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TRAF6 Involvement In Prion Disease: A Possible Crosstalk Among Neurodegenerative Disorders

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DECLARATION

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INDEX

LIST OF ABBREVIATIONS

AD: Alzheimer's disease

ALS: amyotrophic lateral sclerosis

BSE: bovine spongiform encephalopathy

CC1: positively charged cluster 1

CC2: positively charged cluster 2

CJD: Creutzfeldt-Jakob disease

CMA: chaperone-mediated autophagy

^{Ctm}PrP: PrP with the C-terminus facing the ER

CWD: chronic wasting disease

cyPrP: cytosolic PrP

DCs: dendritic cells

Dpl: Doppel

ER: endoplasmic reticulum

ERAD: endoplasmic reticulum associated degradation

ERC: endocytic recycling compartment

EUE: exotic ungulate encephalopathy

fCJD: familial Creutzfeldt-Jakob disease

FFI: fatal familial insomnia

FL-PrP: full-length form of PrP^{C}

FSE: feline spongiform encephalopathy

FTD: frontotemporal dementia

GSS: Gerstmann-Sträussler-Scheinker syndrome

HD: Huntington's disease

HEK293: human embryonic kidney 293 cell line

HSC: hematopoietic stem cells

Hsc70: heat shock-cognate protein of 70 kDa

HSP: heat shock protein

Hsp70: heat-shock protein 70

IDP: intrinsically disordered proteins

IPOD: insoluble protein deposit

JUNQ: juxtanuclear quality control

KO N2a: N2a cell line knock-out for PrP^{C}

LAMP2: lysosome-associated membrane protein type 2

MAVS: mitochondrial antiviral signalling protein

MNDs: motor neuron diseases

MTOC: microtubule organizing centre

MVB: multivesicular body

MVBs: endosomal multivesicular bodies

ND: neurodegenerative disease

^{Ntm}PrP: PrP with the C-terminus facing the cytosol

PD: Parkinson's disease

PK: proteinase-K

PrP^{C}: cellular prion protein

PrPC1: N-terminally truncated PrP fragment anchored to the plasma membrane

PrP-CAA: prion protein cerebral amyloid angiopathy

PrPN1: secreted N-terminally truncated PrP fragment

PrP^{res}: protease-resistant prion protein

PrP^{Sc}: Scrapie PrP

PS1: presenilin 1

RLRs: RIG-1 like receptors

ROS: reactive oxygen species

RRM: RNA recognition motif

SBMA: spinobulbar muscular atrophy

ScN2a: Scrapie infected N2a cell line

^{sec}PrP: secreted PrP

SG: stress granule

SOD: superoxide dismutase

STI1: stress inducible protein 1

SUMO2: small ubiquitin-like modifier 2

TCR: T-cell receptor

TGN: trans Golgi network

TME: transmissible mink encephalopathy

TRAF6: Tumor necrosis factor receptor (TNFR)-associated factor 6

TSE: transmissible spongiform encephalopathy

Ub: Ubiquitin

UBDs: Ub binding domains

vCJD: variant Creutzfeldt-Jakob disease

WT N2a: N2a cell line wild type for PrP^{C}

ABSTRACT

Prion diseases are characterized by the spreading and the accumulation of the pathological isoform (PrP^{Sc}) of the cellular prion protein (PrP^{C}). Accumulation of PrP^{Sc} in the brain results in loss of neurons, astrogliosis, PrP amyloid plaques and spongiform degeneration of the tissue. Despite many advances in the study of neurodegeneration caused by prions, knowledge about the physiological function of the PrP^{C} is still lacking. It is well established that the absence of PrP^{C} rescues the toxic effect of prions. A neuroprotective function of PrP^{C} as well as a role in cell signalling has been suggested. Ubiquitylation is a cellular process to address proteins to different fates, *e.g.* degradation, aggregation, localization, DNA damage repair. It is known that PrP^{C} is ubiquitylated *via* the canonical ubiquitin code and addressed to proteasomal degradation. In this work we investigated the relationship between PrP^{C} and the Tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6), a player of the atypical ubiquitylation that drives ubiquitylated substrates to a different cellular fate. TRAF6 ubiquitylates α-synuclein and huntingtin as well as their pathological isoforms. TRAF6 is found in cellular aggregates of these proteins. Through *in vitro* and *in vivo* experiments we found that full-length and cytosolic form of PrP^{C} are able to interact with TRAF6 and are present in cellular aggregates. Moreover, full-length PrP^{C} is a substrate of TRAF6 ubiquitylation. These findings open the door to the identification of a putative common mechanism for neurodegenerative diseases, suggesting a role for TRAF6 in regulating the fate of PrP^{C} and of other neurodegenerative-associated proteins.

CHAPTER 1

INTRODUCTION

Neurodegenerative diseases are an age-dependent leading cause of death, characterized by the progressive loss of nerve cells and affecting movement or mental functions. With the increase in life expectancy, the number of affected persons is strikingly increased but yet no disease-modifying therapies exist. Because of their enormous social and economic implications, neurodegenerative diseases are considered a public health priority.

Neurodegenerative diseases include Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), frontotemporal dementia (FTD) and motor neuron diseases (MNDs) [e.g., amyotrophic lateral sclerosis (ALS)]. Each disease is characterized by the progressive dysfunction of specific neural populations, associated with extra and intracellular accumulation of misfolded proteins. The clinical manifestations associated to each diseases depend on the anatomical location of the affected neurons and on the nature of the unfolded protein (Figure 1).

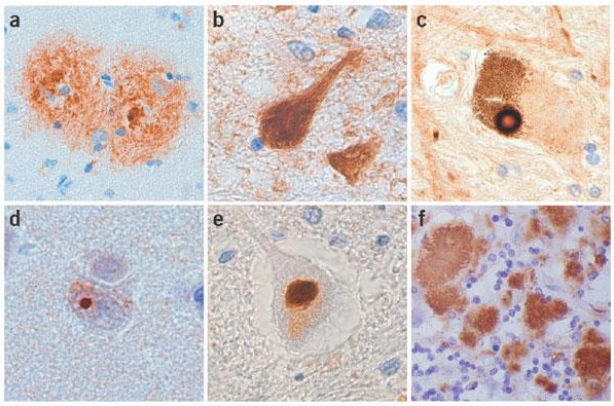

Figure 1. Protein aggregates in neurodegenerative diseases. (**a**) Senile plaques in neocortex of AD. (**b**) Neuro fibrillary tangles in the hippocampus of FTD. (**c**) Lewy body in Substantia Nigra of PD. (**d**) Intranuclear polyglutamine inclusion in neocortex of HD. (**e**) Ubiquitylated inclusion in spinal cord motor neuron of ALS. (**f**) Protease-resistant PrP in cerebellum of Creutzfeldt-Jacob disease (CJD). (Forman, Trojanowski et al. 2004).

At the cellular level, abnormal protein dynamics have to be referred to altered post-translational modification of newly synthesized proteins, deficiency of the ubiquitin-proteasome-autophagy system, oxidative stress, mitochondrial dysfunction, compromised axonal transport (Jellinger 2010). An overlap of neurodegenerative diseases and intraindividual phenotypic diversities have been recently observed, suggesting a synergistic mechanisms between pathological proteins and common pathogenic mechanisms (Fujishiro, Tsuboi et al. 2008, Clarimon, Molina-Porcel et al. 2009).

Appearance of neurodegenerative diseases is mainly attributed to genetic alterations; however, most causes are of unknown or sporadic origin. The discovery of prions has provided an explanation of how a disease can be both infectious and genetic (Prusiner 1993). Neurodegenerative diseases can be now classified as ‘‘protein misfolding’’ diseases (Gregersen 2006, Winderickx, Delay et al. 2008) or proteinopathies (Forman, Trojanowski et al. 2004, Forman, Trojanowski et al. 2007) (Figure 2).

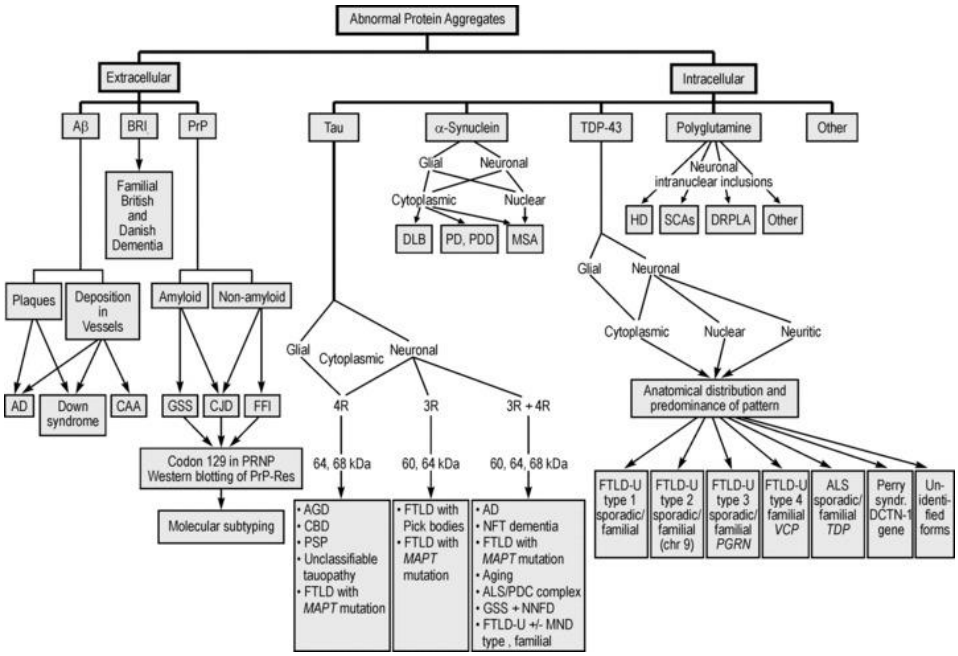

Figure 2. Classification of neurodegenerative diseases with protein deposits (proteinopathies) (Jellinger 2010).

PRION DISEASES

Prion diseases, also termed transmissible spongiform encephalopathies (TSEs) are a group of fatal neurodegenerative diseases that affects both humans and animals. They represent an heterogeneous group of brain disorders. Prion diseases include genetic, transmitted and sporadic forms and display a wide spectrum of clinical phenotypes and histopathological patterns. Human TSEs include Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), kuru, fatal familial insomnia (FFI) and prion protein cerebral amyloid angiopathy (PrP-CAA). Animal TSEs include the most known Scrapie of sheep and goats and the bovine spongiform encephalopathy (BSE) of cattle, the chronic wasting disease (CWD) of elks and deers, the feline spongiform encephalopathy (FSE) of cats, the transmissible mink encephalopathy (TME) of minks and the exotic ungulate encephalopathy (EUE) of nyala and kudu (Table 1).

DISEASE	HOST	MECHANISM OF PATHOGENESIS*
Kuru	Fore people in New Guinea	Infection through ritualistic cannibalism
Creutzfeldt-Jakob disease	Humans	Infection from prion-contaminated human growth hormone, dura mater grafts, and so forth
New variant	Humans	Infection from bovine prions?
Familial	Humans	Germ-line mutations in the *PrP* gene
Sporadic	Humans	Somatic mutation or spontaneous conversion of PrP^{C} into PrP^{Sc}?
Gerstmann-Sträussler-Scheinker disease	Humans	Germ-line mutations in the *PrP* gene
Fatal familial insomnia	Humans	Germ-line mutations in the *PrP* gene (D178N, M129)
Sporadic fatal insomnia	Humans	Somatic mutation or spontaneous conversion of PrP^{C} into PrP^{Sc}?
Scrapie	Sheep	Infection in genetically susceptible sheep
Bovine spongiform encephalopathy	Cattle	Infection with prion-contaminated meat and bone meal
Transmissible mink encephalopathy	Mink	Infection with prions from sheep or cattle
Chronic wasting disease	Mule deer, elk	Unknown
Feline spongiform encephalopathy	Cats	Infection with prion-contaminated beef
Exotic ungulate encephalopathy	Greater kudu, nyala, oryx	Infection with prion-contaminated meat and bone meal

*A question mark indicates that mechanism has not been confirmed. PrP denotes prion protein.

Table 1. List of prion diseases of human and animals. Adapted from (Prusiner, 2001)

The sporadic, infectious and many of the genetically determined human as well as animal forms of prion diseases can be transmitted across and within animal species. The underlying mechanism of all TSEs involves changes in the conformation of the cellular prion protein (PrP^{C}) into a pathogenic PrP conformer (named Scrapie PrP, PrP^{Sc}), which is capable of converting other PrP^{C} molecules into the abnormal form. Conversion in PrP^{Sc} results in a degenerative cascade, with the potential to transmit the disease (Prusiner 1998). Clinical manifestations of TSEs in humans include ataxia, dementia, insomnia, deviant behaviour, paraplegia and paresthesias. These symptoms are determined by a plethora of neuropathological changes rather than by specific and definite features, as they range from minimal to widespread astrocytic gliosis, neuronal loss, PrP amyloid plaques and vacuolation (spongiform appearance of brain tissue) (Soto and Satani 2011). Spongiform vacuolation, which is the most consistent histological abnormality to describe this group of disorders, can vary from focal areas of micro-vacuolation to areas of extensive confluent spongiform changes in the cerebral cortex, frequently observed also in the basal ganglia and thalamus in sporadic CJD, or from plaques with a margin of radiating fibrils in Kuru to 'florid' plaques in variant CJD (vCJD) in the occipital cortex and cerebellum (Parchi, Giese et al. 1999, Ironside, Head et al. 2002). However, vacuolation is not a diagnostic feature sufficient in the diagnosis of GSS , the neuropathology of which varies from absence of spongiform changes to deposits of fibrillar and non-fibrillar PrP in the cerebral and cerebellar parenchyma (Ghetti, Piccardo et al. 1996).

Besides the different conformation of vacuoles, the morphology of PrP deposits as well as the affected brain regions depend on the polymorphisms of the *PRNP* gene and the specific genetic mutation involved. Moreover, the prion strain and the host also contributes to the variability observed prion diseases.

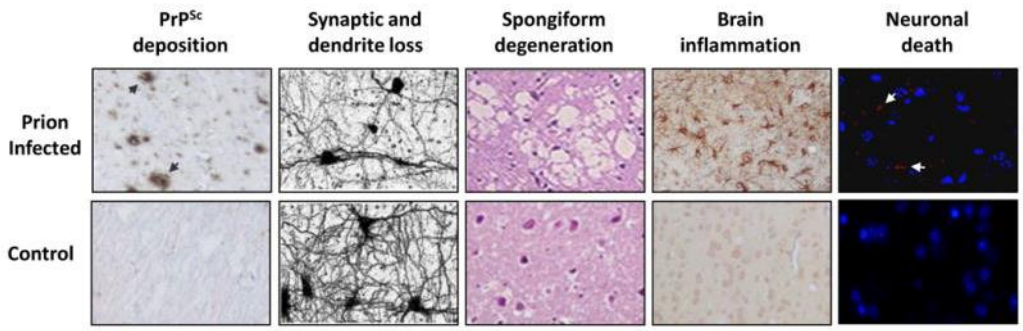

Figure 3. Multiple neurodegenerative pathways are implicated in TSEs. Neuropathological features of infected brains include the accumulation of PrP^{Sc} deposits, synaptic damage and dendrite loss, spongiform degeneration, astroglial activation and neuronal death. (Soto and Satani 2011).

THE PRION CONCEPT

The transmissibility of TSEs was accidentally demonstrated in 1937, after the inoculation of a Scottish sheep with a brain extract tissue against a common virus. The inoculum contained infectious material as the 10% of the flock developed scrapie. The transmission was subsequently demonstrated to sheep and mice (Chandler 1961). In humans, an infectious route was firstly demonstrated for Kuru in the cannibalistic tribes of New Guinea and confirmed in pioneering studies of transmission in chimpanzee (Gajdusek, Gibbs et al. 1966). The same observations were obtained for CJD (Gibbs, Gajdusek et al. 1968) and GSS (Masters, Gajdusek et al. 1981). In 1967, Alper and colleagues demonstrated that the agent causing TSEs was extremely resistant to nucleic acid inactivation, thus excluding a virus or viroid as infectious pathogens (Alper, Cramp et al. 1967). In the same year, J.S. Griffith speculated for the first time that the causative agent could be a protein (Griffith 1967). This launch the so-called 'protein-only' hypothesis of TSE propagation. The 'protein-only hypothesis' has challenged the central dogma of biology. In fact, according to the new hypothesis, proteins themselves (and not only DNA and RNA) could store and propagate biological information. The term "prion" was subsequently introduced by Stanley B. Prusiner in 1982. "Prion" stands for "proteinaceous infectious particle", to underline the exclusively proteinaceous property of this infective agent as primary cause of prion diseases (Prusiner 1982).

Isolation of the protease-resistant prion protein (PrP^{res}) as a 27-30 kDa resistant fragment allowed the determination of its amino-terminal sequence leading to the identification of an endogenous cellular gene (*Prnp* in mouse chromosome 2 or *PRNP* in human chromosome 20), whose translational product was called PrP^{C} (Chesebro, Race et al. 1985, Oesch, Westaway et al. 1985, Basler, Oesch et al. 1986). PrP^{C} was proven to be the product of a single host gene, present endogenously and constitutively expressed by many cell types. The conversion of PrP^{C} into its protease-resistant disease-associated form, named PrP Scrapie (PrP^{Sc}), is the result of a post-translational conformational change. In fact, the α-helical content of the PrP^{C} diminishes at the expense of an increase of β-sheets in PrP^{Sc} (Caughey, Dong et al. 1991, Pan, Baldwin et al. 1993). In inherited prion diseases, mutations occurring in the *PRNP* gene alter protein folding and stability and promote the conversion to PrP^{Sc}. In infective cases, the endogenous PrP^{C} becomes a substrate that is directly converted to PrP^{Sc} by a preformed PrP^{Sc}–aggregate. In sporadic cases, it is likely that PrP^{C} folding is perturbed by environmental conditions favouring pathological conversion (Prusiner 1991, Prusiner 1994, Caughey 2003).

STRAINS

Prions exist as strains able to exhibit distinct prion disease phenotypes. Strains of prions have been defined by the pattern of protein aggregate deposition, histopathological lesion profiles, rapidity of disease progression and specific neuronal targets (Dickinson, Meikle et al. 1968). Mounting evidence suggests that the tropism of a prion strain is dictated by its tertiary or quaternary structure, which influences its ability to bind and interact with specific molecules (Mahal, Baker et al. 2007, Aguzzi 2008). Indeed, the information for the specific conformation of a PrP^{Sc} protein resides in its supramolecular structure, as demonstrated in studies involving the transmission of FFI and familial Creutzfeldt-Jakob disease (fCJD) to transgenic mice (Telling, Parchi et al. 1996, Scott, Groth et al. 1997, Safar, Wille et al. 1998).

These observations suggest that PrP^{Sc} itself is the molecule in which the information of a specific prion strain is encrypted.

PRION NEUROTOXICITY

The mechanisms underlying neurodegeneration in prion diseases remain unknown. The contribution of apoptosis and oxidative stress in TSEs pathology has been described (Milhavet and Lehmann 2002), but little is known about the primary causes that lead to neurodegeneration. PrP^{Sc} was generally assumed to be the neurotoxic specie, acting through a gain-of-toxic-function mechanism. However, PrP^{Sc} itself is not sufficient to trigger disease progression, as the presence of the cellular prion protein is also required (Bueler, Aguzzi et al. 1993, Brandner, Raeber et al. 1996). Removal of PrP^{C} from cell membrane has been shown to inhibit neurotoxicity (Chesebro, Trifilo et al. 2005). Ablation of PrP^{C} *in vivo* reversed prion pathology in prion infected mice (Mallucci, Dickinson et al. 2003). Recently, it has been suggested that replication events are dissociated from prion-induced neurotoxicity (Hill and Collinge 2003, Halliday, Radford et al. 2014). The ability of PrP^{C} to protect neurons from toxicity during prion infection is reduced as a consequence of its conversion to PrP^{Sc} (Roucou and LeBlanc 2005). Therefore, it is still under debate whether prion toxicity is due to a gain-of-function of oligomeric species, to a loss-of-function of PrP^{C} or, most likely, to a combination of the two.

CELLULAR PRION PROTEIN

PrP^{C} is encoded by a chromosomal gene denoted *PRNP*. *PRNP* gene belongs to the *PRN* gene family that includes also Doppel (Moore, Lee et al. 1999) and Shadoo proteins (Watts, Drisaldi et al. 2007). The gene itself is composed by two (in hamster and humans) or three exons (in rat, mouse, bovine, sheep), while the open reading frame of PrP^{C} is encoded within a single exon (Basler, Oesch et al. 1986,

Westaway, Goodman et al. 1987). The other exons contain untranslated sequences including the promoter and the termination sites, to which the control of the *PRNP* gene expression has been attributed. The PrP^C promoter contains GC-rich repeats, canonical binding site for the transcription factor Sp1, driving expression in many different tissues (Puckett, Concannon et al. 1991, Baybutt and Manson 1997). TATA box is absent from the gene. For these reasons *PRNP* is often labelled as a housekeeping gene. Alignment of PrP^C translated sequences reveals a striking degree of conservation not only among mammals, but also in birds, reptiles, amphibians, fish and more primitive organisms such as insects and protozoa (Gabriel, Oesch et al. 1992, Simonic, Duga et al. 2000, Favre-Krey, Theodoridou et al. 2007). This evidence suggests the retention of some important function for PrP^C through evolution.

PrP^C is an N-glycosylated, glycosylphosphatidylinositol (GPI)-anchored protein of 208–209 aa. (Stahl, Borchelt et al. 1987). Mature PrP^C is exported to the cell surface after cleavage of a 22-amino acid (aa) signal peptide and post-translational processing that occurs in the ER and Golgi. PrP^C structure is characterized by a flexible random-coiled N-terminal domain and a C-terminal globular domain (Figure 4). The unfolded N-terminal region consists of residues 23–124 and contains a stretch of several octapeptide repeats (OR) and two positively charged clusters, CC1 (aa 23–27) and CC2 (aa 95–110), linked by a hydrophobic stretch known as the hydrophobic domain (HD, aa 111–129). The globular domain of human PrP^C is arranged in three α-helices (aa 144–154, 173–194 and 200–228), interspersed with antiparallel β-sheets (aa 128–131 and 161–164). A single disulfide bond is found between cysteine residues 179 and 214 (Riek, Hornemann et al. 1996, Riek, Hornemann et al. 1997). Despite low sequence similarity between chicken, turtle and frog PrP^C, and the highly conserved homology between mammalian, PrP^C structural features are remarkably preserved (Calzolai, Lysek et al. 2005). Full-length PrP^C is glycosylated in the Golgi apparatus in residues Asn-181 and Asn-197 in human and Asn-180 and Asn-196 in mice (Haraguchi, Fisher et al. 1989) (Figure 5). The variable occupancy of residues results in non-, mono-, di-glycosylated PrP^C forms. The glycosylation pattern gives rise to a large variety of N-glycans attached

to both the full-length and truncated PrP^C (Rudd, Endo et al. 1999, Pan, Wong et al. 2002) and results in a different distribution of PrP^C in the brain (DeArmond, Qiu et al. 1999, Beringue, Mallinson et al. 2003). The pattern of glycosylation seems to be involved in the susceptibility to conversion and to the diversity of TSEs (Lawson, Collins et al. 2005).

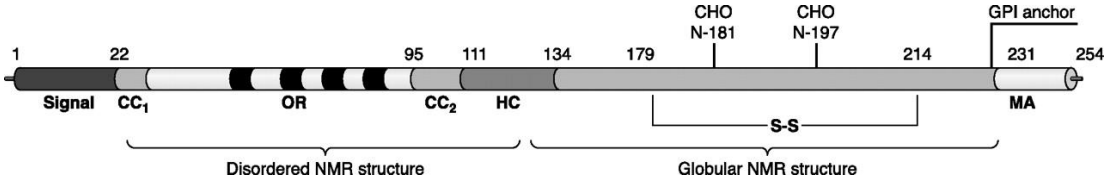

Figure 4. Outline of the primary structure of the cellular prion protein including post-translational modifications. (Aguzzi and Calella 2009)

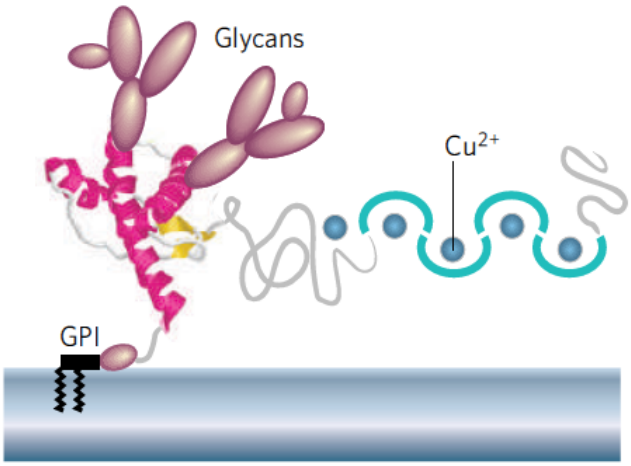

Figure 5. Mature form of PrP^C. PrP^C is a GPI-anchored membrane protein, with a flexible, random coil N-terminus and a globular C-terminal domain. The N-terminal domain of PrP^C contains the stretch of octapeptide repeats (in light blue), which binds divalent cations such as copper ions, the two positively charged clusters, CC1 (aa 23–27) and CC2 (aa 95–110) and the hydrophobic domain (HD). The C-terminal domain of PrP^C contains three alpha-helices (in pink) and two antiparallel β-sheets (in yellow). PrPC can be found in non-, mono-, or di-glycosylated forms (Caughey and Baron 2006)

PrP^C EXPRESSION AND LOCALIZATION

Increase of *PRNP* expression is correlated with brain development, from the initial postnatal weeks till the end of the synaptogenesis, reaching a plateau during adulthood. Moreover, distribution and expression level of PrP^C vary among distinct brain regions, cell types and neurochemical phenotypes, indicating a correlation between expression and neuronal differentiation (Manson, West et al. 1992, Sales, Hassig et al. 2002, Linden, Martins et al. 2008, Benvegnu, Poggiolini et al. 2010). In the central nervous system, PrP^C expression has also been described in glial cells (Radovanovic, Braun et al. 2005, Bribian, Fontana et al. 2012). Moreover, PrP^C is present in non-neuronal tissues, such as blood lymphocytes, gastro-epithelial cells, heart, kidney and muscles (Horiuchi, Yamazaki et al. 1995, Fournier, Escaig-Haye et al. 1998).

