E3 UBIQUITIN LIGASE HRD1 MEDIATES THE RETROTRANSLOCATION OF HUMAN PRION PROTEIN

By

Sarah L. Peters

Department of Neurology and Neurosurgery

McGill University, Montreal

December 2013

A thesis submitted to McGill University in partial fulfillment of the requirements for the degree of Master of Science

TABLE OF CONTENTS

PAGE

ABSTRACT

Prion diseases are fatal neurodegenerative disorders. Ubiquitously expressed Prion protein (PrP) is found at the cell surface and is abundant in brain. However, a small proportion of PrP is found in the cytosol (CyPrP), arising from the ER-associated degradation (ERAD) pathway. CyPrP protects against Bcl2-associated X protein (Bax)-mediated apoptosis and most disease-causing PrP mutants (mPrP) completely or partially lose their ability to prevent Bax-mediated apoptosis through defective retrotranslocation of PrP (Jodoin *et al.*, 2007). The E3 ubiquitin ligase HMG-CoA reductase degradation protein 1 (hrd1p) was found to mediate the retrotranslocation of human PrP in yeast (Apodaca *et al.*, 2006). To determine the mechanism responsible for PrP retrotranslocation in the mammalian CNS, we either overexpressed an eYFP-Hrd1 fusion protein, or silenced endogenous Hrd1 in human CR7 glioblastoma cells and examined resultant levels of CyPrP. Hrd1 overexpression results in an increase in CyPrP, while, conversely, targeted knockdown of Hrd1 mRNA results in a marked decrease in CyPrP. Additionally we examined the effect of mPrP on the retrotranslocation of both PrP. Expression of mPrP substantially decreases CyPrP. Additionally, mPrP may be able to block the retrotranslocation of other model ERAD Hrd1 substrates, such as mutant transthyretin (TTR^{D18G}), indicating the effect is not specific to PrP. The results show that E3 ligase Hrd1 mediates the retrotranslocation of cellular PrP and that mPrP fail to produce CyPrP by interrupting Hrd1-associated retrotranslocation machinery. This disruption also appears to interfere with the retrotranslocation of Hrd1-mediated ERAD substrate $TTR^{D18G.}$ We conclude that familial prion disease pathology could be due to both a loss of neuroprotective CyPrP and an inability to properly degrade other mutated or misfolded proteins through a disruption of the ERAD pathway. This accumulation of misfolded proteins over time, combined with general dysfunctions associated with aging, may explain delayed disease onset in individuals carrying pathogenic PrP mutations.

RÉSUMÉ

Les maladies à Prion sont des maladies neurodégénératives mortelles. La protéine Prion (PrP), ubiquitairement exprimée, se retrouve en abondance dans le cerveau à la surface cellulaire. PrP provenant de la machinerie de dégradation des protéines associées au réticulum endoplasmique (ERAD) est localisée au cytosol (CyPrP). CyPrP protège contre l'apoptose induite par Bax. Aussi, des mutants de PrP (mPrP) causant les maladies ont perdu leurs capacité à prévenir l'apoptose causée par Protéine X associée à Bcl-2 (Bax), dû à une rétrotranslocation défectueuse de PrP (Jodoin *et al.*, 2007). La E3 ubiquitine ligase appelée protéine HMG CoA réductase dégradation 1 (hrd1p) est un rétrotranslocateur de huPrP dans les levures (Apodaca *et al.*, 2006). Pour déterminer le mécanisme responsable de la rétrotranslocation du PrP dans le SNC des mammifères, nous avons soit surexprimé ou soit diminué l'expression de Hrd1 dans les glioblastomes humaines CR7. La surexpression d'eYFP-Hrd1 entraîne une augmentation du CyPrP et la diminution de l'ARN messager de Hrd1 cause une nette diminution de CyPrP. De plus, nous avons étudié les effets des mPrP sur la rétrotranslocation de PrP. La surexpression de mPrP diminue considérablement la présence de CyPrP. Cette perturbation semble interférer avec la retrotranslocation d'un mutant de transthyrétine (TTR^{D18G}), un autre substrat du ERAD médié par Hrd1. Ces résultats démontrent que la ligase Hrd1 est responsable de la rétrotranslocation de CyPrP et que les mPrP sont incapables de produire du CyPrP en bloquant la rétrotranslocation par Hrd1. Nous concluons que la pathologie des maladies familiales à Prion pourrait être due à une perte du CyPrP neuroprotecteur ainsi qu'à une perturbation de la machinerie ERAD. Par conséquent, une accumulation de protéines mal repliées, combinée à des dysfonctions générales associées au vieillissement, pourrait expliquer l'apparition et la progression lente de la maladie chez les individus portants les mutations pathogéniques de PrP.

ACKNOWLEDGEMENTS

First and foremost I would like to thank my advisor, Dr. Andrea LeBlanc, without which none of this would be possible. Thank you for providing me with a challenging project, for your passion and guidance and for supporting me through the ups and downs of graduate student life.

I am grateful to all past and present lab members. In particular I would like to thank *Dr. Raphael Rouget* for taking me under his wing and teaching me so patiently. *Prateep Pakavathkumar*, an incredibly supportive lab mate, as well as a dear friend. I won't say any more for your sake Tips!

Selin Neseliler, my running partner and light of my life, words cannot describe how grateful I am for having met you. *Jasmine Ramcharitar,* thank you for every laugh, every tear, every hug (and every bottle of wine). You are, and will continue to be an inspiration to me. *Stephanie Shumsky,* thank you for being the most amazing friend and roommate imaginable. I am so lucky to have such strong, amazing women in my life. Love you chickens till the end!

Lastly, I would like to thank my family, *Ann, David, Steph, Jamie* and *Shine-O* for providing me with every imaginable kind of support and, most importantly, for believing in me when I didn't. I love you more then I could possibly put into words.

ABBREVIATIONS

129M: Polymorphism of PrP, Methionine at position 129

129V: Polymorphism of PrP, Valine at position 129

Asn: Asparagine

ATF6: Activating Transcription Factor 6

Bax: Bcl-2 associated X protein

Bcl-2: B Cell Lymphoma/Leukemia 2

BiP: Binding immunoglobulin Protein

BFA: Brefeldin A

CJD: Creutzfeldt-Jakob Disease

CNS: Central Nervous System

CyPrP: Cytosolic prion protein

EDTA: Ethylenediaminetetraacedic acid

eIF2α: Eukaryotic Initiation Factor 2 α

eYFP: Enhanced yellow fluorescent protein

ER: Endoplasmic Reticulum

ERAD: Endoplasmic reticulum associated degradation pathway

ERK1/2: Extracellular signal-regulated kinases 1 and 2

ERSE: endoplasmic reticulum stress response elements

fCJD: familial Creutzfeldt-Jakob Disease

FFI: Fatal Familial Insomnia

GFP: Green fluorescent protein

GPI: Glycophosphatidylinositol

GSS: Gerstmann-Strausssler-Sheinker Syndrome

HSF: Heat Shock Factor

Hrd1: Human E3 ligase HMG-CoA reductase degradation protein 1

HSP: Heat shock protein

mHsp70: Mitochondrial heat shock protein 70

mRNA: Messenger ribonucleic acid

NMDA: N-methyl-D-aspartate

P97/VCP: Valosin-containing protein

PCR: Polymerase Chain Reaction

PERK: Protein kinase RNA-like ER kinase

PHN: Primary human neurons

PIPLC: Phosphatidylinositol specific phospholipase C

PK: Proteinase K

PKA: Protein Kinase A

PK^{Res}: Proteinase K-resistant prion protein

PNGase F: Peptide-N-Glycosidase F

PrP: Prion protein

PrP^{C}: Normal cellular prion protein

PrP^{ctm}: Transmembrane prion protein with C-terminal located inside the ER lumen

PrP^{ntm}: Transmembrane prion protein with N-terminal located inside the ER lumen

PrP^{Sc}: Pathological isoform of prion protein, scrapie-like

PrP^{Sec}: Secreted/GPI anchored cell surface prion protein

PRNP: Human prion gene

RING: Really interesting new gene

R-PrP: Recombinant prion protein

sCJD: Sporadic Creutzfeldt-Jakob Disease

SDS: Sodium dodecyl sulphate

SDS-PAGE: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

siRNA: Small interfering ribonucleic acid

SP-CyPrP: Cytosolic prion protein retaining the N- and C-terminal signal peptides

SPRN: Human Shadoo gene

STI1: Stress Inducible protein 1

SYVN1: Synoviolin

sXBP1: Spliced X-box Binding Protein 1

TSE: Transmissible Spongiform Encephalopathy

TM: Tunicamycin

Ub: Ubiquitination

UPR: Unfolded protein response

UPRE: Unfolded protein response element

Val: Valine

vCJD: Variant Creutzfeldt-Jakob Disease

WT: Wild Type

LIST OF FIGURES

PAGE

INTRODUCTION

Prion protein (PrP) is best known for its role as the disease-causing agent in transmissible spongiform encephalopathies (TSEs) (Prusiner, 1982). As a ubiquitously expressed and highly conserved protein, the role of PrP in normal physiology, particularly as a neuroprotective protein, is still under intense investigation (Roucou *et al.*, 2005, Hu *et al.*, 2008, Lin *et al.*, 2008). Our laboratory has shown that PrP protects against cell death in primary human neurons (PHNs) and MCF7 cells by preventing the Bax conformation change that precedes activation, thereby inhibiting Bax-mediated apoptosis (Bounhar *et al.*, 2001a, Roucou *et al.*, 2005). This neuroprotective function has been attributed to cytosolically expressed PrP (CyPrP) as opposed to the more abundantly expressed, GPI-anchored, cell surface or transmembrane forms of PrP (Lin *et al.*, 2008). Familial PrP mutants, block the ability of cells to generate CyPrP in a dominant-negative fashion, resulting in the loss of anti-Bax ability (Jodoin *et al.*, 2007, Jodoin *et al.*, 2009). There are multiple routes by which PrP can become cytosolically expressed, however, anti-Bax CyPrP is generated via the endoplasmic reticulum associated degradation (ERAD) pathway, whereby proteins are retrotranslocated from the ER to the cytosol and targeted to the proteasome for degradation (Roucou *et al.*, 2003, Jodoin *et al.*, 2007).

From these data, we deduced that PrP mutants are interfering with the retrotranslocation complex, preventing the ERAD of both mutant and normal PrP. The aim of the work presented was to determine the mechanism, specifically, which E3 ubiquitin ligase mediates PrP retrotranslocation in mammalian cells. We hope to determine how PrP mutants are interrupting PrP retrotranslocation and if the ERAD of other substrates is affected concurrently. The goal is to test the hypothesis that familial Prion disease pathology is the result of a loss-of-function event, due to an inability to generate CyPrP, combined with the accumulation of mutant, misfolded or

excess proteins through disruption of the ERAD pathway, resulting in ER-stress and cellular apoptosis.

LITERATURE REVIEW

1.1 INTRODUCTION TO PRION DISEASES: CLASSIFICATION, PREVALENCE AND ETIOLOGY

Transmissible Spongiform Encephalopathies (TSEs) or prion diseases are a collection of inherited or transmitted universally fatal disorders of the central nervous system (CNS) (Gains *et al.*, 2007). The unique disease pathology has been reported in multiple species: Bovine Spongiform Encephalopathy in cattle, Chronic Wasting Disease in deer, mule and elk, and Scrapie in sheep (Imran *et al.*, 2011). Human TSEs are diverse and are comprised of Gerstmann-Straussler-Sheinker Syndrome (GSS), Fatal Familial Insomnia (FFI), both associated with mutations of the prion protein gene (*PRNP*) (Collins *et al.*, 2001), and Creutzfeldt-Jakob Disease (CJD), which has distinct sporadic (sCJD), genetic/familial (fCJD) and iatrogenic subtypes (iCJD) (Chesebro, 2003). CJD, being the most common, has affected a total 1-2 individuals per million between 1993 to 2002 of which 84% were sporadic, 10% genetic and 3% of iatrogenic origin with an additional 3% being attributed to variant CJD (vCJD) (Ladogana *et al.*, 2005).

CJD was the first documented human prion disease (Kirschbaum, 1968) and its infectious nature was demonstrated through intracerebral inoculation of brain biopsies obtained from CJD patients into chimpanzees, who manifested a similar syndrome over the course of 13 months (Gibbs Jr *et al.*, 1968). Transmission of infection was further supported by the discovery that the Highlander Tribes of New Guinea developed a form of TSE, later termed “Kuru”, following the cannibalistic ingestion of the dead, part of a funeral ritual common within this population (Collins *et al.*, 2001). TSEs were thrust into the international spotlight during the “mad-cow”

epidemic in the early 1980's, combined with the discovery that dietary exposure to bovine spongiform encephalopathy-infected meat resulted in vCJD, indicating the causative agent of prion disease was able to cross the species barrier (Baker *et al.*, 1996). Most fascinating, this collection of diseases is not the result of a viral infection, as originally thought, but is caused by a misfolded form of an ubiquitous protein able to propagate in the absence of nucleic acid (Prusiner, 1982). Stanley Prusiner named the protein *proteinatious infectious particle,* abbreviated to prion (PrP), thus differentiating TSEs as transmissible neurodegenerative disorders caused by an infectious protein (Prusiner, 1982).

1.2 CLINICAL PRESENTATION AND DISEASE PATHOLOGY

Within each etiologic subtype, disease onset and progression vary dramatically, with age at death for vCJD and iCJD typically less than 39 years, sCJD between 60-79 and those of genetic origin between 50-69 years of age (Ladogana *et al.*, 2005). All forms of CJD tend to have a life expectancy of less then 12 months from time of diagnosis, with a mean survival time of less than 6 months (de Pedro-Cuesta *et al.*, 2006), while GSS and FFI have mean life expectancies of 5-6 years (Goldfarb *et al.*, 1992a), and 13-15 months respectively (Medori *et al.*, 1992). With the exception of vCJD, which, interestingly, can be diagnosed through biochemical analysis of tonsil tissue extracted from patients (Collinge, 2001), a definite diagnosis of human prion disease can only be made by neuropathological examination of biopsied brain tissue post-mortem (Kretzschmar, 1999). However, diagnosis of probable TSEs can be achieved through clinical evaluation of the symptoms associated with each disease pathology (Kretzschmar, 1999, Gains *et al.*, 2007).

