plastic bottom dishes (Ibidi, Germany) were carefully washed and fixed with 2% paraformaldehyde (PFA) for 30 minutes at room temperature. Then, cells were incubated in NH_4Cl 50 mM for 10 minutes at room temperature. Cells were permeabilized with 0.1% Triton X-100 for 2 minutes at room temperature.

In experiments with scCAD-cells, cells were pretreated with guanidium hydrochloride as published by others (*8*), in order to reveal endogenously expressed PrP^{Sc}. Cells were then blocked with 2% BSA in PBS for 30 minutes at room temperature. In the case of immunofluorescence with anti-LBPA antibodies, cells were blocked with 0.2 % BSA. Cells were incubated with primary antibody for 30 minutes, carefully washed three times and incubated with secondary antibody and washed again three times before imaging.

Proteinase K-digestion

CAD-cells and scCAD were lysed in lysis buffer (Tris HCl 10 mM, pH 8, NaCl 100 mM, Triton X-100 0.5%, deoxycholate 0.5%). 500 μg of total protein was digested with 20µg/ml of Proteinase K (Eurobio) for 30 minutes at 37°C. The reaction was stopped with 2 mM PMSF for 10 minutes at room temperature. Lysates were centrifuged at 140000 rpm at 4°C for 1 hour. Pellets were solubilized in lysis buffer and processed for 12% SDS-PAGE and revealed by western blotting with the anti-PrP-antibody SAF61.

Imaging of cells

Cells were imaged by fluorescence videomicroscopy on Spinning Disc-Confocal Systems Perkin-Elmer (Boston, USA) and Andor Revolution (Belfast, Ireland) as specified. Fixed cells were imaged by confocal microscopy (Zeiss LSM 510, Germany), in the case of PrP^{Sc} with a Marianis bright-field microscope (TripleI, Germany). All imaging systems for living cells were equipped with a heated control chamber kept at 37°C (Pecon, Germany). For 3D analysis stacks were taken at 300nm per z-section and where necessary combined utilizing ImageJ. Advanced 3D and 4D-reconstructions were performed utilizing Volocity (Improvision) and Osirix (Osirix Medical Imaging Software) software programmes.

Labelling of PTA-precipitated PrPSc with Alexa-568 nm

Whole brains from Tg20 mice infected with the 139A prion strain (a generous gift from Hubert Laude) were weighed and 10% homogenates were made by dounce homogenization in

1X PBS and subsequent passage through a 22 gauge needle for 10 strokes. Two millilitres of this homogenate was mixed with 0.5 mL of a 4% sarkosyl (Fluka) solution in PBS and incubated for 10 minutes at 37°C with agitation. Next, benzonase (Sigma) was added to a final concentration of 50U/mL and the mixture incubated for 30 minutes at 37°C with agitation. Next, a prewarmed (37°C) solution of 4% phosphotungstic acid (PTA; Sigma) in PBS was added to a final concentration of 0.3% PTA and incubated further for 10 minutes at 37 °C with agitation. Subsequently, this mixture was centrifuged at 18 000 x g for 30 minutes and the supernatant removed. The pellet was resuspended in 0.8 mL of a solution of 0.1% sarkosyl in PBS and passed through a 261/2 gauge needle for 10 strokes. Next 0.6 mL of this suspension was diluted with 0.4 mL of PBS and treated with proteinase K (5μg/mL final; Eurobio) for 1 hour at 37°C with agitation. The reaction was quenched by the addition of PMSF (Fluka) to a final concentration of 2 mM and centrifuged at 18 000 xg at 4°C for 1 hour and resuspended in 0.2 mL PBS. This suspension was sonicated in a flat-bottomed, borosilicate glass vial in a cuphorn sonicator (Sonics, USA) and then brought up to 0.9 mL PBS. Next, 1mg of Alexa fluor 568 or 594 succinimidyl ester (Invitrogen) in 0.1 mL DMSO (Sigma) was added and incubated at room temperature (RT) for 30 minutes with agitation (under subdued light conditions from this point onward). The reaction was quenched with the addtion of glycine in PBS to a final concentration of 50mM and incubated for 30 minutes at RT followed by centrifugation at 18 000 x g for 15 minutes at RT. The supernatant was discarded and the pellet resuspended with 1mL of a solution of 10mM glycine in PBS and incubated for a further 30 minutes at RT, followed by centrifugation at 18 000 x g for 15 minutes at RT. Subsequently, the pellet was washed 3 times by steps of resuspension with 1mL PBS and centrifugation for 15 minutes at 18 000 x g. Finally, the pellet was resuspended with 0.2mL of PBS as the working stock.

Tracking of vesicles

We have developed a computer program for automated 4D tracking of fluorescent vesicles as described elsewhere (*9*). Based on the extracted locations of vesicles, we calculated the mean speed and MSD curves. For detailed description of MSD please refer to Figure Legend S5.

Length measurements of TNTs

The coordinates of the two end points of a tunnel were extracted in order to determine its length. This was done in a semi-automatic way described as follows:

1. Denoising: we applied a denoising algorithm (*10*), which is adapted to eliminate a mixture of photon and readout noises. At the end of this step, the noise is well separated from the image and the signal has better contrast.
2. End-points extraction: in the image treated as described above, the user manually selects the two YZ slices, which delimit the region of the entire tunnel. A binarizing threshold can easily segment the 2D intensity profile of the tunnel ends from these two slices, and the intensity-weighted centroids is calculated for the segmented tunnel ends. In this way, the full 3D coordinates of the end points are obtained from which the length is derived.

Diameter measurements of TNTs

The mean tunnel diameter is measured as the average of the diameters at 5 different positions along the tunnel. For each chosen spot, the corresponding YZ slice information is extracted. We assume that the tunnel itself has a Gaussian intensity profile with a standard deviation parameter sigma0 unknown. Our goal is to estimate this sigma0 and the tunnel diameter is then estimated as 2*sigma0. Toward this goal, we use a Gaussian PSF approximation model for the microscope (*11*). The parameters for the Gaussian PSF approximation can be computed using the microscope configurations utilized for the acquisitions. As the PSF and the tunnel profile are both approximated by Gaussian functions, the observed intensity also has a Gaussian profile. As a result, we fit a 2D Gaussian function to derive the underlying sigma0 from which the estimation of the diameter is computed. Since the image contains unwanted photon and readout noises, the fit is actually done in a variance-stabilizing transform domain. This transform is proposed in (*10*) to stabilize and gaussianize the noise.