PrP^C KNOCK-OUT MICE

Several non-co-isogenic lines of mice lacking PrP^C have been generated by homologous recombination in embryonic stem cells, in order to shed light into PrP^C physiological function. Disruptive modifications restricted to the open reading frame results in the known $Prnp^{o/o}$ [Zürich I] (Bueler, Fischer et al. 1992) or $Prnp^{-/-}$ [Edinburgh] (Manson, Clarke et al. 1994) lines. These mice developed normally, without severe pathologies. As predicted by the protein-only hypothesis, these mice were entirely resistant to prion infections (Bueler, Aguzzi et al. 1993). In contrast, three lines generated afterwards, $Prnp^{-/-}$ [Nagasaki], *Rcm0*, and $Prnp^{-/-}$ [Zürich II], developed ataxia and Purkinje cell loss later in life (Sakaguchi, Katamine et al. 1996, Moore, Lee et al. 1999, Rossi, Cozzio et al. 2001). The discrepancy with the previous lines was clarified with the discovery of *Prnd*, a gene located 16 kb downstream to *Prnp* encoding for a protein called Doppel (Dpl). In $Prnp^{-/-}$ [Nagasaki], Rcm0, and $Prnp^{-/-}$ [Zürich II] a splice acceptor site to the third exon of *Prnp* was deleted, placing the *Prnd* gene under the transcriptional control of the *Prnp* promoter. This resulted in a chimeric *Prnp-Prnd* product and in the overexpression of Dpl in the

brain (Moore, Lee et al. 1999), responsible for the brain phenotype. The effect of Dpl overexpression in these mice was rescued by reintroduction of *Prnp* gene (Nishida, Tremblay et al. 1999, Didonna, Sussman et al. 2012). Moreover, all *Prnp*$^{-/-}$ lines were generated in ES cells derived from the 129 strain of the laboratory mouse and maintained in non-129 backgrounds, except for the *Prnp*$^{-/-}$ [Edinburgh]. Consequently, a systematic genetic confounders happens when *Prnp*$^{-/-}$ and *Prnp+/+* lines are compared, leading to the misleading attribution of altered physiological evidence to the lack of the *Prnp* gene. For example, an hyperphagocytosis of apoptotic cells in primary macrophages from PrP$^{-/-}$ mice (de Almeida, Chiarini et al. 2005) was proved to be the result of a flanking gene problem, rather than of absence of PrP^{C} (Nuvolone, Kana et al. 2013). To finally avoid the above-mentioned problems, Aguzzi group has successfully created a new co-isogenic line of PrP$^{-/-}$ mice [Zürich III] on pure C57BL/6J background.

CELLULAR PROCESSES INFLUENCED BY PrP^{C} EXPRESSION

Although several studies have been carried out to underpin possible functions of PrP^{C} and many lines of transgenic mice carrying mutations and deletions of PrP^{C} have been established, straightforward evidence is still lacking to understand the physiological role and the molecular pathways leading to neurodegeneration in prion diseases. The flexible and unstructured N-terminal domain of PrP^{C} resembles the group of intrinsically disordered proteins (IDP) which partially or entirely lack a globular structure (Dyson and Wright 2005, Uversky and Dunker 2010). This group comprises proteins that have been shown to be involved in cell signalling, protein-protein interactions and regulation of transcription and chromatin remodelling. Examples of IDP are α-synuclein, associated to PD, and p53 and BRCA1 oncogenes, associated to tumor development. PrP^{C} shares with these proteins in particular the

characteristic of molecular recognition of several partners, a feature that allows disordered proteins to switch functions (Tompa, Szasz et al. 2005).

PrP^C has been demonstrated to be involved in a number of physiological cellular processes including regulation of neuronal signalling and synaptic function and neuroprotection (Table 3).

Cell type	Process	Function	Mechanisms, ligands and pathways
Neuron	Neuritogenesis	Adhesion, signalling	Recruits NCAM into rafts to allow it to activate Fyn kinase[35], which mediates intracellular signalling pathways
			STI-1 binding induces activation of mitogen-activated protein kinase[36]
			Binds LRP/LR and HSPG by means of separate sites[60]
			Binds laminin[74]
	Synaptogenesis, polarization	Signalling	PrP^C acts as a growth factor, activating multiple pathways[38]
	Survival, trophic effects	Anti-apoptotic	Interacts with BAX[31], STI1 (ref. 36) and NCAM[75]
		Pro-apoptotic	Binds to anti-apoptotic Bcl-2 (for a review, see ref. 31)
			Crosslinks with anti-PrP antibody[76]
			Increases levels of p53 (reviewed in ref. 31)
	Copper binding	Copper endocytosis	Induces PrP^C to aggregate, exit from rafts and undergo clathrin-dependent endocytosis[77]
		Copper homeostasis	Maintains appropriate copper levels at the presynaptic membrane and during conditions of oxidative stress (reviewed in ref. 34)
		SOD activity*	Copper-bound PrP^C has SOD activity (reviewed in ref. 34)
	Redox homeostasis	Signalling	Induces NADPH-oxidase dependent ROS through Fyn activation[78]
Neural stem cells	Neurogenesis	Unknown	Increases cell proliferation in neurogenic regions[46]
	Differentiation	Unknown	PrP^C levels positively influence differentiation[46]
Haematopoietic stem cells	Long-term renewal	Anti-apoptotic? Homing?	Possible mechanisms: transduce cell survival signals; cell-adhesion activity targets cells to appropriate environment; or function as co-receptors for hormones affecting HSC activity[45]
T cells	Activation	Signalling?	PrP^C upregulation upon mitogen-induced activation[79]
	Development	Antioxidant	Copper binding in thymus[80]
Leukocytes	Differentiation	Unknown	PrP^C expression by lymphocyte/monocyte lineage[81]
	Phagocytosis	Unknown	PrP^C modulates phagocytosis[44]
	Inflammatory response	Homing	PrP^C alters leukocyte recruitment to site of inflammation[44]

*Some argue that PrP does not exhibit SOD activity.

Table 3. Cellular distribution and activities of PrP^C in cell types in which known or putative functions have been described (Caughey and Baron 2006).

PrP^C SIGNALLING IN NEURONAL CELLS

Since PrP^C is mainly localized on the outer leaflet of the cell membrane as a GPI-anchored protein, it has been extensively studied its role as a cell receptor. Upon binding, PrP^C triggers intracellular signals, via its binding to other plasma membrane molecules, including laminin receptor and neural cell adhesion molecules (Gauczynski, Peyrin et al. 2001, Schmitt-Ulms, Legname et al. 2001). Signalling pathways that were found activated i*n vitro* suggest an involvement of PrP^C in neuronal development, differentiation and neurite outgrowth (Zanata, Lopes et al. 2002, Chen, Mange et al. 2003, Kanaani, Prusiner et al. 2005, Santuccione, Sytnyk et

al. 2005). PrP^C has been shown to induce rapid phosphorylation of ERK1/2 (Grewal, York et al. 1999) and its activation through the Fyn kinase cascade (Mouillet-Richard, Ermonval et al. 2000, Schneider, Mutel et al. 2003). As a GPI-anchor protein, PrP^C can transfer and transduce signals from cells to cells (Liu, Li et al. 2002). The activation of the cAMP/protein kinase A (PKA) observed in retinal explants from neonatal mice using a PrP binding peptide (Chiarini, Freitas et al. 2002) and the interaction of PrP^C with the stress inducible protein 1 (STI1) suggest a neuroprotective role for PrP^C in cells, through the activation of the ERK1/2 and PI3K-Akt-mTor pathways (Zanata, Lopes et al. 2002, Roffe, Beraldo et al. 2010). Moreover, interaction with Grb2 adaptor protein in the endosomal compartment further stresses PrP^C involvement in neuronal survival (Spielhaupter and Schatzl 2001). The interaction with STI1 efficiently inhibited Aβ oligomers binding to PrP^C *in vitro* and in mouse primary hippocampal neurons, preventing synaptic loss and neuronal death (Ostapchenko, Beraldo et al. 2013). Following the interaction of Aβ oligomers, PrP^{Sc} and other β-sheets peptides, the neuroprotective function of PrP^C is lost and cells undergo PrP^C-dependent induction of apoptosis via caspase 3 (Resenberger, Harmeier et al. 2011). Moreover, PrP^{Sc} interaction with PrP^C induces a Jun N-terminal kinase dependent pro-apoptotic signalling at the expense of the pro-survival ERK1/2 signalling (Rambold, Muller et al. 2008).

PrP^C ROLE IN SYNAPSES

The presence of PrP^C in pre- (Herms, Tings et al. 1999) and postsynaptic structures (Haeberle, Ribaut-Barassin et al. 2000) argues for an important role of this protein in the normal development of synapses and in maintenance of their functionality (Moya, Sales et al. 2000, Kanaani, Prusiner et al. 2005). Moreover, the finding that some PrP^C glycoforms can be selectively transported along axons suggests a specific presynaptic function (Rodolfo, Hassig et al. 1999). Prion diseases are characterized by synaptic disorganization and loss, irrespective of the presence or absence of spongiform changes (Clinton, Forsyth et al. 1993), phenomenon accompanied by

PrP^{Sc} deposition in synaptic terminals (Kitamoto, Shin et al. 1992, Grigoriev, Escaig-Haye et al. 1999) and reduced exosomes-associated synaptic vesicle proteins in TSEs patients (Ferrer, Rivera et al. 1999). The importance of PrP^{C} in synapses has been corroborated by electrophysiological studies in CA1 hippocampal neurons from $PrP^{-/-}$ mic. In these neurons, excitatory glutamatergic synaptic transmission, GABAA receptor–mediated fast inhibition, long-term potentiation, and late after hyperpolarization were reduced or absent (Carleton, Tremblay et al. 2001, Mallucci, Ratte et al. 2002) when PrP^{C} was missing. Moreover, synaptic dysfunction related to $PrP^{-/-}$ mice could be related to altered circadian rhythms and sleep (Tobler, Gaus et al. 1996) and impaired hippocampal dependent spatial learning (Criado, Sanchez-Alavez et al. 2005).

PROTECTION AGAINST OXIDATIVE STRESS

Increasing evidence suggests that PrP^{C} improves resistance to oxidative stress, through chelation of free copper ions responsible for reactive oxygen species (ROS) generation (Brown 2001). PrP^{C} deficient neurons are more susceptible to oxidative stress than wild-type cells, an evidence explained *in vivo* by a reduction of the Cu/Zn superoxide dismutase (SOD) activity and high levels of oxidative damage to proteins and lipids (Brown, Schulz-Schaeffer et al. 1997, Brown, Nicholas et al. 2002). Injection of copper in the rat hippocampus results in memory impairment, neuronal loss and astrogliosis. This effect was rescued by co-injection of a peptide (PrP59-91) corresponding to the OR of PrP^{C}, thus suggesting a regulatory role of PrP^{C} in copper homeostasis (Chacon, Barria et al. 2003). Moreover, mitochondria morphological alterations have been described in scrapie infected hamsters (Choi, Ju et al. 1998) and mice (Lee, Sohn et al. 1999), as well as in mice lacking PrP^{C} (Miele, Jeffrey et al. 2002). Since mitochondria are important players in oxidative stress as well as in induction of apoptosis, it seems that the two effects are not mutually exclusive.

ANTIAPOPTOTIC FUNCTION

A survival-promoting effect mediated by PrP^C has been observed both *in vitro* and *in vivo* on neuronal and nonneuronal cells. Roucou and co-workers suggested a cytoprotective role of PrP^C against Bax-mediated apoptosis in human primary neurons (Roucou, Guo et al. 2003, Roucou, Giannopoulos et al. 2005). This anti-apoptotic function against Bax-mediated cell death was attributed to the similarity of the Bcl-2 homology domain (BH2) of the Bcl-2 proteins and the OR. Neuroprotection was nullified if the OR region of PrP^C is completely deleted (Bounhar, Zhang et al. 2001). Overexpression of Bax and PrP^C completely abolished Bax-mediated cell death, as well as did the co-expression of Bcl-2. The anti-Bax function requires a mature PrP^C, as treatment of cells with inhibitors of transport through ER and Golgi as well and beyond *trans*-Golgi vesicles prevents PrP^C anti-apoptotic function. However, the GPI anchor is not essential for the anti-Bax function (Roucou, Giannopoulos et al. 2005). Furthermore, expression of PrP^C in the cytosol is sufficient to inhibit Bax-mediated cell death (Roucou, Guo et al. 2003). These results are in line with previous findings on PrP^C rescue from Bax-mediated apoptosis caused by serum deprivation in neuronal cells (Deckwerth, Elliott et al. 1996, Kuwahara, Takeuchi et al. 1999). Protection from apoptosis was also investigated in a screening of gene-expression profile in a cell clone of MCF-7 breast cancer cells resistant to TNFα-induced apoptosis, where PrP^C was found overexpressed. Conversely, MCF-7 cells acquired resistance to TNFα-induced apoptosis after overexpression of PrP^C (Diarra-Mehrpour, Arrabal et al. 2004). However, PrP^C does not protect human neuroblastoma SK-H-SH and BE(2)-M17 cell lines, mouse neuroblastoma N2a cells and human embryonic kidney 293 cells against Bax overexpression (Roucou, Gains et al. 2004). Neuroprotection of PrP^C was confirmed *in vivo* in post-ischemic brain rodents which show a higher PrP^C level compared to controls (Weise, Crome et al. 2004, Shyu, Lin et al. 2005) and in rat brains in which the overexpression of PrP^C improved neurological behaviour after ischemia (Shyu, Lin et al. 2005). Moreover, in a model of focal brain ischemia, $PrP^{-/-}$ mice displayed three times larger infarct size compared to $PrP^{+/+}$ mice (Mitteregger, Vosko et al. 2007). Mice deleting of aa 32-93 had infarct size similar to $PrP^{-/-}$ mice,

identifying the N-terminal domain as player in PrP^C neuroprotection. Increased levels of caspase-3 and reduction of Akt activation were found in the ischemic brains (Spudich, Frigg et al. 2005, Weise, Sandau et al. 2006).

CONTROVERSY AROUND PrP^C ANTIAPOPTOTIC FUNCTION

Depending on the cell type, overexpression of PrP^C could results in a toxic effect rather than in protection. Human embryonic kidney 293 (HEK293), rabbit epithelial Rov9, murine cortical TSM1 cell lines are susceptible to the apoptotic inducer staurosporine when PrP^C is overexpressed (Paitel, Fahraeus et al. 2003). Apoptosis in these cells is mediated through p53 (Paitel, Sunyach et al. 2004). These results, that are in contrast with the Bax-mediated anti-apoptotic function of PrP^C, could be explained by several causes. PrP^C overproduction may interfere with the production of essential proteins in these cells. PrP^C overexpression may lead to the formation of the transmembrane form of PrP, ^{Ctm}PrP, characterized by the N-terminal in the cytoplasm and the C-terminal facing the ER lumen. ^{Ctm}PrP induces neuronal cell death *in vitro* and *in vivo* (Hegde, Mastrianni et al. 1998). Cytosolic PrP^C, retro-translocated from the ER, could be toxic in N2a cell line and cerebellar granule neurons in mice (Ma and Lindquist 2001, Yedidia, Horonchik et al. 2001, Ma, Wollmann et al. 2002) or non- toxic in HEK293, COS-1, BE(2)-M17 and SK-N-SH cells and human primary neurons (Drisaldi, Stewart et al. 2003, Heller, Winklhofer et al. 2003). In light of this, it can be postulated that the effect of PrP^C depends on the cell type. Alternatively, when overexpressed, PrP^C could be converted in a lethal protein. As Bcl-2 and Bcl-xL cleavage by endogenous caspases results in a proapoptotic carboxy-terminal fragment (Cheng, Kirsch et al. 1997, Clem, Cheng et al. 1998), PrP^C cleavage in the caspase-like site at aa145 could generate a toxic fragment. Indeed, the PrP 145_{STOP} mutation is associated to a vascular form of prion disease (Kitamoto, Iizuka et al. 1993, Ghetti, Piccardo et al. 1996).

NEUROPROTECTION AGAINST Dpl AND N-TERMINALLY TRUNCATED PrP^C MEDIATED CELL DEATH

As mentioned above, re-expression of PrP^C in mice depleted from *Prnp* and overexpressing Dpl, rescues cerebellar Purkinje cells loss and ataxia (Nishida, Tremblay et al. 1999). Two mechanisms could explain the neuroprotective function of PrP^C. PrP^C and Dpl could compete for the same ligand, and the overexpression of Dpl prevents survival mechanisms activated by the binding of PrP^C (Behrens and Aguzzi 2002). Alternatively, Dpl could cause oxidative stress, an effect counteracted by the presence of the anti-oxidant property of PrP^C (Wong, Liu et al. 2001). Mice expressing a truncated NH2-terminal PrP^C (PrP Δ32-134) died of severe ataxia 1-3 months after birth. Re-expression of a single PrP^C allele was sufficient for rescue the disease. The PrP^C deleted domain is absent in Dpl, thus suggesting a common mechanism in the induction of the disease. In absence of PrP^C, PrPΔ32-134 could still bind to a putative ligand, but would not results in a survival signals (Shmerling, Hegyi et al. 1998).

HD DOMAIN AS THE PrP^C NEUROPROTECTIVE FRAGMENT

The HD domain has acquired increasing interest because of its role in different modulations of PrP^C. Besides membrane integration of minor topological isoforms, it has been found to be involved in α-cleavage and dimerization. Human PrP^C is physiologically β- and α-cleaved at amino acids 89/90 and 110–111/112, while a cleavage at amino acids 228/229 results in ectodomain shedding (Chen, Teplow et al. 1995, Mange, Beranger et al. 2004, Taylor, Parkin et al. 2009). α-cleavage produces a 17 kDa, N-terminally truncated fragment (PrPC1), anchored to the plasma membrane and a secreted 11kDa fragment (PrPN1) (Chen, Teplow et al. 1995, Mange, Beranger et al. 2004) (Figure 6). Mice overexpressing PrPC1 do not show a neurotoxic phenotype (Westergard, Turnbaugh et al. 2011) and PrPC1 is associated with protection against infection with the prion strain M1000 in cultured cells (Lewis, Hill et al. 2009). Moreover, PrPC1 acts as a dominant-negative inhibitor

of PrP^{Sc} conversion *in vivo* (Meier, Genoud et al. 2003, Westergard, Turnbaugh et al. 2011). Neuroprotection is also attributed to PrPN1 against *in vivo* ischemic stress and against oligomeric amyloid-β (Aβ)-associated cell death in primary neurons (Guillot-Sestier, Sunyach et al. 2009, Resenberger, Harmeier et al. 2011). The presence of amorphous aggregates of Aβ and PrPN1 and the increased levels of PrP α-cleavage in post mortem brain tissues from AD patients (Beland, Bedard et al. 2014) strongly support a neuroprotective mechanism mediated by the α-cleavage. In favour of this neuroprotective hypothesis, PrP^{Sc} and toxic PrP^{C} mutants are impaired in their α-cleavage (Oliveira-Martins, Yusa et al. 2010). Several studies demonstrate that the HD of PrP^{C} is essential for α-cleavage (Bremer, Baumann et al. 2010, Oliveira-Martins, Yusa et al. 2010). Interestingly, the HD is also essential for PrP^{C} homodimerization on the cell surface, a process that regulates PrP^{C} physiological neuroprotective/neurotrophic activities (Rambold, Muller et al. 2008). Stimulation of PrP^{C} dimerization in cell culture increases both PrP^{C} trafficking to the cell surface and the formation of all extracellular PrP^{C} metabolites: PrPN1, PrPC1 and shed PrP^{C} (Beland, Motard et al. 2012). Deletion of HD does not prevent PrP^{C} trafficking to the plasma membrane (Winklhofer, Heller et al. 2003), suggesting a non-essential role of dimerization for PrP^{C} trafficking. Roucou and colleagues proposed a model with a constitutive and dimerization-independent pathway for PrP^{C} secretion and a pathway regulated by dimerization (Figure 6). With this model, the cells are allowed to quickly respond to toxic insults by increasing the levels of protective PrP^{C} metabolites (Beland and Roucou 2013).

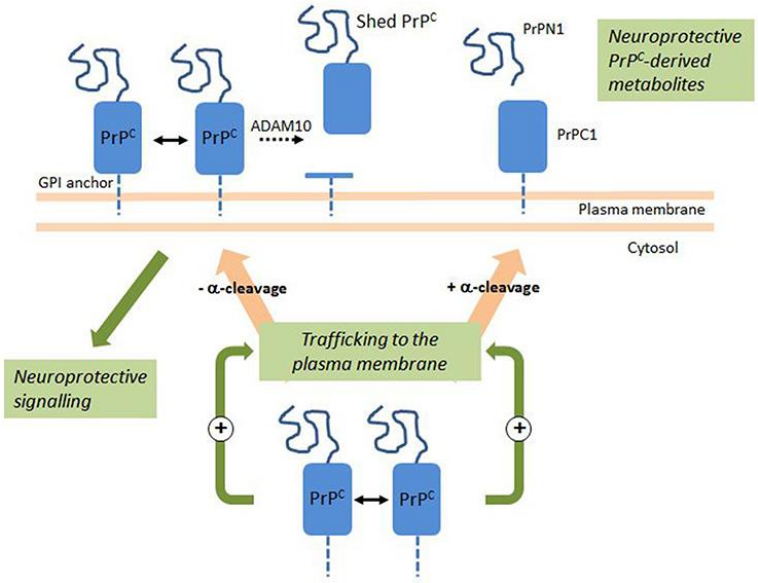

Figure 6. PrP^C dimerization activates a neuroprotective signalling pathway and the production of neuroprotective PrP^C-derived metabolites (Roucou 2014).

IMMUNE SYSTEM

Expression of PrP^C is differently regulated among the immune system, suggesting a specific role on the basis of the cellular type. However, the lack of PrP^C does not affect the maturation of immune cell compartments (Isaacs, Jackson et al. 2006). PrP^C sustains the self-renewal of hematopoietic stem cells (HSC) under stress conditions (Zhang, Steele et al. 2006) and is highly expressed in lymphocytes (Cashman, Loertscher et al. 1990). This evidence confers a putative role to PrP^C in mediating immune responses. PrP^C co-localizes with the T-cell receptor (TCR) in lipid rafts (Stuermer, Langhorst et al. 2004) and cooperates with TCR in T cell activation (Thomas, DeGasperi et al. 1991, Mattei, Garofalo et al. 2004). Within the T-cell compartment, CD8+ cells are characterized by a higher PrP^C expression level than CD4+ cells. CD3+ cells co-expressing the activation marker CD56 (N-CAM) exhibited significantly higher levels of PrP^C if compared to CD56 counterparts (Durig, Giese et al. 2000). In dendritic cells (DCs) PrP^C has been found to increase during maturation, when it co-localize with MHC class II (Burthem, Urban et al. 2001, Martinez del Hoyo, Lopez-Bravo et al. 2006) However, PrP^C expression it is not required for full

maturation of DCs. The importance of PrP^C in the regulation of the immunological synapse between DC and T-cell was strongly evidenced (Ballerini, Gourdain et al. 2006). Several studies have highlighted that prion infection occurs and propagate within in the immune system (McBride, Eikelenboom et al. 1992, Jeffrey, McGovern et al. 2000, Klein and Aguzzi 2000, Beringue, Couvreur et al. 2002, Prinz, Montrasio et al. 2002). While circulating lymphocytes do not show any detectable infectivity, B cells in lymphoid organs are able to accumulate prions in a PrP^C-dependent fashion, (Raeber, Klein et al. 1999). Susceptibility to peripheral prions in lymph nodes was observed even in the absence of follicular dendritic cells. Therefore, it is possible that poorly defined immune cells or stromal precursor cells might be capable of replicating prions (Kaeser, Klein et al. 2001, Prinz, Montrasio et al. 2002).

PrP^C TRAFFICKING

ENDOPLASMIC RETICULUM (ER) TRANSLOCATION

During translation, PrP^C is translocated to the ER following the recognition of an N-terminal signal peptide, that is cleaved in the ER lumen. The signal sequence is characterized by a "weak" efficiency of translocation if compared to other proteins and results in the segregation of different PrP^C populations. The ^{Ntm}PrP, the C-terminus of which faces the cytosol, and the secreted PrP (^{sec}PrP) are characterized by the N-terminus in the ER lumen. On the contrary, the ^{Ctm}PrP, with the N-terminus facing the cytosol, and the cytosolic PrP(cyPrP) have the N-terminus in the cytoplasm (Figure 7). PrP^C ER translocation is regulated by the HD domain, which acts as an internal signal sequence that competes with the N-terminal signal of PrP^C (Hegde and Lingappa 1999). Nascent chains, that fail to rapidly target the translocon after synthesis of the N-terminal signal, could target via the HD. The result is the generation of ^{Ctm}PrP (Kim, Rahbar et al. 2001, Kim and Hegde 2002). Indeed, in vitro synthesized ^{Ctm}PrP contains an uncleaved signal sequence (Stewart, Drisaldi et al. 2001). This feature is beneficial during ER stress since it reduces the translocation

into the ER, thus preventing PrP aggregation in the secretory pathway (Kang, Rane et al. 2006). However, it is detrimental in the context of genetic mutation in prion diseases. Indeed, natural point mutants in human PrP^C that present ^{Ctm}PrP are associated with GSS, in the case of PrP(A117V) and PrP(G131V) (Tateishi, Kitamoto et al. 1990, Hsiao, Cass et al. 1991, Panegyres, Toufexis et al. 2001), as well as with CJD, as for PrP(G114V) (Liu, Jia et al. 2010). The common feature of all these mutant forms of PrP^C is an increased HD hydrophobicity, which influence the efficiency and timing of the post targeting function of the signal sequence in initiating N-terminus translocation (Kim and Hegde 2002). Substitution of the signal sequence of PrP^C with the more efficient prolactin one in mice, revert the effect of GSS mutants improving the formation of ^{sec}PrP and rescuing animals from the disease (Rane, Chakrabarti et al. 2010).