1.2.1 General Neuropathological Changes in Prion Diseases

Post-mortem analysis of human brain tissue and transmission studies in animals have identified three major neuropathological features which, together, form the clinical definition of prion disease: spongiform degeneration, neuronal loss and astrocytic gliosis, with the occasional detection of amyloid plaques comprised of proteinase K (PK) resistant PrP (Bell *et al.*, 1993, Kretzschmar, 1999). Spongiform degeneration, also referred to as vacuolation, consists of multiple vacuoles ranging in size from 2-20 μM. Vacuoles tend to be localized in the neuropil, generally within cell processes of neurons, oligodendrocytes, and astrocytes, but can occasionally be detected within the soma and myelin sheath (Tateishi *et al.*, 1980). While spongiform changes are more widespread in animal TSEs, they are consistently found in human prion diseases (Bell *et al.*, 1993). Often these fine vacuoles become confluent, distorting the cytoarchitecture and resulting in neuronal cell death (Bell *et al.*, 1993, Kovacs *et al.*, 2004). Studies have shown that neuronal cell loss appears to be the result, rather than the cause of the pathological process and is caused by an inability of the cell to perform its normal function, indicating general degeneration as opposed to necrosis/apoptosis as the root cause (Gains *et al.*, 2007). It has also been shown that the dendrites, dendritic spines and synapses degenerate before the loss of cell bodies (Mallucci, 2009).

1.2.2 Creutzfeldt-Jakob Disease (CJD)

Clinically, sCJD presents with a rapidly progressive multifocal dementia, myoclonic twitching, cerebellar disturbances, pyramidal and extrapyramidal dysfunction and, finally, akinetic mutism shortly before death (Kretzschmar, 1999, Collinge, 2001, Venneti, 2010). While having nearly identical presentation to sCJD, iCJD is linked to an identifiable source of PrP exposure, the most common causes being contaminated neurosurgical tools, meningeal implants and pituitary hormone therapy (Kretzschmar, 1999). vCJD, on the other hand, is dominated by

psychiatric and behavioral symptoms, specifically depression (Collinge, 2001), as well as cerebellar syndrome and ataxia, with myoclonus and dementia quickly following (Kretzschmar, 1999, Collinge, 2001). While spongiform changes are generally detected in deeper cortical layers (Bell *et al.*, 1993), degeneration is often seen in cerebral grey matter as well as in basal ganglia, the thalamus, visual cortex and cerebellum in sCJD. Iatrogenic CJD shows a similar pattern but with pronounced spongiform changes in the granule layer of the cerebellar cortex (Bell *et al.*, 1993, DeArmond *et al.*, 2008). Most CJD patients show evidence of both cortical and cerebellar atrophy with, as mentioned above, cerebellar cell loss more pronounced in iCJD cases, although this is variable from case to case (Ribadeau-Dumas *et al.*, 1974, Tateishi *et al.*, 1980, Mallucci, 2009). Astrogliosis tends to be severe and widespread in CJD, particularly in brain regions where neuronal cell loss is also detected (Gains *et al.*, 2007).

1.2.3 Gerstmann-Straussler Scheinker Syndrome (GSS)

Clinical presentation of familial TSEs vary depending on the type of disease-causing mutation. The mental deterioration seen in GSS is far more mild that that seen in forms of CJD (Kretzschmar, 1999), instead it is dominated by progressive ataxia, cerebellar syndrome, pyramidal features, and spastic paraparesis (Collins *et al.*, 2001). Spongiform degeneration of grey and white matter is quite variable and appears to depend on the type of disease-causing mutation and the particular ancestral lineage. However, degeneration is consistently found in the motor cortex and the molecular layer of the cerebellum (Budka, 2003). White matter degeneration is most severe within the substantia nigra, subcortical telencephalic nuclei as well as thalamic and anterior horn cells (Masters *et al.*, 1981). However, some degeneration is found in the cerebral cortex, cerebellum and spinal cord and occasionally in the basal ganglia. Intuitively, gliosis tends to correspond to areas of neuronal death, particularly in the cerebellum

where substantial Purkinje cell loss is observed (Bugiani *et al.*, 2000). Unlike other prion diseases, GSS is almost always associated with widespread, multi-centric prion positive amyloid plaques, found with particular high density in the cerebellum and cerebral cortex although occasionally seen in the basal ganglia, subcortical nuclei and brainstem (Masters *et al.*, 1981, Bell *et al.*, 1993, Budka, 2003, DeArmond *et al.*, 2008). However, plaques are highly variable in size and distribution and are sometimes found in sCJD, drawing their use as a diagnostic tool into question (Budka, 2003).

1.2.4 Fatal Familial Insomnia (FFI)

The pathophysiology of FFI is fascinatingly unique, with patients developing irregular circadian rhythms, followed by profound disturbances in the sleep-wake cycle (insomnia), autonomic and motor disturbances, dementia, and eventually, rigidity and mutism (Medori *et al.*, 1992, Kretzschmar, 1999, Collins *et al.*, 2001). Spongiform changes are consistently found in the cerebral cortex and often in the hippocampus of patients (Medori *et al.*, 1992). The pattern of neuronal cell loss seen in FFI is very precise, with diffuse cerebral atrophy, loss of cerebellar Purkinje cells, and some brainstem neuronal loss (Medori *et al.*, 1992, Almer *et al.*, 1999, Capellari *et al.*, 2011). Most striking, however, is the massive degeneration of the mediodorsal and anteroventral nuclei of the thalamus, showing greater than 95% cell loss combined with severe depletion of the inferior olive complex and corresponding astrogliosis (Silburn *et al.*, 1996, Almer *et al.*, 1999). Degeneration seen in these specific thalamic nuclei has been linked to the disordered sleep associated with disease pathology.

1.3 THE PROTEIN ONLY HYPOTHESIS

With the clinical pathology well defined, all that remained was to isolate the causative agent. One of the earliest described CJD patients was diagnosed with a “slow virus”, based on previous

work with scrapie-infected sheep (Prusiner, 1998). In an effort to confirm its viral identity, the unidentified particle was treated with agents known to degrade nucleic acids such as extreme pH and ionizing radiation, all of which failed to influence its infectivity, while those known to denature proteins such as Proteinase K (PK) and sodium dodecyl sulphate (SDS) were successful (Alper *et al.*, 1967, Prusiner, 1982, 1998). Using antiserum raised against PrP, purified from scrapie-infected hamster brains, an identical protein was observed in human CJD brain tissue and found to possess the same characteristics of PK resistance (PK^{Res}) and a propensity towards aggregation (Bockman *et al.*, 1985). It is important to note that an accumulation of recent evidence supports the existence of a fraction of PK-sensitive PrP^{Sc} in infected tissue, which retains its ability to convert PrP^{C} to the pathological isoform (Safar *et al.*, 1998, Pastrana *et al.*, 2006, Sajnani *et al.*, 2012). Of equal importance, it was shown that in normal, healthy brains, mRNA encodes prion protein, which is not infectious and is sensitive to PK digestion (Chesebro *et al.*, 1985, Basler *et al.*, 1986). Based on these data, Stanley Prusiner claimed that PrP was the agent necessary for the conversion of the normal cellular PrP (PrP^{C}) into its pathological form, termed PrP "Scrapie"(PrP^{Sc}) (Prusiner, 1982). The theory postulates that PrP conversion involves a PrP^{Sc}-induced conformational change from approximately 3% β-sheet content, found in the healthy isoform, to 40-45% β-sheets, resulting in a protein more prone to aggregation, as well as amyloid and fibril formation (Prusiner, 1998). However, some groups maintain the data is unconvincing and claim that the particle's resistance to nucleic acid-destroying agents relates to its proteinaceous coating (Narang *et al.*, 1988, Brown *et al.*, 1991, Narang, 1996). Additionally they claim that serial passage studies, in which the particle retains its original amino acid sequence as opposed to acquiring that of the host, combined with the existence of multiple prion "strains" (defined as differential transmissible states caused by the same protein) presents a

major challenge to the protein-only theory (Narang, 1996, Tanaka *et al.*, 2004). While quick to point out that PrP^{Sc} is the only *known* element of the infectious particle, the Prusiner group argues that the existence of multiple strains is the result of various post-translational modification of the protein (Prusiner, 1998), while other proponents claim that a single protein possesses the ability to misfold into multiple pathological conformations, resulting in observed strain differences (Tanaka *et al.*, 2004). Regardless, the protein-only hypothesis is now widely, if not universally, accepted and the discovery of PrP^{Sc} as the infectious agent causing/ human and animal TSEs provides a unique molecular marker for the disease.

1.4 THE GENETICS OF HUMAN PRION DISEASE

1.4.1 Introduction to Familial Prion Disease

Of all human prion diseases, approximately 15% are inherited and can be attributed to over 30 different autosomal dominant mutations of *PRNP* (Dlouhy *et al.*, 1992, Goldfarb *et al.*, 1992a, Medori *et al.*, 1992, Gabizon *et al.*, 1993, Prusiner, 1998, Almer *et al.*, 1999). While some mutations are associated with a very specific clinical outcome, there is striking phenotypic variability within inherited prion disease (Mead, 2006). Pathological mutations are associated with all three disease classifications, CJD, GSS and FFI, and are caused by insertions or deletions in the N-terminal octapeptide repeat region as well as missense and non-sense point mutations throughout the coding region of *PRNP,* but with a particularly high density in the C-terminal α-helix 3 region (Collinge, 1997, Wadsworth *et al.*, 2003). These mutations are believed to increase the probability that PrP^{C} will convert into PrP^{Sc}, although this has not been proven concretely (Riek *et al.*, 1998). While insertions in the octapeptide repeat region lead to an exposed N-terminal, which is more susceptible to oxidative damage (Yin S, 2006), authors have additionally shown that familial mutations lead to an increase in the thermodynamic stability of

an intermediate in the normal folding pathway of PrP, however, this was not found in all mutant proteins and therefore cannot be the only mechanism through which mutations lead to disease pathology (Apetri *et al.*, 2004). While, admittedly, there is variability within the age of onset, those afflicted with familial prion disease tend to present with symptoms at an earlier median age, compared to sporadic or iatrogenic disease subtypes (Masters *et al.*, 1979). Since the mutation is present since birth, delayed disease onset could be attributed to general dysfunctions of the aging brain, such as an inability to effectively degrade aberrant or misfolded proteins.

1.4.2 Prion Gene Polymorphism and Disease Susceptibility

While there have been several polymorphisms reported in *PRNP*, the most common is at position 129, which can encode either a methionine or a valine residue (Zimmermann, 1999) This polymorphism has been shown to influence the clinical phenotype, age of onset and progression of inherited, sporadic and iatrogenic forms of the disease (Lloyd *et al.*, 2011). While found worldwide, within those of European decent, approximately 51% are heterozygous (129M/V), 38% are homozygous for methionine (129M), and 11% homozygous for valine (129V) (Zimmermann, 1999). Homozygosity appears to be a genetic risk factor with most sCJD and all vCJD patients being 129M (Palmer *et al.*, 1991, Bratosiewicz-Wąsik *et al.*, 2012). In fact, the most dramatic example of the influence of the polymorphism is illustrated in the D178N mutation, where substitution of an aspartate for asparagine residue associated with 129M leads to FFI, whereas that associated with a 129V leads to fCJD (Goldfarb *et al.*, 1992b). Interestingly, heterozygosity is a protective factor and has been associated with longer incubation times in sCJD, and within inherited forms of the disease, a delayed age of onset (Baker *et al.*, 1991). The protective effect of heterozygosity is most dramatically demonstrated within the Fore peoples of Papua New Guinea, where, during the Kuru epidemic, a strong favorable selective pressure was

placed on women bearing the resistant alleles, allowing them to survive into child-bearing years, thus, the majority of Fore woman, middle-aged and older, are currently heterozygous at position 129 (Mead, 2006).

1.4.3 Familial CJD Mutations

fCJD has been associated with 15 single point mutations and is, additionally, linked to both deletions and insertions in the octapeptide repeat encoding region (Gambetti *et al.*, 2003, Kong *et al.*, 2004, Capellari *et al.*, 2011). While not all will be discussed here, two mutations deserve particular attention. The E200K coupled with 129M is the most common mutation associated with inherited prion disease and results in a PK^{Res} conformer of PrP with reduced trafficking to the cell surface (Kong *et al.*, 2004, Capellari *et al.*, 2011). It is found in Jews of Libyan/Tunisian descent one hundred times more then normal, making it the most common cause of CJD in the world (Brown, 2002). The D178N coupled to 129V is also of note as the same mutation leads to FFI when coupled to 129M (Monari *et al.*, 1994). The substitution of a glutamic acid for a lysine residue leads to altered glycosylation patterns (likely due to the proximity of the mutation to a glycosylation site) and a reduction in unglycosylated cell surface PrP. The resultant protein shows altered stability of the β-sheet conformations, PK^{Res}, increased aggregation and retention in both the endoplasmic reticulum (ER) and Golgi apparatus (Kong *et al.*, 2004, Capellari *et al.*, 2011).

1.4.4 GSS Mutations

GSS is associated with 12 distinct single point mutations of *PRNP* (Kong *et al.*, 2004). The resultant protein is either full length, N- or C-terminally truncated or both (Brown, 2002). P102L is the most common cause of inheritable prion disease after E200K and can be coupled with either a 129M or 129V (Kong *et al.*, 2004). Of note, the Y145STOP mutant, also associated

with GSS, results in a prematurely terminated protein, which forms PrP deposits around blood vessels resulting in "cerebral amyloid angiopathy" This mutation has a very high penetrance with pathological symptoms developing in the 4th or 5th decade of life. It is associated with two PrP^{Sc} fragments, 21 kDa and 8 kDa, both derived from the mutant allele and are frequently used as a molecular marker for the disease (Kong *et al.*, 2004).