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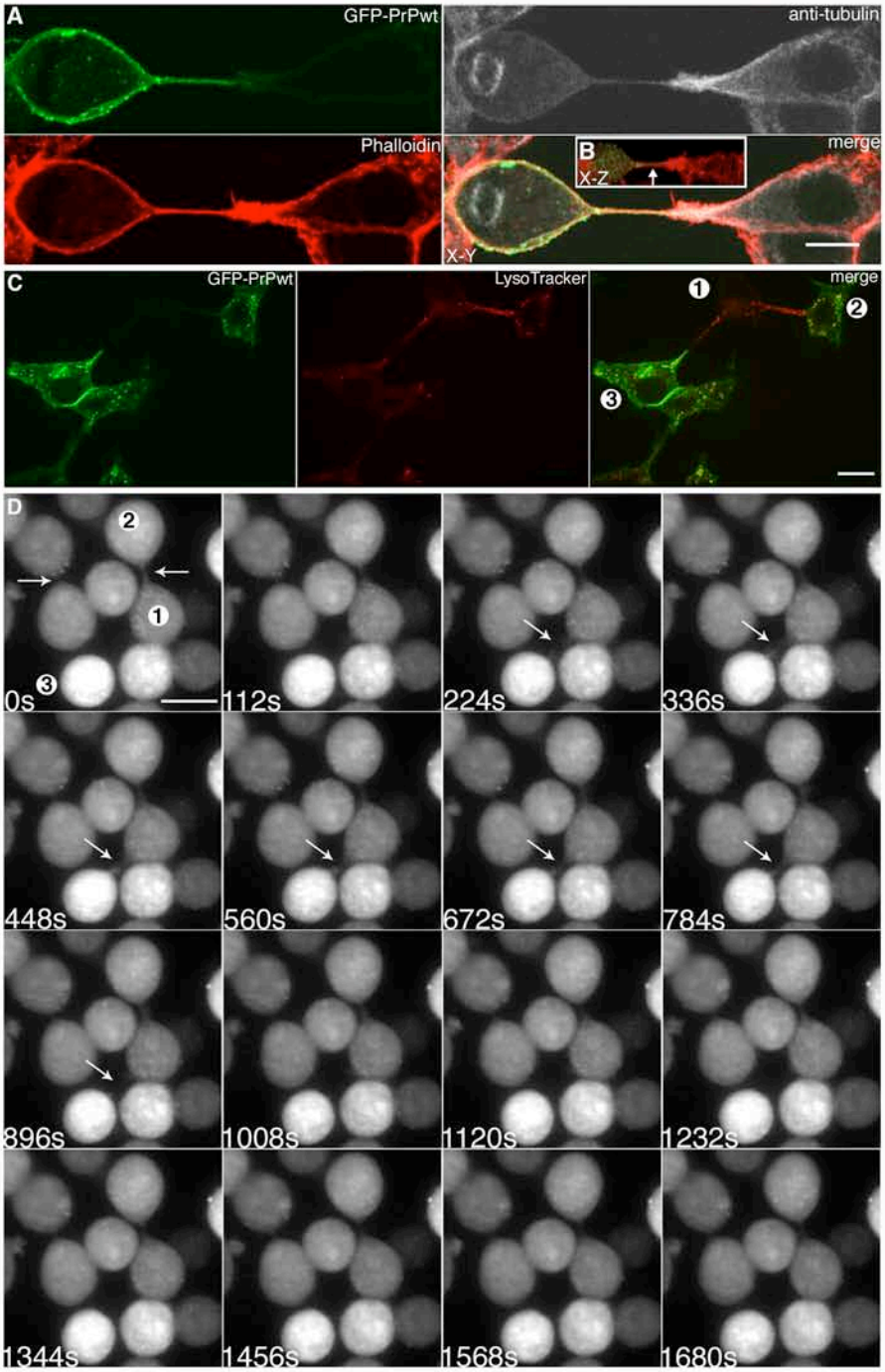
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2
3
D
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112s
224s
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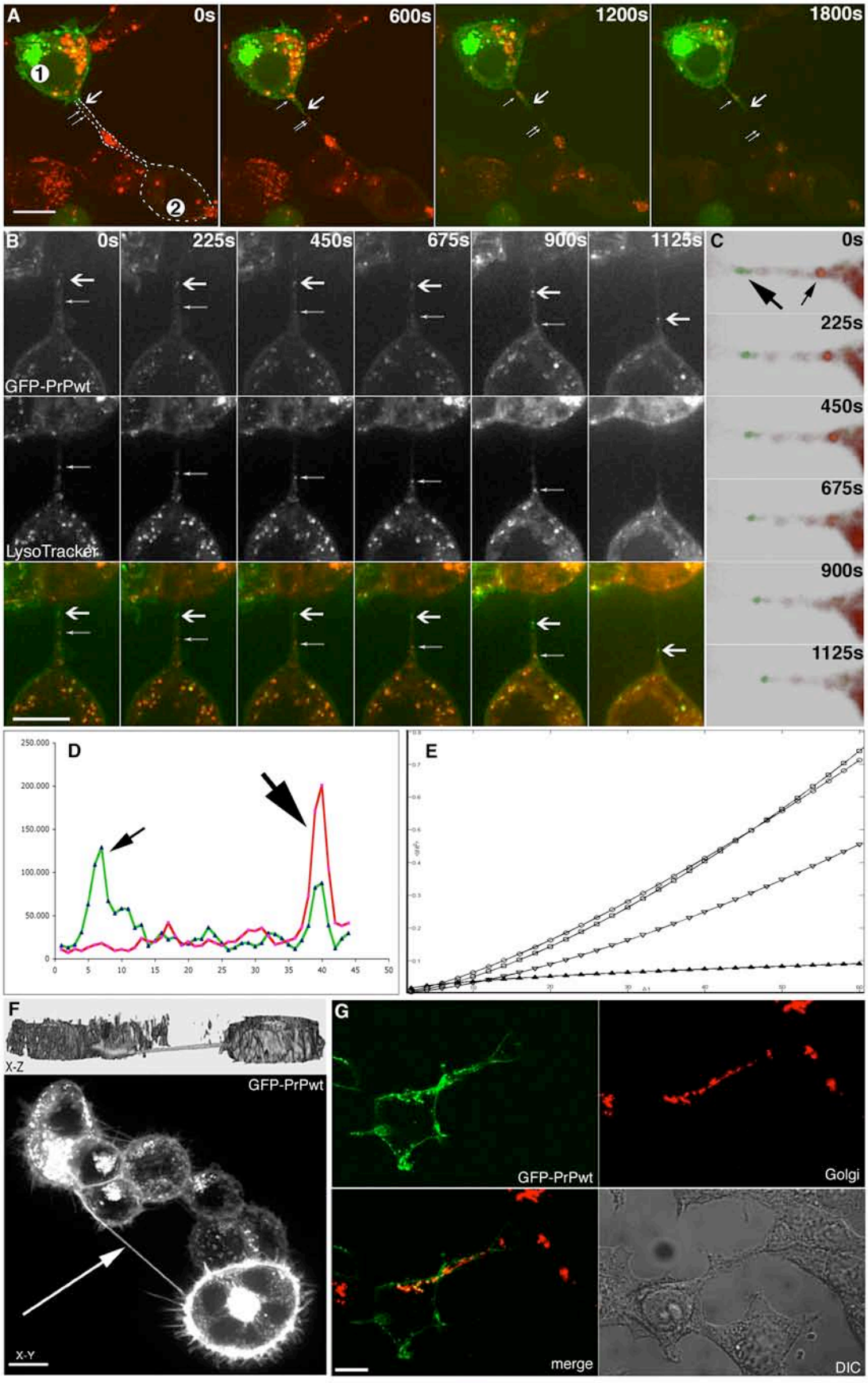
Fig.1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C
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0
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30
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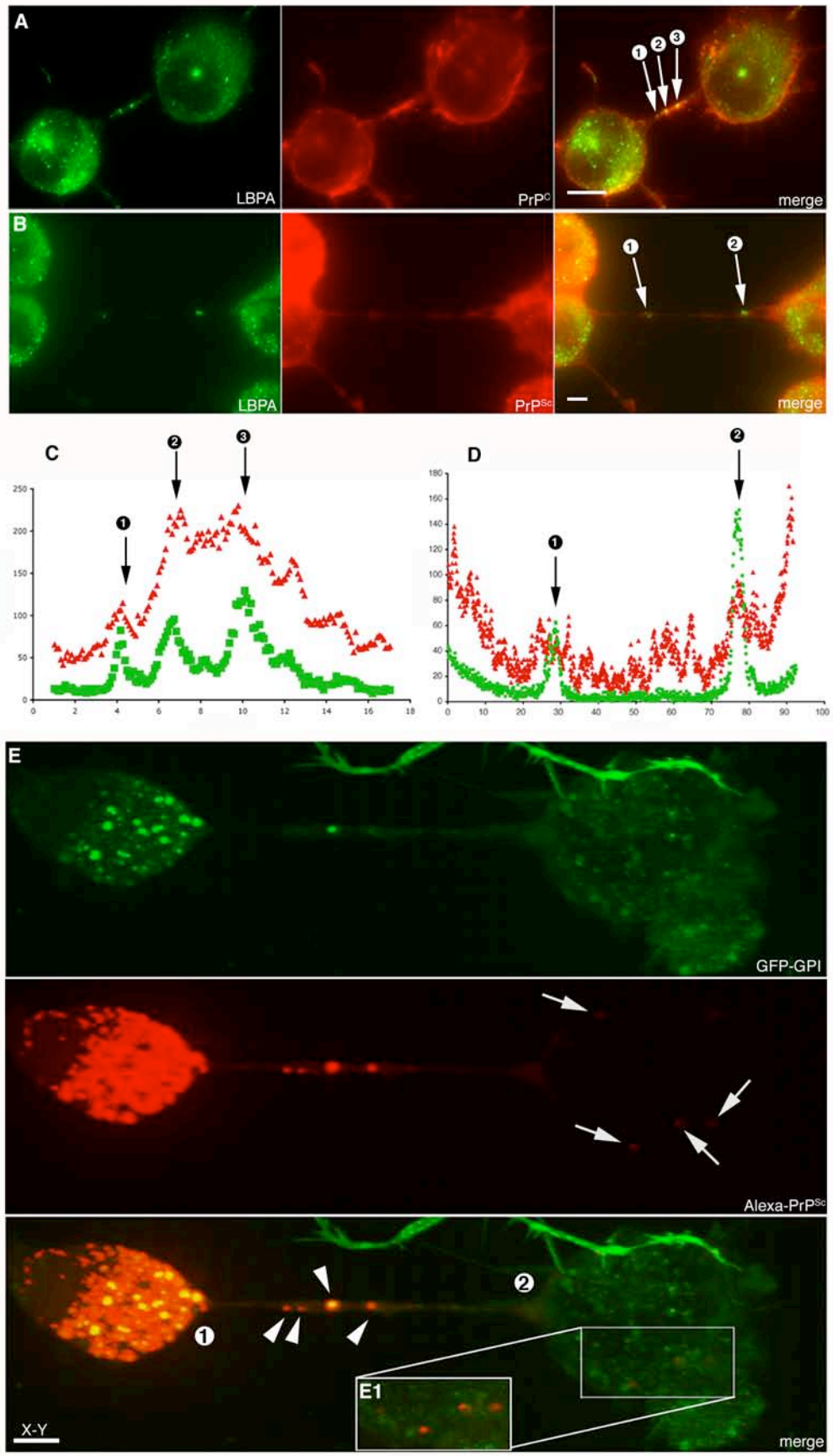
Fig.2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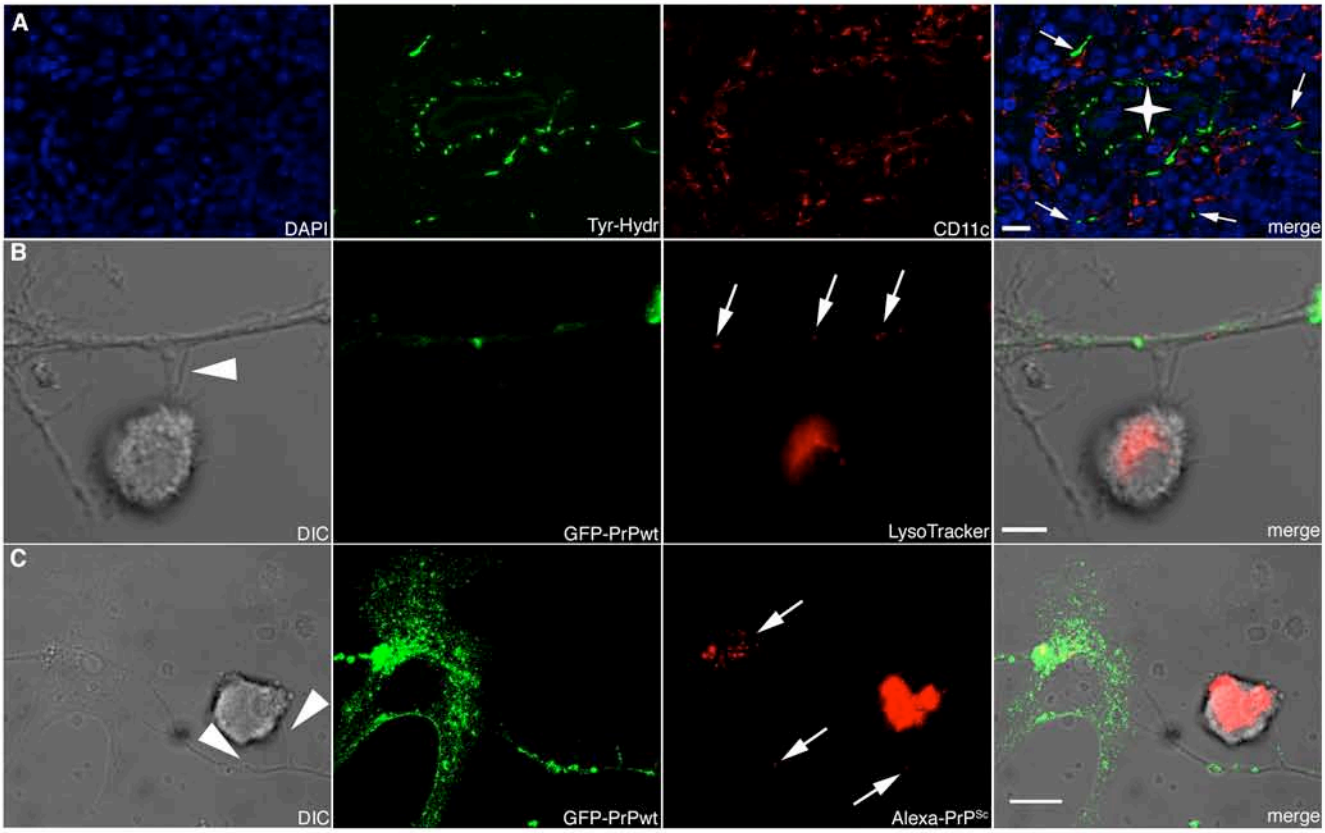
merge