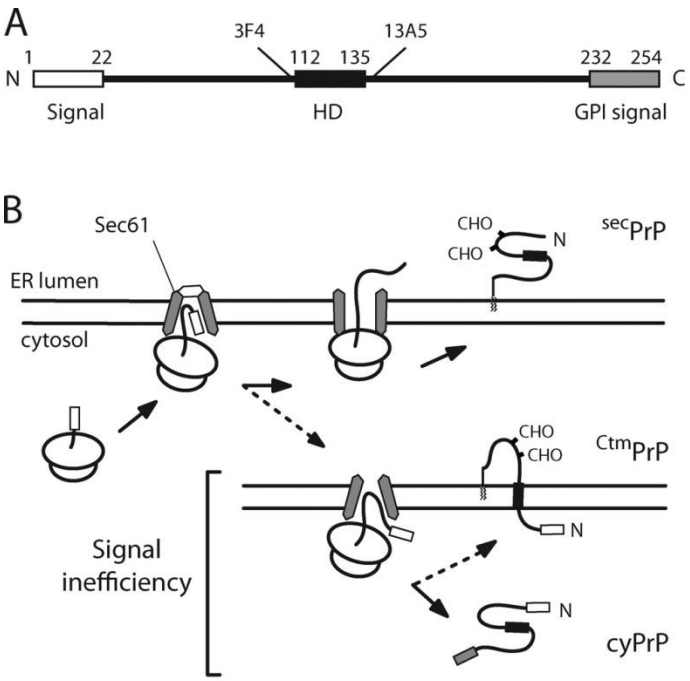

Figure 7. **Signal efficiency mediated formation of different PrP^C populations.** (A) Line diagram showing elements involved in PrP^C translocation. (B) Steps in PrP^C translocation. PrP^C is targeted to a Sec61 translocon via its N-terminal signal sequence. Translocation initiates when Sec61 interacts with gates and open the channel. Further protein synthesis results in complete translocation into the ER lumen to generate ^{sec}PrP. This pathway is followed by the majority of PrP^C polypeptides synthesized. Bottom: intrinsic inefficiencies in the signal sequence interaction with the translocon can cause a small proportion of PrP^C polypeptides to fail at the crucial gating/initiation steps. In the

first case, the polypeptide is expelled into the cytosol to generate cyPrP. Alternatively, the central HD, particularly if carrying a mutation that increases hydrophobicity, can engage the nearby translocon to generate ^{Ctm}PrP (Rane, Chakrabarti et al. 2010).

MATURATION IN THE SECRETORY PATHWAY

During passage in the ER, PrP^{C} undergoes N-glycosylation of null, one or two glycans to the two asparagine residue in position 181 and 197, in human. Several modifications are made on the N-linked glycan chains and a disulphide bond is formed between its two cysteines (C180 and C214). Once added of the GPI anchor, PrP^{C} is translocated to the Golgi apparatus, where the association with distinct membrane rafts is necessary for the correct fording of the protein (Sarnataro, Campana et al. 2004). Finally, the protein is exocytosed to the cellular membrane where is usually localized in lipid rafts domains (Lewis and Hooper 2011).

ENDOCYTOSIS

PrP^{C} could re-enter the cell with different ways of endocytosis: the most common lipid rafts, or caveolae-like domains, that drive also the internalization of PrP^{Sc} (Vey, Pilkuhn et al. 1996) and the clathrin-coated vesicles (Peters, Mironov et al. 2003). Alternatively, endocytosis of PrP^{C} has been observed upon interaction with laminin receptor (Gauczynski, Peyrin et al. 2001). It is likely that small GTP binding Rab proteins could control PrP^{C} trafficking (Zerial and McBride 2001). Rab7 was found to co-localize with PrP^{Sc} in late endosomes and lysosomes (Magalhaes, Baron et al. 2005), while Rab6 is involved in the retrograde transport of the PrP^{Sc} and may also regulate trafficking of PrP^{C} (Beranger, Mange et al. 2002). Only a small fraction of the endocytosed PrP^{C} is degraded by the lysosomes, while a large fraction returns to the cell surface. Moreover, in the intracellular trafficking of PrP^{Sc}, part of the recycled PrP^{C} is secreted to the extracellular space with exosomes (Fevrier, Vilette et al. 2004, Porto-Carreiro, Fevrier et al. 2005, Robertson, Booth et al. 2006).

Endocytosis should not be only investigated as a way for the internalization and propagation of PrP^{Sc} (Laszlo, Lowe et al. 1992), but also for putative PrP^{C} physiological roles. PrP^{C} could be seen as a broad-spectrum sensor at the cell surface. PrP^{C} ligand-mediated internalization could serve as transport and homeostasis of different ligands, including copper and hemin (Vassallo and Herms 2003, Lee, Raymond et al. 2007). It may regulate intracellular signalling after extracellular stimulation (Prado, Alves-Silva et al. 2004, Americo, Chiarini et al. 2007, Caetano, Lopes et al. 2008)or control PrP^{C} degradation (Kiachopoulos, Heske et al. 2004).

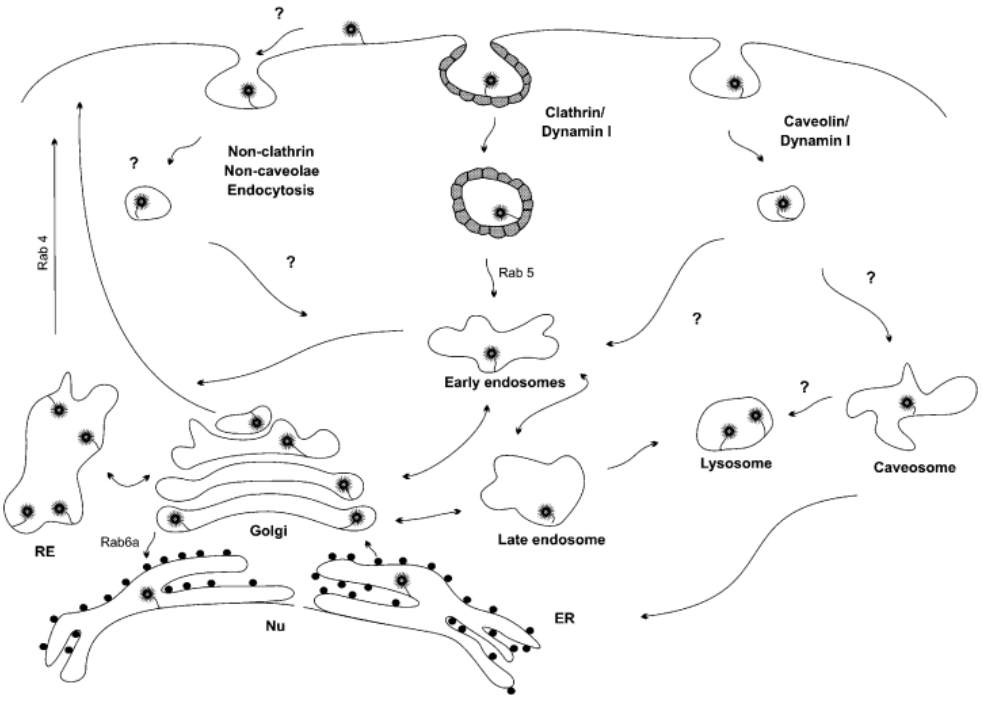

Figure 8: Endocytosis and subcellular localization of PrP^{C} (Prado, Alves-Silva et al. 2004).

CYTOSOLIC PrP

A variable amount of PrP^{C} has been found in the cytoplasm after *Prnp* overexpression but also in a subset of cerebellar neurons (Mironov, Latawiec et al. 2003).In physiological conditions, when ER quality control and UPS systems are fully functional, PrP^{C} is rarely detected in the cytoplasm. Instead, under ER-stress

conditions, translocation of nascent PrP^C into the ER is prevented, thus resulting in increased levels of PrP^C in the cytosol (Kang, Rane et al. 2006, Orsi, Fioriti et al. 2006). Moreover, when the activity of the proteasome system is compromised, PrP^C rapidly accumulates in the cytoplasm, suggesting the existence of a constant flux of unfolded or misfolded PrP^C to this compartment. In this view, PrP^C would appear in the cytoplasm when aging or environmental stresses compromise the system (Ma and Lindquist 2001). In prion pathology, two mutations related to the GSS, the Q217R and $Y145_{STOP}$, generate mutated forms of PrP^C that accumulate in the ER and other membrane-bound compartments when proteasome activity is blocked (Zanusso, Petersen et al. 1999). Interestingly, prion-infected mice have cytoplasmic PrP^C aggregates (Kristiansen, Messenger et al. 2005). However, it is still under debate if the presence of PrP^C in the cytosol is directly associated to neuronal pathogenesis (Ma and Lindquist 2001, Ma, Wollmann et al. 2002, Wang, Wang et al. 2005, Campana, Sarnataro et al. 2006, Orsi, Fioriti et al. 2006, Wang, Wang et al. 2006) or it exerts a physiological/ neuroprotective function (Roucou, Guo et al. 2003, Fioriti, Dossena et al. 2005, Jodoin, Laroche-Pierre et al. 2007), as discussed before.

MECHANISMS OF ACCUMULATION AND DEGRADATION

The fate of PrP^C inside the cells is still subjected to intense study. Presently, the mechanisms leading to the formation of PrP^C aggregates are unknown. Most importantly, it still needs to be clarified the role of aggregates both in the physiological settings and in the pathological conditions. First of all, it has to be clarify that protein aggregation is a continuous process in cells. Many physiological processes depend on the transient aggregation of proteins. In other cases, protein aggregates result from misfolding caused by stress conditions. Damaged proteins are degraded by the proteasome or by chaperone-mediated autophagy when processed by single peptides units. Undissolved large protein aggregates are

degraded by macroautophagy (aggrephagy). The degradation system becomes compromised in conditions of overwhelm of misformed proteins or toxic pathological forms. However, it is still not clear if the formation of aggregates is a cytoprotective mechanism, aimed at sequestering toxic proteins to avoid cellular damage.

PATHOLOGICAL AGGREGATES

In neurodegenerative diseases, classical protein aggregation follows the model of mass action and energy minimization proposed by Dobson and Lansbury (Jarrett and Lansbury 1993, Dobson 2003): aggregation prone proteins are present in the cell as monomers, that misfold randomly in a process devoid of biological function. In this model, the formation of aggregates is dependent on the amount of starting material and by the HD, which determines the propensity of the protein to aggregate. Oligomers and fibrils that derive from this process overcome the action of chaperones and of the proteasomal and autophagic systems, resulting in cell death and inflammation (Figure 9). Broader neurodegeneration is explained in this way as the amyloid cascade (Selkoe and Schenk 2003). This model well suits for example the cases of PD and prion diseases, where aggregates of misfolded α-synuclein or PrP^{Sc} are found in neuronal cells throughout the CNS.

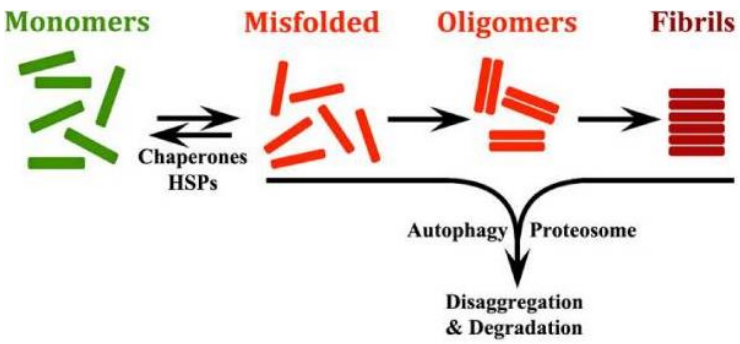

Figure 9: conventional model for degenerative disease based on mass action and hydrophobic interactions. Monomeric proteins randomly misfold. The chaperone system, including heat shock

proteins (HSPs), can reverse the misfolding, and produce normal, functional proteins. However, the misfolded proteins are prone to random oligomerization. The oligomers aggregate further to form fibrillar aggregates. Formation of oligomers and fibrils is considered to lack normal protein biological functions. These oligomers and fibrillar aggregates can be removed by degradation, which occurs through the actions of the autophagic system and the ubiquitin proteasomal system. (Wolozin 2012)

OTHER CELLULAR AGGREGATES

The classical model of pathological aggregation contrasts with the highly regulated and reversible aggregation that occurs in the biology of RNA binding proteins. Many of these proteins have been found to be involved in motor neuron diseases. These proteins contain a hydrophobic glycine motif, that mediates their reversible aggregation, and a RNA recognition motif (RRM)(Kim, Kuwano et al. 2007). Aggregation of RNA binding proteins with bound RNA form an insoluble macromolecular structure, named stress granule (SG). The function of SG is to shift quickly RNA translation towards cytoprotective proteins (Kedersha, Cho et al. 2000). SG formation is well understood in pathway of ER- stress conditions (Kedersha and Anderson 2007). The yeast Sup35 protein, a termination factor that assists in proper protein translation, aggregates in an analogous way. Moreover, Sup35 undergoes self-perpetuating changes in conformation and is epigenetically inherited upon cell division or by mating (Wickner 1994). In an elegant study conducted by the group of Vorberg, it was demonstrated that cyPrP and C-terminal PrP^{C} (PrP 90-231) are able to aggregate in a Sup35-like fashion, as a spontaneous event in the cell, rather than depending on sequestration in aggregation-prone proteins (Krammer, Suhre et al. 2008). Independently from its intrinsic ability to aggregate, cyPrP inhibits the formation of SGs by sequestering the RNA into aggresomes (Beaudoin, Goggin et al. 2008) and block Hsp70 synthesis. Inhibition of the stress-response could be the explanation of the toxicity of cyPrP observed *in vivo* and the reason of a short cyPrP permanence in the cytosol (Roucou 2009). To conclude, this evidence suggests that aggregate formation is not necessarily dictated by misfolded proteins.

A second type of aggregates is represented by aggresomes. Aggresomes have a juxtanuclear localization, at the centrosome (or microtubule organizing centre , MTOC), are positive for γ-tubulin and are surrounded by a cage of vimentin filaments. Aggresome formation is regulated by the microtubule-associated histone deacetylase 6 (HDAC6). Upon binding to their Lys63-polyubiquitylated chains (Hook, Orian et al. 2002, Du, Wang et al. 2014), HDAC6 recruits misfolded proteins to dynein motors on microtubules (Johnston, Ward et al. 1998, Hubbert, Guardiola et al. 2002). Deposition of protein aggregates into aggresomes has been proposed as a common mechanism in neurodegenerative diseases (Johnston, Ward et al. 1998). The regulation of HDAC6 in the formation of cytoplasmic aggregates in PD, HD (Tran and Miller 1999) and its presence in Lewy bodies (McNaught, Shashidharan et al. 2002) suggests a link between the formation of aggresomes and pathological aggregates. Lys63-linked polyubiquitylation promoted by the E3 ubiquitin ligase Parkin results in HDAC6-mediated transport into aggregates under condition of proteasomal impairment (Olzmann, Li et al. 2007, Tan, Wong et al. 2008). Moreover, it underlies HDAC6 function in reducing the effect of misfolded protein-induced stress (Taylor, Tanaka et al. 2003). Indeed, formation of aggregates may have a cytoprotective function (Tanaka, Kim et al. 2004, Chesebro, Trifilo et al. 2005, Cohen, Bieschke et al. 2006, Douglas, Treusch et al. 2008), and might also facilitate the efficiency of aggregate removal by autophagic clearance (Taylor, Tanaka et al. 2003, Kaganovich, Kopito et al. 2008, Tan, Wong et al. 2008), as seen in models of PD, HD and spinobulbar muscular atrophy (SBMA) (Webb, Ravikumar et al. 2003, Iwata, Riley et al. 2005, Pandey, Nie et al. 2007, Su, Shi et al. 2011). Cytotoxicity of PrP106-126 peptide is regulated by HDAC6 through the induction of autophagy and activation of the PI3K-Akt-mTOR axis (Zhu, Zhao et al. 2016). Mutants that are not fully translocated in the ER (PrP V203L, E211Q and Q212P) are found in aggresomes in M17 human neuroblastoma cells (Mishra, Bose et al. 2003). However, cyPrP (PrP 90-231) is found in aggresomes in N2a cells, but not in Hela, Cos-7 and Huh-7 cells, where it is present in dispersed aggregates, that do not share characteristic features of aggresomes (Beaudoin, Goggin et al. 2008). This evidence

suggests that aggresomes are not the only final product of protein misfolding, and that other mechanisms of protein sequestration have to be investigated.

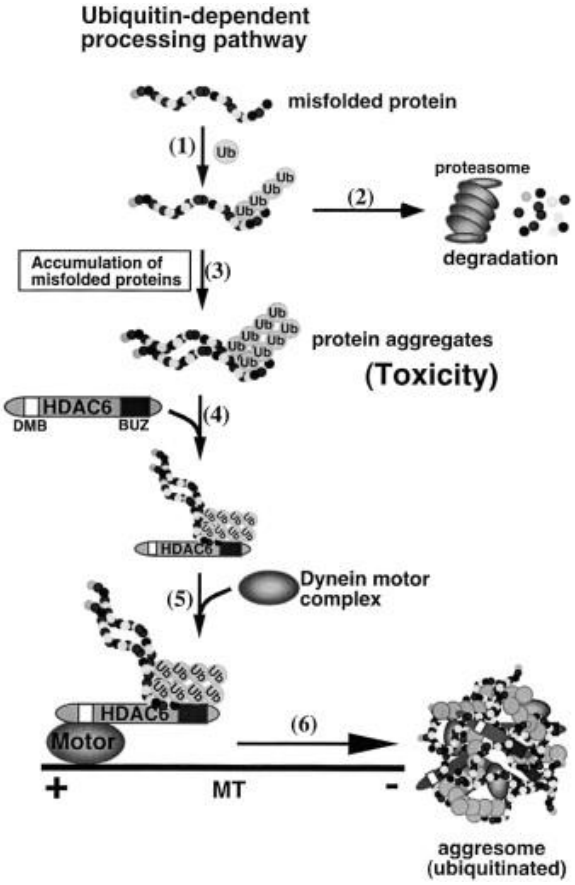

Figure 10. Model for HDAC6-dependent processing of misfolded proteins and aggresome formation. (Kawaguchi, Kovacs et al. 2003)

Another way to sequester misfolded proteins is represented by the accumulation in sequestosome, by the action of SQSTM-1/p62. Polyubiquitylated proteins are sequestered and degraded by the autophagolysosomal pathway (Bjorkoy, Lamark et al. 2005, Komatsu, Waguri et al. 2007, Pankiv, Clausen et al. 2007, Ichimura, Kumanomidou et al. 2008) or linked to the proteasome (Seibenhener, Babu et al. 2004). SQSTM-1 is present in neurofibrillary tangles in the early phase of AD (Kuusisto, Salminen et al. 2002). In vitro evidence for p62-mediated pathological PrP^{C} degradation is found in the clearance of PrP^{Sc} (Homma, Ishibashi et al. 2014) as well as of cyPrP and PrP-PG14, a mutant with an extra nine-octapeptide insertion

associated to CJD in HEK293 cells (Xu, Zhang et al. 2014). Sequestosomes are thought to be the equivalent of aggresomes in neurodegenerative diseases (Shin 1998, Zatloukal, Stumptner et al. 2002).

Another kind of distinction has to be done in order to define two subcellular compartments: the juxtanuclear quality control (JUNQ) and the insoluble protein deposit (IPOD). JUNQ is distributed along the ER, where cellular quality control components could efficiently clear misfolded proteins that are retrotranslocated from the ER. Ubiquitin, chaperones and the 26S proteasome components are present in the proximity of JUNQ. On the contrary, IPOD are inert aggregates of insoluble not-ubiquitylated sequestered proteins (Kaganovich, Kopito et al. 2008). Involvement of IPOD in the disaggregation of the prion protein has also been postulated (Muchowski and Wacker 2005).

CLEARANCE MECHANISMS

The autophagic system and the ubiquitin/proteasome system (UPS) are the main proteolytic systems involved in the cellular protein quality control (Ciechanover 2005). Molecular chaperones and cargo-recognition molecules are involved in defining the fate of degradation of cellular proteins (Douglas, Summers et al. 2009).

THE AUTOPHAGIC SYSTEM

In the autophagic system, the catalytic component is represented by the lysosome. The lysosomal lumen is reached by cytosolic proteins for degradation via three different mechanisms: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA) (Lynch-Day, Mao et al. 2012). In macroautophagy, a whole region of the cytosol is sequestered into a double membrane vesicle that fuses with lysosomes. In microautophagy, invagination of the lysosomal membrane traps regions of the cytosol that are internalized into the lysosomal lumen as single

membrane vesicles. Lastly, in chaperone-mediated autophagy, a targeting motif in the substrate proteins is recognized by a cytosolic chaperone that delivers it to the surface of the lysosome. Binding of the substrate to a lysosomal receptor leads to its multimerization and to the formation of a translocation complex. A luminal chaperone mediates the translocation of the substrate protein into the lumen where it is rapidly degraded (Figure 11).

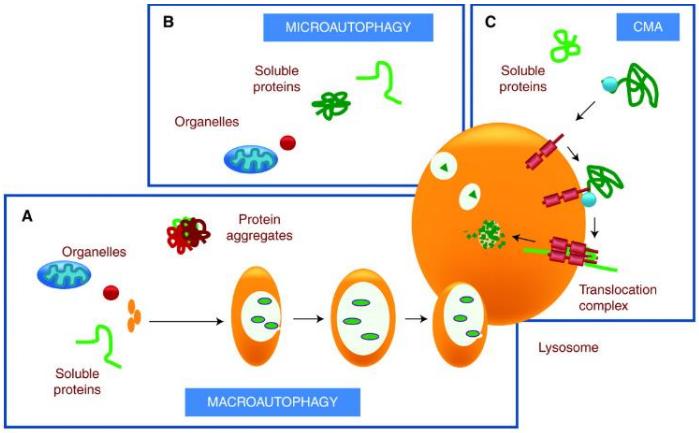

Figure 11. Autophagic pathways. (Wong and Cuervo 2010)

Hsc70, the heat shock-cognate protein of 70 kDa, is the constitutive chaperone involved in CMA. Once bound to Hsc70, the substrate is targeted to the lysosome where it interacts with the single-span membrane protein lysosome-associated membrane protein type 2 (LAMP2) (Cuervo and Dice 1996). Increased levels of α-synuclein and LRRK2 beyond a tolerable threshold results in toxic effect on CMA (Cuervo, Stefanis et al. 2004, Orenstein, Kuo et al. 2013). In chronically-infected cells, autophagy appears to be the major route of delivery of PrP^{Sc} into lysosomes (Heiseke, Aguib et al. 2010, Yao, Zhao et al. 2013) Indeed, late endosomes, lysosomes and autophagic vesicles increase greatly in prion-infected brain tissue (Boellaard, Kao et al. 1991, Sikorska, Liberski et al. 2004, Liberski, Sikorska et al. 2010). In Figure 12 the pathway of PrP^{Sc} cell entrance, replication and degradation is shown

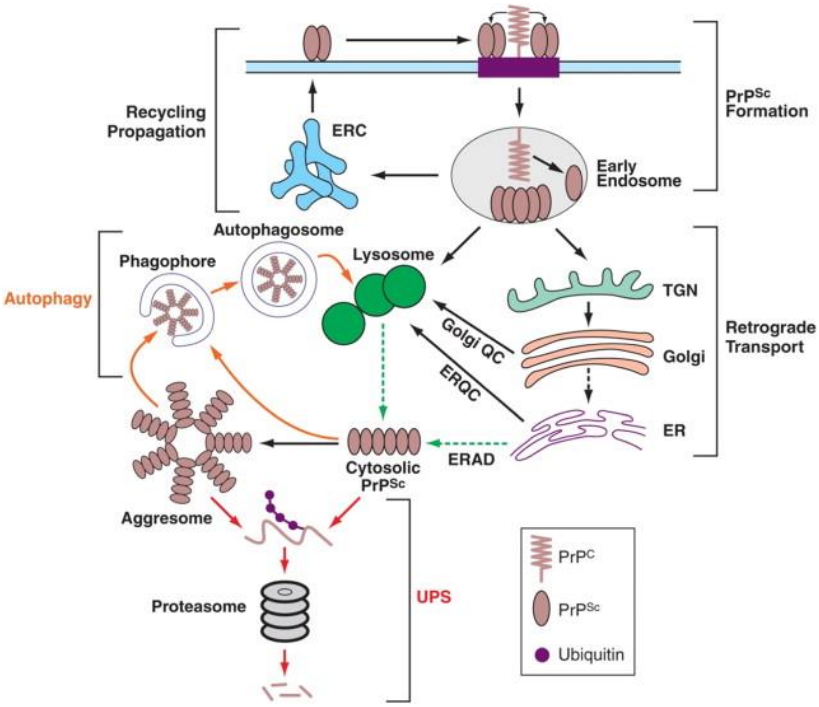

Figure 12. PrP^{Sc} formation, trafficking and degradation. Schematic illustration of the PrP^{Sc} metabolism. PrP^{Sc} forms at the plasma membrane or shortly after endocytosis in endosomes, endocytic recycling compartment (ERC) or lysosomes. Prion propagation is allowed by recycling of PrP^{Sc} to the plasma . Newly formed PrP^{Sc} undergoes retrograde transport to the trans Golgi network (TGN) and Golgi where it is subject to the Golgi quality control and trafficked to lysosomes for degradation. More mature forms of PrP^{Sc} are trafficked to lysosomes via the endolysosomal and autophagic pathways. PrP^{Sc} may reach the cytosol through lysosomal rupture or ERAD, and accumulates in aggresomes when the proteasomal activity is impaired. Proteasomal degradation follows aggregates unfolding and ubiquitylation Aggresomal PrP^{Sc} and smaller insoluble forms are engulfed by phagophores and degraded by autophagic pathways (Goold, McKinnon et al. 2015)

THE UBIQUITIN-PROTEASOME SYSTEM

The UPS regulates the degradation of extra-lysosomal cytosolic, nuclear and ER-residing proteins. The tagging molecule involved in UPS-mediated degradation is ubiquitin. Once tagged to the substrate protein, ubiquitin leads to its degradation by the 26S proteasome. The 26S proteasome is found next to the intermediate filaments of the cytoskeleton (Scherrer and Bey 1994) and in association with the cytoplasmic side of the ER membrane (Palmer, Rivett et al. 1996, Reits, Benham et

al. 1997). A close relationship exists between UPS activity and ER stress: when the proteasome is inhibited, it induces ER stress and *vice versa* (Lee, Iwakoshi et al. 2003, Menendez-Benito, Verhoef et al. 2005). Impairment or overload of the UPS are likely to be the major contributors to the aggregation of ubiquitylated proteins in neuronal inclusion bodies. Indeed, in protein conformational disorder, the proteasome is directly targeted by the pathogenic proteins. Huntingtin, tau and α-synuclein inhibit proteasome activity by clogging the entrance of other substrates (Keck, Nitsch et al. 2003, Landles and Bates 2004, Bennett, Bence et al. 2005, Betarbet, Sherer et al. 2005). Oligomers of PrP^{Sc} are able to inhibit proteasome activity by blocking the β subunits of the 26S proteasome (Kristiansen, Messenger et al. 2005) (see the "UBIQUITYLATION OF PrP^{C}" section for further in-depth analysis of the UPS involvement in PrP^{C} degradation).