1.4.5 FFI Mutation

FFI is associated with a single point mutation, D178N coupled to a 129M, distinguishing it from the fCJD phenotype caused by the same mutation (Monari *et al.*, 1994). It is the third most common cause of inheritable prion disease (Kong *et al.*, 2004). The mutation is located in the C-terminal region and the resultant protein shows an increased tendency towards aggregation but, unlike other familial disease subtypes, lacks transmissibility into rodents (Capellari *et al.*, 2011). Interestingly there have also been reports of sporadic cases of FFI, termed sporadic fatal insomnia, which lack any identifiable gene mutation, but whose neuropathological presentation is nearly identical to that of its familial counterpart. All recorded cases are associated with the 129M polymorphism (Parchi *et al.*, 1999).

1.5 THE PRION GENE AND PRION PROTEIN

1.5.1 The Prion Gene, *PRNP*

PRNP is a single copy gene mapping to the *Prn* locus on the short arm of chromosome 20 in humans (Pastore *et al.*, 2007, Watts *et al.*, 2007b). While its expression is variable and developmentally regulated, *PRNP* is ubiquitously expressed with consistently high expression shown in both brain and testes (Makrinou *et al.*, 2002). The gene is comprised of two exons separated by a 12,696 bp intron. The short exon is 134 bp and remains untranslated, while the long exon is 2,355 bp and contains the coding region, including the 759 bp open reading frame

(Makrinou *et al.*, 2002, Lloyd *et al.*, 2011). The *PRNP* promoter region is located within the short, untranslated exon and has been shown to lack both a canonical TATA box and initiation element (Mahal *et al.*, 2001). Importantly, *PRNP* has been shown to be upregulated during ER stress and, consequently, the promoter region of *PRNP* contains four endoplasmic reticulum stress response elements (ERSE), three classical ERSE and one novel ERSE, termed "ERSE-like 26" (Déry *et al.*, 2013, Misiewicz *et al.*, 2013). The classical ERSE elements have been shown to bind unfolded protein response (UPR) transcription factors Activating Transcription Factor 6 (ATF6) and spliced X-box Binding Protein 1 (sXBP1), however, preliminary findings suggest that the ERSE-like 26 element is regulated only by sXBP1 (Déry *et al.*, 2013, Misiewicz *et al.*, 2013). The PRNP promotor has additionally been shown to bind to and be regulated by a number of other transcription factors, including Specificity Protein 1 (SP1, a transcription factor known to be important in gene regulation during early development), signal dependent protein 53 (a tumor suppressor protein), Nuclear Factor-Interleukin 6 (indicating a possible role for cytokines IL-1/6 and TNF-α in *PRNP* regulation), heat shock factor (HSF) and metal transcription factor 1 (MTF1) (Mahal *et al.*, 2001).

Of note, there have been three additional structurally and biochemically similar members of the "prion gene family" identified. *PRND*, encoding the protein Doppel is located 23kb downstream of *PRNP*, *PRNT*, located 3kb downstream of *PRNP*, which either does not encode a protein or encodes a yet unidentified protein, and *SPRN*, not part of the *Prn* locus, encodes the protein Shadoo, located on chromosome 2 and 10 in humans and mice respectively (Makrinou *et al.*, 2002, Watts *et al.*, 2007a, Watts *et al.*, 2007b).

1.5.2 The Prion Protein, PrP^C

1.5.2.1 Structural Features of PrP

PRNP encodes a 253 amino acid sialoglycoprotein with a molecular weight ranging from 23-37 kDa (Goldmann, 1993). PrP^C is expressed most abundantly in brain but is also found in numerous peripheral tissues (Goldmann, 1993, Pastore *et al.*, 2007). The protein is divided into two distinct regions, containing a number of conserved structural elements (**Figure 1)**. The flexible and unstructured N-terminal contains an ER-directing signal peptide (amino acids 1-22), five glycine or proline rich octapeptide repeats between amino acids 51-91 and a central hydrophobic transmembrane domain spanning residues 106-126 (Stahl *et al.*, 1987, Stahl *et al.*, 1991, Prusiner, 1998). Interestingly, the last four repeats of the octapeptide repeats show similarity to the B Cell Lymphoma/Leukemia 2 (Bcl-2) Homology domain found in Bcl-2 family member proteins, hinting at a possible role in cell survival (Yin *et al.*, 1994, Kurschner *et al.*, 1996, LeBlanc, 1998). Conversely, the C-terminal is highly structured in the form of a globular fold comprised of three α-helices and two short β-sheets. It contains two N-linked glycosylation sites at asparagines (Asn) 180 and 196, important for intracellular trafficking and protein folding, and a disulphide bridge connecting helix 2 and helix 3 through cysteines 178 and 213 respectively. Lastly this region contains a signal peptide for post-translational glycophosphatidylinositol (GPI)-anchor attachment, allowing expression of the protein at the outer leaflet of the plasma membrane (Stahl *et al.*, 1991, Prusiner, 1998, Mehrpour *et al.*, 2010).

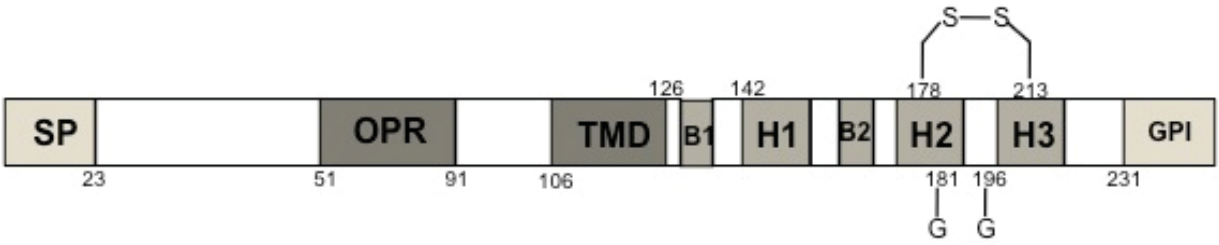
1.5.2.2 Post Translational Modification of PrP

Within the ER lumen, the C-terminal signal peptide is cleaved and GPI-transamidase catalyzes an amide linkage between the ethanolamine head of the GPI anchor and the newly exposed carboxyl group at the C-terminal of PrP (Mayor *et al.*, 2004). PrP^C contains two sites for

Figure 1. Normal cellular Prion protein contains conserved elements.

PrP^C possesses an N-terminal signal peptide (SP) required for targeting to the endoplasmic reticulum, an octapeptide repeat region (OPR), hydrophobic transmembrane domain (TMD) and at the C-terminal, a glycosylphosphatidyl-inositol anchor signal (GPI). In addition, the protein contains two short beta sheets (B1, B2), three alpha-helices (H1, H2, H3), two N-linked glycosylation sites (G) and a disulfide bridge (s) connected H2 and H3. Depicted sites are highly conserved amongst mammals.

Figure 1.


S—S
126
142
178
213
SP
OPR
TMD
B1
H1
B2
H2
H3
GPI
23
51
91
106
181
196
231
G
G

Asn-linked glycosylation at residues 180 and 196, where complex oligosaccharides are attached, a process critical for proper folding and trafficking of the protein (Stahl *et al.*, 1991, Lawson *et al.*, 2005). Analysis of PrP^{C} by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), shows a molecular weight ranging from 23-37 kDa, indicative of multiple glycosylation "states" resulting from post-translational processing (Lawson *et al.*, 2005). Indeed, treatment of cellular extracts from PHN with deglycosylating agents, results in a migrational shift to a single 25 kDa band, further supporting the notion that PrP^{C} is modified during trafficking (Lawson *et al.*, 2005, Jodoin *et al.*, 2007). Within the ER, high mannose oligosaccharide chains are attached and are further modified in the Golgi apparatus by addition of sialic acid, while N-acetylneuraminic acid is added to the GPI anchor (Stahl *et al.*, 1992, Harris, 2003). PrP^{C} contains two endoproteolytic cleavage sites, one within the GPI anchor, suggesting PrP^{C} can be released from the membrane, and one within the hydrophobic core, between amino acids 111 and 112 (Chen *et al.*, 1995), which, according to the Harris group, represents a major processing incident with possible physiological relevance, although the significance of these cleavage events are not currently fully understood (Harris *et al.*, 1993). PrP also contains multiple phosphorylation motifs, which are highly conserved amongst mammals (Negro *et al.*, 2000, Giannopoulos *et al.*, 2009). They are located throughout the sequence and of the ten predicted sites, nine possessed a high probability for phosphorylation (Giannopoulos *et al.*, 2009). Of note, phosphorylation of the serine residue at position 43 has been shown to induce the conformational change to, and possess the properties of PrP^{Sc} (Giannopoulos *et al.*, 2009). Lastly, protein disulphide isomerase catalyzes the formation of a disulphide bridge, shown to be critical for proper protein folding (Harris, 2003).

1.5.2.3 Topology and Intracellular Trafficking of PrP

1.5.2.3.1 Major topological variants of PrP

The topology of a protein encompasses both it's orientation and subcellular localization, a schematic of which is depicted in **Figure 2** (Ott *et al.*, 2002). There are four major topological variants of PrP^{C}, secreted PrP (PrP^{Sec}) two transmembrane forms (PrP^{ntm} and PrP^{ctm}) and cytosolic PrP (CyPrP) (Harris, 2003).

1.5.2.3.2 Topogensis due to differential intracellular trafficking of PrP

PrP^{Sec} accounts for the vast majority (90%) and is found attached via GPI-anchor to the outer leaflet of the plasma membrane following normal transit through the secretory pathway (Borchelt *et al.*, 1990). As translation occurs, the emerging signal peptide is recognized, and bound, by the signal recognition particle at the rough ER, resulting in the temporary cessation of translation (Higy *et al.*, 2004). The polypeptide-ribosome complex is then directed to the signal recognition particle receptor, found in complex at the ER membrane with the heteromeric Sec61 translocon (Harris, 2003, Higy *et al.*, 2004). Here the signal peptide is co-translationally cleaved by signal peptidase, an intramembrane aspartyl protease, as the nascent chain emerges into the ER (Harris, 2003, Heller *et al.*, 2003). Following the addition of Endoglycosydase H-sensitive high mannose glycans and GPI anchor attachment, PrP^{Sec} transits to the Golgi in COPII vesicles, where N-linked glycans are modified by the addition of sialic acid to become resistant to Endoglycosydase-H digestion, followed by transport to the cell surface (Harris, 2003). PrP^{Sec} has been shown to be associated with detergent-resistant lipid rafts at the plasma membrane, typical for a GPI-anchored protein (Naslavsky *et al.*, 1997, Harris, 2003). Raft localization is mediated by cholesterol, an N-terminal internal raft-localization domain (Walmsley *et al.*, 2003), and the disulphide bridge-mediated binding to sphingolipids (Mahfoud *et al.*, 2002). Once at the cell surface, PrP^{Sec} has a half life of three to six hours and regularly cycles from the cell surface to endosomes and lysosomes and back through endocytosis mediated by clathrin-coated pits or

Figure 2. Topology and Subcellular Trafficking of Cellular Prion Protein.

1. *PRNP* gene transcription. **2.** PrP^{C} undergoes post-translational modifications and transits normally through the secretory pathway. **3.** GPI-anchored, cell surface PrP accounts for 90% of total cellular PrP**. 4.** Recycling and endocytosis of cell surface PrP mediated by clathrin-coated pits, caveolae and, possibly, copper binding. **5.** Proteolytic cleavage by PIPLC to generate secreted PrP. **6.** Generation of SP-CyPrP, retaining both the N- and C-terminal SP through inefficient integration of the signal peptide into the ER-membrane. **7.** CyPrP, lacking both the N- and C-terminal signal peptide resulting from the retrotranslocation by ERAD from the ER to the cytosol. **8.** Transmembrane PrP resulting from the integration of the hydrophobic transmembrane domain into the ER membrane. **Abbreviations:** PrP: Cellular Prion protein, GPI: glycosylphosphatidylinositol, PIPLC: Phosphatidylinositol phospholipase C, SP: Signal Peptide, ER: Endoplasmic Reticulum, ERAD: Endoplasmic Reticulum Associated Degradation

Figure 2.