Fig.3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Tyr-Hydr
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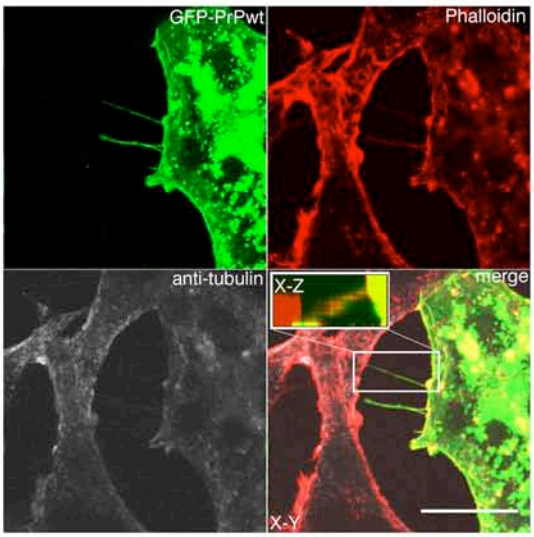
Fig.4



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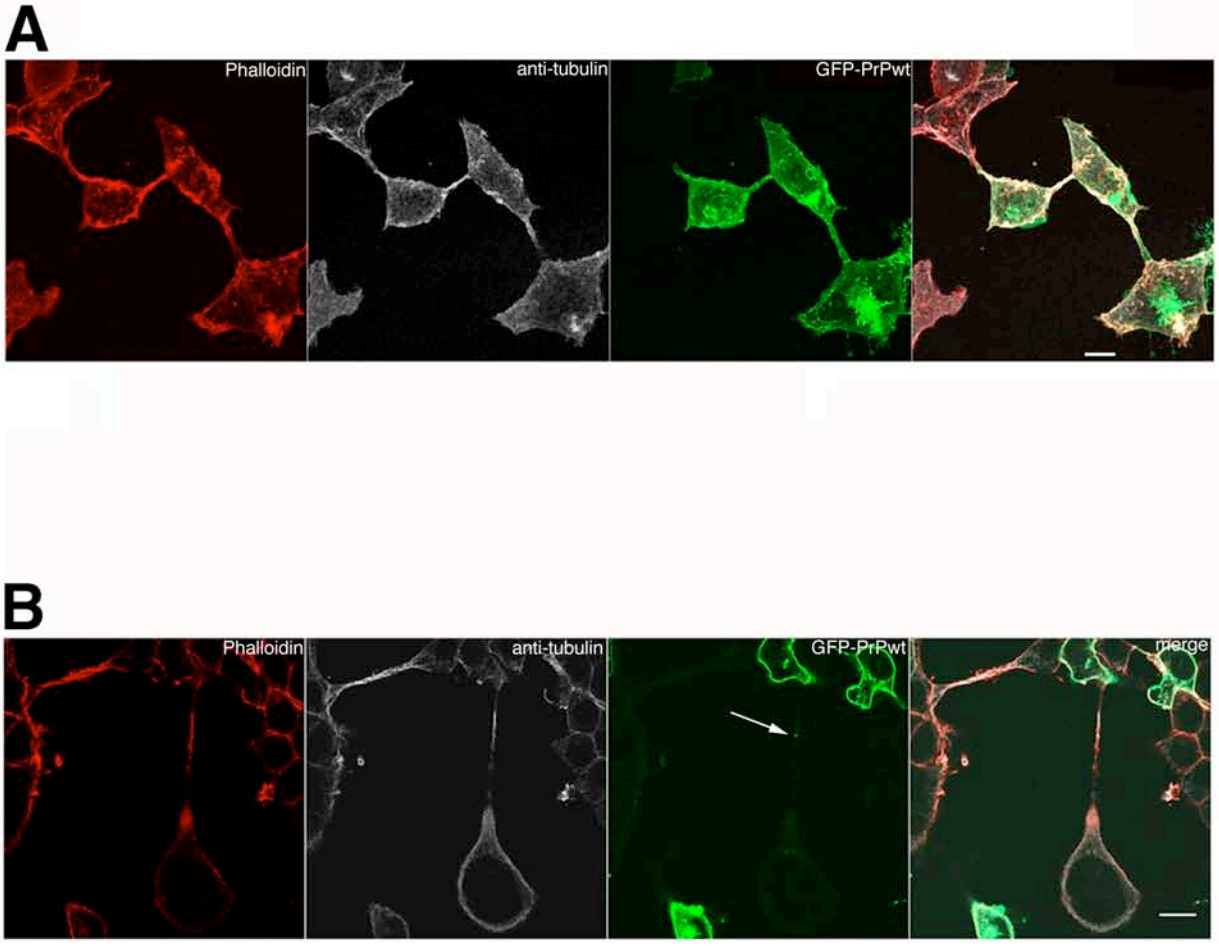
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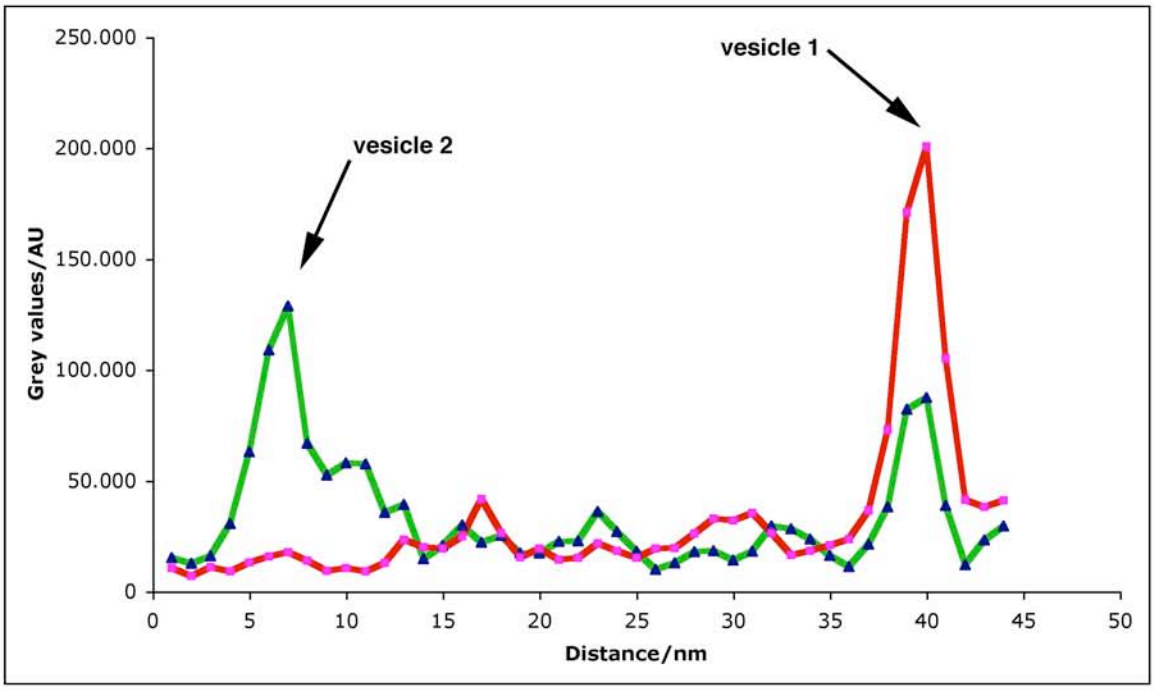
GFP-PrPwt
merge