It is likely that in neurodegenerative conditions, an interconnection between different quality control systems and the various nature of toxically aggregated or misfolded proteins, as well as mutations in quality control players, results in the impairment of cell clearance and in the formation of aggregates. As previously described, this could be a way that the cells utilize to overcome the degenerative process.

THE UBIQUITIN CODE

Ubiquitin (Ub) is a highly stable molecule of 8 kDa, evolutionarily conserved from yeast to men. The essential role of ubiquitin is to generate a code, which triggers specific outcomes in the cell. According to linkage and fate, ubiquitin is covalently attached to lysine (Lys) residues of a target protein (ubiquitylation). Ubiquitylation is the result of the subsequent action of three different enzymes. The E1 Ub-activating enzyme forms a thioester bond between its catalytic cysteine and a Ub molecule in an ATP-dependent manner. Ub is then transferred to the catalytic cysteine of an E2 Ub-conjugating enzyme. An E3 ligating enzyme (ubiquitin ligase) is responsible for the transfer and covalent binding of the Ub from the E2 to the

substrate protein. Two different type of E3 proteins exists. The RING E3 ubiquitin ligase, that binds to both the Ub-charged E2 and the protein substrate to mediate transfer of the Ub C-terminus to a substrate lysine to form an isopeptide bond, resulting in protein ubiquitylation. Conversely, HECT E3 ligases accept first the Ub onto a catalytic cysteine and then transfer the C-terminus of Ub to a substrate lysine via a isopeptide bond. (Pickart and Eddins 2004). The ubiquitin pathway is characterize by a high versatility. First, ubiquitylation mode is affected by the combination of different E2 and E3 proteins, that catalyse the formation of distinct protein-Ub structures (Deshaies and Joazeiro 2009, Ye and Rape 2009). Second, Ub binding proteins possess different Ub binding domains (UBDs) that selectively recognize structural characteristics on a Ub molecule near its lysine (Lys) residues (Komander 2009, Ikeda, Crosetto et al. 2010). Third, the kind of Ub linkage formed can result in a compact conformation if Lys 48, Lys6 and Lys11 residues are involved, or in an open conformation when Met1 or Lys63 residues are involved. The resulting conformational flexibility/rigidity raises the possibility that UBDs remodel chains to increase interaction interfaces or to improve specificity (Sims and Cohen 2009, Bremm, Freund et al. 2010, Matsumoto, Wickliffe et al. 2010, Virdee, Ye et al. 2010).

CLASSIFICATION OF CLASSICAL AND ATYPICAL UB CHAINS

The classical known polyubiquitin chain is formed via Lys48 and discovered as a destruction tag for proteasomal degradation. However, several kinds of Ub chains exists (Figure 13). Homotypic chains are formed by the conjugation of a single type of Lys residue in sequential Ub molecules, whereas mixed-linkage chains are assembled through distinct Lys residues on Ub molecules. Moreover, a bifurcations could arise in mixed-linked chains, resulting in Lys6/11, Lys29/48 or Lys29/33 linked chains (Kim, Kim et al. 2007). Further modifications are applied by Ub-like modifiers such as SUMO and NEDD8, resulting in heterologous chains. Also, many mono Ubs attached to the substrate in a spatially packed proximity can be considered as

atypical Ub signals. Atypical ubiquitylation is involved in different non proteolytic signals, regulating signaling pathways, trafficking and localization of substrate proteins, as well as substrate's activity.

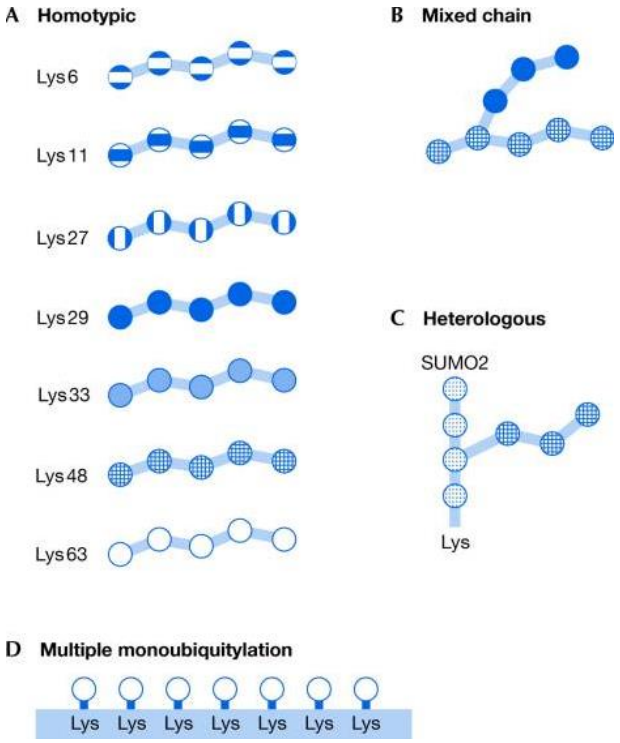

Figure 13. Schematic model of possible ubiquitin chain formations on a target protein. A) Homotypic atypical chains. B) Mixed-linkage atypical chains in which the use of different Lys for sequential Ub conjugation leads to the formation of bifurcated chains. C) Heterologous chains are formed between Ub and ubiquitin-like proteins, for example the small ubiquitin-like modifier 2 (SUMO2). D) multivalent chain-like Ub signals: multiple monoubiquitylation. (Ikeda and Dikic 2008).

PHYSIOLOGICAL ROLE OF ATYPICAL CHAINS

Regulation of protein activity is well explained for the NF-κB signaling, which involves different kind of Ub chains. NF-κB activation by TNF-α evokes different responses in the cells, include innate immune responses, cell survival, cell death and inflammation (Locksley, Killeen et al. 2001). Lys48 and Lys27 chains are necessary for the assembly of protein complexes, Lys48 chain for the degradation of the NF-κB inhibitor, E3 TRAF6 mediated Lys63 chain, cIAP1 mediated mixed Lys63/11 chain and LUBAC mediated Met1 chain are used for activation of IKK and its binding to the

TAK1 kinase complex (Deng, Wang et al. 2000, Wang, Deng et al. 2001, Dynek, Goncharov et al. 2010). Changes in localization of p53 protein is deciphered by multimonoubiquitylation (Li, Brooks et al. 2003). Regulation in protein interactions are likely to happen via Lys63 chains, as observed in the stabilization of polysomes (Spence, Gali et al. 2000), DNA damage conditions (Al-Hakim, Escribano-Diaz et al. 2010) or SMAD proteins interaction (Dupont, Mamidi et al. 2009). Moreover, Lys63 chain formation regulates the multivesicular body (MVB) pathway and is responsible for the sorting of receptors for endocytosis and their subsequent degradation through the endo-lysosomal pathway (Dunn and Hicke 2001, Lauwers, Jacob et al. 2009). Lys11 chain represents an independent proteasomal degradation signal (Kirkpatrick, Hathaway et al. 2006, Jin, Williamson et al. 2008). Lys27, Lys29 and Lys33 are instead involved in the clearance of damaged mitochondria (Geisler, Holmstrom et al. 2010, Glauser, Sonnay et al. 2011). Lys27 ubiquitylation of Jun and Lys29 ubiquitylation of Dexter result in the recruitment of autophagic and/or storage vesicles (Chastagner, Israel et al. 2006, Ikeda and Kerppola 2008). Recently, Lys27 has been found to trigger the host immune response after microbial DNA invasion (Wang, Liu et al. 2014). Instead, Lys33 has been found to be implicated in post-Golgi protein trafficking (Yuan, Lee et al. 2014).

UBIQUITYLATION OF PrP^C

Until now, the study of PrP^C ubiquitylation has been only focused on PrP^C degradation by the proteasome. For example, the endoplasmic reticulum associated degradation (ERAD) system has been found to be implicated in the ubiquitylation of the GSS associated mutant Q217R (Jin, Gu et al. 2000) and of $cyPrP^C$ constructs (Yedidia, Horonchik et al. 2001). Recently, the ER-associated gp78 E3 ubiquitin ligase has been found to ubiquitylate unglycosylated PrP^C (Shao, Choe et al. 2014). Furthermore, the co-chaperone Stub1 with E3 ubiquitin ligase activity has been identified as a PrP^C interactor (Gimenez, Richter et al. 2015). All the above evidence is focused on the degradation of PrP^C. Moreover, the ubiquitin-specific protease 14

(USP14), a deubiquitylating enzyme associated to the proteasome that catalyses trimming of polyubiquitin chains, has been shown to prevent the degradation of both PrP^{C} and PrP^{Sc} (Homma, Ishibashi et al. 2015). However, up to now, a defined scenario about PrP^{C} ubiquitylation and fate is still missing.

TRAF6

THE TRAF FAMILY

The tumor necrosis factor receptor (TNF-R)-associated factor (TRAF) family of intracellular proteins were originally identified as signalling adaptors that bind the cytoplasmic regions of receptors of the TNF-R superfamily (Inoue, Ishida et al. 2000, Wajant, Henkler et al. 2001, Ha, Han et al. 2009). In mammals, the TRAF family is composed by six members, with a high degree of similarity. All TRAFs are characterized by a C-terminal Ig-like meprin and TRAF homology domain (MATH), a coiled coil central domain (CC) and, with the exception of TRAF1, an N-terminal RING domain, followed by four zinc-finger motifs (RZF) (Wajant, Henkler et al. 2001, Ha, Han et al. 2009, Ostuni, Zanoni et al. 2010) (representative image of TRAF6 is shown in Figure 14). The C-terminal and central regions contribute to TRAF oligomerization and mediates the interaction with signalling proteins (Chung, Lu et al. 2007). The N-terminal domain comprises the core of the ubiquitin catalytic domain (Ostuni, Zanoni et al. 2010). Thus, TRAFs function in the regulation of signalling both as adaptor proteins and E3 ubiquitin ligases. Further studies have revealed TRAFs involvement in the signalling transduction of a variety of other receptor families, over than TNF-Rs, including receptors of the innate and adaptive immunity and cytokine C-type lectin receptors. The alteration of TRAFs leads to autoimmune diseases, cancers and immunodeficiencies (Hildebrand, Yi et al. 2011, Namjou, Choi et al. 2012, Netea, Wijmenga et al. 2012).

TRAF6

TRAF6 is unique among the TRAFs family because of its pleiotropic role in participating in the signal transduction of both the TNF-R and the interleukin-1(IL1-R)/Toll like receptor (TLR) superfamily. many receptor systems (Cao, Xiong et al. 1996, Lomaga, Yeh et al. 1999, Aderem and Ulevitch 2000, Chung, Park et al. 2002). Structurally, dimerization of the RING domain induces higher ordered oligomerization of the full TRAF6 protein. The constant shift between dimeric and trimeric symmetry of TRAF6 may be a way to facilitate multiple ligand-dependent signalling transduction (Yin, Lin et al. 2009). Structural difference of the C-terminal domain between TRAF6 and the other TRAFs is responsible of TRAF6 unicity in regulating signal transduction in adaptive and innate immunity and bone homeostasis (Ye, Arron et al. 2002).

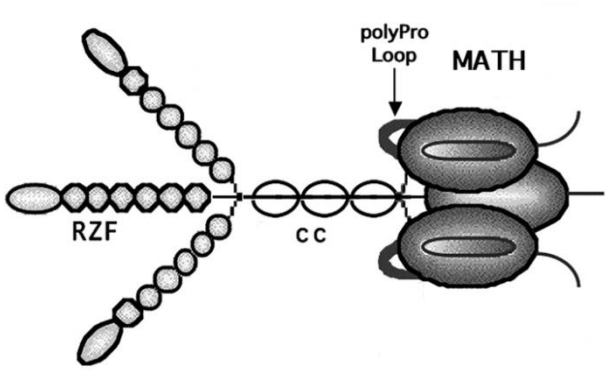

Figure 14. TRAF6 structure. TRAF6 is usually found as a dimer or trimer. In this schematic figure TRAF6 trimeric structure in shown. RZF: N-terminal Ring and zinc finger domain; CC: central coiled-coil region; MATH: C-terminal Ig-like meprin and TRAF homology domain Adapted from: (Wang, Wara-Aswapati et al. 2006).

Genetic ablation of TRAF6 ($TRAF6^{-/-}$) in mice results in perinatal death, with severe osteopetrosis, splenomegaly and thymic atrophy multiple organ abnormalities, indicating the essential role of TRAF6 in early development. (Lomaga, Yeh et al. 1999, Naito, Azuma et al. 1999).

TRAF6 IN CELL SIGNALLING

TRAF6 has been widely shown to act as mediator at the cross-road of diverse cell signalling pathways. The classical TLR-dependent activation of NF-κB is regulated by the interaction of MyD88 and stimulation of TRAF6 E3 ubiquitin ligase activity. After recruitment to signalling rafts, TRAF6 catalyses the attachment of Lys63-linked polyubiquitin chains onto its substrates, including itself (Lamothe, Besse et al. 2007, Keating and Bowie 2009, Ostuni, Zanoni et al. 2010) and synthesizes free unanchored Lys63 chains. TRAF6 functions as a scaffold to activate TAK1, resulting in the activation of NF-κB (Xia, Sun et al. 2009). TRAF6 is also involved in the signalling down-stream IL-1R/TLR. Upon ligand binding, IL-1R/TLR activate p38 and JNK pathways, via TRAF6 and TAK1 (Yamashita, Fatyol et al. 2008). Moreover, inhibition of the p38-JNK pathway by p62 and HDAC6 occurs through a negative regulation of the signalling complex MyD88-TRAF6 (Into, Inomata et al. 2010). In cellular responses to bacterial infection, TRAF6 has been shown to translocate to the mitochondria and increase the amount of ROS through ubiquitylation (West, Brodsky et al. 2011). During viral infection, the activation of the RIG-1 like receptors (RLRs) family of cytosolic RNA helicases triggers the direct binding of TRAF6 to mitochondrial antiviral signalling protein (MAVS), which in turn promotes the activation of the p-38, JNK and NF-κB (Yoshida, Takaesu et al. 2008, Konno, Yamamoto et al. 2009). In the immune response, TRAF6 is also involved in the signalling pathways activated by IL-17 receptor (Zhu, Pan et al. 2010), INF receptors (Yang, Murti et al. 2005), TGFβ receptor (Wang, McPherson et al. 2012), IL-2 receptor (Motegi, Shimo et al. 2011). Moreover, TRAF6 is implicated in the activation and proliferation of T cells (Bidere, Snow et al. 2006, Xie, Liang et al. 2013), in the maturation of dendritic cells (Yang, Chen et al. 2008) and in DNA damage response (Hinz, Stilmann et al. 2010).

In the brain, TRAF6 has been demonstrated to interact with NGF-activated TrkA and $p75^{NTR}$ receptors (Khursigara, Orlinick et al. 1999, Geetha, Jiang et al. 2005). This binding is mediated by p62 (Sanz, Diaz-Meco et al. 2000), after receptor dimerization and endocytic internalization (Grimes, Beattie et al. 1997, Riccio,

Pierchala et al. 1997). TRAF6 mediated Lys63 polyubiquitylation of TrkA in the juxtamembrane region of the receptor results in the activation of the TrkA-MAPK signalling (Traverse, Gomez et al. 1992, Yang, Wang et al. 2009). Overexpression of TRAF6 mediates activation of PI3K, actin polymerization and growth of long filopodia in transfected cells (Wang, Wara-Aswapati et al. 2006). The TRAF6 recognition sequence (Pro-X-Glu-X-X) binds to Src tyrosine kinase, which in turn activates PI3K (Wong, Besser et al. 1999). TRAF6-Src interaction is independent from sequestosomes, arguing that the IL1 signalling for actin remodelling results in the TRAF6 relocalization in the cytoplasm (Wang, Wara-Aswapati et al. 2006),. Although it has been reported that TRAF6 has a cytoplasmic localization before activation and translocation to the cell membrane (Force, Glass et al. 2000, Hostager, Catlett et al. 2000, Zapata, Pawlowski et al. 2001, Ha, Kwak et al. 2003), for some TNFR and TIR, TRAF6 seems to be associated to large membrane-bound sequestosomes (Puls, Schmidt et al. 1997, Sanz, Diaz-Meco et al. 2000, Seibenhener, Babu et al. 2004).

Substrates (Lys residues of ubiquitination)	Receptor signaling
TRAFs	
TRAF6	TLRs, IL-1R
Receptors	
IL-17R	IL-17
p75 (Lys274, 280 and 283)	NGF
TβRI	TGFβ
Kinases	
TAK1 (Lys158)	TNF-R1/2 and IL-1R
IRAK1 (Lys134 and 180)	TLR7, TLR9, IL-1R
Akt (Lys8 and 14)	IL-1R, IGF-1R
Fyn (K63)	TLR4
Adaptor proteins	
NEMO (Lys285, 321, 325, 326 and 399)	TLRs, IL-1R, NOD2
TRIF	TLR3, TLR4
NESCA	TrkA and p75
LAT (Lys88)	TCR
Other E3 ligases	
cIAP1/2	TLR4-, IL-1R-induced autophagy
Transcription factors	
IRF7 (Lys444, 446, and 452)	TLR7, TLR8, TLR9, LMP1, RIG-I
IRF5 (Lys410 and 411)	NOD2, TLR7, TLR9
Autophagy proteins	
Beclin 1 (Lys117)	TLR4-, IL-1R-induced autophagy
NDP52	TLR3-induced autophagy
Regulators of ROS production	
ECSIT	TLR1, 2, 4-induced ROS production

Table 3: Substrates of the E3 ligase activity of TRAF6. Adapted from (Xie 2013).

TRAF6 IN NEURODEGENERATIVE DISEASES

The first evidence of TRAF6 implication in neurodegeneration was revealed by its co-localization with Tau in AD post mortem brains (Babu, Geetha et al. 2005). More recently, in AD pathology TRAF6-dependent Lys63 ubiquitylation of presenilin 1 (PS1) has been found to regulate PS1 function as a passive ER Ca^{2+} leak channel, independently of its γ-secretase activity (Yan, Farrelly et al. 2013). Interestingly,

TRAF6 is also involved in the pathogenesis of PD and HD. Indeed, TRAF6 is able to promote an atypical ubiquitylation of mutant DJ-1 and α-synuclein in PD and huntingtin in HD via Lys6, Lys27 and Lys29, but not with its conventional Lys63 ubiquitylation. Through ubiquitylation, TRAF6 stimulates the formation of aggregates of the disease associated proteins (Zucchelli, Codrich et al. 2010, Zucchelli, Marcuzzi et al. 2011). Moreover, TRAF6 is present in post-mortem brains of sporadic PD patients (Zucchelli, Codrich et al. 2010).

AIM

Neurodegeneration caused by prions has been extensively studied. However, despite various evidence of PrP^C involvement in cell physiology, knowledge about its physiological function is still lacking. Moreover, confusion surrounding the evidence of cytosolic forms of PrP^C and the related functions has been increased by the use of many different deleted constructs, the cellular type and the animal background chosen. Genetic mutations further enrich the scenario in which subpopulations of PrP^C play a different role in the cell and where the GPI-membrane anchored PrP^C is able to interact with other PrP^C molecules or to mediate cellular signalling.

In this work, we focus our attention on TRAF6, an E3 ubiquitin ligase known to mediate signalling cascades upon receptor triggering. Recent evidence suggests the involvement of TRAF6 in the aggregation and atypical ubiquitylation of normal and mutated neurodegenerative-associated proteins. We investigated the interplay of TRAF6 on PrP^C in the context of protein binding, localization and aggregation.

CHAPTER 2

MATERIALS AND METHODS

CONSTRUCTS

Wild type (WT) and dominant negative (DN) FLAG and GFP tagged TRAF6 constructs and HA-ubiquitin WT were kindly provided by the laboratory of prof. Gustincich (SISSA, Trieste, Italy). Full length PrP^C (FL PrP^C) construct was kindly provided by Dr. J.R. Requena (University of Santiago de Compostela, Santiago de Compostela, Spain). The cytosolic PrP^C 40-231 (cyPrP^C) construct was obtained from FL PrP^C (Giachin, Mai et al. 2015)). Restriction Free (RF) cloning methodology was used to insert a FLAG sequence into the FL PrP^C construct between amino acids 40-41, a region that does not interfere with the ER translocation sequence and the octarepeat region (Figure 1).

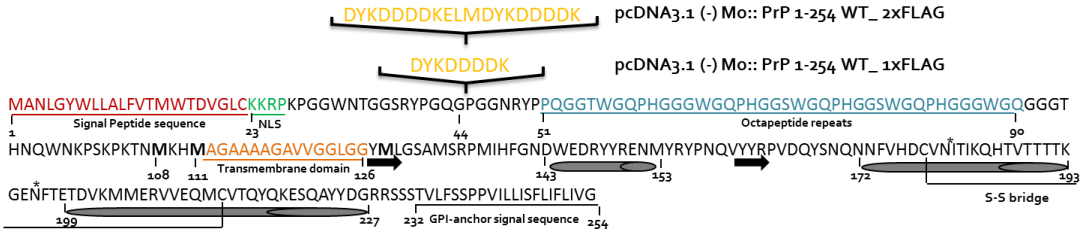

Figure 1. RF cloning insertion of 2xFLAG sequence into FL PrP^C sequence. Different functional regions of the PrP^C sequence are highlighted. A-helices and β-sheets are also evidenced. Two-step insertion of the FLAG sequence is shown.

2xFLAG sequence insertion was allowed with a two-step RF cloning reaction, in order to respect all parameters for a successful cloning procedure (Unger, Jacobovitch et al. 2010). The RF procedure is a universal cloning method allowing a precise insert of a DNA fragment into any desired position within a circular plasmid.

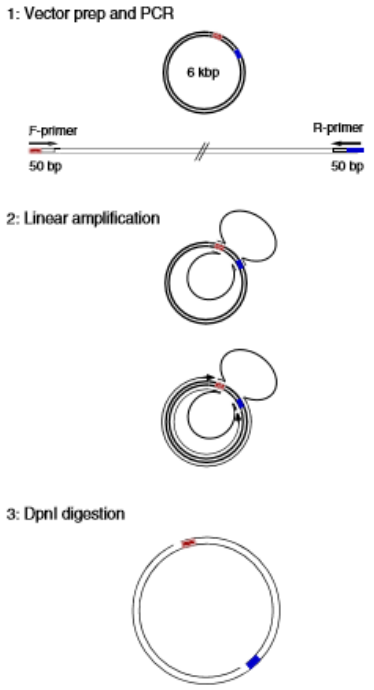

Figure 2. Schematic representation of the restriction-free (RF) cloning. The RF cloning is a two-steps process. Initially, the gene of interest is amplified along with primers containing complimentary sequences (25-30 bp) to the flanking sites of integration in the target vector. The two strands of the PCR product obtained in the first stage are used as mega primers for the second stage of amplification. Adapted from (van den Ent and Lowe 2006).

The 2xFLAG sequence (ATGGACTACAAGGACGACGATGACAAG GAATTG ATGGACTACAAGGACGACGATGACAAG) was inserted in two steps into the pcDNA3.1(-)::MoPrP1-254 construct, between aa 40-41. Primers were designed with sequences overlapped to the region chosen for 2xFLAG insertion (Table 2, Step1). Reaction of Step 1 was performed as follow:

	Starting concentration	Final concentration	To be added
Phusion Buffer			10 µL
$MgCl_2$	50mM	2mM	2 µL
dNTPs	25mM	0,2mM	0,4 µL
pcDNA3.1(-)::MoPrP 1-254	10ng/µL	40ng	4 µL
Primer Fw	0,2µg/µL	200ng	1 µL
Primer Rv	0,2µg/µL	200ng	1 µL
H_2O			30,8 µL
5x Phusion polymerase	2U/µL		0,8 µL

Table 1: Reaction mixture for the insertion of 1xFLAG sequence into pcDNA3.1(-)::MoPrP1-254 construct. This reaction was repeated for the insertion of the second FLAG sequence into pcDNA3.1(-)::MoPrP1-254 1xFLAG construct, with the primers indicated in Table2, Step2.