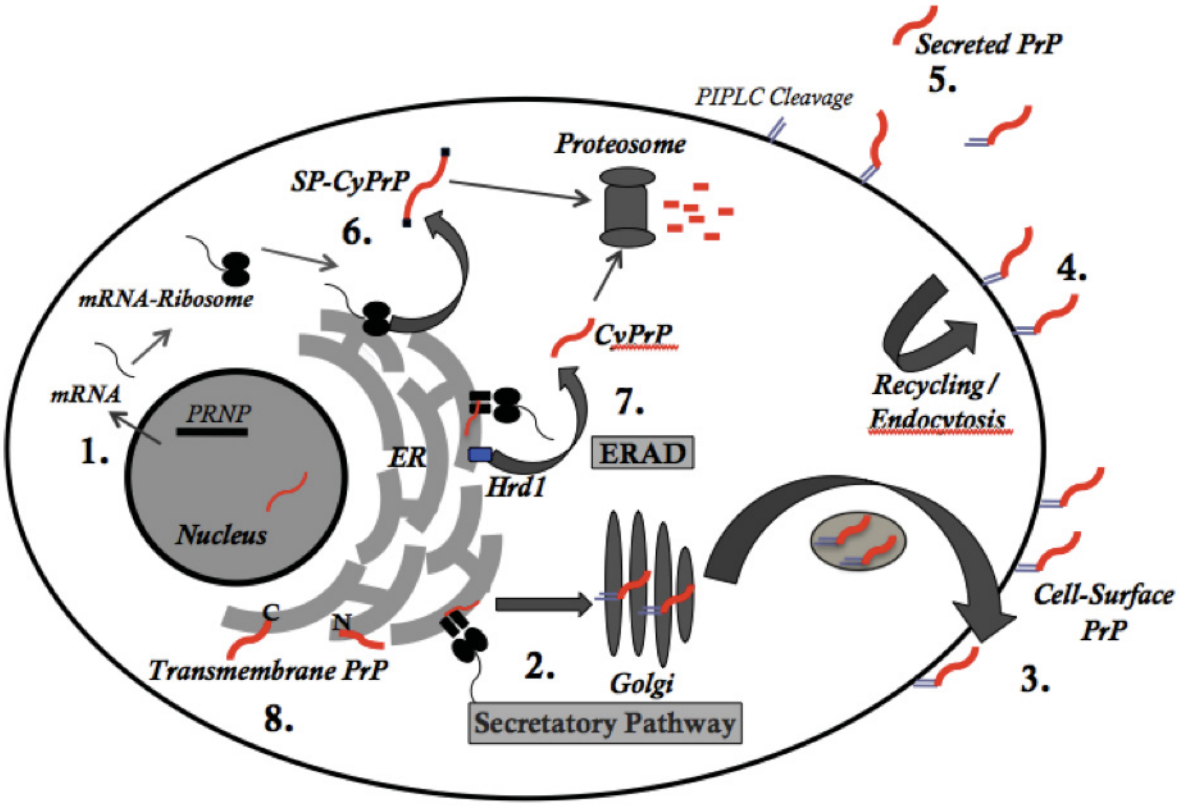


Figure adapted from: (Gains et al., 2007)

specialized lipid rafts called caveolae (Caughey *et al.*, 1989, Borchelt *et al.*, 1992). The process mediating, and the physiological significance of, PrP internalization is still a subject of intense debate. It has been shown to be copper-mediated in both N2a mouse neuroblastoma cells and the human SH-SY5Y cell lines (Pauly *et al.*, 1998, Sunyach *et al.*, 2003), however, endocytosis does not require copper in PHN (Shyng *et al.*, 1994), indicating multiple pathways for PrP internalization in mammalian cells.

Proteolytic cleavage within the GPI anchor of PrP^{Sec}, possibly by phosphatidylinositol specific phospholipase C (PIPLC), can lead to an extracellular form of PrP (Borchelt *et al.*, 1992, Harris *et al.*, 1993). Lastly, a variant lacking both the N-terminal signal peptide and GPI anchor has been found in the nucleus of both healthy and diseased cells, likely resulting from two nuclear localization signal found within the protein sequence (Mehrpour *et al.*, 2010).

Transmembrane PrP^{ntm} and PrP^{ctm} account for less then 10% and result from the insertion of the transmembrane domain into the ER membrane. The two variants have opposite orientations from which they derive their names, one with the N-terminal located inside the ER lumen and one with the C-terminal in the lumen, designated PrP^{ntm} and PrP^{ctm}, respectively (Hegde *et al.*, 1998, Harris, 2003). PrP^{ntm} lacks both a N-terminal signal peptide and C-terminal GPI anchor, while PrP^{ctm} contains both. This is likely because the N-terminal does not enter the ER where signal peptide cleavage occurs, rather, the hydrophobic domain acts as a signal anchor sequence and the C-terminal portion enters the ER where its signal peptide is replaced by a GPI anchor, which, subsequently results in attachment to the inner leaflet of the ER lumen (Stewart *et al.*, 2001, Harris, 2003).

CyPrP, accounting for less then 10% of total PrP^{C} (Yedidia *et al.*, 2001), has multiple routes through which it can become cytosolically expressed. Cell-surface PrP can be retained in

the cytosol after undergoing endocytosis, in which case it would likely possess the GPI anchor. It has also been shown that incomplete translocation of PrP into the ER, due to a weak signal peptide, leads to the accumulation of PrP, retaining its N- and C-terminal signal peptide (SP-CyPrP) in the cytosol (Rane *et al.*, 2004). When PrP is overexpressed in experimental models via transfection, it has been shown to bypass the secretory pathway, possibly due to overloaded translation machinery, to be synthesized in the cytosol thus retaining both the N- and C-terminal signal peptides (Drisaldi *et al.*, 2003). Finally, CyPrP can be generated through the endoplasmic reticulum associated degradation (ERAD) pathway, where misfolded or excess endogenous PrP, following normal translocation into the ER, is retrotranslocated back into the cytosol and targeted to the proteasome for degradation (Ma *et al.*, 2001, Yedidia *et al.*, 2001, Apodaca *et al.*, 2006). Indeed, numerous studies have reported the accumulation of PrP in the cytosol of PHNs and multiple cell lines treated with inhibitors of the proteasome (Zanusso *et al.*, 1999, Ma *et al.*, 2001, Yedidia *et al.*, 2001, Gu *et al.*, 2003, Mironov *et al.*, 2003, Roucou *et al.*, 2003, Roucou *et al.*, 2005, Laroche-Pierre *et al.*, 2009) as well some populations of neurons in the hippocampus, cortex and thalamus (Mironov *et al.*, 2003). However, due to the possibility of detachment of GPI-anchored PrP^{Sec} from membranes during cryogenic processing, it is unclear which topological type of PrP authors were examining (Mironov *et al.*, 2003).

1.6 PROPOSED PHYSIOLOGICAL ROLE OF THE PRION PROTEIN:

The presence of PrP^{C} is critical for the pathological conversion to PrP^{Sc}, and, as a result, it has been proposed that the elimination of endogenous PrP is a potential cure for TSEs (White *et al.*, 2008). Indeed, $PRNP^{-/-}$ mice survive till old age, and aside from a conferred resistance to developing prion disease, show no gross phenotypic or structural abnormalities (Büeler *et al.*, 1992). However, *PRNP* is highly conserved across mammalian species (Schätzl *et al.*, 1995), has

been shown to perform a number of important physiological roles in the human CNS and, therefore, PrP^{C} knockdown, as a therapeutic strategy, should be approached with caution (Roucou *et al.*, 2005, Hu *et al.*, 2008).

1.6.1 Signal Transduction and Synaptic Transmission

PrP^{C} has been shown to bind a variety of ligands whose signal transduction pathways modulate growth, proliferation and cell survival (Mouillet-Richard *et al.*, 2000, Spielhaupter *et al.*, 2001). Antibody cross-linking studies performed in murine cells have shown that a caveolin-1-dependent coupling of PrP^{C} to Src family member tyrosine kinase Fyn, leads to its phosphorylation in neurites and mediates cell proliferation, growth and survival (Mouillet-Richard *et al.*, 2000). PrP^{C} has additionally been shown to bind to Stress Inducible Protein 1 and this interaction mediates cell survival in the neuroblastic layer of retinal explants and, along with its interaction with laminin, has been linked to neuritogenesis in hippocampal neurons (Graner *et al.*, 2000, Zanata *et al.*, 2002, Lopes *et al.*, 2005). Binding to adaptor protein Growth factor receptor-bound protein 2 as well as dimerization of PrP^{C} at the plasma membrane have been shown to activate the Extracellular Signal-Regulated Kinases 1 and 2 (ERK1/2) and Mitogen-activated protein kinase (MAPK) pro-survival signaling pathways (Spielhaupter *et al.*, 2001, Monnet *et al.*, 2004, Grenier *et al.*, 2006). Lastly, in retinal explants from wild type (WT) but not $PRNP^{-/-}$ mice, a PrP^{C}-binding peptide inhibited apoptosis by activating cyclic adenosine monophosphate (cAMP), cAMP-dependent protein kinase A (PKA) and ERK1/2 pro-survival pathways. This effect was abolished by treatment with PIPLC (resulting in cleavage of the GPI anchor and release of PrP^{Sec} from the outer membrane) or a PKA inhibitor, demonstrating the importance of cell surface PrP and resultant signal transduction via the PKA pathway (Chiarini *et al.*, 2002). Interestingly, Phosphoinositide-3-kinase, which is regulated by PKA and Ras to

modulate cell survival, was shown to correlate with PrP^C levels in mouse neuroblastoma cells as well as protect hippocampal cells from serum deprivation-induced apoptosis (Vassallo *et al.*, 2005). Endogenous PrP has been proposed to play roles in synaptic transmission (Hu *et al.*, 2008). A yeast two-hybrid study demonstrated an interaction between PrP^C and the phosphoprotein Synapsin Ib, a protein expressed at nerve terminals on the membranes of synaptic vesicles and this interaction has been thought to be involved in synapse formation and the regulation of neurotransmitter release (Spielhaupter *et al.*, 2001). PrP null mice have also shown disrupted synaptic transmission (Collinge *et al.*, 1994), however not all studies support this finding (Lledo *et al.*, 1996).

1.6.2 Copper Metabolism and Homeostasis

It was observed early on that treatment of both rats and guinea pigs with copper chelator cuprizone led to TSE-like degeneration of the rodents brains (Carlton, 1969). It was later demonstrated that, at neutral pH and within physiological concentration range, the highly conserved octapeptide repeat region of PrP^C was able to bind four extracellular copper ions (Brown *et al.*, 1997a, Whittal *et al.*, 2000, Kramer *et al.*, 2001). Incidentally, studies in $PRNP^{-/-}$ mice have shown lower overall levels of copper in brain, and, correspondingly, reduced copper/zinc -dependent superoxide dismutase activity, versus their WT counterparts, thus leaving them more vulnerable to oxidative stress (Brown *et al.*, 1997a, Brown *et al.*, 1999b). In addition, studies in neuroblastoma cells established that extracellular copper, upon binding to PrP, was able to induce its rapid endocytosis, indicating PrP is potentially serving as a receptor to facilitate copper intake from the extracellular environment to accumulate in perinuclear organelles (Pauly *et al.*, 1998, Lee *et al.*, 2001). However, not all studies support a protective role for PrP-mediated

copper binding. Indeed, copper was shown to be necessary and sufficient for the aggregation and neurotoxicity of the short PrP peptide encompassing residues 106-126 (Jobling *et al.*, 2001).

1.6.3 Evidence for Disturbances in PrP Knockout Mice

As discussed previously, no gross anatomical or phenotypic abnormalities have been observed in PrP knockout models, however, multiple subtle disturbances in sleep, memory formation, cognition and synaptic transmission have been consistently reported. Disturbances were reported in both the period length and activity of circadian rhythms of *PRNP*$^{-/-}$ mice, who also displayed fragmented sleep patterns and a more dramatic response to sleep deprivation (Tobler et al., 1996). It was later shown that knockout models have a decreased capacity for non-rapid eye movement and slow wave sleep activity, with an increased percentage of short waking episodes (Tobler *et al.*, 1997), and that these disruptions could be reversed by exogenous introduction of PrP^{C} into neurons (Tobler *et al.*, 1996).

PrP^{C} is abundantly expressed in both the hippocampus, important for learning and memory, and the cerebellum, which is important for motor coordination (Steele *et al.*, 2007), posing the question: does PrP play a role in elements of cognition? In fact, Craido and colleagues, demonstrated that *PRNP*$^{-/-}$ mice showed specific deficits in hippocampal-dependent spatial learning, a phenotype rescued by crossbreeding with mice expressing *PRNP* under a neuron specific promoter (Criado *et al.*, 2005). Deficits have also been noted in motor coordination and balance in PrP null mice, with altered granule cell proliferation due to disruptions in excitability and plasticity of neurons, which corresponds to the cerebellar ataxia commonly seen in GSS patients (Nazor *et al.*, 2007, Prestori *et al.*, 2008) In addition, deficits in long-term potentiation were observed in hippocampal neurons extracted from these same mice (Criado *et al.*, 2005, Maglio *et al.*, 2006). Consequently, neurons derived from the CA1 region of

the hippocampus of knockout mice show disturbances in calcium-mediated potassium channels, known to be critical for long-term potentiation and memory formation (Maglio *et al.*, 2006). Furthermore, PrP was shown to be critical for both short-term memory formation and, subsequent consolidation into long-term memory through its interactions with STI1 and laminin (Coitinho *et al.*, 2006, Coitinho *et al.*, 2007). However, some groups disagree and report no long-term memory or other cognitive problems in knockout mice (Büeler *et al.*, 1992).