Fig. S2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200.000
150.000
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30
35
40
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Distance/nm

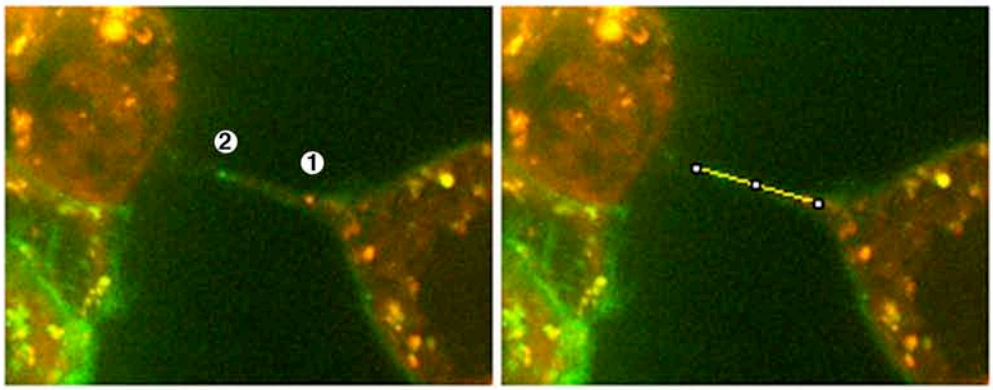
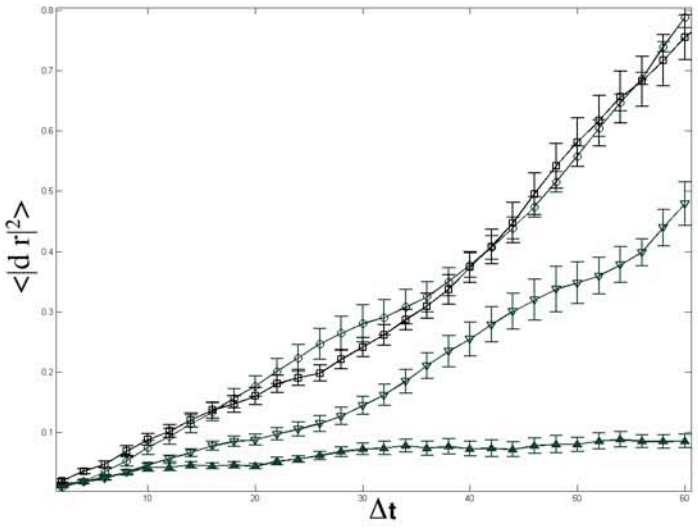

2
1

Fig.S3

A



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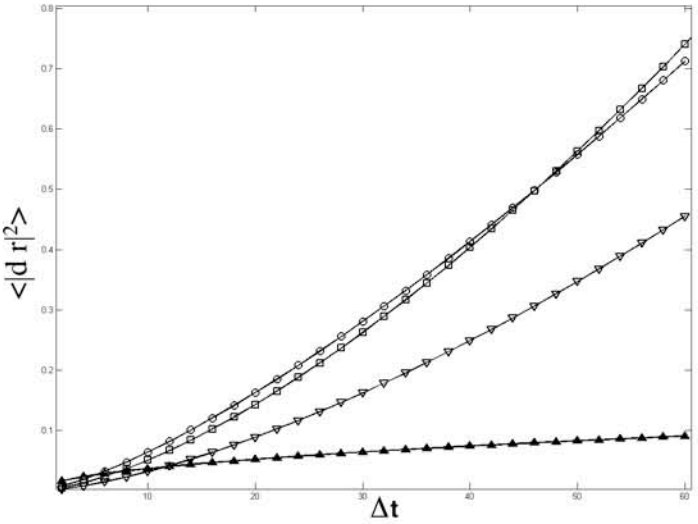


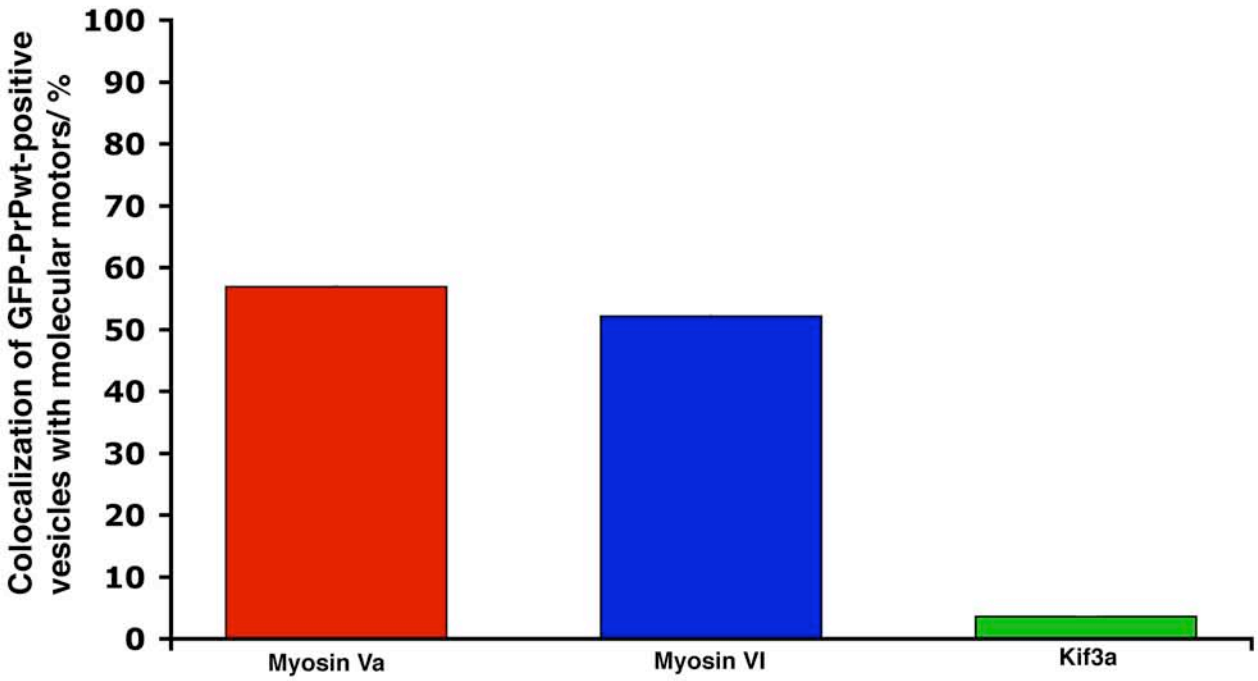
MSD(t) = C (Dt)^a
Green vesicle:
Part1 (C, a) = (0.0115, 0.5042)
Part2 (C, a) = (0.0029, 1.3443)
Part3 (C, a) = (0.0010, 1.4881)
Double-positive vesicle
Part1 (C, a) = (0.0016, 1.4962)

a < 1 confined motion
a = 1 Brownian motion
a > 1 directed motion

A) raw data measurements
B) fitted data after application of algorithm

closed triangles: MSD of the green vesicle in part I (0'-10') of the film
open triangles: MSD of the green vesicle in part II (10'-20') of the film
circles: MSD of the green vesicle in part III (20'-30') of the film
rectangles: MSD of the double-positive vesicle in part I (0'-10') of the film

Fig. S4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80
70
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40
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10
0
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Kif3a

Fig. S5

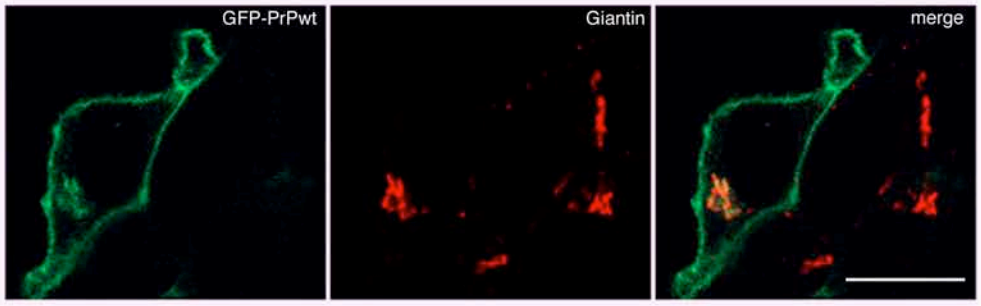
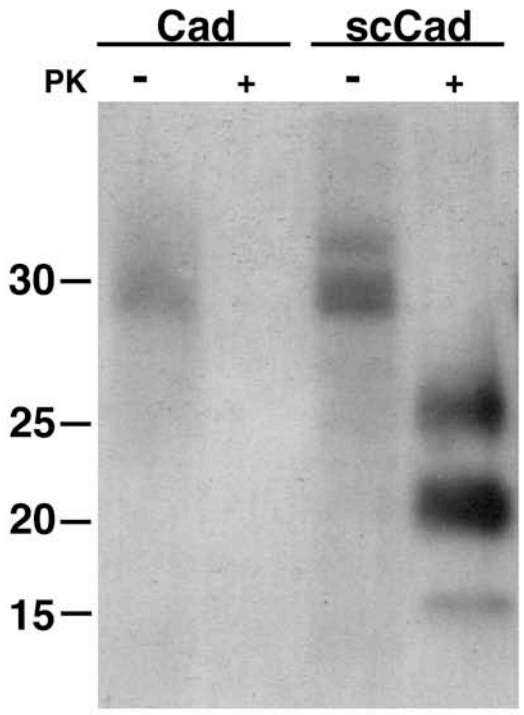