Step1	Fw_Flag1: CGGTATCCCGGGCAGGGAATGGACTACAAGGACGACGATGACAAGGAAAGCCCTGGAGGCAACCGT
	Rv_Flag1: ACGGTTGCCTCCAGGGCTTTCCTTGTCATCGTCGTCCTTGTAGTCCATTCCCTGCCCGGGATACCG
Step2	Fw_Flag2:GACGACGATGACAAGGAATTGATGGACTACAAGGACGACGATGACAAGAGCCCTGGAGGCAACCGT
	Rv_Flag2:ACGGTTGCCTCCAGGGCTCTTGTCATCGTCGTCCTTGTAGTCCATCAATTCCTTGTCATCGTCGTC

Table 2. Primer design for RF cloning insertion of 2xFLAG sequence into wild type FL PrP^C sequence.

The PCR cycle was as follow: Denaturation (95°C 30”), 30x [Denaturation (95°C 30”), Annealing (60°C 1’), Elongation (72°C 5’)], Elongation (72°C 7’), Store (4°C ∞). Dpn1 digestion was necessary for the digestion of the parental DNA template (Dpn1 is specific for methylated or hemimethylated DNA) Digestion with DpnI ensures that none of the colonies obtained in subsequent transformation contain the parental plasmid and therefore reduce the transformation background. Transformation of DH5α bacteria was performed as follow with different conditions tested. Transformation with 0,5µL of control plasmid, 5µL or 10µL from the PRC reaction. After incubation in ice for 20’, transformed bacteria were heat-shocked at 42°C for 90”. 900 µL of Luria-Bertani (LB) broth (composition for 1L: 10g Bacto-tryptone, 5g yeast extract, 10g NaCl. pH 7.5) were added prior to bacterial plating on Amp-resistant LB plates. Mini-prep of chosen isolated colonies were performed with the QIAprep Spin Miniprep kit (27106, QIAGEN). Nanodrop quantification was followed by BamH1 digestion, to check for the correct insertion of 1xFLAG sequence into the

pcDNA3.1(-)::MoPrP1-254 construct. The procedure described in Step1 was repeated for the insertion of the second couple of primers (Table 2, Step2), to obtain a 2xFLAG FL PrP^C construct. The obtained sequences were sequenced by the BMR Genomics.

A detailed list of utilized construct is found in Table 1 of Results section.

CELL CULTURE

Human embryonic kidney 293T (HEK293T) cell line was chosen for experimental continuity reason with the works of Zucchelli et al. (Zucchelli, Codrich et al. 2010, Zucchelli, Marcuzzi et al. 2011). Moreover, HEK293T cells are suitable because they do not express endogenous PrP^C. Human embryonic kidney (HEK293T) cells were cultured at 37°C, 5% CO2 atmosphere in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (SIGMA). Cells were transiently transfected with the amplified DNA constructs listed in Results, Figure 2A with polyethylenimine linear (23966,Polysciences) and collected 48 or 72 hours after transfection.

Mouse neuroblastoma (N2a) cell line wild type for PrP^C (WT N2a) and RML infected (ScN2a) (Enari, Flechsig et al. 2001) were maintained in culture with MEM (SIGMA), supplemented with Glutamax, 10% fetal bovine serum, Non-Essential Amino Acid (NEAA).

IMMUNOPRECIPITATION AND WESTERN BLOT

For co-immunoprecipitation experiments, HEK293T cells were transfected with TRAF6 and the indicated PrP^C constructs. After transfection, cells were either left untreated or incubated for 16 h with 5 μM of the reversible proteasomal inhibitor MG132 (C2211, SIGMA) or 10 μM of the irreversible proteasomal inhibitor Lactacystin (L6785, SIGMA). 48 hours post transfection was chosen as the best time point for co-immunoprecipitation. Cells were lysed in TRAF6 buffer (200 mM NaCl,

50 mM Tris pH 7.5, 0.5% NP40, 10% glycerol) supplemented with anti-protease inhibitors cocktail (Roche). Cell lysates were incubated for two hours with anti-FLAG M2 agarose resin (A2220, SIGMA) or over-night with anti-mouse PrP W226 or anti-mouse PrP DE10 antibodies followed by two hours incubation with protein A/G Sepharose resin (GE Healthcare). Resin was washed with three subsequent solutions (Buffer B: 0.01M Tris HCl pH 7.6, 2mM EDTA pH 8.0, 3.5mM NaCl; buffer C: 0.01M Tris HCl pH 7.6, 2mM EDTA pH 8.0, 12.5mM NaCl, buffer D: 0.01M Tris HCl pH 7.6). After washing, proteins were eluted with 2× sodium dodecyl sulfate (SDS) Laemli buffer, boiled and processed for standard Western Blot protocol (WB). A 10% acrylamide gel was used for SDS-PAGE electrophoresis. Proteins were transferred on a nitrocellulose membrane. After blocking in 5% milk TBS-T,

Membranes were blocked with 5% (w/v) milk in 1x Tris buffered saline containing 0.1% Tween (TBS-T) at room temperature for 1 hour. For GFP detection, blocking was performed in 5% (w/v) bovine serum albumin (BSA) in PBS was used. Membranes were then incubated over-night with the following antibodies: anti-rabbit FLAG (1:2000) (F7425, SIGMA), anti-mouse GFP 1:1000 (AB290, Abcam), anti-human PrP D18 (1:1000), anti-rat β-tubulin (1:1000) (ab6160, Abcam). For detection, protein A-HRP (18-160, Millipore), goat anti-mouse HRP (P044701, Dako), goat anti-human HRP (11829150, Thermo Scientific) and goat anti-rat HRP (31470, Thermo Scientific) conjugated secondary antibodies were used. WB image acquisition was performed using the ECL detection kit (Amersham) and the Alliance 4.7 software (UVITECH).

IN VITRO UBIQUITINATION ASSAY

HEK293T cells were transfected with HA–Ub, FL PrP^C or cy PrP^C and FLAG-TRAF6 WT or DN constructs. After transfection, cells were incubated with 10 µM MG132 for 3 h and lysed at 48 hours post transfection with radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris pH 7.5, 1% Triton X-100,1% deoxycholic acid and 0.1% SDS) supplemented with anti-protease inhibitors cocktail (Roche). Samples were briefly sonicated and centrifuged 10000 rpm for 10min at 4°C. Cell

lysates were incubated over-night with anti-mouse PrP W226 antibody. The following day, incubation with protein A/G Sepharose resin (GE Healthcare) for two hours was performed. Resin was washed with three subsequent solutions (Buffer B: 0.01M Tris HCl pH 7.6, 2mM EDTA pH 8.0, 3.5mM NaCl; buffer C: 0.01M Tris HCl pH 7.6, 2mM EDTA pH 8.0, 12.5mM NaCl, buffer D: 0.01M Tris HCl pH 7.6). After washing, proteins were eluted with 2× sodium dodecyl sulfate (SDS) Laemli buffer, boiled and processed for standard WB protocol, as previously described. Co-immunoprecipitated ubiquitin was revealed with anti-rabbit HA (1:1000) (71-5500, Invitrogen) antibody. The following antibodies were used for WB detection of lysates: anti-rabbit FLAG (1:2000) (F7425, SIGMA), anti-human PrP D18 (1:1000), anti-rat β-tubulin (1:1000) (ab6160, Abcam). For detection, protein A-HRP (18-160, Millipore), anti-human HRP (11829150, Thermo Scientific) and goat anti-rat HRP (31470, Thermo Scientific) conjugated secondary antibodies were used. WB image acquisition was performed using the ECL detection kit (Amersham) and the Alliance 4.7 software (UVITECH).

IMMUNOFLUORESCENCE

Transfected HEK293T cells were cultured on 15mm glass coverslips and fixed in 4% paraformaldehyde after 48 or 72 hours. Cells were blocked in 5% NGS (005-000-121; Jackson ImmunoResearch), 0.3% Triton-X100 in PBS and incubated over-night with the following antibodies. For double transfection with cyPrP^{C} and FLAG-TRAF6, cells were incubated over-night with anti-mouse PrP (W226) (1:1000) and anti-rabbit FLAG (1:1000) (F7425, SIGMA) antibodies. Signals were detected with goat anti-mouse IgG (H+L) Alexa Fluor 488 (1:500) (A-11001, Life Technologies) and goat anti-rabbit IgG (H+L) Alexa Fluor 594 (1:500) (A-11012, Life Technologies) fluorescent-conjugated secondary antibodies. For double transfection with FLAG-FL PrP^{C} and GFP-TRAF6, incubation with rabbit anti-FLAG (F7425, SIGMA) was performed, followed by incubation with goat anti-rabbit IgG (H+L) Alexa Fluor 594 (1:500) (A-11012, Life Technologies) fluorescent-conjugated secondary antibody.

GFP was detected by auto fluorescence at 488 laser. p62 staining on cells transfected with FLAG-FL PrP^C and GFP-TRAF6 was performed with anti-mouse p62 antibody (1:500) (610833, BD Transduction Laboratories), followed by incubation with goat anti-Mouse IgG (H+L) Biotin antibody (1:100) (SAB4600004, SIGMA) and Alexa Fluor® 647 Streptavidin (1:100) (S21347, Life Technologies. For triple transfection with FLAG-FL PrP^C, GFP-TRAF6 and HA-Ub, cells were incubated with anti-mouse FLAG (1:1000) (F3165, SIGMA) and anti-rabbit HA (1:1000) (71-5500, Invitrogen) antibodies. Antibody staining was revealed with goat anti-Mouse IgG (H+L) Alexa Fluor® 594 conjugate (1:500) (A-11005, Life Technologies) fluorescent-conjugated secondary antibody and goat anti-Rabbit IgG (H+L) Biotin antibody (1:100) (SAB4600006, SIGMA) followed by incubation with Alexa Fluor® 647 Streptavidin (1:100) (S21347, Life Technologies). GFP was detected by auto fluorescence at 488 laser. 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI, SIGMA) was used for nuclear staining. N2a cells were fixed in 4% paraformaldehyde, treated with 6N guanidine hydrochloride (GdnHCl) for 10 minutes and subjected to the protocol described previously. Cells were incubated over-night with anti-mouse PrP (W226) (1:1000) and goat anti-rabbit TRAF6 (1:500) (sc7221, Santa Cruz) antibodies. Signals were revealed with goat anti-mouse IgG (H+L) Alexa Fluor 488 (1:500) (A-11001, Life Technologies) and goat anti-rabbit IgG (H+L) Alexa Fluor 594 (1:500) (A-11012, Life Technologies) fluorescent-conjugated antibodies. Fluorescent images (1024x1024 pixels) were acquired with the C1 Nikon confocal with a 40x Oil N.A: 1.30 objective, and additionally magnified 4x. 0.7µm steps were chosen for z-stacks (n. 10-15 per cell).

MICE

Zurich III wild type PrP^C (ZH3 $PrP^{+/+}$) mice were generously provided by Adriano Aguzzi, Institute of Neuropathology, University Hospital of Zurich (Zurich, Switzerland) (Nuvolone, Hermann et al. 2016). The colony was maintained ad SISSA animal facility (Trieste, Italy). Mice had access to food and water *ad libitum* and

maintained in a 12:12 light:dark cycle with controlled temperature and humidity. All experiments employing animals were carried out in accordance with European regulations [European Community Council Directive, November 24, 1986 (86/609/EEC)] and approved by the local authority veterinary service and by the Ethics Committee of the Scuola Internazionale Superiore di Studi Avanzati (SISSA), Trieste.

CO-IMMUNOPRECIPITATION FROM BRAIN SAMPLES

ZH3 mice were euthanized with an excess of CO_2 and cervical dislocation . Brains were extracted and homogenized in modified TRAF6 buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 0.5% NP40, 10% glycerol) supplemented with anti-protease inhibitors cocktail (Roche), sonicated and centrifuged 10000 rpm for 10min at 4°C. Brain lysates were incubated over-night with anti-rabbit TRAF6 antibody (sc7221, Santa Cruz) or control IgG (12-370, Millipore), followed by four hours incubation with protein A/G Sepharose (GE Healthcare). Resin was washed with three subsequent solutions (Buffer B: 0.01M Tris HCl pH 7.6, 2mM EDTA pH 8.0, 3.5mM NaCl; buffer C: 0.01M Tris HCl pH 7.6, 2mM EDTA pH 8.0, 12.5mM NaCl, buffer D: 0.01M Tris HCl pH 7.6). After washing, proteins were eluted with 2× sodium dodecyl sulfate (SDS) Laemli buffer, boiled and processed for standard WB protocol, as previously described. Proteins were detected on WB with anti-rabbit TRAF6 antibody (sc7221, Santa Cruz) followed by incubation with Protein A-HRP (18-160, Millipore) and with anti-human PrP (D18) antibody followed by incubation with anti-human HRP conjugated antibody. WB image acquisition was performed using the ECL detection kit (Amersham) and the Alliance 4.7 software (UVITECH).

CHAPTER 3

RESULTS

TRAF6 SEQUENCE ANALYSIS

In this work human TRAF6 WT and DN constructs were used to study the interaction with mouse PrP^C constructs. The use of human TRAF6 constructs was allowed by the high sequence homology (88%) of the human and the mouse TRAF6 sequences. In particular the 95% of sequence homology is shared by the C-terminal MATH domain, responsible for substrate binding (Figure 1).

Range 1: 1 to 530 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
932 bits(2408)	0.0	Compositional matrix adjust.	464/530(88%)	490/530(92%)	8/530(1%)

```
Hu TRAF6    MSLLNCENSCGSSQSESDCCVAMASSCSAVTKDDSVGGTASTGNLSSSFMEEIQGYDVEF  60
            MSLLNCENSCGSSQS SDCC AMA+SCSA  KDDSV G+ASTGNLSSSFMEEIQGYDVEF
Mo TRAF6    MSLLNCENSCGSSQSSSDCCAAMAASCSAAVKDDSVSGSASTGNLSSSFMEEIQGYDVEF  60
                                RING-domain
Hu TRAF6    DPPLESKYECPICLMALREAVQTPCGHRFCKACIIKSIRDAGHKCPVDNEILLENQLFPD  120
            DPPLESKYECPICLMALREAVQTPCGHRFCKACIIKSIRDAGHKCPVDNEILLENQLFPD
Mo TRAF6    DPPLESKYECPICLMALREAVQTPCGHRFCKACIIKSIRDAGHKCPVDNEILLENQLFPD  120

Hu TRAF6    NFAKREILSLMVKCPNEGCLHKMELRHLEDHQAHCEFALMDCPQCQRPFQKFHINIHILK  180
            NFAKREILSL VKCPN+GCL KMELRHLEDHQ HCEFAL++CPQCQRPFQK  +N HI++
Mo TRAF6    NFAKREILSLTVKCPNKGCLQKMELRHLEDHQVHCEFALVNCPQCQRPFQKCQVNTHIIE  180
                                Zn-finger domain
Hu TRAF6    DCPRRQVSCDNCAASMAFEDKEIHDQNCPLANVICEYCNTILIREQMPNHYDLDCPTAPI  240
            DCPRRQVSC NCA SMA+E+KEIHDQ+CPLAN+ICEYC TILIREQMPNHYDLDCPTAPI
Mo TRAF6    DCPRRQVSCVNCAVSMAYEEKEIHDQSCPLANIICEYCGTILIREQMPNHYDLDCPTAPI  240

Hu TRAF6    PCTFSTFGCHEKMQRNHLARHLQENTQSHMRMLAQAVHSLSVI--------PDSGYISEV  292
            PCTFS FGCH+KMQRNHLARHLQENTQ HMR+LAQAVH++++          P  G   E
Mo TRAF6    PCTFSVFGCHQKMQRNHLARHLQENTQLHMRLLAQAVHNVNLALRPCDAASPSRGCRPED  300

Hu TRAF6    RNFQETIHQLEGRLVRQDHQIRELTAKMETQSMYVSELKRTIRTLEDKVAEIEAQQCNGI  352
             N++ETI QLE RLVRQDHQIRELTAKMETQSMYV ELKRTIRTLEDKVAE+EAQQCNGI
Mo TRAF6    PNYEETIKQLESRLVRQDHQIRELTAKMETQSMYVGELKRTIRTLEDKVAEMEAQQCNGI  360
                                TRAF-domain
Hu TRAF6    YIWKIGNFGMHLKCQEEEKPVVIHSPGFYTGKPGYKLCMRLHLQLPTAQRCANYISLFVH  412
            YIWKIG FGMHLK QEEE+PVVIHSPGFYTG+PGYKLCMRLHLQLPTAQRCANYISLFVH
Mo TRAF6    YIWKIGKFGMHLKSQEEERPVVIHSPGFYTGRPGYKLCMRLHLQLPTAQRCANYISLFVH  420

Hu TRAF6    TMQGEYDSHLPWPFQGTIRLTILDQSEAPVRQNHEEIMDAKPELLAFQRPTIPRNPKGFG  472
            TMQGEYDSHLPWPFQGTIRLTILDQSEA +RQNHEE+MDAKPELLAFQRPTIPRNPKGFG
Mo TRAF6    TMQGEYDSHLPWPFQGTIRLTILDQSEALIRQNHEEVMDAKPELLAFQRPTIPRNPKGFG  480

Hu TRAF6    YVTFMHLEALRQRTFIKDDTLLVRCEVSTRFDMGSLRREGFQPRSTDAGV  522
            YVTFMHLEALRQ TFIKDDTLLVRCEVSTRFDMG LR+EGFQPRSTDAGV
Mo TRAF6    YVTFMHLEALRQGTFIKDDTLLVRCEVSTRFDMGGLRKEGFQPRSTDAGV  530
```

Figure 1. Sequence alignment of human and mouse TRAF6 sequences results in 88% of homology. TRAF6 N-terminal RING, central Zn-finger and C-terminal TRAF domains are highlighted as in the cartoon (95% of sequence homology is shared by the C-terminal MATH (aa 350-499) domain, 80% by the RING and Zn-finger domains).

CLONING FLAG-PrP^{C} AND CONSTRUCTS

FL PrP^{C} sequence was cloned with the RF cloning technique for the insertion of a 2xFLAG sequence at its N-terminal portion. The more suitable region for FLAG insertion was identified between aa 40-41. This region does not interfere with the signal peptide sequence (aa 1-22), necessary for ER translocation, and the nuclear localization sequence (NLS, aa 23-26), as well as with the octapeptide region of PrP^{C}. Restriction Free (RF) cloning methodology was chosen for some of its advantages, in particular: any vector of choice can be used, there is no need for restriction enzyme digestion, cloning can be performed at any desired position and without the addition of unwanted extra residues. This universal cloning method allows the precise insertion of a DNA fragment into any desired position within a circular plasmid. Cloned FLAG-FL PrP^{C} and all the other constructs listed in Figure 2A were amplified, sequenced and used for transient transfection in HEK293T cells. Position of the FLAG insertion in both TRAF6 and FL PrP^{C} constructs is depicted in Figure 2B.

A)

	Constructs	Abbreviation	Molecular weight (kDa)
PrP^C	pcDNA3.1 (-) Mo::PrP 1-254 WT FL PrP	FL PrP^C	26, 33, 37
	pcDNA3.1 (-) Mo::PrP 40-231 WT * 40 231 cyPrP	cyPrP^C	21.5
	pcDNA3.1 (-) Mo:: PrP 1-254 WT_ 2xFLAG 1 253 FLAG FL PrP	FLAG-FL PrP^C	32, 39, 43
TRAF6	pcDNA3.1 (-) Hu::TRAF6 WT_ 2xFLAG FLAG TRAF6	FLAG-TRAF6 WT	66
	pcDNA3.1 (-) Hu::TRAF6 DN_ 2xFLAG FLAG TRAF6	FLAG-TRAF6 DN	35
	pEGFP Hu::TRAF6 WT GFP TRAF6	GFP-TRAF6 WT	87
	pEGFP Hu::TRAF6 DN GFP TRAF6	GFP-TRAF6 DN	56
Ubiquitin	Prk5 Hu::HA-ubiquitin HA Ub	HA-Ub	17.5

B)

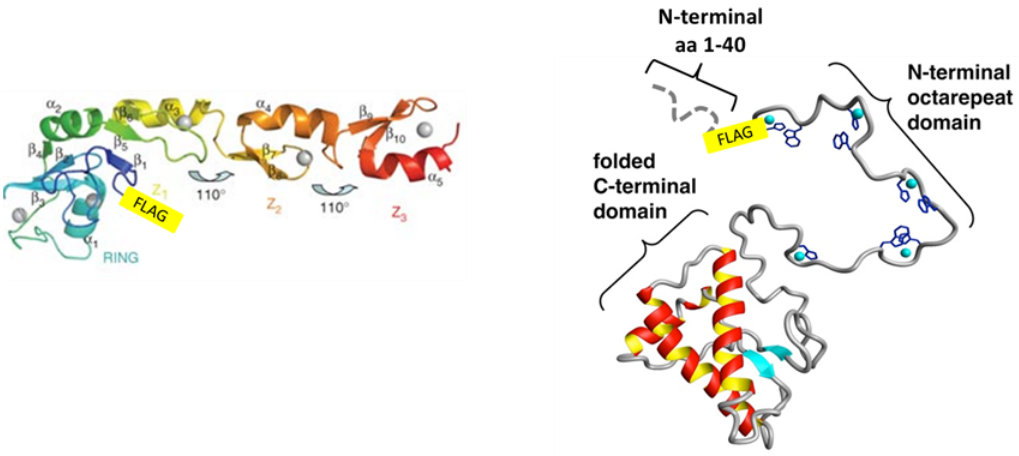

Figure 2. Detailed list of all constructs used in the experiments. A) Description of PrP^C, TRAF6 and ubiquitin constructs used. * This cytosolic form of PrP^C (cyPrP^C) is highly aggregation prone (cytosolic puncta) and poorly degraded by the proteasome (Chakrabarti, Rane et al. 2011). In comparison with the cytosolic PrP^C 22-231, rapidly degraded by the proteasome, for its resident time in the cytosol this construct is more suitable for the aim of this project. B) Cartoon images of flagged TRAF6 and FL PrP^C constructs. FLAG was inserted at the N-terminal portion of FL PrP^C between aa 40-41. Adapted images from (Yin, Lin et al. 2009) and (Burns, Aronoff-Spencer et al. 2003).

INTERACTION BETWEEN TRAF6 AND cyPrP

The aggregation prone neuropathological-associated proteins α-synuclein and huntingtin are normally present in the cell cytoplasm (Force, Glass et al. 2000, Hostager, Catlett et al. 2000) and are characterized by an intrinsically tendency to misfold, a feature exacerbated in presence of protein mutations (Dobson 2001). Given the TRAF6 ability to bind the physiological forms of these proteins (Zucchelli, Codrich et al. 2010, Zucchelli, Marcuzzi et al. 2011), we decided to investigate its interplay with a cytosolic form of PrP^C (PrP^C 40-231, hereafter named $cyPrP^C$). $cyPrP^C$ is found in the cytosol in puncta and is less degraded by the proteasome as other cytosolic PrP^C molecules (Chakrabarti, Rane et al. 2011). In order to assess if TRAF6 is able to bind $cyPrP^C$, HEK293T cells were kept in culture and transfected with flagged TRAF6 WT (FLAG-TRAF6 WT) and $cyPrP^C$ constructs. Cells were left untreated or treated with a reversible (MG132) or irreversible (Lactacystin) proteasomal inhibitors and lysed after 48 hours from transfection. Immunoprecipitation of FLAG-TRAF6 WT (n.=6) revealed the co-precipitated $cyPrP^C$ only when the activity of the proteasome was blocked (Figure 3A). To confirm this result, we performed a reverse co-immunoprecipitation of HEK293T cells transfected with $cyPrP^C$ and FLAG-TRAF6 WT, left untreated or treated with proteasome inhibitors and lysed as previously described. Immunoprecipitation of $cyPrP^C$ (n.=2) was performed with two different anti-PrP antibodies: the N-terminal anti-PrP DE10 antibody, which recognizes the N-terminal epitope 41-56 (Didonna, Venturini et al. 2015), and the C-terminal anti-PrP W226 antibody, mapped at epitope: 144-152 of the C-terminal portion of PrP^C (Petsch, Muller-Schiffmann et al. 2011). Immunoprecipitation with the anti-PrP DE10 antibody resulted in the detection of the co-immunoprecipitated FLAG-TRAF6 WT. On the contrary, when the anti-PrP W226 antibody was used, no co-precipitated FLAG-TRAF6 WT was observed (Figure 3B). It is plausible that $cyPrP^C$-TRAF6 WT binding involves the $cyPrP^C$ C-terminal portion, thus explaining why with the anti-PrP W226 antibody the co-precipitated TRAF6 WT is not visible (Figure 3D). A weak $cyPrP^C$-TRAF6 WT interaction could explain the loss of TRAF6 binding after the formation of an immune complex between the anti-PrP W226 antibody and the PrP^C molecule.