Pronounced and prolonged N-methyl-D-aspartate (NMDA) evoked currents due to upregulation of NMDA receptors lead to increased excitability and subsequent glutamate excitotoxicity in hippocampal neurons from PrP null mice, an effect reversed by the inhibition of NMDA receptors by re-introduction of PrP^{C} (Khosravani *et al.*, 2008). However not all groups agree, with unimpaired excitability and synaptic transmission reported in both hippocampal neurons and the Purkinje cells of the cerebellum (Herms *et al.*, 1995, Lledo *et al.*, 1996). Finally, alterations in the response to brain injury have been observed in PrP knockout mice, showing an increased susceptibility to seizure activity (Walz *et al.*, 2005), and greater lesion volume following traumatic brain injury (Hoshino *et al.*, 2004)

1.6.4 Neuroprotective Role of Prion Protein

1.6.4.1 Oxidative Stress

While somewhat controversial, PrP^{C} has consistently been shown to be protective against oxidative stress (Brown *et al.*, 1997b, Brown *et al.*, 1997c, Brown *et al.*, 1999a), and while the exact mechanism is currently unknown, it has been suggested that the, previously discussed, role of PrP in copper binding and metabolism could contribute to this phenomenon (Brown *et al.*, 1997a, Roucou *et al.*, 2005). Indeed, a marked increase in oxidation proteins and lipids was detected in the brains of $PRNP^{-/-}$ mice (Wong *et al.*, 2001) with a corresponding decrease in the

ability to protect against copper-mediated oxidative stress (Vassallo *et al.*, 2003), possibly due to a reduction in copper/zinc-superoxide dismutase activity (Brown *et al.*, 1999a). In astrocytes derived from PrP null mice, an increase in reactive oxygen species was reported and when PrP^C was put into a cell free system, a nearly 70% reduction in oxidation was observed (Bertuchi *et al.*, 2012). Recent evidence shows that this protective ability of PrP^C could be important in development, with knockout mice embryos presenting with altered copper homeostasis, increased oxidative stress, the development of a hypoxic environment and subsequent placental dysfunction and reduced litter size (Alfaidy *et al.*, 2013). While PrP^C levels have been shown to correlate with superoxide dismutase activity and glutathione levels (Brown *et al.*, 1997c, Bertuchi *et al.*, 2012), it has been suggested that opposed to possessing superoxide dismutase-like activity itself, PrP^C could be modulating activity of the endogenous enzyme (Sakudo *et al.*, 2003)

1.6.4.2 Doppel and Truncated PrP

While *PRNP*$^{-/-}$ mice survive to adulthood with no major abnormalities, some models display Purkinje cell loss and corresponding cerebellar ataxia (Weissmann *et al.*, 2003). Further investigation led to the discovery that this was, in fact, not due to the loss of PrP^C expression, but to the upregulation of downstream *PRND*, which, due to alterations in the open reading frame, was under the transcriptional control of *PRNP* (Moore *et al.*, 1999). This unintended overexpression led to defective myelination, degeneration and swelling of Purkinje axons, which was protected by re-introduction of PrP^C (Moore *et al.*, 1999). Additionally, it has been shown that Doppel overexpression stimulates the production of nitric oxide through activation of nitric oxide synthases *in vivo* (Cui *et al.*, 2003). Interestingly, deletion of proapoptotic Bcl-2-associated X protein (Bax) partially rescued from Doppel-induced toxicity (Heitz *et al.*, 2007) and this

effect was counteracted by Bcl-2 expression (Heitz *et al.*, 2008), indicating Doppel can act through Bax-dependent and independent pathways. In an attempt to elucidate which region of PrP^C was most important to its normal physiological the function, Schmerling *et al.,* generated and expressed amino-truncated forms of PrP^C in mice. They found that amino acid deletions encompassing 32-121 or 32-134 resulted in cerebellar ataxia due to massive degeneration of the granule cell layer (Shmerling *et al.*, 1998). Furthermore, this phenotype was rescued by re-introduction of *PRNP* (Shmerling et al., 1998) and Caspase-3-dependent cell death cascades were delayed by overexpression of Blc-2 (Nicolas *et al.*, 2007). This evidence appears to indicate a role for PrP^C in protection against Bax-mediated apoptosis.

1.6.4.3 Apoptosis

Apoptosis, often referred to as "cell suicide" or "programmed cell death", is a process by which, damaged or dying cells are disposed of in a controlled manner so as not to disrupt the surrounding cellular environment (Kerr *et al.*, 1972). In general terms, this is accomplished through fractionation of the cell into distinct membrane-bound compartments followed by ingestion by neighboring cells and lysosomal-mediated degradation (Kerr *et al.*, 1972). While multiple apoptotic pathways exist, our focus will be on Bax-mediated apoptosis. The initiation of apoptotic events leads to the upregulation of Bax and other Bcl-2 homology domain-containing proteins (Puthalakath *et al.*, 2002). Once Bax is activated, it translocates to the mitochondria where it undergoes a conformational change to expose a hydrophobic C-terminal domain, which, subsequently, leads to its oligomerization (Wolter *et al.*, 1997, Puthalakath *et al.*, 2002). This process results in the release of cytochrome *c* through pores in the mitochondrial membrane, apoptosome formation through the association of cytochrome *c* with caspase-9 and the

subsequent activation of caspase-3 and downstream executioner caspases followed by the initiation of irreversible cell death (Wolter et al., 1997).

A. Anti-Bax Function of PrP

While PrP is not a member of the Bcl-2 family of proteins (Bounhar *et al.*, 2006), the octapeptide repeat region, as previously discussed, shares striking similarity with the Bcl-2 homology domain of anti-apoptotic Bcl-2. It was first observed that hippocampal neurons from *PRNP*$^{-/-}$ mice, exposed to conditions of serum deprivation, underwent apoptosis readily, whereas WT neurons remained healthy, and the former was rescued by overexpression of Bcl-2 or PrP (Kuwahara *et al.*, 1999), indicating that PrP could be involved in mediating the intrinsic apoptosis pathways in the cell. It has been shown that WT PrP protects against Bax-mediated apoptosis in PHNs (Bounhar *et al.*, 2001b, Roucou *et al.*, 2003), arguably the most relevant model for studying human neurodegenerative disease. This protective effect was shown to be mediated by the octapeptide repeat region, with microinjection of this domain alone providing protection against Bax-mediated cell death (Bounhar *et al.*, 2001b). However, subsequent studies have showed a cell-type specificity of the anti-Bax function of PrP with the retention of protection in human MCF7 breast adenocarcinoma cells (Roucou *et al.*, 2005), yeast *Saccharomyces Cerevisiae* (Bounhar et al., 2006) but a failure to protect against Bax in mouse N2a neuroblastoma cells, human BE(2)-M17 and SK-N-SH neuroblastoma cell lines and human embryonic kidney 293 (HEK293) cells (Roucou *et al.*, 2004). A positive correlation was observed, *in vivo*, between overall levels of PrP and the ability to resist ethanol-induced cell death (Gains *et al.*, 2006).

Investigation into the mechanism behind inhibition of Bax-mediated apoptosis by PrP lead to the discovery that, similar to Bcl-2, PrP prevented the conformation change of Bax into

its pro-apoptotic form in both PHN and MCF7 cells (Roucou *et al.*, 2005). Indeed, cytochrome *c* release was blocked in these cells, but this protective ability was limited to the Bax conformational change, as evidenced by an inability to prevent downstream caspase-3 or -6-mediated apoptosis, once initiated (Roucou *et al.*, 2005). It was proposed that PrP could be directly interacting with either Bcl-2 or Bax. Interestingly, it was shown in a yeast-two-hybrid system that PrP interacted directly with Bcl-2 but not Bax (Kurschner *et al.*, 1996), however, these proteins were tagged, introducing the possibility of artificial interaction. Indeed, immunoprecipitation studies using endogenous proteins were unable to replicate these results (Lin *et al.*, 2008). Our group investigated the possibility of the involvement of other Bcl-2 proteins as interacting partners in yeast, which lack expression of mammalian Bcl-2 homologs. Expression of exogenous human Bax induced apoptotic death and cell-cycle arrest and this pathological process was prevented when Bax was co-expressed with PrP, indicating that PrP is able to inhibit Bax in the absence of other Bcl-2 family members (Bounhar *et al.*, 2006). However, the majority of PrP is located at the cell surface, whereas Bax is localized to either the cytosol or the mitochondria. In fact, cell-free and subcellular localization experiments confirmed an indirect interaction between PrP and Bax to be responsible for its anti-apoptotic effect (Roucou *et al.*, 2005), indicating they do not need to be localized within the same subcellular compartment. However, investigation into the topological variant responsible for the observed anti-Bax function of PrP revealed it was attributed to CyPrP, while transmembrane and secreted forms were ruled out (Lin *et al.*, 2008). As previously mentioned, a small percentage of PrP is expressed in the cytosol, and this CyPrP has, in fact, been shown to prevent Bax-mediated cell death in neurons (Roucou *et al.*, 2003). This was quite a controversial finding at the time as a large body of previous work argued that CyPrP induced degeneration of the cerebellar granule

layer and were neurotoxic in mouse N2a cells (Ma *et al.*, 2002a, Ma *et al.*, 2002b). This is likely due to the previously demonstrated cell-type specificity of the PrP anti-Bax function.

B. The Neuroprotective Role of Cytosolic PrP

While only 10% of total PrP resides in the cytosol, there has been continuing controversy with multiple groups attributing either a neurotoxic or neuroprotective function to CyPrP. Evidence suggested, even early on, that CyPrP was derived from cellular PrP retrotranslocated from the ER via ERAD, as evidenced by PrP accumulation upon proteasome inhibition (Zanusso *et al.*, 1999, Ma *et al.*, 2001, Yedidia *et al.*, 2001). Later it was found that, following proteasome inhibition, N2a cells showed decreased viability, indicating a possible neurotoxic role for CyPrP (Ma *et al.*, 2001). In addition, overexpression of PrP lacking both the N- and C-terminal signal peptides, thereby resulting in cytosolic expression, caused ataxia as a result of massive cerebellar degeneration in transgenic mice (Ma *et al.*, 2001, Ma *et al.*, 2002b). Some report this neurotoxic effect is specific to the cerebellum due to an interaction with the hydrophobic lipid core of the cellular membrane, and is not observed in other brain regions (Wang *et al.*, 2006). Indeed, in PHN derived from fetal brains, CyPrP is not only not toxic, but protects against Bax-mediated cell death (Roucou *et al.*, 2003). Furthermore, Jodoin *et al,* showed that both PHN and MCF7 cells expressing CJD and GSS-associated PrP mutants lost the ability to protect against Bax and that this loss was associated with a concurrent reduction in CyPrP expression (Jodoin *et al.*, 2007). Interestingly, loss of anti-Bax function was rescued by co-expression of CyPrP but not its full-length counterpart, indicating that PrP mutants are disrupting the retrotranslocation of PrP (Jodoin *et al.*, 2007) and thus PrP-mutant-associated pathology could represent a loss-of-function event due to an inability to generate neuroprotective CyPrP.

1.7 ENDOPLASMIC RETICULUM ASSOCCIATED DEGRADATION

1.7.1 The Role of the Endoplasmic Reticulum in Protein Trafficking

It is of critical importance for newly synthesized proteins to receive proper post-translational modifications and to assume correct tertiary and quaternary structure. The ER is a major cellular organelle surrounding the nucleus but also extending throughout the cytoplasm of the cell. The most important role of the ER is that of a port for nearly all proteins destined for the secretory and endocytic pathways (Tsai *et al.*, 2011). About a third of all unfolded proteins enter either co- or post-translationally into the ER lumen (Rapoport, 2007). It is within this highly regulated environment that early protein maturation occurs, including, but not limited to, addition of N-linked glycans, chaperone-mediated folding, formation of disulphide bonds and protein complex formation (Kostova *et al.*, 2003, Vembar *et al.*, 2008). However, protein modification and folding are imperfect processes and when not properly processed, the ER is able to retrotranslocate proteins back into the cytoplasm, where they are targeted to the proteasome for degradation. Because proper protein maturation is so important to the health and proper function of the cell, the ER possesses an army of quality-control machinery to ensure proper protein trafficking and delivery to target sites (Määttänen *et al.*, 2010).

1.7.2 ER Stress and the Unfolded Protein Response (UPR)

When any system becomes stressed, it responds to maintain homeostasis. Within the ER, any disruptions leading to imbalances in synthesis, modification, trafficking, and degradation of proteins, results in ER stress (Schröder *et al.*, 2005, Tsai *et al.*, 2011). In the context of disease, mutant proteins are often unable to fold correctly and, when this happens, are retained within the ER for longer then normal periods of time, engaging in cycles of folding and refolding (Kim *et al.*, 1998). The resultant system overload, due to the buildup of proteins, leads to the induction of

the unfolded protein response (UPR) as the ER attempts to alleviate the burden (Kim *et al.*, 1998, Schröder *et al.*, 2005). The UPR is an adaptive response initiated by transmembrane sensors, which monitor the state of the ER. In the mammalian cell these proteins are Protein Kinase RNA-like ER Kinase (PERK), Inositol-requiring Protein 1 (IRE1), and ATF6, which, under non-stressed conditions are bound on their luminal side by the heat shock protein Binding immunoglobulin Protein (BiP). When stressed, BiP is recruited to bind to the exposed hydrophobic domains of improperly folded proteins within the ER lumen, thus activating ER stress sensors (Schröder *et al.*, 2005). While not discussed in detail here, the end result is the eukaryotic Initiation Factor 2 (eIF2α)-mediated inhibition of protein translation. Briefly, upon dissociated from BiP, PERK dimerizes and phosphorylates eIF2α, which acts to limit translation of proteins by inhibiting polypeptide chain initiation (Schröder *et al.*, 2005). Futhermore, there is an upregulation of foldases and chaperones and a global inhibition of protein translation mediated by binding of transcription factors ATF6 and sXBP1 to the ERSE elements of genomic promoters. Simultaneously, upon activation, ATF6 is cleaved to release its cytosolic domain, which transits to the Golgi where it is spliced to generate to 50 kDa fragment, which can then enter the nucleus and act as a transcription factor, binding to ERSE elements (Yoshida *et al.*, 2001, Lee *et al.*, 2002). In addition, the UPR results in an increase in the clearance of proteins via the ERAD pathway, indeed, ATF6 has been shown to induce expression of BiP, C/EBP Homology Protein (CHOP) and XBP1 (Yoshida *et al.*, 2001, Schröder *et al.*, 2005). Lastly, once activated, IRE1 dimerizes, autophosphorylates and initiates the splicing of XBP1 mRNA through its RNase activity. Spliced XBP1 (sXBP1) is translated in the cytosol and transits to the nucleus, where it acts as a potent transcription factor, binding to ERSE elements within genomic promoters (Yoshida *et al.*, 2001, Lee *et al.*, 2002). The end result of this process is an

upregulation of chaperones and foldases to increase proper folding and trafficking of proteins, a downregulation of protein transcription and translation and an increase in the clearance of proteins via the ERAD pathway (Schröder *et al.*, 2005).

1.7.3 Endoplasmic Reticulum Associated Degradation (ERAD)

The primary role of ERAD is to maintain cellular homeostasis by facilitating the removal and degradation of excess or misfolded proteins from the ER. This is accomplished through the recognition, ubiquitination, retrotranslocation and proteasomal degradation of aberrant proteins through a tightly regulated pathway (Vembar *et al.*, 2008).