Fig.S6



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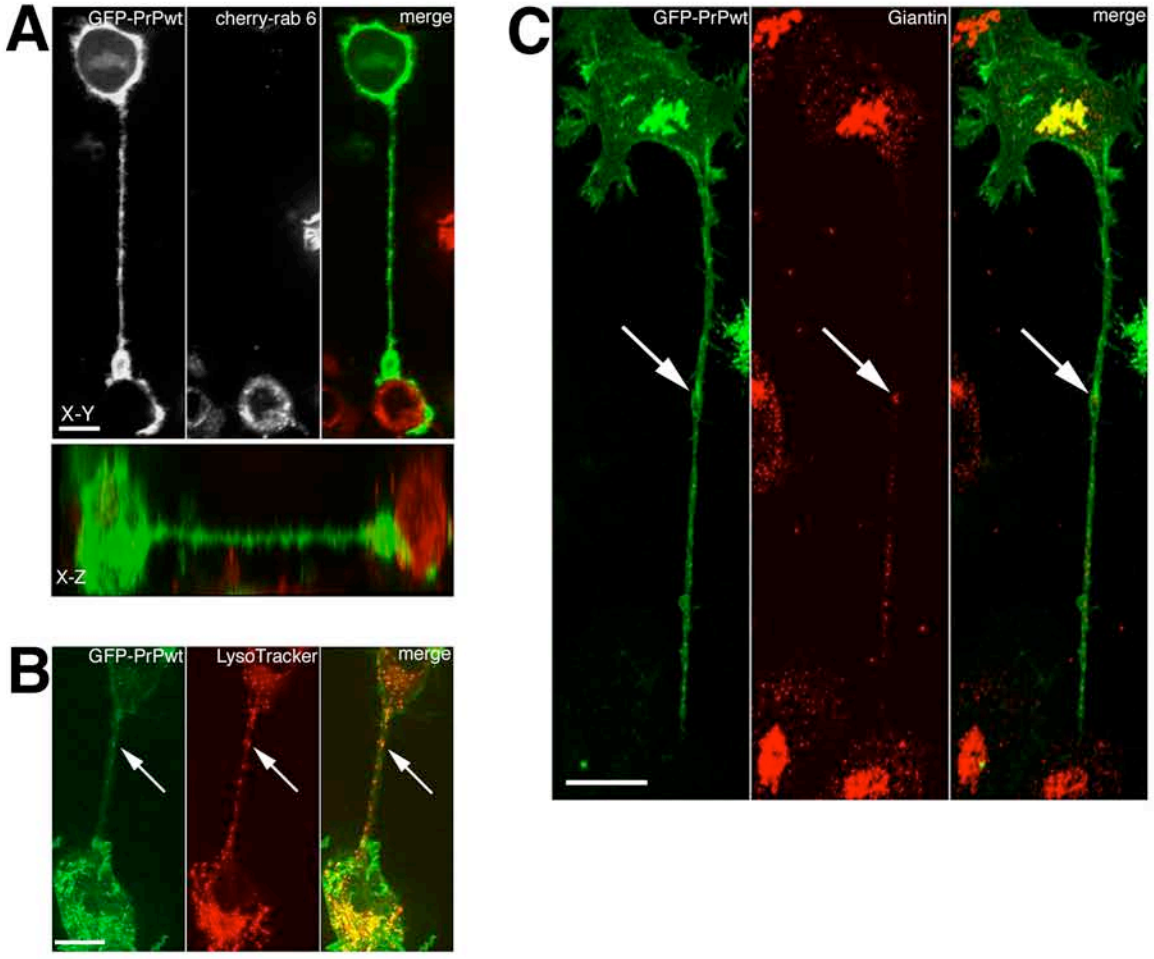
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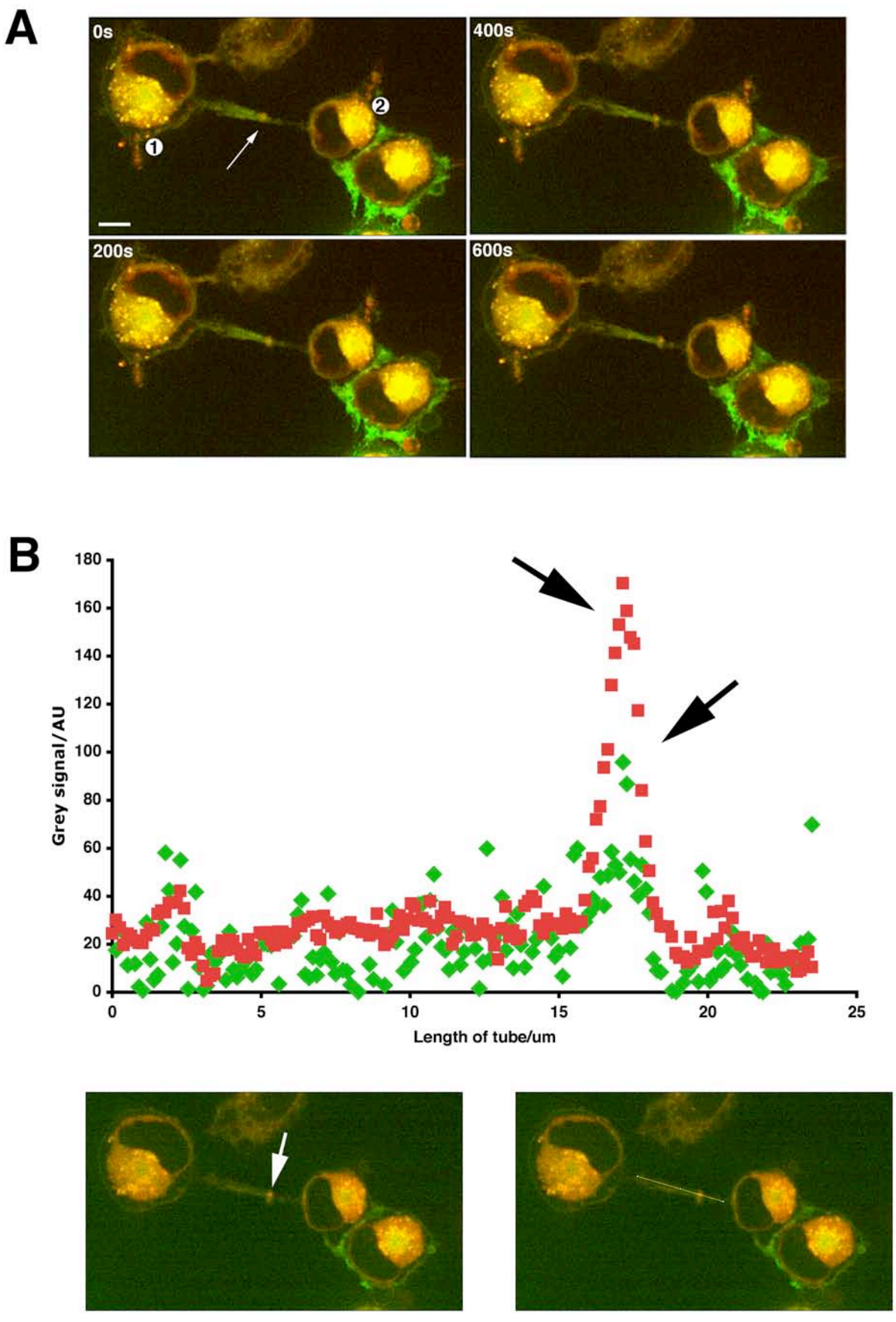
Fig. S8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B
Grey signal/AU
Length of tube/um
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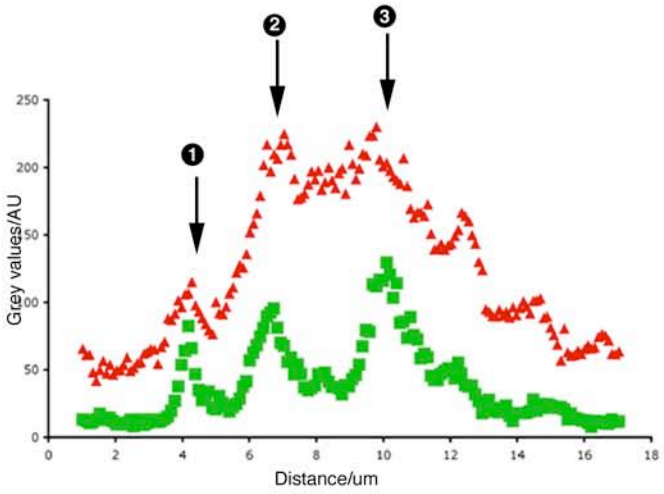
Fig. S9