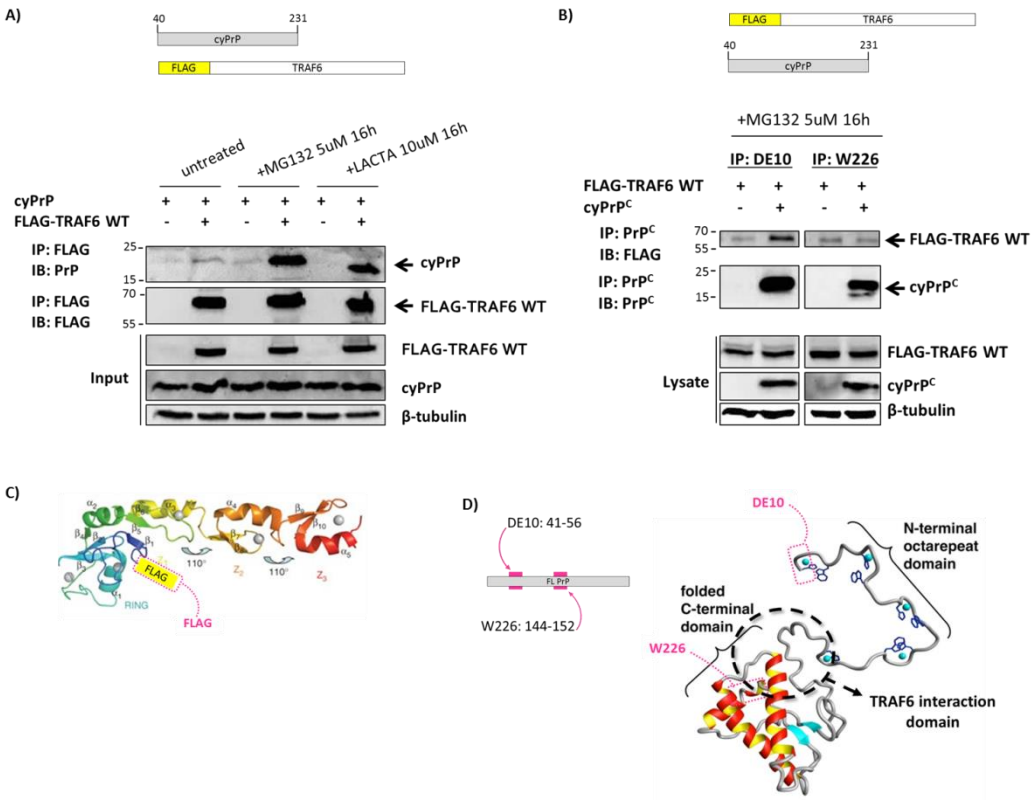

Figure 3: TRAF6 interacts with cyPrP^C. A) Direct co-immunoprecipitation of FLAG-TRAF6 WT and cyPrP^C (n.=6). HEK293T cells were transfected with cyPrP^C alone or with FLAG-TRAF6 WT and left untreated or incubated with 5µM MG132 or 10µM Lactacystin for 16 h. After 48 hours lysates were immunoprecipitated (IP) with anti-FLAG agarose resin and bound proteins were revealed by immunoblot (IB) with anti-PrP D18 and anti-FLAG antibodies. Lysates were tested for the expression of FLAG-TRAF6 WT and cyPrP^C proteins. β-tubulin was used as loading control. B) Reverse co-immunoprecipitation with cyPrP^C and FLAG-TRAF6 WT constructs (n.=2). Experiment was performed as in (A) with HEK293T cells treated with MG132 and Lactacystin. Immunoprecipitation was performed with the N-terminal anti-PrP DE10 antibody (epitope: 41-56) or with the C-terminal anti-PrP W226 antibody (epitope: 144-152). Bound proteins were revealed by IB with anti-FLAG and anti-PrP D18 antibodies. Co-Immunoprecipitation of FLAG-TRAF6 WT was revealed only when the anti-PrP DE10 antibody was used in IP. C) Cartoon image of FLAG-TRAF6 WT structure showing the binding site of anti-FLAG antibody at the N-terminal of the protein. D) Cartoon images showing the epitopes of anti-PrP DE10 and W226 antibodies. Anti-PrP DE10 antibody binding did not affect FLAG-TRAF6 WT binding to cyPrP^C. On the contrary, anti-PrP W226 antibody binding resulted in a negative co-immunoprecipitation result as seen in (B). This led to hypothesise the binding site of FLAG-TRAF6 WT in the C-terminal portion of cyPrP^C.

TRAF6 CO-LOCALIZE WITH cyPrP^{C} IN DISCRETE PERINUCLEAR REGIONS IN AGGRESOME-LIKE STRUCTURES

HEK293T cells were plated and transfected with FLAG-TRAF6 WT and cyPrP^{C} constructs. After treatment with 5µM MG132 for 16 hours, cells were fixed at 48 hours post transfection an stained with anti-FLAG and anti-PrP antibodies. The cyPrP^{C} characteristic localization in puncta (Chakrabarti, Rane et al. 2011) was revealed. Staining of FLAG-TRAF6 WT showed a diffused cytoplasmic localization as well as the presence of small aggregates in the cytosol and a bigger TRAF6 WT aggregate in the perinuclear region. Partial cyPrP^{C}-FLAG-TRAF6 WT co-localization is observed in the perinuclear region (Figure 4).

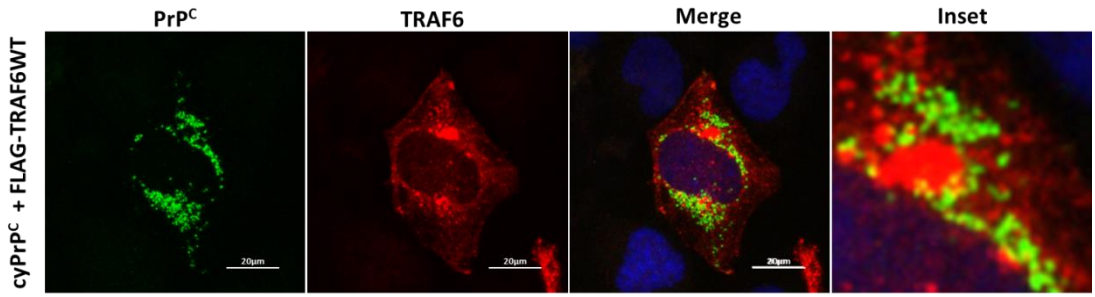

Figure 4: Co-localization of TRAF6 and cyPrP^{C}. HEK293T cells were plated on 15mm coverslips, transfected with FLAG-TRAF6 WT and cyPrP^{C} and incubated with 5 µM MG132 for 16 hours before fixation at two days in culture. Double-immunofluorescence was performed incubating the cells with anti-PrP W226 antibody and FLAG antibody. Overnight primary incubation was revealed with Alexa Fluor 488 and 594 conjugated fluorescent secondary antibodies. Nuclei were counterstained with DAPI. Merged images reveal the partial co-localization of the FLAG-TRAF6 WT and cyPrP^{C} in the perinuclear region (n.=3). Scale bar: 20µm.

cyPrP^{C} IS NOT UBIQUITYLATED

Given the ability of TRAF6 to ubiquitylate the substrate α-synuclein and huntingtin (Zucchelli, Codrich et al. 2010, Zucchelli, Marcuzzi et al. 2011), we decided to investigate cyPrP^{C} ubiquitylation, as a consequence of TRAF6 WT binding. Little

evidence relative to the ubiquitylation of PrP^C is found in literature and its study is mostly addressed to proteasomal degradation. HEK293T cells were transfected with Ha-Ub, $cyPrP^C$ and FLAG-TRAF6 WT and dominant negative (DN). TRAF6 DN retains the C-terminal MATH domain for substrate binding but lacks the N-terminal RING domain for TRAF6 auto-ubiquitylation and substrate-ubiquitylation. Surprisingly, when co-transfected with HA-Ub, $cyPrP^C$ is not ubiquitylated, even in absence of FLAG TRAF6 WT (n.=3, Figure 5A). This evidence allowed us to postulate the localization of the PrP^C ubiquitin binding site at the PrP^C N-terminal domain comprised by aa 1-39. Indeed, three lysine residues are localized in this domain and are absent in the $cyPrP^C$ construct. In figure 5B a comparison between the full length PrP^C (FL PrP^C, here indicated as PrP^C 1-254) and the $cyPrP^C$ (indicated as PrP^C 40-231) is shown. Three lysine residues that are present in position 23, 24 and 27 are absent in the $cyPrP^C$ construct. Cartoon images in figure 5C exemplifies the putative ubiquitylation of FL PrP^C and the lack of ubiquitylation of the $cyPrP^C$ construct, once established TRAF6 WT binding to the PrP^C protein.

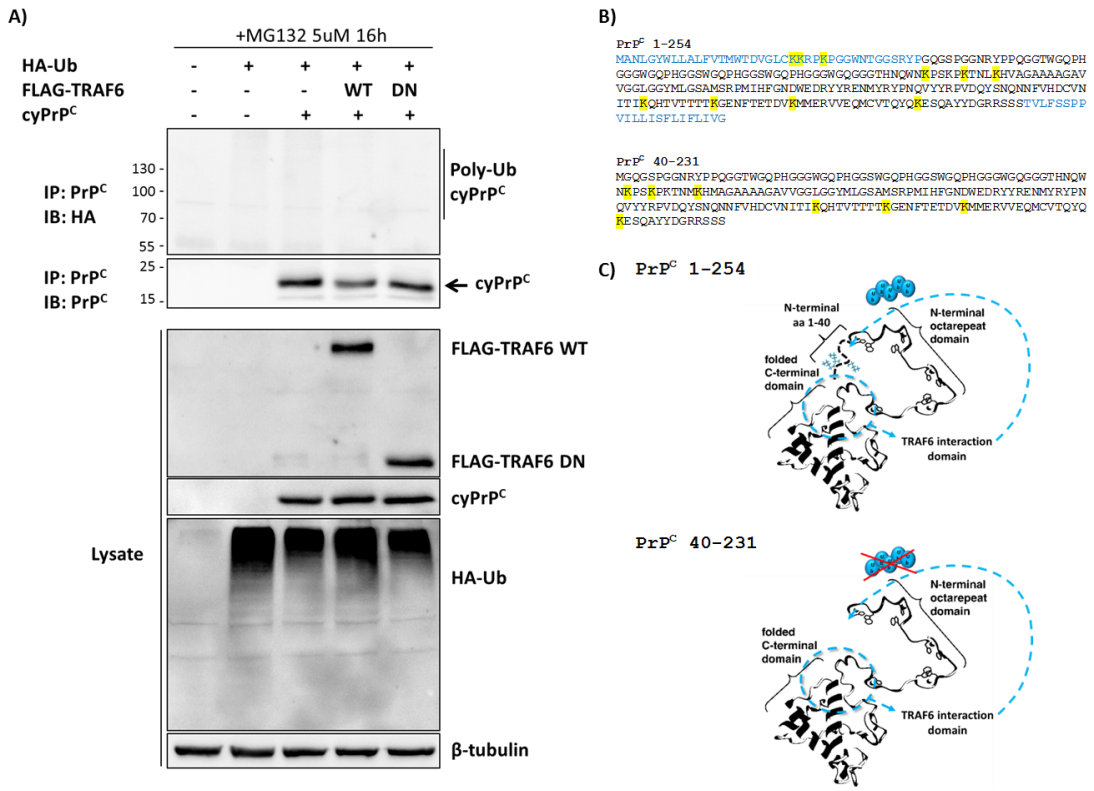

Figure 5: lack of $cyPrP^C$ ubiquitylation. A) HEK293T cells were transfected with HA-Ub, $cyPrP^C$ and FLAG-TRAF6 WT or DN constructs, treated with 10µM MG132 for 3 hours and lysed after 48 hours

post transfection. Immunoprecipitation (IP) of cyPrP^C was performed with anti-PrP W226 antibody. The use of W226 was allowed since it does not interfere with PrP-ubiquitin binding. Anti-HA antibody was used for HA-Ub detection. No co-precipitated HA-Ub was revealed. Lysates were tested for the expression of HA-Ub, cyPrP^C and FLAG-TRAF6. β-tubulin was used as loading control. B) Sequence analysis of FL PrP^C (PrP^C 1-254) and cyPrP^C (PrP^C 40-231). In blue: Amino acids that are absent in PrP^C 40-231 are depicted in blue. Lysine residues are highlighted in yellow. C) Cartoon images showing the putative TRAF6 C-terminal binding site to the C-terminal of PrP^C and the lysine residues at the N-terminal of PrP^C (23, 24 and 27) that are absent in the cyPrP^C construct. Ubiquitin proteins cannot be linked to the cyPrP^C protein due to the lack of the first N-terminal 1-39 amino acids.

INTERACTION BETWEEN TRAF6 AND FL PrP^C

The evidence of TRAF6 WT-cyPrP^C interaction and the absence of cyPrP^C ubiquitylation, led us to focus our attention to the FL PrP^C protein. FL PrP^C is normally localized to the plasma membrane in lipid rafts, but is also found in endosomal vesicles during recycling (Kiachopoulos, Heske et al. 2004) and in a little amount in the cell cytoplasm, due to its re-routing from the ER (Kang, Rane et al. 2006, Orsi, Fioriti et al. 2006). TRAF6 is found in the sub-membrane region of lipid rafts, where it is involved in signalling (Lewis and Hooper 2011), besides being present in the cytoplasm. In order to verify is TRAF6 WT is also able to bind to the FL PrP^C, HEK293T cells were transfected with FLAG-TRAF6 WT and FL PrP^C. Cells were left untreated or treated with a reversible (MG132) or irreversible (Lactacystin) proteasomal inhibitors and lysed after 48 hours from transfection. Immunoprecipitation of FLAG-TRAF6 WT (n.=11) resulted in the detection of co-precipitated FL PrP^C even in absence of proteasomal inhibition. FLAG-TRAF6 WT and FL PrP^C interaction was enhanced when the activity of the proteasome was blocked (Figure 6A). This data is in line with the previous observation of Zucchelli et al., where the amount of co-precipitated TRAF6 WT substrate is increased during proteasomal inhibition (Zucchelli, Codrich et al. 2010, Zucchelli, Marcuzzi et al. 2011). To confirm the interaction between FLAG-TRAF6 WT and FL-PrP^C and to avoid possible artefacts due to the FLAG insertion, reverse co-immunoprecipitation was performed. HEK293T cells were transfected with FLAG-FL PrP^C and GFP-TRAF6

WT and treated as previously described (n.=6, Figure 6B). Immunoprecipitated FLAG-FL PrP^{C} was detected with anti-FLAG antibody, while the co-precipitated GFP-TRAF6 WT was revealed by anti-GFP antibody. Also in this case, the amount of co-precipitated protein was increased during proteasome inhibition. In figure 6C the FLAG epitope recognized by the anti-FLAG antibody at the N-terminal of both FLAG-TRAF6 and FLAG-FL PrP^{C} constructs is indicated.

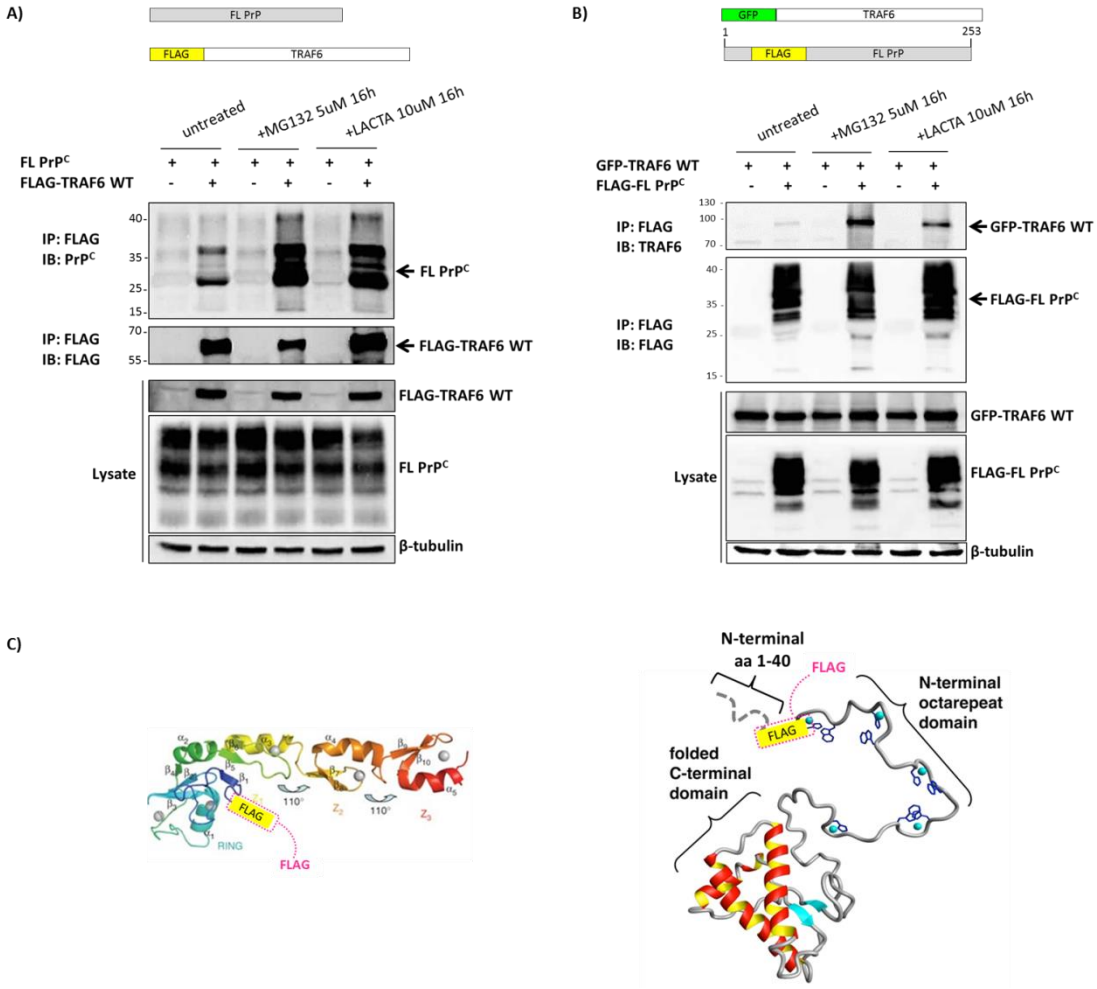

Figure 6: TRAF6 interacts with FL PrP^{C}. A) Direct co-immunoprecipitation of FLAG-TRAF6 WT and FL PrP^{C} (n.=11). HEK293T cells were transfected with FL PrP^{C} alone or with FLAG-TRAF6 WT and left untreated or incubated with 5μM MG132 or 10μM Lactacystin for 16 h. After 48 hours lysates were immunoprecipitated (IP) with anti-FLAG agarose resin and bound proteins were revealed by immunoblot (IB) with anti-PrP D18 and anti-FLAG antibodies. Lysates were tested for the expression of FLAG-TRAF6 WT and FL PrP^{C} proteins. β-tubulin was used as loading control. B) Reverse co-immunoprecipitation with FLAG–FL PrP^{C} and GFP-TRAF6 WT constructs (n.=6). Experiment was

performed as in (A) with HEK293T cells treated with MG132 and Lactacystin. Bound proteins were revealed by IB with anti-GFP and anti-FLAG antibodies. C) Cartoon images show the inserted FLAG sequence in the FLAG-TRAF6 WT and the FLAG-FL PrP^C constructs, as the site of immunoprecipitation with the FLAG resin.

In order to further validate these data we used an unflagged FL PrP^C construct for reverse co-immunoprecipitation of HEK293T cells transfected with FL PrP^C and FLAG-TRAF6 WT. As previously done for immunoprecipitation of cyPrP^C, two different antibodies for PrP^C detection were used. Again, immunoprecipitation of FL PrP^C resulted in the detection of the co-precipitated FLAG-TRAF6 WT only when the N-terminal anti-PrP DE10 antibody was used (n.=3). However, if compared to Figure 3B, the detected FLAG-TRAF6 WT band after immunoprecipitation with the anti-PrP DE10 antibody is lower (Figure 7A). The absence of the co-precipitated substrate further confirms the putative binding site of TRAF6 WT to the C-terminal portion of PrP^C, in a zone that the steric hindrance of protein binding determines the loss of protein interaction in favour of the immune binding of the anti-PrP W226 antibody. Moreover, the difficulty in detecting a clear band of the co-precipitated FLAG-TRAF6 WT when the anti-PrP DE10 antibody is used, suggests that the PrP^C N-terminal 1-39 amino acids could influence in its intrinsically flexible nature, the binding of TRAF6 to PrP^C (Figure 7B). Cartoons in figure 7C show the comparison among FL PrP^C, FLAG-FL PrP^C and cy PrP^C. Insertion of the FLAG sequence into the FL PrP^C molecule (between aa 40-41) results in a change in conformation of the N-terminal portion, allowing the FLAG antibody not to interfere with PrP^C-TRAF6 binding, as observed in Figure 6C. Similarly, deletion of amino acids 1-39 at the N-terminal portion of cyPrP^C has a similar effect of the FLAG insertion into the FL PrP^C sequence, thus allowing the anti-PrP DE10 antibody to bind to cyPrP^C without being an obstacle for cyPrP^C-TRAF6 interaction.

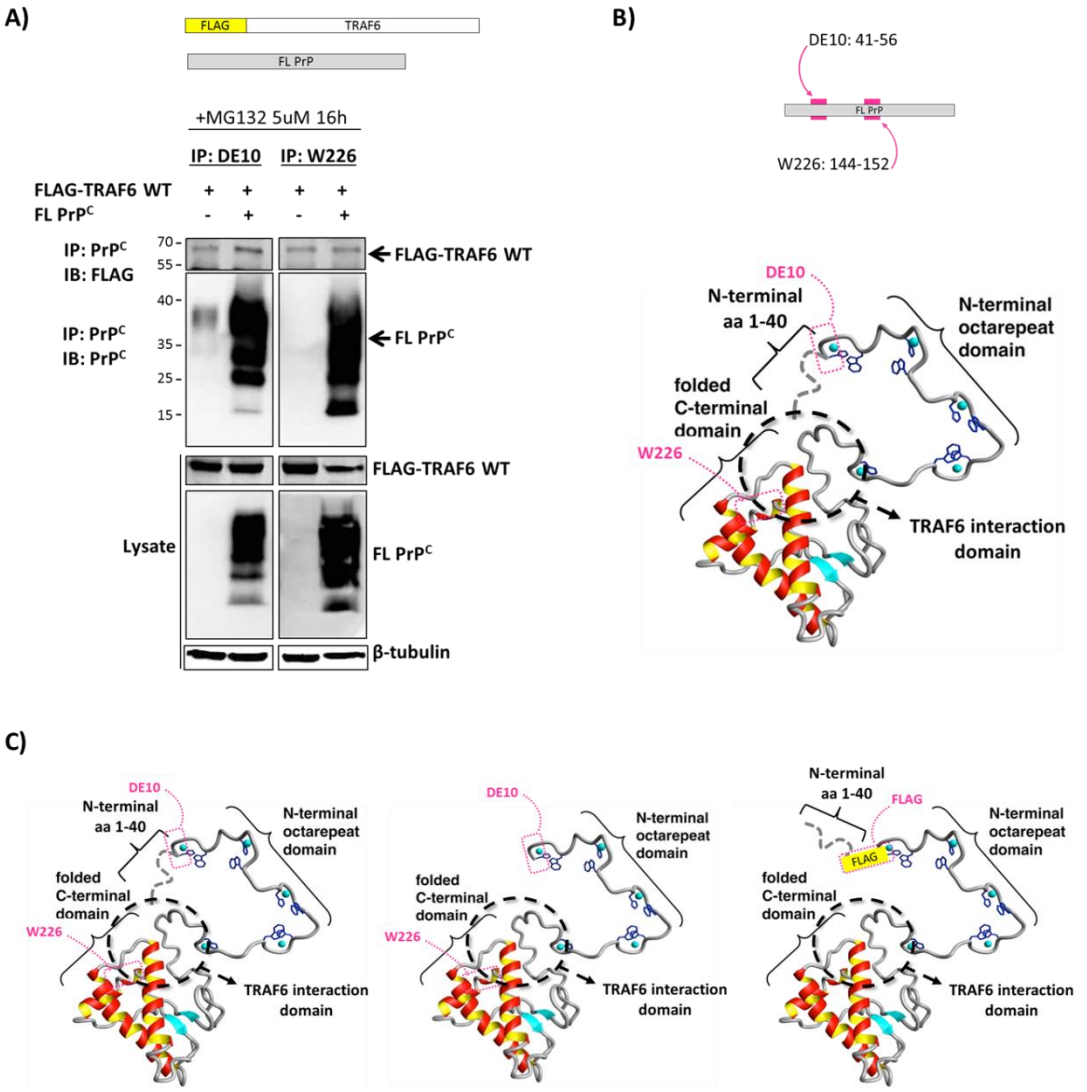

Figure 7: Putative influence of the PrP^C N-terminal in the TRAF6 WT binging to PrP^C. A) Reverse co-immunoprecipitation of unflagged FL PrP^C and FLAG-TRAF6 WT revealed a faint band of co-immunoprecipitated FLAG-TRAF6 WT only when the anti-PrP DE10 antibody is used in immunoprecipitation. HEK293T cells were transfected with FLAG-TRAF6 WT alone or with unflagged FL PrP^C and left untreated or incubated with 5µM MG132 for 16 h. After 48 hours lysates were immunoprecipitated (IP) with anti-PrP DE10 antibody (n.=3) or with anti-PrP W226 antibody(n.=6). Bound proteins were revealed by immunoblot (IB) with anti-FLAG and anti-PrP D18 antibodies. Lysates were tested for the expression of FL PrP^C and FLAG-TRAF6 WT proteins. β-tubulin was used as loading control. B) Cartoon images show the epitopes recognised by anti-PrP DE10 antibody at the N-terminal portion of PrP^C and by anti-PrP W226 antibody at the C-terminal portion of FL PrP^C. C) Putative influence of the N-terminal residues 1-39 of PrP^C in the TRAF6-PrP^C binding. Absence of 1-39 residues in the cyPrP^C or FLAG insertion in the FL PrP^C sequences results in a strong detection of co-precipitated TRAF6 as seen in figure 3B (IP: DE10) and 6B (IP: FLAG). The hardly detectable co-

precipitated observed in A) (IP: DE10) suggests a role of interference of amino acids 1-39 in the TRAF6 binding to FL PrP^C.

ENDOGENOUS PrP^C AND TRAF6 INTERACT IN THE MOUSE BRAIN

Interaction of PrP^C and TRAF6 was investigated in the mouse brain. Six month old ZH3 $PrP^{+/+}$ mice brains were lysed an subjected to immunoprecipitation of endogenous TRAF6. Interestingly, co-precipitated PrP^C was detected, thus confirming our *in vitro* results (n.=3).