The ERAD pathway has been described in detail elsewhere (Vembar *et al.*, 2008). Briefly, a $GlcNAc_2$-Man_9-Glc_3 moiety is added to Asn residues, within a specified consensus sequence, of most proteins entering the ER. The terminal glucose is then trimmed by glucosidases I and II and can then be recognized by lectin-like chaperones calnexin and calreticulin, which, in association with PDI ERp57, facilitate proper disulphide bond formation and folding. If a protein is incorrectly folded, specific properties, such as exposed hydrophobic patches, can be recognized by UDP-glucose:glycoprotein glucosyltransferase (UGGT), which is able to catalyze the addition of a glucose moiety from UDP-glucose to N-linked glycans so the protein can re-enter the folding cycle. Eventually, possibly due to successive mannose trimming, terminally misfolded proteins are recognized by ER degradation enhancer mannosidase alpha-like 1 (EDEM1) and, in conjunction with lectin proteins Osteosarcoma Amplified 9 (OS9) and XTP3-transactivated gene β protein (XTP3β), targeted to the retrotranslocation complex at the ER membrane (Vembar *et al.*, 2008).

The retrotranslocation machinery facilitates the ubiquitination and dislocation of ERAD substrates from the ER to the cytosol. Briefly, upon delivery, misfolded proteins are recognized

and are then pulled through the putative retrotranslocation channel by AAA-ATPase p97/Valosin-containing protein (p97/VCP) (Jarosch *et al.*, 2002, Meyer *et al.*, 2002). Proteins are then poly-Ub through the actions of E1 ubiquitin activating, E2 ubiquitin conjugating and E3 ubiquitin ligating enzymes to lysine residues via an isopeptide bond (Pickart, 2001). Following ubiquitantion, proteins are deglycosylated and escorted to the 26S proteasome by RAD23/DSK2, where they are deubiquitinated and degraded (Rao *et al.*, 2002). There has been considerable debate in the literature as to the identity of the proposed retrotranslocon (Meusser *et al.*, 2005). One possibility is the Sec61 translocon, the protein responsible for importing nascent protein chains into the ER, is also the retrotranslocation channel. Indeed, the retrotranslocation of some model ERAD substrates has been linked to Sec61, including the Δ508 Cystic fibrosis transmembrane conductance regulator mutant (Bebök *et al.*, 1998, Schäfer *et al.*, 2009). While it is possible that differential substrate binding re-engineers the channel, it cannot be the only mechanism as mutations of the protein does not completely retard retrotranslocation (Yabal *et al.*, 2003). The Derlin family of proteins has also been implicated as a potential retrotranslocation channel, and is, currently, the most promising candidate. Derlin1 has been shown to interact with components of the ubiquitination and targeting machinery, including p97/VCP (Ye *et al.*, 2004) and has been implicated in the retrotranslocation of numerous substrates including the cholera toxin (Bernardi *et al.*, 2008). Lastly, the E3 ligase HMG-CoA reductase protein 1 (Hrd1) would provide an elegant means of coupling Ub machinery to the retrotranslocation channel and has been implicated in the degradation of numerous ERAD substrates and has been shown to interact with proposed retrotranslocon Derlin1 (Kikkert *et al.*, 2004, Schulze *et al.*, 2005, Omura *et al.*, 2006, Bernardi *et al.*, 2010, Kaneko *et al.*, 2010).

1.7.4 E3 Ligase HMG CoA Reductase Degradation Protein 1 (Hrd1) Gene and Protein

1.7.4.1 *SYVN1* Encodes the Hrd1 Protein in Mammalian Cells

Originally identified in yeast as Hrd1p, the gene encoding the human homolog, Hrd1, is also known as synoviolin (*SYNV1*), due to its high expression in synovial cells and role in rheumatoid arthritis pathology (Amano *et al.*, 2003). Henceforth the gene will be referred to as *SYVN1*, and its encoded protein, Hrd1. In humans, *SYVN1* maps to the long arm of chromosome 11 and is comprised of 16 coding exons separated by 15 introns, with an open reading frame 1854bp long. Alternative splicing of *SYVN1* mRNA yields three distinct isoforms, producing two short forms of the protein lacking either amino acids 127-177 and amino acid 411 or 411 alone and the full-length canonical sequence (NCBI Gene Database, Gene ID: 84447). As would be expected of a protein involved in the UPR and ERAD, the *SYVN1* promoter contains a single ERSE element as well as an unfolded protein response element (UPRE). In addition its mRNA has been shown to be upregulated in cells treated with ER-stress inducing drugs (Kaneko *et al.*, 2002) and in response to overexpression of IRE1-sXBP1 and ATF6 (Kaneko *et al.*, 2007).

1.7.4.2 Hrd1 is an Endoplasmic Reticulum-Resident E3 Ubiquitin Ligase

First characterized as an E3 ligase in yeast (Deak *et al.*, 2001), the 617 amino acid long human canonical splice variant is expressed in multiple peripheral tissues, as well as in brain (Omura *et al.*, 2008a). Hrd1p gained its name from its role in the degradation of HMG-CoA reductase, an important enzyme in the biosynthesis of cholesterol and a model ERAD substrate (Bays *et al.*, 2001, Nadav *et al.*, 2003, Kaneko *et al.*, 2007). The canonical variant results in a 617 amino acid protein and is expressed most abundantly in liver, kidney and pancreas, as well as, albeit at lower levels, in brain (Nadav *et al.*, 2003, Kaneko *et al.*, 2007, Omura *et al.*, 2008a). Co-localization studies show the expression pattern of Hrd1 to overlap with that of the classical

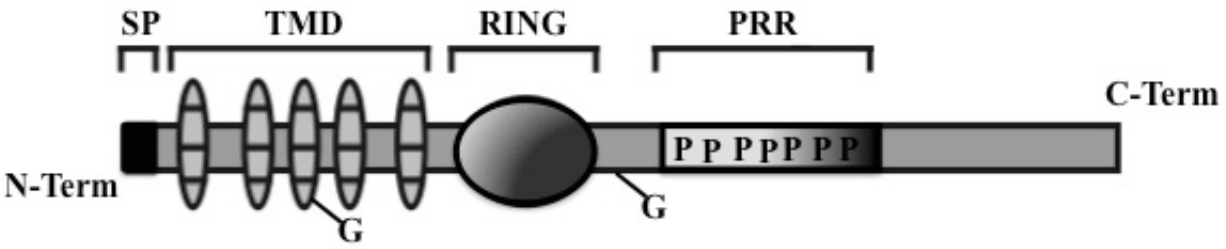
ER proteins PERK (Kaneko *et al.*, 2002) and Calnexin (Kikkert *et al.*, 2004) confirming its subcellular location. In addition to a cleavable signal peptide (amino acids1-17), the N-terminal portion of the protein contains five transmembrane domains, which anchor the protein in the ER membrane and have been shown to be critical to the stability of Hrd1 (**Figure 3)** (Omura *et al.*, 2008a). Notably, Hrd1 contains a RING (Really Interesting New Gene)-H2 finger motif, residing between amino acids 291 and 329 (Kaneko *et al.*, 2002, Nadav *et al.*, 2003, Kikkert *et al.*, 2004).

RING motifs are composed of 8 conserved cysteine and histidine residues which bind two zinc atoms forming a cross-braced globular structure characterized by two β-sheets and a central α-helix and is responsible for the E3 ubiquitin ligase activity of the enzyme (Jackson *et al.*, 2000). The Hrd1 RING motif acts as a scaffold, binding its cooperating E2 ubiquitin conjugating enzyme UBC7 and facilitating the transfer of ubiquitin to the substrate. Indeed, it has been demonstrated, both *in vitro* and *in cellulo*, that Hrd1 possesses E3 ubiquitin ligase activity mediated by its RING finger domain (Kaneko *et al.*, 2002, Kikkert *et al.*, 2004). Located at the C-terminal region of the protein are proline-rich clusters, generally known to be important for protein interactions and, in addition to luminal portions of the transmembrane domain, is a proposed site for substrate recognition. Interestingly, Nadav *et al*. (2003), showed that Hrd1 possesses the ability to autoubiquitinate, possibly to target itself to the proteasome for degradation. Lastly, when Hrd1 was initially characterized, two putative glycosylation sites were found in its sequence, although it has since been shown not to possess N-glycans *in vitro* (Kikkert *et al.*, 2004).

Figure 3. Hrd1 is an ER-resident, RING-finger-containing, E3 Ubiquitin Ligase.

The N-terminal portion of Hrd1 contains an endoplasmic reticulum-targeting signal peptide (SP) followed by five hydrophobic transmembrane domains (TMD) for insertion into the ER-membrane. The C-terminal region of the protein contains the enzymatic RING-finger domain composed of highly conserved cysteine and histidine residues and is necessary for the ligase activity (RING). Lastly the protein contains a region rich in proline residues (proline rich region, PRR), thought to be important in substrate recognition and binding. Two putative N-linked glycosylation sites (G) were identified in the sequence, but have since been shown not to be glycosylated *in vivo.*

Figure 3.



1.7.4.2.1 Hrd1-Mediated Retrotranslocation

Hrd1 is part of a multi-protein complex found at the ER membrane, linking the luminal ERAD pathway with cytosolic degradation machinery (Iida *et al.*, 2011). Inserted into the membrane alone, Hrd1 would be rapidly turned over, however, it is found to be associated with adaptor protein Sel1L, UPR-induced, UBL regulatory protein Herp and putative retrotranslocation channel Derlin1/2, which, together form a stable complex (Cattaneo *et al.*, 2008, Kny *et al.*, 2011).

Upon release from the Calnexin/Calreticulin folding cycle by EDEM1, terminally misfolded proteins are thought to be recognized by lectins OS9, XTP3-B and/or chaperone Grp94, which, have been shown to interact with the Hrd1-Sel1L complex (Christianson *et al.*, 2008). Upon delivery, it has been proposed that the proline-rich domain of Hrd1 is responsible for substrate recognition and binding, while the transmembrane domain is critical for retrotranslocation to the cytosol (Omura *et al.*, 2008a). However, there is ongoing debate as to the identity of the substrate recognition motif, with at least one group showing the transmembrane domain to be responsible for substrate binding in yeast (Sato *et al.*, 2009). It is more then likely that multiple domains of Hrd1 are able to differentially bind substrates. A cleft in the RING domain, formed between the two zinc-containing loops is responsible for binding the cooperating E2 enzymes UBC7 (Kikkert *et al.*, 2004) or UBE2J1 (Burr *et al.*, 2011), followed by the transfer of Ub to the bound substrate (Deshaies *et al.*, 2009). Poly-Ub substrates are then pulled into the cytosol by p97, where they are targeted to the 26S proteasome for degradation. It is important to note that p97 is found bound to the cytosolic face of both Hrd1 and Derlin-1 (Ye *et al.*, 2005).

Figure 4. **Model of the Hrd1 retrotranslocation complex.** E3 ubiquitin ligase Hrd1 binds directly to Sel1L, UBC7, Derlin1/2/3 and p97. Adaptor protein Sel1L stabilizes Hrd1 in the ER membrane. Derlin 1/2/3 are currently the best candidate for the elusive retrotranslocation channel. E2 ubiquitin conjugating enzyme UBC7 cooperates with Hrd1 to facilitate the transfer of ubiquitin to the bound substrate. AAA-ATPase p97 interacts with almost all components of the retrotranslocation complex and acts to pull the ubiquitinated substrate from the retrotranslocon and direct it to the proteasome for degradation. Lastly, Herp binding is thought to initiate retrotranslocation, whereas Vimp recruits p97 to the complex and acts as a scaffold, bridging the connection. Red arrows represent direct interactions between proteins.

Figure 4.

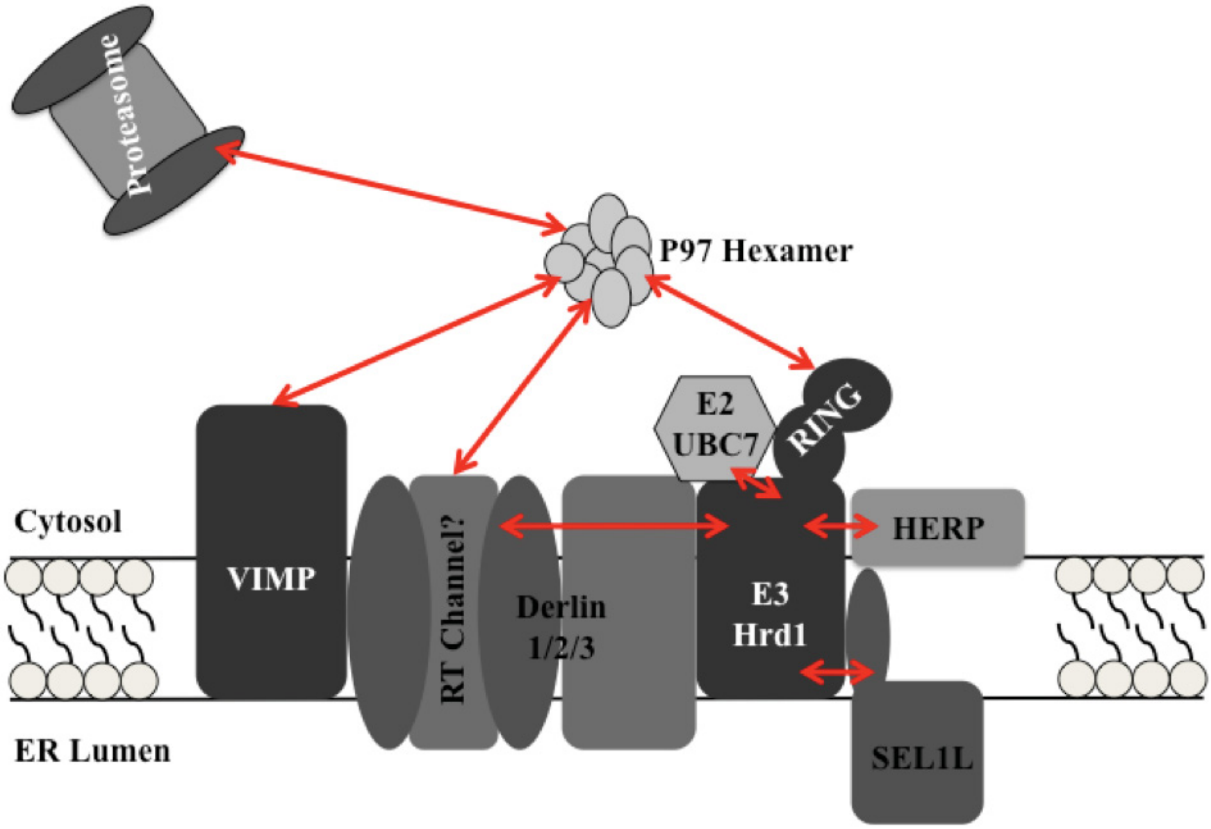


Figure adapted from: (Schulze et al., 2005, Määttänen et al., 2010)