A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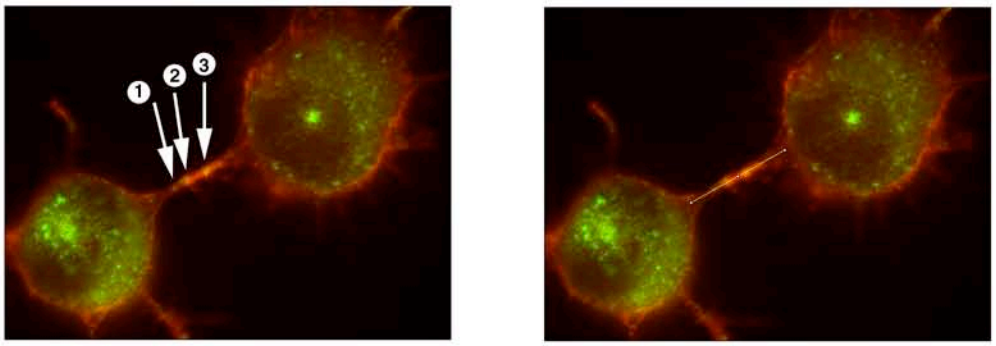
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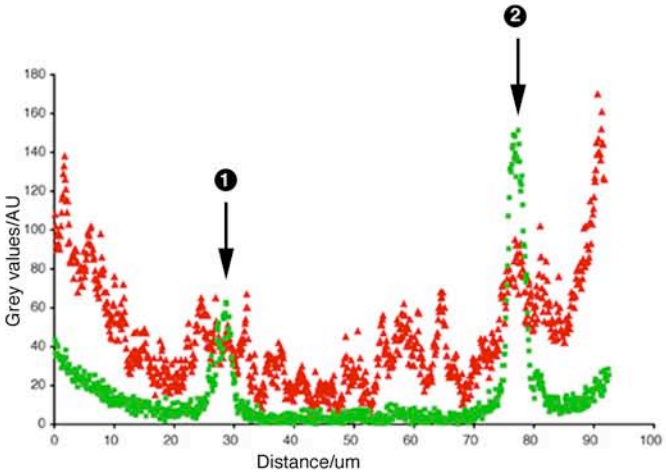
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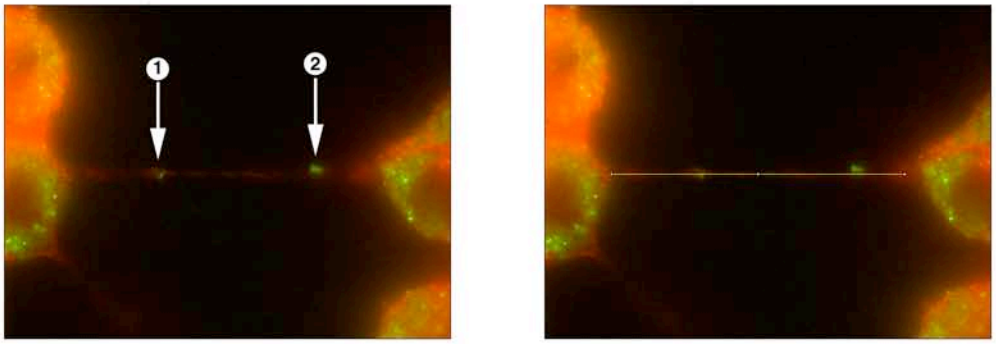
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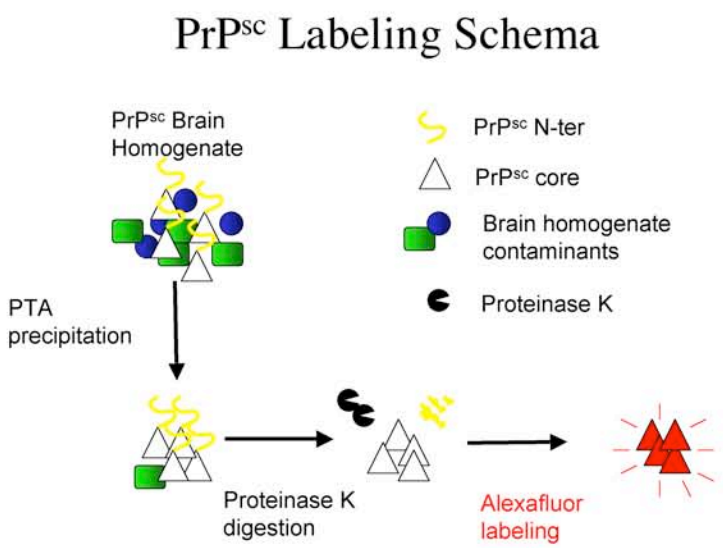
Fig. S10

A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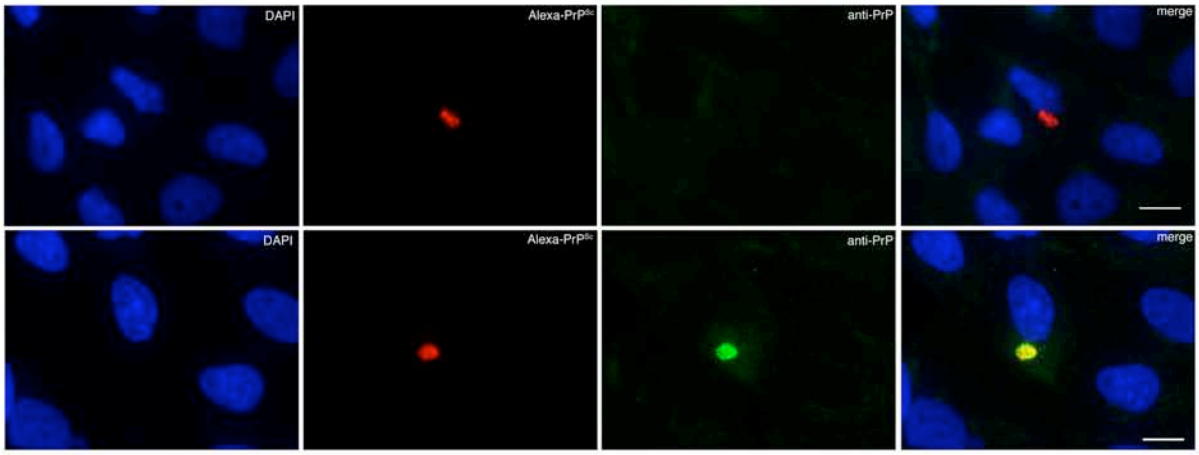
B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anti-PrP
merge
DAPI
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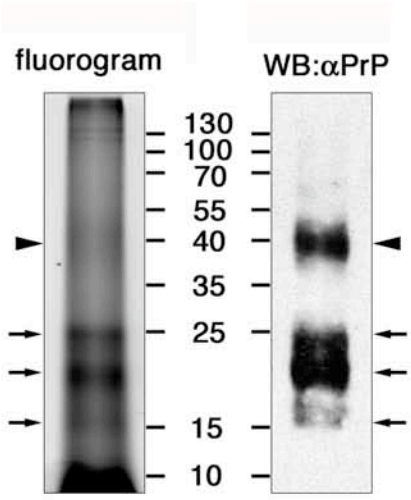
anti-PrP
merge

C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100
70
55
40
35
25
15
10

Fig.S11

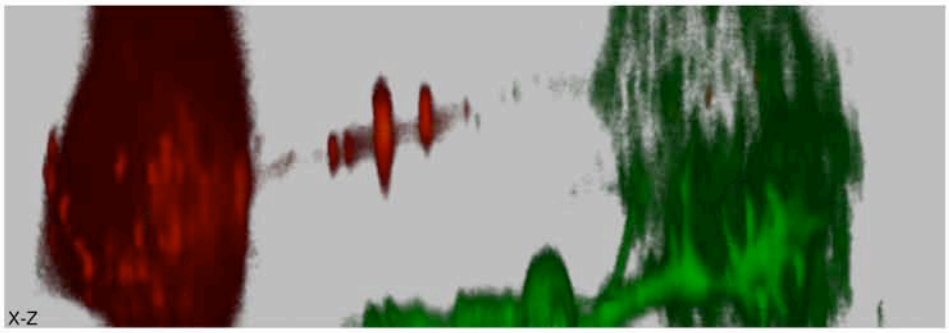
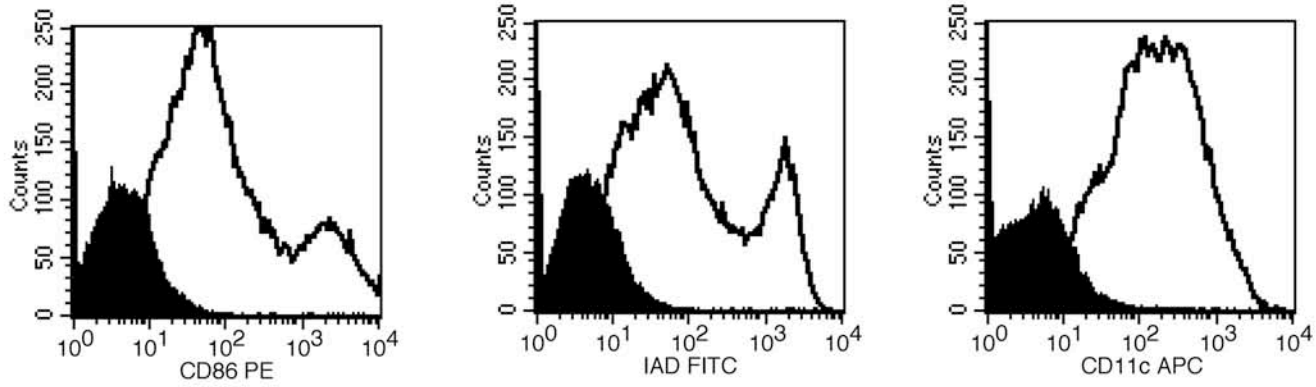