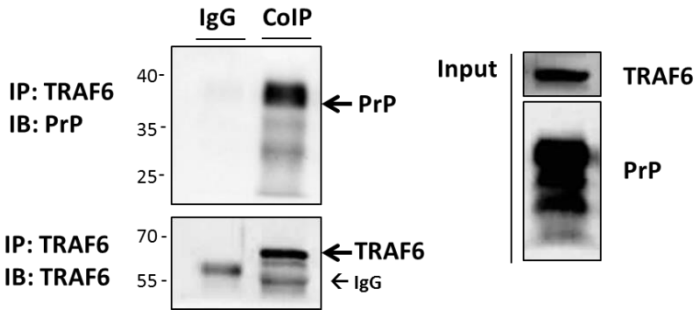

Figure 8: PrP^C and TRAF6 interacts in the mouse brain. Six month old ZH3 mouse brains were lysed with modified TRAF6 buffer and immunoprecipitated (IP) for endogenous TRAF6 (n.=3). WB analysis with anti-PrP D18 antibody revealed the presence of co-immunoprecipitated PrP^C (Co-IP lane). IgG were used as internal control. Lysates were tested for the expression of TRAF6 and PrP^C.

EXPRESSION OF TRAF6 AND FL PrP^C AT DIFFERENT TIME POINTS

Various works have suggested the presence of PrP^C in the cytosol (Mironov, Latawiec et al. 2003, Kang, Rane et al. 2006, Orsi, Fioriti et al. 2006). Our co-immunoprecipitation data strongly support this evidence for both the $cyPrP^C$ and the FL PrP^C. Prior to evaluate the co-localization of TRAF6 WT and FL PrP^C, HEK293T cells were single transfected with FLAG-FL PrP^C or GFP-TRAF6 WT at two different time points (48 and 72 hours) in order to check for possible differences in the localization of FLAG-FL PrP^C in its trafficking to the membrane as well as changes in GPF-TRAF6 WT localization in the cell (n.=2). At 48 hours FLAG-FL PrP^C is expressed on the cellular membrane and in cellular compartments as at 72 hours post transfection (Figure 9A). This data excludes the possibility that at the defined time point chosen for immunoprecipitation (48 hours), FLAG-FL PrP^C could be present only in the cytoplasm, bound to TRAF6. Moreover, it suggests that FLAG insertion doesn't interfere with FL PrP^C routing to the membrane. No significant changes were observed in GFP-TRAF6 WT localization at the different time points analysed (Figure 9B).

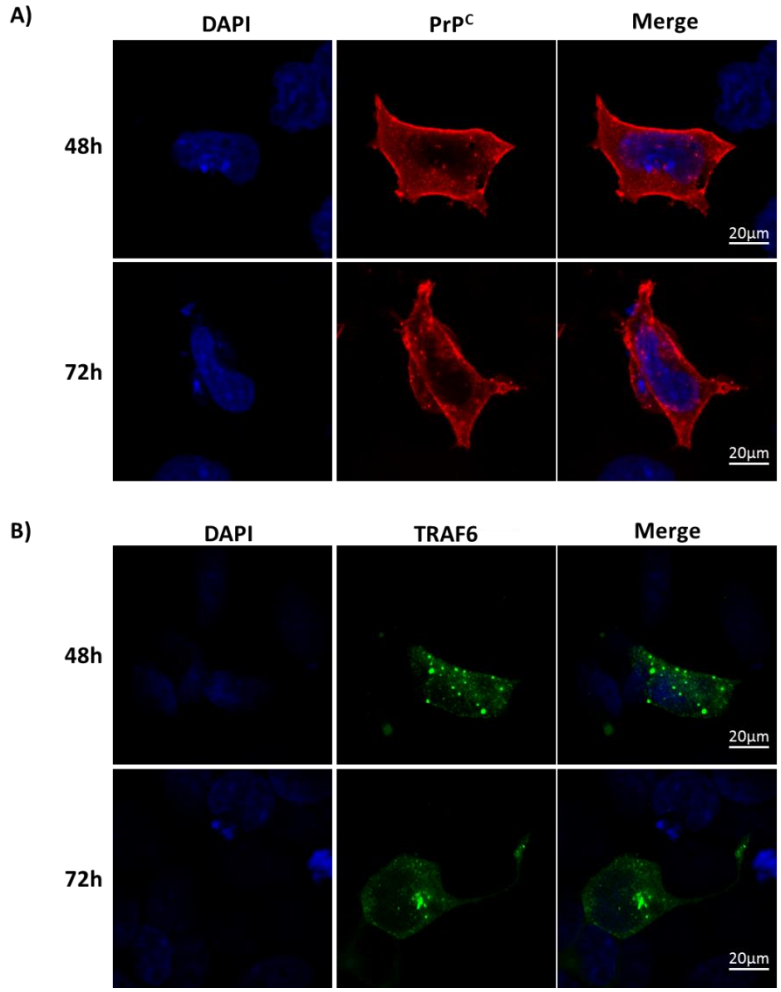

Figure 9: Expression of FL PrP^C and TRAF6. HEK293T cells were transfected with FLAG-FL PrP^C (A) or GFP-TRAF6 WT (B) and incubated with 5 µM MG132 for 16 hours before fixation at two or three days in cultures. Immunofluorescence was performed incubating the cells with anti-FLAG antibody. Overnight primary incubation was revealed with Alexa Fluor 594 conjugated fluorescent secondary antibody. GFP-TRAF6 WT was revealed by GFP autofluorescence. Nuclei were counterstained with DAPI. No differences were observed at the two different time points analysed in the FL PrP^C localization: the protein reached the membrane even at 48 hours post transfection. Moreover, it showed a cytoplasmic localization. No differences were found in the localization of GFP-TRAF6 as diffused in the cytoplasm and present in small aggregates (n.=2). Scale bar: 20µm.

TRAF6 CO-LOCALIZE WITH FL PrP^C AND IS FOUND IN AGGRESOME-LIKE STRUCTURES IN THE PERINUCLEAR REGION

HEK293T cells were plated and co-transfected with FLAG-FL PrP^C and GFP-TRAF6 WT and fixed at 48 and 72 hours post transfection, after treatment with MG132 proteasomal inhibitor. Co-localization of FLAG-FL PrP^C and GFP-TRAF6 WT into cell aggregates was observed at both the two time points analysed. Moreover, a different aggregates distribution was visible. Representative images in Figure 10 show the presence of small well-defined GFP-TRAF6 WT aggregates, some of which fully co-localized with FLAG-FL PrP^C aggregates. Alternatively, a widespread distribution of both proteins was observed, with a co-localization signal into one big perinuclear aggregate. Last but not the least, small and diffused aggregates of co-localizing proteins are found in the cell cytoplasm.

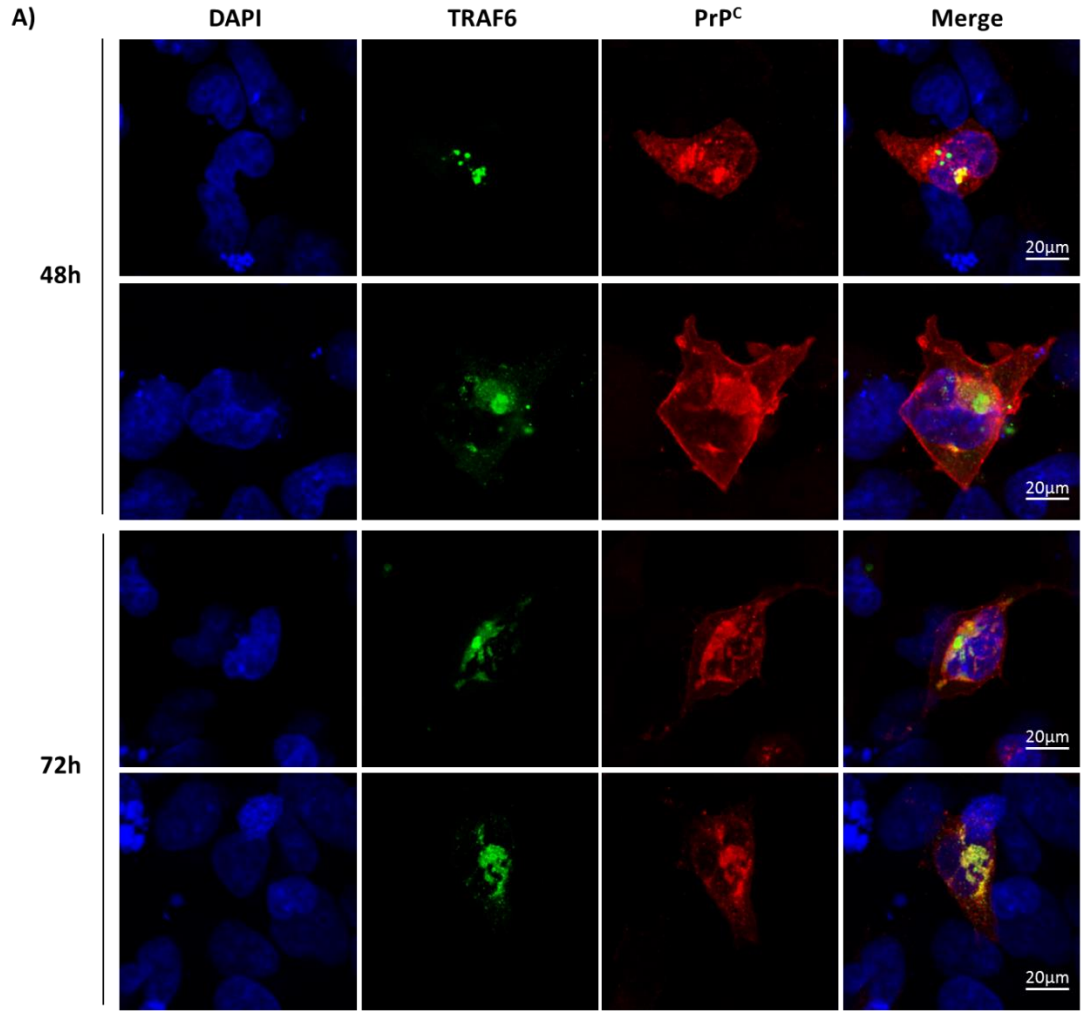

Figure 10: Co-localization of TRAF6 and FL PrP^C in aggresomes-like structures. Cells were transfected with FLAG-FL PrP^C and GFP-TRAF6 WT, treated with 5 µM MG132 for 16 hours and fixed after 48 hours from transfection. FL PrP^C was detected by anti-FLAG antibody followed by incubation with Alexa Fluor 594, GFP-TRAF6 WT was revealed by GFP autofluorescence. Nuclei were counterstained with DAPI. Merged images revealed protein co-localization into cell aggregates independently of the time point chosen. The first panel shows co-localization of FLAG-FL PrP^C with some small GFP-TRAF6 WT aggregates. The second and third panels show a more diffused staining with co-localization of the two proteins into one bigger aggregate. The fourth panel shows co-localization into small dispersed aggregates (n.=3). Scale bar: 20µm.

Stack analysis of FLAG-FL PrP^C and GFP-TRAF6 WT co-transfected cells revealed again the different pattern of aggregates formation. Insight of the representative cell in Figure 11A showed the co-localization of FLAG-FL PrP^C and GFP-TRAF6 WT into small cytoplasmic aggregates and the presence of FLAG-FL PrP^C and GFP-TRAF6 WT aggregates that never co-localize. Two different stacks were chosen to

underline that protein co-localization was not due to the merge of all z-stack that compose the image. On the contrary, the cell in Figure 11B is characterized by not co-localizing FLAG-FL PrP^C and GFP-TRAF6 WT aggregates, by two aggregates that partially overlap (Figure 1B, white arrow) and by other two aggregates that are in close contact but never co-localize (Figure 1B, black arrow). The different distribution observed could be explained by the different moment of cell cycle of each cell in culture.

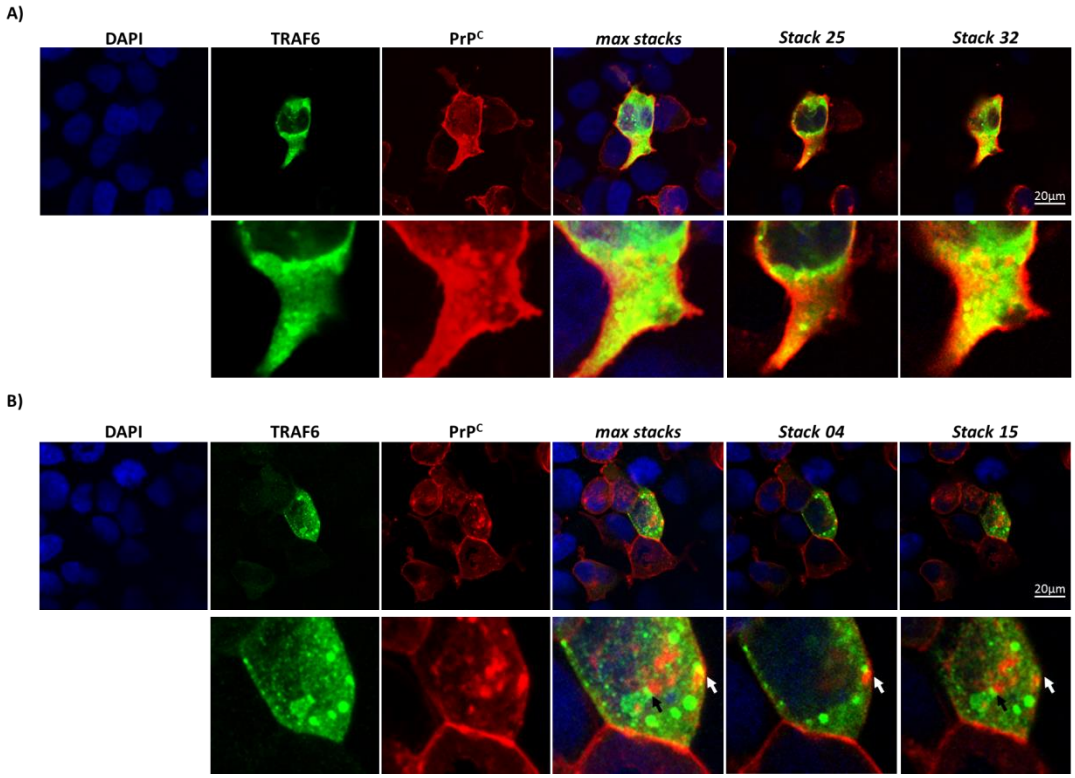

Figure 11: Stack analysis of TRAF6 and FL PrP^C aggregates. HEK293T cells were co-transfected and staining was revealed by immunofluorescence as in Figure 10. A) Co-localization of FLAG-FL PrP^C and GFP-TRAF6 WT was observed in aggregates in the cell cytoplasm. Some aggregates of FLAG-FL PrP^C are not positive for GFP-TRAF6 WT and *vice versa*. Two different z-stacks show the different accumulation of aggregates in the cell. B) Aggregates of FLAG-FL PrP^C and GFP-TRAF6 WT are separate identities in the cell. White arrow: partial overlap of two aggregates. Black arrow: two aggregates of FLAG-FL PrP^C and GFP-TRAF6 WT in close contact. Scale bar: 20µm.

TRAF6 AND FL PrP^C AGGREGATES ARE POSITIVE FOR THE SEQUESTOSOME MARKER p62

A way to sequester proteins is represented by accumulation in cellular sequestosomes, by the action of p62. The interaction of endogenous TRAF6 with substrate receptors is mediated by p62 (Sanz, Diaz-Meco et al. 2000), after receptor dimerization and endocytic internalization (Grimes, Beattie et al. 1997, Riccio, Pierchala et al. 1997). Moreover, TRAF6 was found associated to large membrane-bound sequestosomes (Puls, Schmidt et al. 1997, Sanz, Diaz-Meco et al. 2000, Seibenhener, Babu et al. 2004). In order to define the nature of FL PrP^C and TRAF6 WT aggregates, HEK293T cells transfected with FLAG-FL PrP^C and GFP-TRAF6 WT were stained for FLAG-FL PrP^C, GFP-TRAF6 WT and endogenous p62. In absence of GFP-TRAF6 WT, cytoplasmic FLAG-FL PrP^C co-localize with p62. When also the GFP-TRAF6 WT construct was present, the co-localization was further confirmed in aggregates positive for FLAG-FL PrP^C, GFP-TRAF6 WT and p62 (Figure 12). From this evidence we could assume that the aggregates observed are cellular sequestosomes.

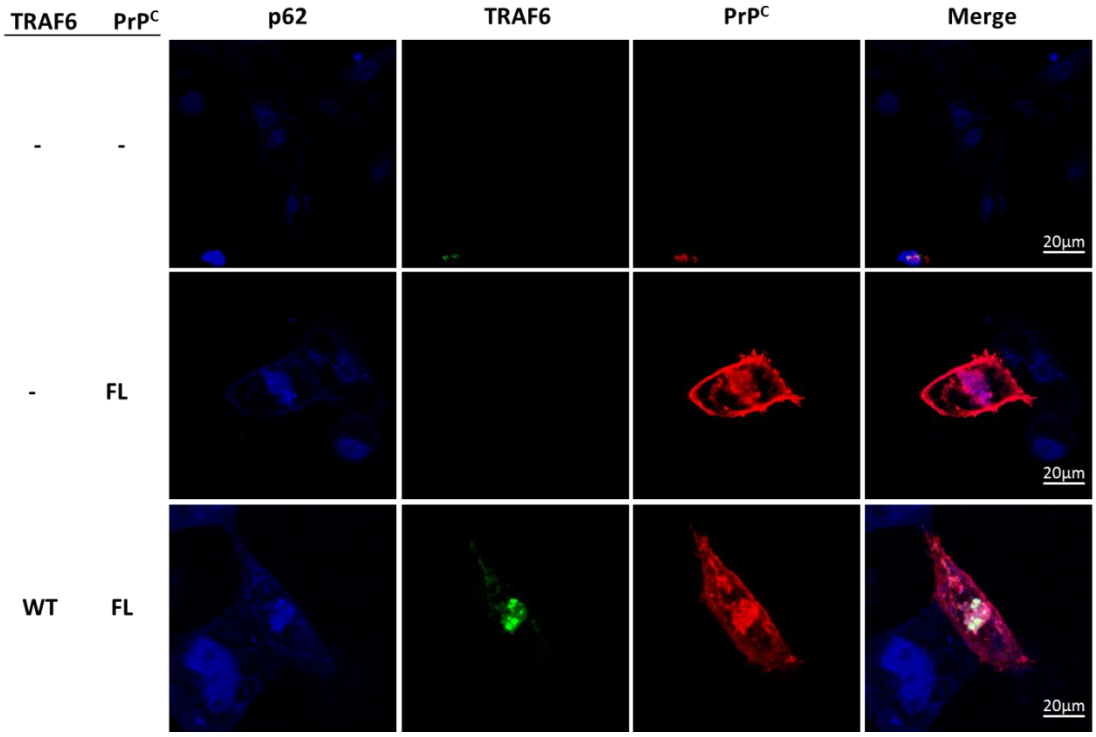

Figure 12: TRAF6 and FL PrP^C co-localize in cellular sequestosomes. HEK293T cells were single or co-transfected with GFP TRAF6 WT and FLAG-FL PrP^C and treated with 5 µM MG132 for 16 hours before fixation at 48 hours post transfection. Endogenous p62 was detected by anti-mouse p62 antibody followed by biotin-streptavidin 647 conjugated secondary antibody. FLAG-FL PrP^C was detected by anti-FLAG antibody followed by incubation with Alexa Fluor 594. GFP-TRAF6 WT was revealed by GFP autofluorescence. Merged images showed that in absence of GFP-TRAF6 WT, FLAG-FL PrP^C is present as diffuse staining that co-localize with endogenous p62 in a defined region of the cell. Triple staining revealed the co-localization of GFP TRAF6 WT, FLAG-FL PrP^C and p62. Increased intracellular staining of FL PrP^C was observed in presence of GFP TRAF6 WT. Scale bar: 20µm.

FL PrP^C IS UBIQUITYLATED EVEN IN ABSENCE OF TRAF6

In order to verify if TRAF6 WT is able to ubiquitylate the FL PrP^C after binding, HEK293T cells were transfected with Ha-Ub, FL PrP^C and FLAG-TRAF6 WT and DN. TRAF6 DN is a perfect internal control, as it excludes artefacts simply due to over-expression. As previously discussed, TRAF6 ubiquitin ligase activity needs the RING domain to ubiquitylated substrate proteins and this domain is absent in the TRAF6 DN construct. However, we observed the ubiquitylation of FL PrP^C in presence of

HA-Ub, independently of TRAF6 expression (Figure 13A). This evidence allowed us to confirm the observation on the ubiquitin binding site of the FL PrP^C protein. The presence of lysine residues in the N-terminal 1-39 portion of the FL PrP^C is necessary for ubiquitin binding (Figure 13B). The absence of evident differences when FLAG-TRAF6 WT is expressed suggested that other ligases are involved in FL PrP^C ubiquitylation.

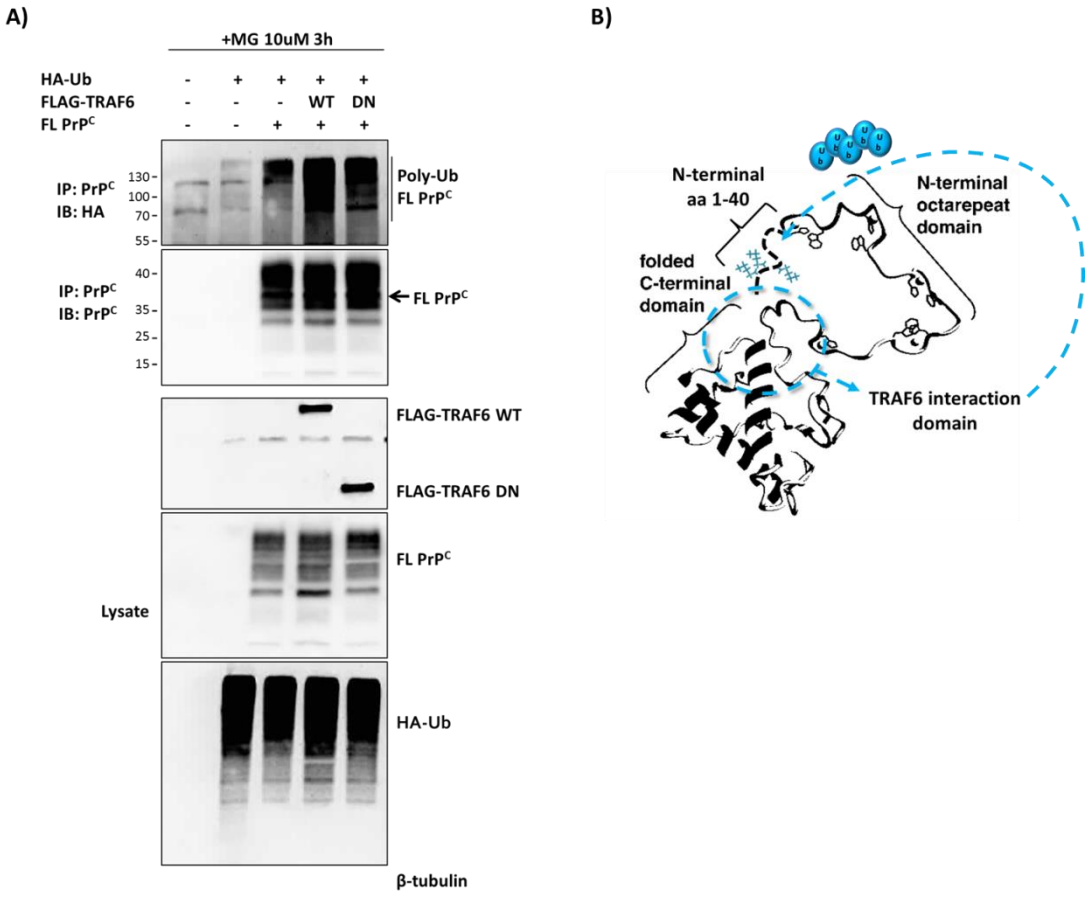

Figure 13: FL PrP^C is ubiquitylated. A) HEK293T cells were transfected with HA-Ub, FL PrP^C and FLAG-TRAF6 WT or DN constructs, treated with 10μM MG132 for 3 hours and lysed after 48 hours post transfection. Immunoprecipitation (IP) of FL PrP^C was performed with anti-PrP W226 antibody. Anti-HA antibody was used for detection of co-precipitated ubiquitin. Lysates were tested for the expression of HA-Ub, FL PrP^C and FLAG-TRAF6. β-tubulin was used as loading control. B) The cartoon image shows the putative TRAF6 C-terminal binding site and the N-terminal lysine residues that are ubiquitylated on the FL PrP^C (n.=4).

FL PrP^C IS FOUND IN CELLULAR AGGREGATES POSITIVE FOR UBIQUITIN AND TRAF6 WT

Presence of ubiquitin was then investigated in HEK293T cells transfected with Ha-Ub, FLAG-FL PrP^C, and GFP-TRAF6 WT or DN. FLAG-FL PrP^C showed a membrane and partially cellular localization when expressed alone or with HA-Ub (Figure 14A). Addition of GFP-TRAF6 WT resulted in the formation of cellular aggregates of FLAG-FL PrP^C, positive for HA-Ub and for GFP-TRAF6 WT (Figure 14A-B, inset). As magnified in Figure 14B (max stack and two separated z-stacks), smaller aggregates positive for FLAG-FL PrP^C and HA-Ub (little violet) as well as positive for GFP-TRAF6 WT and HA-Ub (little blue) were present. No aggregates were observed when TRAF6 DN was used (Figure 14A). Cell count positive for Ha-Ub, FLAG-FL PrP^C and GFP-TRAF6 WT revealed that the 59% of cells contained aggregates of HA-Ub and GFP-TRAF6 WT were negative for FL PrP^C, that maintained prevalently a membrane localization. The remaining 41% was characterized by aggregates positive also for FL PrP^C (n. cell counted= 128, from two different experiments).