1.7.4.2.2 Hrd1 Mediates the ERAD of Multiple Substrates

Since it's initial characterization, Hrd1 has been shown to not only ubiquitinate and translocate model ERAD substrates such as T-cell receptor subunits TCR-α and CD3-δ (Kikkert *et al.*, 2004) but also to mediate the retrotranslocation of many proteins involved in disease pathology, including, but not limited to mutant Hedgehog (Chen *et al.*, 2011), mutant α1-anti-trypsin (Christianson *et al.*, 2008), the Pael receptor (Omura *et al.*, 2006), amyloid precursor protein (Kaneko *et al.*, 2010), and polyglutamine-expanded Huntingtin (Yang *et al.*, 2007). Accumulation of these aberrant proteins is devastating and leads to, or is associated with, congenital brain malformations, liver and lung disease, Parkinson's disease, Alzheimer's disease and Huntington's disease pathology, respectively. Hrd1 has also been linked to the ERAD of other E3 ligases including gp78 (Shmueli *et al.*, 2009) and itself through autoubiquitination (Nadav *et al.*, 2003). Interestingly, unlike its yeast homolog, mammalian Hrd1 does not mediate the retrotranslocation of its namesake protein HMG-CoA reductase (Kikkert et al., 2004). Lastly a recent paper implicated Hrd1 in the retrotranslocation of the D18G mutant of Transthyretin (TTR^{D18G}), a luminal non-glycosylated model ERAD substrate (Christianson *et al.*, 2011). TTR^{D18G} results in CNS amyloidosis due to its reduced ability to form homotetramers. The monomeric mutant is extremely prone to aggregation and fibril formation, eventually resulting in CNS degeneration (Hammarström *et al.*, 2003). Authors found that Hrd1 knockdown stabilized TTR^{D18G} levels in pulse-chase experiments, while over-expression of Hrd1 and/or Sel1L enhanced its degradation (Christianson *et al.*, 2011). Fluorescently-tagged model ERAD substrates such as TTR^{D18G} provide an excellent tool with which to test the dominant negative effect of PrP mutants on protein retrotranslocation.

1.8 RATIONALE: EVIDENCE FOR DEFECTIVE PRION PROTEIN RETROTRANSLOCATION IN FAMILIAL PRION DISEASE AND THE PUTATIVE ROLE OF HRD1

Our group has shown that PrP, specifically CyPrP, protects against Bax-mediated cell death in PHN and MCF7 cells by preventing the conformational change to its pro-apoptotic form (Bounhar *et al.*, 2001b, Roucou *et al.*, 2003, Roucou *et al.*, 2005). If the protection offered by CyPrP is somehow impaired, it could represent an interesting loss-of-function explanation for TSE pathology. However, the Ma group proposes an opposing gain-of-function hypothesis through repeated reports that CyPrP is a toxic species, which contributes to the conversion of PrP to PrP^{Sc} in both sporadic and inherited disease pathologies (Ma *et al.*, 2002b, Wang *et al.*, 2006, Wang *et al.*, 2009). They have shown extensive loss of cerebellar granule neurons and degeneration in the forebrain of mice expressing a transgene encoding CyPrP, with a corresponding phenotype consistent with the associated familial mutation (Ma *et al.*, 2002b, Wang *et al.*, 2009). In addition, they showed that in the reducing environment of the cytosol, a large proportion of PrP formed aggregates and becomes PK^{Res}, echoing the conformational change to PrP^{Sc} occurring in disease (Ma *et al.*, 1999, Ma *et al.*, 2002b). While these findings have been replicated by several other groups both *in vivo* and *in cellulo* (Rane *et al.*, 2004, Grenier *et al.*, 2006, Rambold *et al.*, 2006), other groups support the work of the LeBlanc lab by showing, both in cell lines and primary cultures, that CyPrP is, in fact, not toxic, but protective (Fioriti *et al.*, 2005, Crozet *et al.*, 2006).

It has previously been shown that neuronal apoptosis occurs in all familial forms of human TSEs (Dorandeu *et al.*, 1998, Gray *et al.*, 1999, Liberski *et al.*, 2004) and other groups have confirmed the involvement of the Ub-proteasome system in the turnover of both mutant

and WT PrP (Ma *et al.*, 2001, Yedidia *et al.*, 2001, Wang *et al.*, 2005b). With this in mind, Jodoin *et al*., investigated the anti-Bax function of 11 fCJD, 1 GSS and the FFI PrP mutations, as well as the differential impact of the 129M/V in MCF7 cells and PHN (Jodoin *et al.*, 2007). Results showed that, compared with full length WT PrP coupled to the corresponding polymorphic allele, all mutants, save two, partially or completely lose their anti-Bax function in MCF7 cells. A total of 83% of mutants examined almost completely lost their protective function and the 129M was associated with increased apoptosis over 129V. A strong negative correlation was found between cell death and levels of CyPrP (R^2 =.85), indicating that mutants generating the lowest levels of CyPrP showed the highest percentage of cell death. Lastly, in both MCF7 cells and PHN, co-expression of WT CyPrP or the corresponding mutant CyPrP (i.e., PrP containing the same familial mutation but lacking the N- and C-Terminal signal peptides, thus being synthesized in the cytosol) restored the anti-Bax function, while co-expression with full-length WT PrP did not. This was especially evident in PHN, which express very high levels of endogenous PrP. Overall this study showed that retrotranslocation of disease-causing PrP mutants is disrupted and this effect is dominant negative, meaning that, not only is mutant protein prevented from generating CyPrP, but it also blocks the retrotranslocation of PrP^C. A mechanism for this effect has not yet been elucidated.

Using a yeast model, Apodaca *et al.* (2006) demonstrated that human PrP is a substrate of the Hrd3-Hrd1-mediated ERAD pathway (Hrd3 is the yeast homolog to Sel1L). Briefly, the N-terminal signal peptide of human PrP was replaced with a yeast ER-targeting sequence and, when transfected into yeast, the human PrP protein showed normal trafficking through the secretory pathway. To determine if human PrP was a substrate of the ERAD pathway, both Cue1 (an E2 enzyme) and Der1 (the retrotranslocation channel in yeast) were mutated, and when

human PrP was expressed in these cells, a marked reduction in its degradation was observed. There are, however, several established ERAD pathways in yeast, with E3 ligases Doa10 and Hrd1 responsible for degrading the majority of substrates. Both pathways were mutated and it was shown that human PrP was not only not Ub, but degradation was stabilized only in cells lacking Hrd3-Hrd1, indicating it is dependent on this complex for retrotranslocation. Lastly immunoprecipitation was employed to demonstrate a direct interaction between human PrP and Hrd1. This was the first and only study to establish a clear mechanism for PrP retrotranslocation and provides compelling clues as to the identity of the protein responsible for the generation of protective CyPrP in mammalian cells.

RESEARCH HYPOTHESIS

We submit the hypothesis that the E3 Ubiquitin Ligase Hrd1 mediates the endoplasmic reticulum-associated degradation of prion protein in human central nervous system cells through ubiquitination and retrotranslocation to the cytosol, where it is subsequently degraded by the proteasome. It is proposed that this process is dependent on the enzymatic activity of the RING-finger domain of Hrd1, while either the proline rich region or the luminal portion of the transmembrane domain is believed to be responsible for the recognition and binding of PrP in the endoplasmic reticulum. Lastly, we submit the hypothesis that pathological disease-causing PrP mutants block the retrotranslocation of WT PrP, thus abolishing the neuroprotective effects of cytosolic PrP, by irreversibly binding to and saturating Hrd1, resulting in the retention of WT PrP and other Hrd1 substrates within the endoplasmic reticulum.

MATERIALS AND METHODS

Cell cultures

CR7 human glioblastoma cells were maintained in Opti-MEM reduced serum media (Invitrogen,

Burlington, Ontario, Canada) supplemented with 2.4 g/L $NaHCO_3$ and 5% fetal bovine serum (FBS, Thermo Fisher Scientific, Mississauga, Ontario, Canada). MCF-7 cells (American Type Culture Collection (ATCC), Manassas, VA) and N2a cells (ATTC) were grown in Roswell Park Memorial Institute (RPMI) or Dulbecco's Minimum Essential Media (DMEM) respectively, both supplemented with 1.7 g/L $NaHCO_3$ and 10% FBS. All cells were maintained at 37°C in 5% CO_2 with 95% air.

Induction of ER Stress and other Pharmacological Treatments

CR7 cells were treated with for 16 hours with 1 μM Tunicamycin (Biomol, Plymouth Meeting, PA); conditions optimized to enhance ER stress while limiting toxicity. To examine retrotranslocation, CR7 cells were treated for 18 hours with 1 μM Epoxomicin (Enzo Life Sciences, Farmingdale, NY) to inhibit the proteasome and 5 μg/mL Brefeldin A (BFA, Sigma, Oakville, Ontario, Canada) to disaggregate the Golgi and enhance retrotranslocation of proteins from the ER to the cytosol.

Isolation of Total RNA and Reverse-Transcriptase Polymerase Chain Reaction

Total RNA was extracted from CR7 cells using the TRIZOL reagent (Invitrogen) following the manufacturer's instructions and quantified using a ND-1000 spectrophotometer (Thermo Fisher Scientific). cDNA was prepared using avian myeloblastosis reverse transcriptase (AMV-RT, Roche, Laval, Quebec, Canada), following the manufacturer's protocol. Briefly, 2 μg total RNA was used for cDNA synthesis by AMV-RT with poly dT primers, and 1 μg (*PRNP, HPRT1*) or 2 μg (*SYVN1*) of cDNA was used as a template for subsequent PCRs using the following primers. Forward 5'ACGCGGATCCCAAGAAGCGCCCGAAGCCT-3' and reverse 5'-GCCGCTCGAG GCTCGATCCTCTCTGGTA-3' for *PRNP*, forward 5'-CTGGCTCTGCCCCAGAGGCTGGC CCT-3' and reverse 5'-GTGGGCAACAGGAGACTCCAGCTTCTGCAGG-3' for *HPRT1* and

forward 5'-CCTGGCGTCGTGATTAGTGAT -3' and reverse 5'-AGACGTTCAGTCCTGTCC ATAA-3' for *HPRT1*. *HPRT1* was used as a housekeeping gene because it has been empirically shown to be the least affected by ER stress (Misiewicz *et al.*, 2013). DNA is double stranded; forward and reverse primers represent the sequence needed to enhance both the DNA stand (forward) as well as its complementary strand (reverse).

Peptide -N-Glycosidase F digestion

CR7 cells were lysed in ice-cold NP-40 lysis buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% NP-40, 5mM EDTA pH 8) supplemented with protease inhibitors (Sigma, 38 μg/mL 4-(2-Aminoethyl) Benzenesulfonyl Fluoride Hydrochloride (AEBSF), 0.5 μg/mL Leupeptin, 0.1 μg/mL Pepstatin and 0.1 μg/mL Nα-Tosyl-Lys Chloromethyl Ketone (TLCK)) for 20 minutes on ice. SDS was adjusted to 0.5% final concentration, samples were boiled for 10 min, and then digested with 2 U of Peptide-N-glycosidase F (PNGase F, New England Biolabs, Pickering, Ontario, Canada) for 18 h at 37°C. The reactions were terminated by the addition of Laemli's sample buffer followed by boiling for 2 minutes.

Description of Clones

Four mammalian expression constructs were utilized in the completion of experiments included in this thesis: eYFP-Hrd1 (a generous gift from Dr. Fang, University of Maryland, College Park, MA), pcDNA3.1(-)-TTR^{D18G}-GFP (a kind gift from Dr. Kopito, Standford University, Standford, CA) and pCEP4β-V210I^{129V} or pCEP4β-M232R^{129V} PrP mutants (Jodoin *et al.,* 2007). Briefly, authors cloned full-length human Hrd1 cDNA (KIAA1810) purchased from KAZUSA DNA Research Insitute (Japan) into the peYFP-C1 vector between the XhoI and BamHI restriction sites under the control of the human cytomegalovirus immediate-early promoter using the following primers: Forward 5'-ATCGCCTCGAGCCATGTTCCGCACGGCAGTGATG-3' and

reverse 5'-ACTAGGGATCCCGGTGGGCAACAGGAGACTCCA-3'. Upon expression in mammalian cells, the resultant protein is comprised of untagged, full-length human Hrd1 with enhanced yellow fluorescent protein (eYFP) fused to the N-terminal as described previously (Ballar *et al.*, 2006). The Transthyretin D18G mutant was cloned by authors into a pcDNA3.1 expression vector with an eGFP moiety fused to the C-terminus of the resultant protein as previously described (Christianson *et al.*, 2011). Lastly, PrP mutants were subcloned from existing constructs between the BamHI and HindIII restriction sites under the control of the cytomegalovirus promoter of the pCep4β vector as described previously (Jodoin *et al.*, 2007).