Fig. S12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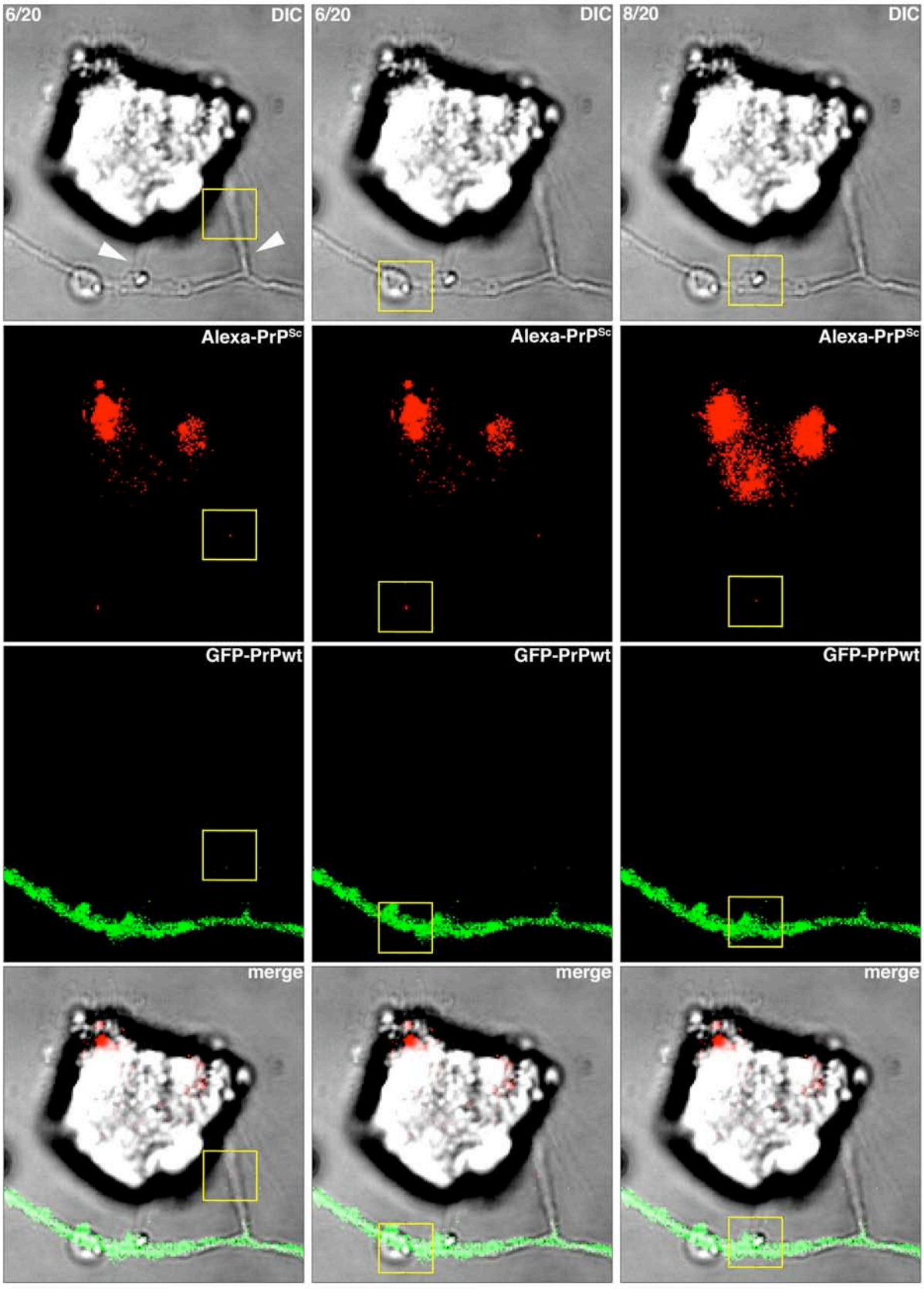
IAD FITC
CD11c APC

Fig. S13


6/20
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DIC
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GFP-PrPwt
GFP-PrPwt
merge
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Fig. S14

VI. Conclusion and Perspectives

In the first part of my thesis I mimicked heterozygosity, as found in the majority of inheritable TSE-cases, by co-expressing wild-type and different mutant PrP-forms in cell culture. This was performed in order to study the reciprocal effect of each form and possibly find new causes for pathology. In my experiments I found that:

1) Co-expression does not affect the glycosylation pattern of the two different PrP-forms.
2) In the case of two mutants (A116V, E199K) no significant change in cellular localization (Golgi-apparatus, plasma membrane) occurred. A third mutant, T182A, which is blocked intracellularly retained also the co-expressed wild-type intracellularly.
3) Upon co-expression significantly higher amounts of wild-type and mutant proteins localize to detergent resistant membranes. This phenotype is not found when two different wild-type are co-expressed.
4) Co-expressed wild-type and mutant protein interact closely as shown by FRET intracellularly and on the plasma membrane.

These data indicate that wild-type and mutant forms interact intracellularly and on the plasma membrane and that this might affect the function and/or behaviour of the wild-type proteins.

The most clinically relevant question resulting from the work on co-expressing mutant and wild-type PrP-proteins is whether the aforementioned results on DRM-sequestration are limited to *in vitro* cell systems or whether these results are applicable to *in vivo* scenarios. To answer this question one might perform experiments in transgenic mice, either expressing the human PrP^C and its mutant counterpart or expressing the murine version of the human mutation. In light of the conflicting results obtained with different cell lines utilized by different groups in their experiments, the described murine-based approach may or may not provide important information and should therefore be utilized with caution. Carrying out similar experiments to ours on post-mortem tissues obtained from deceased heterozygous patients, might overcome these problems; however ethical concerns renders such investigation complicated. Additional research on the function of PrP^C and the implication of its presence in lipid domains would also help in further interpreting of our data.

In the second part of my thesis I wanted to study the mode of intercellular spreading of prions and the role of dendritic cells (DCs) in this process. By using cocultures of differently labelled cells (HEK 293, CAD) and cocultures of primary DCs and hippocampal neurons I could show that:

1) Ectopically expressed GFP-PrPwt transfers *via* TNTs by "surfing" on the plasma membranes and inside vesicles, probably involving actin-based molecular motors.
2) Endogenous PrP^{C} and PrP^{Sc} transfers via TNTs contained in vesicles between immortalized neuronal cells.
3) Fluorescently labelled, purified PrP^{Sc} transfers *via* TNTs between immortalized neuronal cells and from bone-marrow derived dendritic cells to primary neurons *in vitro*.

These data shed light on a novel mechanism of intercellular prion-propagation and emphasizes the possible role of dendritic cells in this process.

The findings of structures resembling TNTs in organisms as diverse as *Drosophila melanogaster* (Hsiung et al., 2005; Ramirez-Weber and Kornberg, 1999), sea urchins (Malinda et al., 1995) murine (Rustom et al., 2004) and human cells (Watkins and Salter, 2005) suggests that this is a widespread phenomenon in nature. Interestingly, similar-shaped structures have already been reported for Gram-negative bacteria in the 1950s with the advent of electron microscopy (EM). These structures, called fimbriae by some (Duguid et al., 1955) and pili by others (Brinton, 1959; Brinton, 1965), are subdivided into several subtypes of which subtype IV enables bacteria such as Escherichia coli, Neisseria spp. and Pseudomonas spp. to exchange DNA-information (e.g. virulence factors) between each other (Telford et al., 2006).

The finding that pathogens as diverse as viruses, bacteria and prions can exploit TNTs for invading eukaryotic cells, implies an ancient evolutionary origin for these structures.

As often occurs in biological research the attempt to answer a question produces several new ones. The description of TNTs and their exploitation by pathogens provides several avenues of further research: The mechanism of how cells find and connect with each other, why different cytoskeletal elements are utilized, which molecular motors serve for plasma membrane and vesicular transfer and most importantly how pathogens can be kept from exploiting these structures for spreading throughout affected organisms are fascinating questions for the coming years.