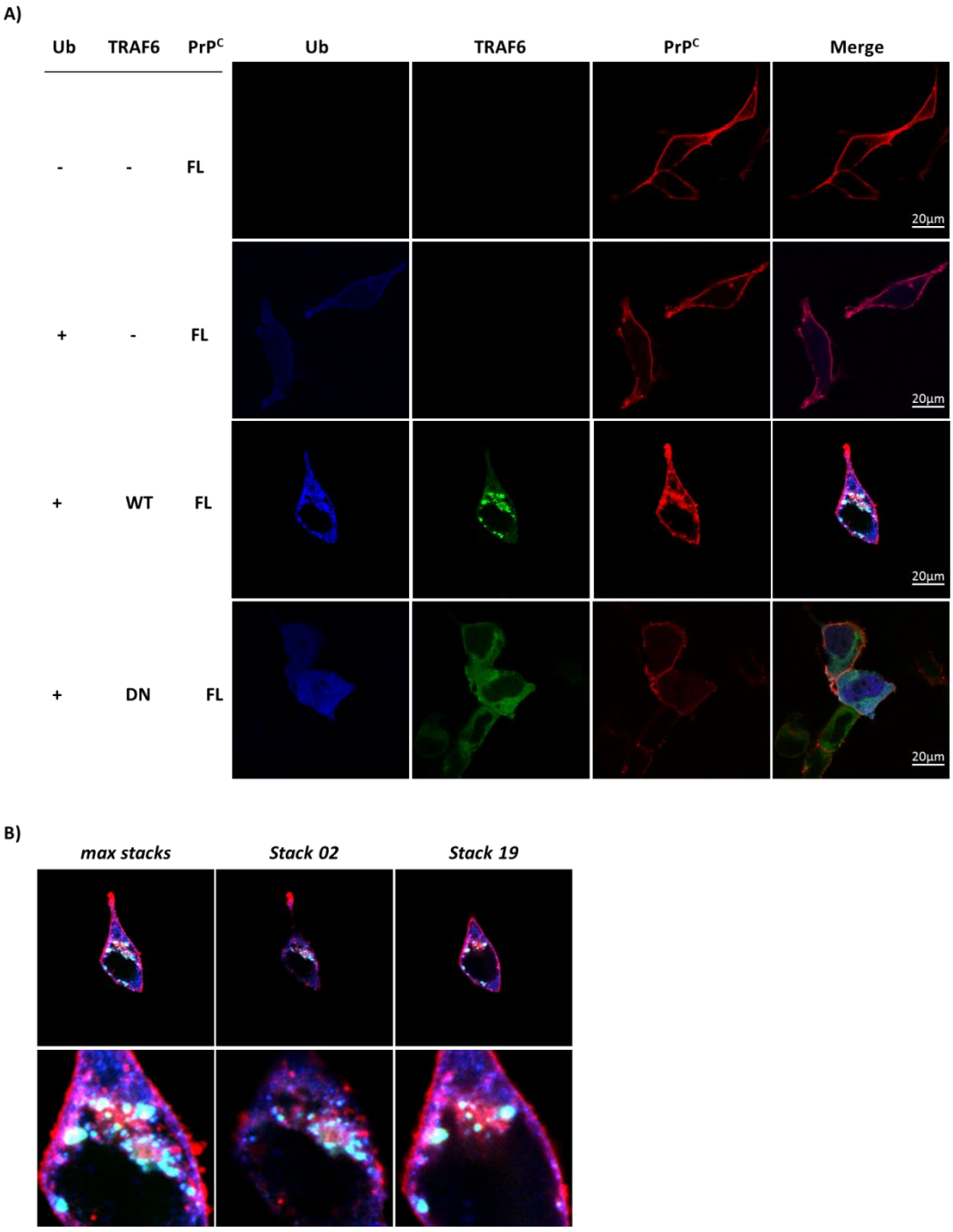

Figure 14: TRAF6 WT promotes the accumulation of FL PrP^C in cellular aggregates A) Cells were single transfected with FLAG-FL PrP^C or in combination with HA-Ub or HA-Ub and GFP-TRAF6, treated with 5 µM MG132 for 16 hours and fixed after 48 hours from transfection. Double immunofluorescence was performed with anti-FLAG and anti-HA antibodies followed by incubation with Alexa Fluor 594 and biotin-streptavidin 647 conjugated secondary antibodies. GFP-TRAF6 was revealed by GFP autofluorescence. In presence of GFP-TRAF6 WT, FLAG-FL PrP^C was found in cellular aggregates positive for TRAF6 and ubiquitin. This was not observed when GFP-TRAF6 DN was expressed. B) Inset of FLAG FL PrP^C aggregates in presence of HA-Ub and GFP-TRAF6 WT from panel

A. Partial triple co-localization (white) was observed (max stack and Stack 19). Smaller GFP-TRAF6 WT and HA-Ub positive aggregates (little blue) surrounded a core of FLAG-FL PrP^C (red), clearly visible in Stack 02. FLAG-FL PrP^C and HA--Ub aggregates (little violet) are present throughout the cell (n.=2).

PrP^C AND TRAF6 CO-LOCALIZATION IN THE N2a CELL MODEL

Murine N2a cells were used as a second *in vitro* model to study the interplay between TRAF6 and PrP^C. These cells are frequently used for the investigation of the pathological form of PrP (PrP^{Sc}), since infected with the RML strain of prion. The use of this cell model would give a validation of the over-expression system used with HEK293T cells and a first pathological evidence of the interplay of TRAF6 and PrP^C. We first checked for endogenous TRAF6 expression in N2a cells, knock-out for PrP^C expression (KO N2a), expressing the wild type PrP^C (WT N2a) or its pathological form (ScN2a). Cells were plated and fixed after 48 hours in culture. Protein expression was revealed after staining with anti-PrP W226 and anti-TRAF6 antibodies. TRAF6 was endogenously expressed in N2a cells (Figure 15, upper panel). Co-localization of PrP^C and TRAF6 was observed in WT N2a cells as diffused staining, and clearly detected in ScN2a cells. ScN2a cells were characterized by aggregates of both PrP^{Sc} and TRAF6 that showed a certain degree of co-localization. Not all the ScN2a cells showed an increased PrP expression and accumulation. In these cells, also the TRAF6 staining revealed a diffused and not aggregated localization, similar to the one observed in WT N2a cells (Figure 15B, inset). This data suggests a tendency of endogenous TRAF6 to aggregate in presence of PrP^{Sc}.

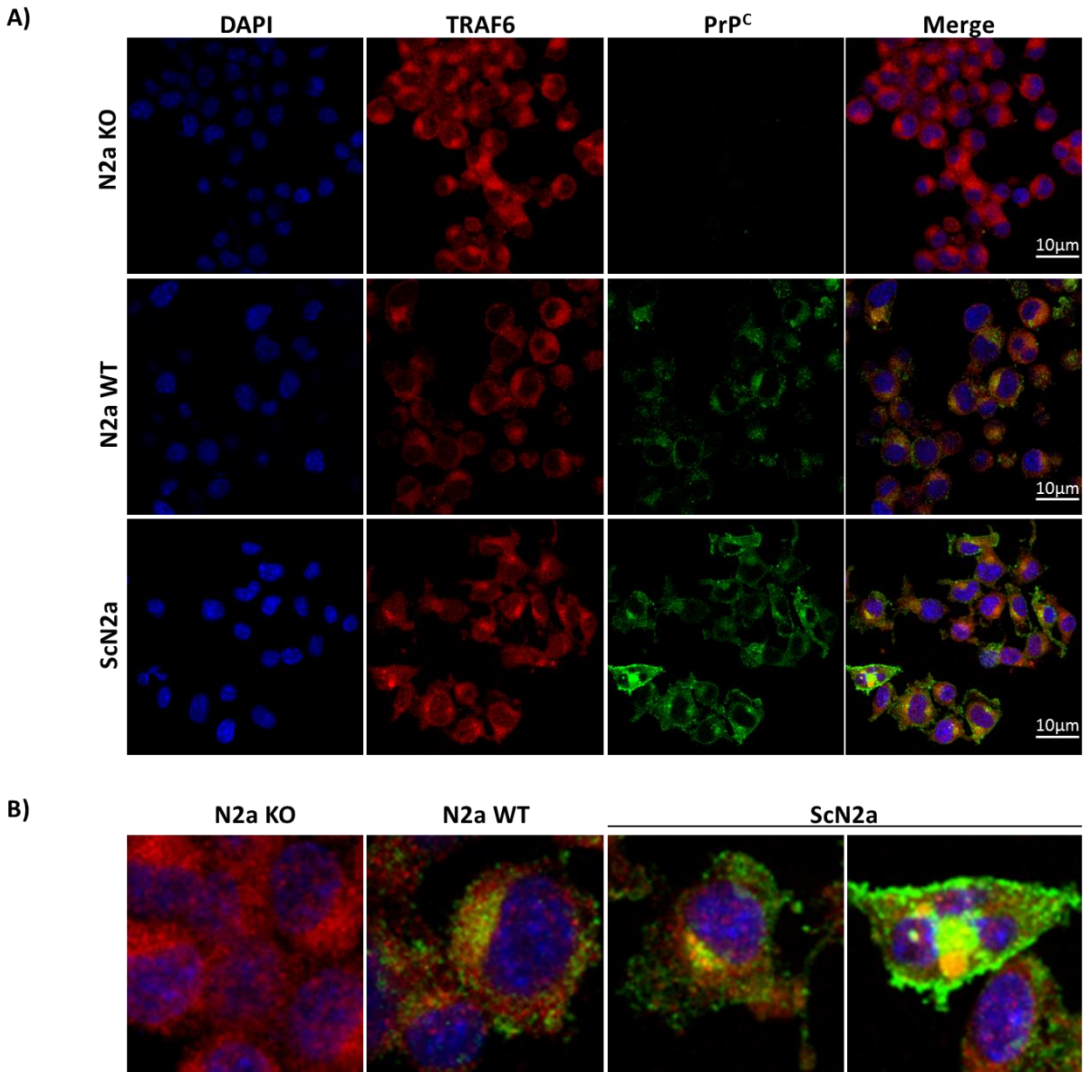

Figure 15: PrP^C and TRAF6 expression and localization in N2a cells. A) N2a cells were fixed after 48 hours in culture and treated with GdnHCl for 10 minutes. Double immunofluorescence was performed with anti TRAF6 and W226 anti-PrP antibodies, followed by incubation with secondary Alexa Fluor 594 and 488 antibodies. Nuclei were counterstained with DAPI. N2a cells were positive for TRAF6. Co-localization of PrP and TRAF6 was clearly seen only in ScN2a cells (n.=2). Scale bar: 10µm. B) Zoom-in images from panel (A) revealed a variable pattern of co-localization.

CHAPTER 4

DISCUSSION

Great attention has been addressed to the study of the pathological isoform of PrP^C until the discovery of being the causative agent of transmissible encephalopathy (Prusiner 1991). Indeed, investigation of the mechanisms of aggregation and spreading has added a conspicuous amount of information to the field. However, a complete knowledge about PrP^C physiological functions is still lacking. Evidence suggests a role for PrP^C as a receptor involved in intracellular signalling for neuronal development (Gauczynski, Peyrin et al. 2001, Schmitt-Ulms, Legname et al. 2001), (Zanata, Lopes et al. 2002, Chen, Mange et al. 2003, Kanaani, Prusiner et al. 2005, Santuccione, Sytnyk et al. 2005) and signalling transfer from cell to cell (Liu, Li et al. 2002). Moreover, neuronal survival has been observed due to PrP^C interaction with Grb2 in the endosomal compartment (Spielhaupter and Schatzl 2001). An increased number of evidence point to an anti-apoptotic function of PrP^C correlated to its presence in the cytosol (Deckwerth, Elliott et al. 1996, Kuwahara, Takeuchi et al. 1999, Roucou, Guo et al. 2003, Roucou, Giannopoulos et al. 2005). However, opposing results were obtained when different models and different cytoplasmic PrP^C constructs were used (Ma and Lindquist 2001, Yedidia, Horonchik et al. 2001, Ma, Wollmann et al. 2002, Paitel, Fahraeus et al. 2003). In order to clarify this uncertain scenario, Hegde and colleagues demonstrated that, in the processing of PrP^C, a small percentage of the protein fails to enter into the ER or is retrotranslocated from the ER into the cytoplasm, where it can exert its protective function prior to be degraded. Pathological point mutations also results in cytoplasmic residence of PrP^C (Hegde and Lingappa 1999, Kim and Hegde 2002). This cytoplasmic PrP^C population is targeted by the UPS for proteasomal degradation (Jin, Gu et al. 2000, Lee, Iwakoshi et al. 2003, Shao, Choe et al. 2014) and shouldn't be confused with PrP^C endocytosed from and recycled to the cell membrane or degraded by the autophagic system. Autophagy of PrP has been investigated for its pathological relevance in presence of PrP^{Sc} (Boellaard, Kao et al. 1991, Sikorska,

Liberski et al. 2004, Heiseke, Aguib et al. 2010, Liberski, Sikorska et al. 2010, Yao, Zhao et al. 2013). PrP^{Sc} aggregates are found in infected cells and degraded by p62-mediated degradation (Homma, Ishibashi et al. 2014). Instead, cytotoxic form of cytosolic PrP constructs are present in dispersed aggregates, that do not share characteristic features of aggresomes (Beaudoin, Goggin et al. 2008). Further investigation of the cellular mechanisms involved in the processing of both physiological and pathological isoforms of PrP^{C} is necessary.

In this work we focus our attention to the TRAF6 protein. TRAF6 is involved in the regulation of signalling both as an adaptor protein and as an E3 ubiquitin ligase. Its atypical substrate ubiquitylation viaLys63 was deeply investigated in the activation and signalling of NF-κB (Xia, Sun et al. 2009). This led to address to TRAF6 a principal role in the inflammatory and immune responses. Moreover, involvement of TRAF6 Lys63 atypical ubiquitylation in actin polymerization and cytoskeletal rearrangement was also observed (Wang, Wara-Aswapati et al. 2006). A role for TRAF6 in protein aggregates formation was also hypothesized. Indeed, TRAF6 was found in aggregates of Tau protein in AD (Babu, Geetha et al. 2005) and of α-synuclein in sporadic PD (Zucchelli, Codrich et al. 2010) post-mortem brains. Interestingly, the investigation of TRAF6 binding and ubiquitylation of substrate α-synuclein and huntingtin revealed a different ubiquitylation code, in which Lys6, Lys27 and Lys29 are involved (Zucchelli, Codrich et al. 2010, Zucchelli, Marcuzzi et al. 2011). Aggregates formation in presence of TRAF6 ubiquitylation activity was observed for both the pathological and the physiological α-synuclein and huntingtin proteins (Zucchelli, Codrich et al. 2010, Zucchelli, Marcuzzi et al. 2011). In this work, we analysed the interplay of TRAF6 with the physiological form of PrP^{C}, in order to unveil a common cellular response to naturally aggregation-prone proteins.

We found that TRAF6 and PrP^{C} interact when co-expressed *in vitro*. We used a FL-PrP^{C} (mouse, aa 1-253) construct in order to mimic the physiological PrP^{C} and a cyPrP^{C} (mouse, aa 40-231), that is not processed as the FL-PrP^{C} and resides in the cytosol in puncta. This cytosolic form of PrP^{C} is not rapidly degraded by the proteasome (Chakrabarti, Rane et al. 2011). The use of this two constructs was

justified by the cytosolic localization of TRAF6 (Force, Glass et al. 2000, Hostager, Catlett et al. 2000). At first we expected a positive immunoprecipitation result only in case of cyPrP^{C}. Indeed, co-immunoprecipitation of cyPrP^{C} was observed after immunoprecipitation of FLAG-TRAF6. cyPrP^{C}-TRAF6 interaction was further increased under condition of transient and irreversible proteasomal inhibition (Figure 3A). The reverse co-immunoprecipitation was positive only when an N-terminal PrP antibody was used (DE10, epitope: 41-56). Reasonably, the interaction of the C-terminal portion of TRAF6 (substrate binding domain) and PrP^{C} involves the C-terminal PrP domain. The formation of the immune complex between PrP and the C-terminal PrP W226 antibody (epitope: 144-152) undermines the PrP^{C}-TRAF6 binding and results in a negative co-immunoprecipitation result (Figure 3B). Surprisingly, PrP^{C}-TRAF6 interaction was also observed when the FL-PrP^{C} construct was used. Direct co-immunoprecipitation using flagged TRAF6 and FL PrP^{C} was confirmed by reverse co-immunoprecipitation of GFP-tagged TRAF6 and flagged FL PrP^{C}. PrP^{C}-TRAF6 interaction was further increased under condition of transient and irreversible proteasomal inhibition (Figure 6). Also in this case, immunoprecipitation of an unflagged FL PrP^{C} construct results in the negative co-immunoprecipitation of TRAF6 when the W226 antibody was used. A positive even if faint result was obtained with the DE10 antibody (Figure 7A). This data confirms the previous evidence for the site of PrP^{C}-TRAF6 interaction and suggests that the truncation of aa 1-39 as well as the insertion of a FLAG sequence between aa 40-41 of PrP^{C} allows the detection of PrP^{C}-TRAF6 interaction. In this model, we can assume that the very first part of the PrP^{C} N-terminal flexible domain is not involved in the interaction and that the FLAG insertion modifies the structure in the way of a more open conformation to allow protein binding and formation of the immune complex with anti-FLAG antibody.

It could be argued that the interaction observed with the FL PrP^{C} is the result of an artefact due to overexpression of the construct and subsequent accumulation in the cell cytosol, where it can interact with TRAF6. Probably, the amount of FL PrP^{C} in the cytosol is increased in our cellular model, allowing us to investigate an effect that physiologically is probably strictly kept under control. The presence of all

glycosylated isoform of PrP^C in western blot and the correct membrane localization (Figure 9A) of FL PrP^C construct suggests that its cytosolic localization could be the result of recycling from the cell membrane. However, we can also assume that a portion of FL PrP^C originates from ER retro-translocation. It is more plausible that free cytosolic FL-PrP^C rather than vesicle-contained FL PrP^C interacts with TRAF6, even if a sub-membrane localization of TRAF6 has been demonstrated for TRAF6 signalling recruitment to lipid rafts (Inoue, Ishida et al. 2000, Wajant, Henkler et al. 2001, Lamothe, Besse et al. 2007, Ha, Han et al. 2009, Xia, Sun et al. 2009). Involvment of TRAF6 in the internalization of FL PrP^C, normally associated to lipid rafts (Vey, Pilkuhn et al. 1996) should not be excluded. To validate our *in vitro* results, co-immunoprecipitation of PrP^C and TRAF6 was observed in the mouse brain at six month of age. We chose this time point hipothesising that in the brain of a mature aged mouse the UPS system is not perfect as in early stage of life. In fact, at two months of age we observed contrasting results (data not shown). It is curious to note that the endogenous PrP^C co-precipitated prevalently in its diglycosilated isoform.

Immunofluorescence experiments revealed PrP^C-TRAF6 co-localization. Overexpression of cyPrP^C and TRAF6 resulted in the formation of small aggregates of cyPrP^C that partially co-localized with bigger TRAF6 aggregates (Figure 4). Moreover, a clear co-localization was observed when FL PrP^C was co-transfected with TRAF6 (Figure 10). Interestingly, despite the time point chosen, we observed different type of aggregation of FL PrP^C and TRAF6: small co-localizing aggregates or amorphous bigger co-localizing aggregates of FL PrP^C and TRAF6 are observed in (Figure 10). However, we also observed partial co-localization or total absence of co-localization of FL PrP^C and TRAF6 aggregates that could be in close contact or in separated cytosolic domains (Figure 11). We can hypothesize that TRAF6 is in some extent responsible for PrP^C aggregation. Alternatively, TRAF6 could be involved in the accumulation of FL PrP^C aggregates in the cytosolic compartment providing an inert stock of molecules or marking them for autophagic degradation. This is in line with the ability of TRAF6 to promote an atypical ubiquitylation of the substrate proteins and with its interaction with p62 (Sanz, Diaz-Meco et al. 2000).

Furthermore, our data are in line with the demonstrated interaction and aggregate localization of TRAF6 with α-synuclein and huntingtin (Zucchelli, Codrich et al. 2010, Zucchelli, Marcuzzi et al. 2011). It is interesting to note a parallelism among α-synuclein, huntingtin and the prion protein, despite the cellular localization. The natural tendency of these proteins to misfold is increased in presence of mutations that affect protein structure. Moreover, presence of a respective "seed" or fibrillar substrate enhance the misfolding process. All togheter, this evidence strongly supports a common role for TRAF6 in aggregate formations of these neurodegenerative-associated proteins, suggesting a control mechanism at first in the processing of their physiological forms.

Interestingly, we found a co-localization of PrP^C and TRAF6 with the sequestosomes marker p62 (Figure 12). In sequestosomes, polyubiquitylated proteins are sequestered prior to be degraded by the autophagolysosomal pathway (Bjorkoy, Lamark et al. 2005, Komatsu, Waguri et al. 2007, Pankiv, Clausen et al. 2007, Ichimura, Kumanomidou et al. 2008) or linked to the proteasome (Seibenhener, Babu et al. 2004). Moreover, as mentioned before, TRAF6 is able to interact with p62 (Sanz, Diaz-Meco et al. 2000) and associates to large membrane-bound sequestosomes during activation of TNFR and TIR receptors (Puls, Schmidt et al. 1997, Sanz, Diaz-Meco et al. 2000, Seibenhener, Babu et al. 2004). Our result suggests that PrP^C and TRAF6 aggregate in sequestosomes. This data is supported by the evidence of a co-localization of FL PrP^C and TRAF6 WT with ubiquitin in cellular aggregates (Figure 14B). We found that FL PrP^C is ubiquitylated in presence of TRAF6 WT. FL PrP^C ubiquitylation was not observed in absence of TRAF6 or when the ligase-incompetent TRAF6 DN was expressed. Only the 41% of the cells analysed, transfected with all three constructs, contined aggregates positive for FL PrP^C, TRAF6 WT and HA-Ub. In the remaining 59% aggregates were positive only for TRAF6 WT and HA-Ub (Figure 14). A representative image in Figure 14B shows the combination of staining that could be observed with the transfected proteins. FL PrP^C ubiquitylation is in line with previous works focused on the role of PrP^C ubiquitylation and proteasomal degradation for cytosolic forms of PrP^C (Shao, Choe et al. 2014), GSS-related cytosolic PrP^C mutants (Jin, Gu et al. 2000) and

unglycosilated PrP^C constructs (Zhang, Wang et al. 2012). Co-localization of TRAF6 WT and ubiquitin is justified by TRAF6 auto-ubiquitylation activity after oligomerization (Ostuni, Zanoni et al. 2010). The fact that less than the half of the analysed cells contain FL PrP^C-ubiquitin aggregates also positive for TRAF6 WT leads us to speculate that TRAF6 has a specific role in PrP^C ubiquitylation and it involves the formation of PrP^C positive sequestosomes. This evidence is further supported by the ubiquitylation assay result. Also in absence of TRAF6 or in presence of the ligase-incompetent TRAF6 DN, the FL PrP^C is ubiquitylated (Figure 13A). It should be interesting to investigate the code of atypical ubiquitylation involved in the TRAF6 ubiquitylation of FL PrP^C. We can postulate a Lys6, Lys27 and Lys29 atypical ubiquitylation as for α-synuclein, DJ-1 and huntingtin (Zucchelli, Codrich et al. 2010, Zucchelli, Marcuzzi et al. 2011), rather than the Lys63 dependent ubiquitylation observed *e.g* in the NF-κB pathway (Lamothe, Besse et al. 2007, Keating and Bowie 2009, Ostuni, Zanoni et al. 2010). However, Lys63 dependent FL PrP^C ubiquitylation should not be excluded taking into account the role of PrP^C in cellular signalling (Mouillet-Richard, Ermonval et al. 2000, Schmitt-Ulms, Legname et al. 2001, Kanaani, Prusiner et al. 2005). Further analysis should be done to clarify if TRAF6 WT could in some way influence the amount of FL PrP^C in the cytosol. In presence of the ligase-incompetent TRAF6 DN, as well as when TRAF6 is not expressed, FL PrP^C seems to be localized prevalently on the cellular membrane and is not present in cytosolic aggregates (Figure 14A). Moreover, we have observed an increased FL PrP^C expression in HEK293T cells when co-transfected with TRAF6 WT, if compared to cells only transfected with FL PrP^C (data not shown).

We have further identified the putative ubiquitin PrP^C binding site. Ubiquitylation of $cyPrP^C$ was not observed nor in presence neither in absence of TRAF6. Deletion of amino acids 1-39 in the $cyPrP^C$ construct results in the lost of Lys23, Lys24 and Lys27. It is reasonable that one of this three lysine residues present in the first part of the N-terminal of PrP^C is the substrate of ubiquitylation.

N2a cells were used to investigate PrP-TRAF6 interaction in another cell line model. N2a cells that expresse the endogenous PrP^C do not show a clear co-localization

result. This is in line with the hypothesis that in physiological condition and in a not overexpressing system the amount of cytosolic resident PrP is low. However, the expression of the pathological PrP^{Sc} results in formation of TRAF6 aggregates that partially co-localize or are in proximity of PrP^{Sc} aggregates (Figure 15). This data suggests the involvement of TRAF6 in the aggregation of pathological PrP^{Sc} and gives further light to a common cellular response to neurodegenerative-associated proteins.

In order to confirm the data on $N2a^{Sc}$ cells, PrP-TRAF6 interaction should be analized *in vivo* on RML infected mouse brains. This data would be a further evidence for TRAF6 involvement in pathological conditions, as seen for the presence of TRAF6 in Tau-containing intracellular aggregates in AD post-mortem brains (Babu, Geetha et al. 2005) and in Lewy bodies in DA neurons of PD post-mortem brains (Zucchelli, Codrich et al. 2010).

In this work we have highlighted the involvement of TRAF6 in the targeting, aggregation and ubiquitylation of cytosolic and full length PrP^{C}. The interaction *in vivo* further confirms our data. Moreover, our *in vitro* evidence of a co-localization of aggregated forms of PrP^{Sc} and TRAF6 allow us to postulate a common mechanism in the cellular response to neurodegeneration.

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