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VIII. Summaries

VIII.1: Zusammenfassung

Transmissible spongiforme enzephalopathien (TSEs) ist eine Gruppe von tierischen Krankheiten mit infektiöser, sporadischer oder genetischer Ätiologie, die durch die pathologisch-missgefaltete Form des PrP-Proteins (Prion oder PrP^{Sc}) ausgelöst werden. Mutationen im *prnp*-Gen, welche die Missfaltung des Proteins begünstigen, bilden die Untergruppe der vererbbaren Erkrankungen. Diese Krankheiten werden an Nachkommen autosomal-dominant weitervererbt, wobei die Mehrheit der Patienten heterozygot sind, d.h. eine gesunde und eine fehlgebildtete Form des Proteins in Zellen exprimieren. Im ersten Teil meiner Dissertation simulierte ich diese Situation *in vitro* indem ich gesunde (PrPwt) und mutierte (PrPmut) Proteine in FRT-Zellen koexprimierte. Da die Proteine an verschiedene fluoreszierende Varianten der GFP-Familie gebunden waren, konnte man sie mikroskopisch unterscheiden. Desweiteren enthielten die mutierten Versionen einen sogenannten „tag“, genannt 3F4, um sie auch per Antikörper unterscheiden zu können. Mit diesen Mitteln wollte ich untersuchen, ob phenotypische Veränderungen (biochemisch und/oder mikroskopisch) auftreten, die auf die Interaktion der beiden Proteine zurück zu führen sind. Aufgrund von positiven FRET-Ergebnissen, erscheint es wahrscheinlich, daß die koexprimierten Proteine eng miteinander interagieren. Die koexprimierten Proteine kolokalisierten in der Zelle im Golgi-Apparat und auf der Plasmamembran, scheinbar ohne den jeweiligen Phänotypen zu beinflussen. Veränderungen im Phänotyp wurden jedoch auf subzellulärer Ebene gefunden: Die Koexpression von wild-typ und mutierten Proteinen führte zu einer signifikanten Erhöhung der beiden Proteine in sogenannten DRMs (detergent resistant membranes). Dies konnte nur festgestellt werden, wenn wild-typ und Mutante koexprimiert wurden, aber nicht in einer Kontrolle, die zwei unterschiedliche wild-typ Proteine exprimierte. Im Ganzen betrachtet, bestätigen diese Ergebnisse die bereits vorgeschlagene Bedeutung von DRMs in der Pathologie von TSE-Krankheiten, allerdings durch einen neuen und vormals unbekannten Aspekt. Unser Ergebnis lässt uns vermuten, daß die beschriebenen Erhöhungen in DRM-Sequestration zur Pathologie in heterozygoten Patienten beitragen könnte.

Im zweiten Teil meiner Dissertation wendete ich mich der Fragestellung zu, wie Prionen von Zelle zu Zelle weiter wandern und wie Prionen dadurch in das periphere Nervensystem (PNS) eindringen können um letztendlich in das zentrale Nervensystem (ZNS) vorzudringen. TNTs (tunneling nanotubes) sind unlängst beschriebene, filigrane, membranäre interzelluläre Zellverbindungen, die vermutlich der Zellkommunikation dienen. Durch mikroskopische Studien an fixierten und lebenden Zellen epithelialer und neuronaler Abstammung, konnte ich zeigen, daß Prionen TNTs für ihre eigene Verbreitung mißbrauchen. Anhand von primären gemischten Zellkulturen konnte ich zeigen, daß dendritische Zellen (DCs) mit hippokampalen Neuronen über TNTs kommunizieren und, daß in diesem Prozess Lysosomen sowie aufgereinigtes, fluoreszentes PrP^{Sc} von DCs zu Neuronen per TNTs transferieren. Die erfolgreichen Experimente mit primären Zellen bekräftigte meine vorherigen Untersuchungen an Zelllinien und führt zu meiner Hypothese, daß dieses Szenario ähnlich *in vivo* ablaufen könnte. Dies wird desweiteren untermauert durch Immunohistofluoreszenz-Experimente an murinem Milzgewebe, wo ich DCs sehr nahe mit Neuronen assoziert fand. Dieser Teil meiner Dissertation liefert einen neuen Mechanismus, wie Prionen aus der Peripherie eines oral infizierten Organismus ins ZNS vordringen können. Informationen zur temporären Blockierung dieser Strukturen könnte sich als eine vielversprechende Möglichkeit zur Inhibierung der Verbreitung von Prionen im Organismus herausstellen.

VIII.2: Resumé

Les maladies causées par des prions (ESST, encéphalopathie subaiguës spongiformes transmissibles) représentent un ensemble de maladies neurodégénératives fatales affectant différentes espèces animales. Dans ces infections, la protéine PrP^{C} s'accumule sous une forme anormale dite PrP^{Sc} ou prions, forme supposée infectieuse. Le sous-groupe des ESST héréditaires est lié à des mutations délétères dans le gène codant pour la protéine PrP^{C} (*prnp*). La majorité des patients affectés par des ESST héréditaires sont hétérozygotes, exprimant alors une version saine (PrPwt) et une version mutée (PrPmut) dans les cellules de leur organisme. Dans la première partie de ma thèse, j'ai reproduit cette situation *in vitro* en co-exprimant un plasmide codant pour une protéine sauvage

(dite «wild-type») avec des plasmides codants pour différentes versions mutées (trouvées dans des ESST héréditaires). Toutes les protéines ont été couplées à des étiquettes fluorescentes GFP, rendant leur visualisation possible en microscopie. De plus, afin de distinguer la forme saugave versus mutantes, ces dernières ont été liées avec une étiquette supplémentaire nomée 3F4 et reconnue par un anticorps spécifique. Nos expériences de FRET suggèrent une interaction entre les protéines co-exprimées et colocalisent dans l'appareil Golgi et sur la membrane plasmique, ne manifestant aucune différence si comparées avec les contrôles.

Des changements ont été trouvés au niveau sub-cellulaire: la co-expression des protéines entraine à une augmentation significative des protéines sauvages et mutantes dans les «DRMs» (detergent resistant membranes). Cet effet était visible que dans le cas de co-expression de formes sauvages et mutantes mais pas dans le cas contrôle de la co-expression de deux versions sauvages différentes. Ainsi nos résultats soulignent le rôle des «DRMs» dans les maladies ESST et suggèrent que les changements décrits de séquestration dans les DRMs décrits contribueraient à la pathologie dans le cas des patients hétérozygotes par un mécanisme inconnue.

Dans la deuxième partie de ma thèse, je me suis intéressé à la question de la dissémination des prions entres les cellules et particulièrement à leur envahissement du système périphérique neuronal (SPN). Les formes acquises sont probablement causées en grande partie par l'ingestion d'aliments contaminés par des prions, Leur dissémination intercellulaire après absorption intestinale reste énigmatique. Les «TNTs» (tunneling nanotubes) ont récemment été décrits comme des tubes membranaires très fins, dont les cellules se servent vraisemblablement pour la communication intercellulaire. Basé sur des études de microscopique, je montre que les prions exploitent les TNTs pour leur dissémination intercellulaire. En utilisant des cellules primaires, j'ai réussi à montrer que les cellules dendritques (CDs) derivées de la moëlle osseuse interagissent avec des neurones de l'hippocampe par la voie des TNTs et qu'elles transfèrent des lysosomes et de la protéine PrP^{Sc} fluorescente par ces structures. Des expériences de fluorescence immunohistologique sur la rate murine ont montré une interaction étroite entre des CDs et neurones, suggèrant l'existence des TNTs *in vivo*. Cette partie de mon travail propose un nouveau mécanisme par lequel les prions pourraient se disséminer entre les cellules et envahir le SPN.

VIII.3: Summary

Transmissible spongiform encephalopathies (TSEs) consist of a group of diseases of infectious, sporadic and genetic origin, found in higher organisms and caused by the pathological form of the prion protein (PrP^{Sc}). The inheritable subgroup of TSEs are linked to deleterious changes in the prion gene *prnp*, which favour its misfolding and are passed on to offspring in an autosomal-dominant fashion. The majority of patients from this group of diseases are heterozygous, leading to the co-expression of the wild-type form (PrP^{C}) and the mutant form (PrPmut) in the same cells. In the first part of my thesis I mimicked this situation *in vitro* by ectopically co-expressing plasmids encoding for wild-type and various PrP-mutants in FRT-cells. Since the proteins were linked to different fluorophores of the GFP-family and also contained protein-tags we could distinguish between wild-type and mutant forms and were interested to find phenotypical changes caused by the interaction of the two proteins by biochemistry and microscopy. The co-expressed proteins occupied the same cellular sites (such as Golgi-apparatus and plasma membrane) and probably interact (as seen by FRET) with no apparent clear change of phenotype when compared with the single-expressing controls. However significant changes were found on a subcellular level. Co-expression lead to an increased segregation of wild-type and mutant forms into detergent resistant membranes (DRMs). This was only seen in the case of co-expressed mutants but not with a control expressing two wild-type proteins. Taken together these results underline once more the importance of DRMs in TSE-pathology and suggest that the described changes in DRM-sequestration could contribute to pathology in heterozygous carriers by a yet to be defined mechanism.

In the second part of my thesis I focused on the question how prions propagate intercellularly and how prions invade the peripheral neuronal system. Specifically the infectious forms are believed to be mainly caused by oral exposure to contaminated foodstuffs. A still enigmatic process is how prions, upon intestinal uptake, transfer from cell to cell and how they invade the peripheral nervous system in order to be retrogradely transported to the central nervous system. Based mainly on microscopic techniques, we could show in fixed and living cell lines of epithelial and neuronal origin that prions exploit for intercellular spread a novel cell-to-cell communication mechanism consisting of fine intercellular membrane tubes, termed tunneling nanotubes (TNTs). Additionally, utilizing primary cell cultures, we show that bone marrow derived dendritic cells interact with hippocampal neurons *via* TNTs and that organelles such as lysosomes, as well as Alexa-568nm-labelled purified PrP^{Sc} can transfer through these membrane connections intercellularly. The experiments with primary cells substantiate our findings with immortalized cell lines and lead us to hypothesize that this scenario could also occur *in vivo* when immune cells, infected with prions, interact closely with the peripheral sympathetic nervous system in secondary lymphoid organs (as we could see by immunohistofluorescence of murine spleen). This part of my work provides a new mechanism by which prions could propagate from the peripheral entry site to the central nervous system. Knowledge on ways how to temporarily inhibit formation of TNTs could serve as a powerful means to block the spread of prions upon acute exposure.