DNA Transfection and RNA interference

Both DNA transfection and RNA interference by siRNA were carried out using the Amaxa Nucleofection system (Lonza, Mississauga, Ontario, Canada). CR7 cell-specific parameters were optimized using Amaxa Cell Line Optimization Nucleofector Kit (Lonza). Briefly, CR7 cells were transfected using two different solutions (Solutions V and L) across nine different programs for a total of 18 reactions. Solution L and program T-030 were chosen because they represented the most favorable ratio of transfection efficiency to cell death. Transfection was achieved using 1 μg of peYFP-Hrd1, 1 μg of control pMAX-GFP (included with Amaxa Nucleofector Kit), 1 μg of pCEP4β-V210I^{129V} or pCEP4β-M232R^{129V} PrP mutants or 1 μg of pcDNA3.1(-)-TTR^{D18G}-GFP and for double transfections, 0.25 μg of pcDNA3.1(-)-TTR^{D18G}-GFP and 0.75 μg of either PrP mutant on 1 X 10^6 CR7 cells using kit L, program T-030 . For siRNA interference, 250 nm of ON-TARGET Plus Human *SYVN1*- or Non-Targeting SMART pool siRNA (Dharmacon, Thermo Fisher Scientific) on 2 X 106 CR7 cells.

Subcellular Fractionation

Subcellular fractionation was performed as described previously (Jodoin *et al.,* 2007) with

modifications. Three wells of a 6-well plate, representing approximately 3 million cells, were used per experimental condition. Forty-two hours post-transfection and 18 hours post-treatment with BFA and epoxomicin, CR7 cells were collected in 0.5% Trypsin-EDTA (Invitrogen) and spun at 500 x g for 5 minutes at 37°C. Cells were washed 3 times in 10 mL of ice-cold phosphate buffered saline and suspended in 1 mL of fractionation buffer (8% sucrose (w/v), 20 mM HCl-Tricine, I mM EDTA) supplemented with protease inhibitors (Sigma, 38 μg/mL AEBSF, 0.5 μg/mL Leupeptin, 0.1 μg/mL Pepstatin and 0.1 μg/mL TLCK). Cells were lysed with 22 stokes in a 2 mL Dounce Homogenizer (Kontes, VWR, Mississauga, Ontario, Canada), with 50 μL set aside as crude cell extract. The homogenate was spun at 2000 x g for 10 minutes at 4°C to pellet nuclei and unbroken cells (Sorval RT6000D centrifuge, H1000B rotor). The resultant supernatant was collected and further spun at 100,000 x g for 1 hour at 4°C (Sorval M120 Ex micro-ultracentrifuge, RP100-AT-286 rotor). The supernatant (containing cytosolic proteins) was removed and the pellet (containing membrane proteins) was washed 2 times in fractionation buffer and resuspended in 200 μL of membrane lysis buffer (150 mM NaCl, 2 mM EDTA, 0.5% Triton-X (v/v), 0.5% sodium deoxycholate (w/v), 50 mM Tris-HCl, pH 7.5). Proteins were quantified using the Bradford Reagent (BioRad) and equal amounts of proteins from matched fraction pairs (i.e. 100 μg of untreated versus 100 μg of epoxomicin-treated cytosolic proteins) were precipitated overnight in 4 volumes of ice-cold methanol. Proteins were then pelleted, methanol aspirated and the pellet was dried and re-suspended in Lammeli Buffer in preparation for western blotting.

Western blot analysis

Proteins were separated on either 15% (PrP) or 7.5% (Hrd1) SDS-PAGE gels followed by transfer to polyvinylidene fluoride membranes at 15 volts over 10 minutes (1.5mm gel, "High

MW" program) using the Trans-blot Turbo Transfer System (Bio-Rad, Mississauga, Ontario, Canada). PrP was detected using the 3F4 antibody (Kascsak *et al.,* 1987) at 1:10,000, Hrd1 using anti-Syvn1 C-terminal (Abgent, San Diego, CA) at 1:250, mitochondrial heat shock protein 70 using anti-mt-Hsp70 (Thermo Fisher Scientific) at 1:500, Extracellular-signal-regulated kinases using anti-ERK 1/2 (Cell Signalling, Danvers, MA) or anti-ERK-2 (Santa Cruz Biotechnology, Santa Cruz, CA) both at 1:1,000, cytosolic heat shock protein 70 using anti-Cy-Hsp70 (Enzo Life Sciences) at 1:1,000, green fluorescent protein (GFP) using anti-GFP at 1:1,000 (Clone B-2, Santa Cruz Biotechnology) and anti-β-Actin (Sigma, Clone AC-15) at 1:2,500. Immunoreactivity was detected using 1:5,000 anti-mouse (GE-Healthcare, Baie d'Urfe, Quebec, Canada) or 1:5,000 anti-rabbit (Dako, Burlington, Ontario, Canada) IgG secondary antibodies conjugated to horseradish peroxidase using chemiluminescence (GE Healthcare).

Microscopy

Prior to visualization, chromatin were stained for 30 minutes at 37°C using 1 μg/mL Hoechst 33342 (Immunochemistry Technologies) added directly to media. Subcellular localization of the eYFP-Hrd1 fusion protein and retrotranslocation of the pcDNA3.1(-)-TTR^{D18G}-GFP was determined with a Nikon (Mississauga, Ontario, Canada) eclipse Ti microscope. eYFP-Hrd1 and TTR^{D18G}-GFP were detected using a FITC filter and Hoechst staining was detected using a DAPI filter. Pictures were taken using NIS-Elements Software (Version 3.1, Nikon). Transfection efficiency was measured by counting eYFP or GFP positive versus total cells in across several randomly chosen visual fields, until a total of 100 cells were counted.

Densitometry and statistical analysis

Quantification of western blot analysis was carried out using ImageJ (National Institutes of Health, Version 1.47t (64-bit), Bethesda, MD). Statistical significance of the results was

analyzed, where indicated, by either independent samples *t*-test or two-way analysis of variance (ANOVA) to test if data were sampled from populations following a Gaussian distribution followed by Dunnett's multiple comparisons tests to evaluate differences between treatment conditions using Prism (Graphpad Software, Version 6.0, San Diego, CA). A *p*-value of < 0.05 was used to determine statistical significance.

RESULTS

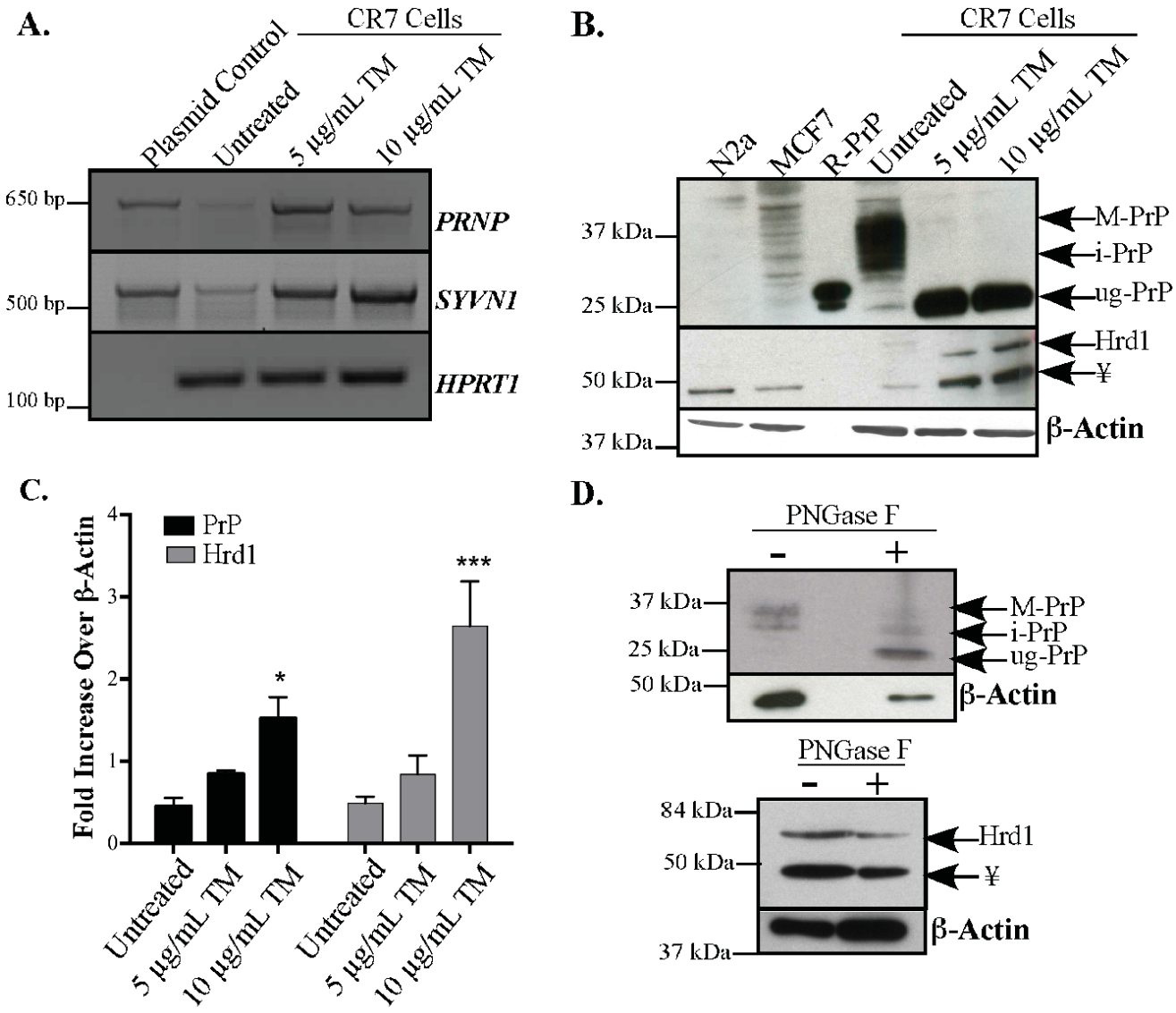
PrP and Hrd1 are endogenously expressed, undergo normal intracellular trafficking and are regulated by ER-stress in CR7 human glioblastoma cells

In experimental models, overexpressed PrP has been shown to bypass the secretory pathway to be synthesized in the cytosol (Drisaldi *et al.*, 2003). To examine retrotranslocation of endogenous PrP, as opposed to artifacts of overexpression, the human CR7 glioblastoma cell line was selected. This line is of astrocytic origin, known to express high levels of endogenous PrP (Arantes *et al.*, 2009). The expression of PrP and Hrd1 was examined at both the mRNA and protein levels, in normal conditions and under ER-stress, which was pharmacologically induced using N-linked glycosylation inhibitor tunicamycin. Tunicamycin was chosen, not only because it is a potent inducer of ER stress, but to provide a preliminary examination of the glycosylation status of the proteins of interest. RT-PCR analysis revealed both *PRNP* and *SYVN1* are endogenously expressed in CR7 cells and, as expected, are strongly upregulated by ER-stress (**Figure 5A**). This effect is particularly robust in the case of *SYVN1*, which shows a very strong dose-dependent increase under conditions of ER-stress, while *PRNP*, seems to plateau, with no further increase in expression at higher tunicamycin concentrations (**Figure 5A**). Immunoblotting against PrP in proteins extracted from CR7, PrP-negative MCF7 and Hrd1-negative N2a cells revealed an accumulation of immature (28-33 kDa) and mature (34-36 kDa)

along with a small amount of unglycosylated (25 kDa) PrP in untreated CR7 cells (**Figure 5B**). In addition, N2a cells act as a negative control for the antibody because 3F4 only detects human PrP, not endogenous mouse PrP. Only unglycosylated PrP was detected following treatment with both concentrations of tunicamycin, migrating as a single 25kDa band. Non-specific immunoreactivity detected in MC7 cells probed with anti-PrP 3F4 was likely due to the cells becoming slightly stressed during culturing, thus inducing expression of PrP combined with the large amount of loaded protein **(Figure 5B**). Lastly, recombinant PrP ran slightly higher then ug-PrP (**Figure 5B**). This is expected, as the protein is full-length WT PrP, which has been translated in an *Escherichia coli* system, thereby retaining both the N- and C-terminal signal peptides and, subsequently, running 3-5 kDa higher then ug-PrP, which has passed through the secretory system. The lower band at approximately 25 kDa likely represents either degradation of the recombinant protein or is the result of an incomplete start site during translation in *Escherichia coli.* Immunoblotting against Hrd1 revealed its expression was below the detectable limit in untreated CR7 cells, however, Hrd1 showed a strong, dose dependent increase as a result of tunicamycin-induced ER stress (68 kDa band), mirroring RT-PCR results (**Figure 5B).** Treatment with tunicamycin did not, appear to induce a migratory shift, suggesting Hrd1 is not glycosylated (**Figure 5B**). A band, mimicking the expression pattern of Hrd1, was consistently detected migrating just below 50 kDa, which will henceforth be referred to as the ¥-band (**Figure 5B**). It could represent an, as yet, unidentified cleavage fragment or splice variant of Hrd1, however, it does not appear to be a glycosylation species of the protein as treatment with tunicamycin did not affect the migration of either band, with Hrd1and the ¥-band continuing to migrate at 68 kDa and 45 kDa, respectively. Treatment with tunicamycin for 16 hours resulted in a significant induction of both PrP and Hrd1 expression, at higher concentrations of the drug,

Figure 5. **Endogenous expression, response to ER stress and glycosylation pattern of PrP and Hrd1 in CR7 cells. A**. Ethidium bromide agarose gel of *SYVN1* and *PRNP* amplicons obtained by RT-PCR from CR7 cells treated with 0, 5, or 10 µg/mL Tunicamycin (TM) for 16 hours **B.** Western blot analysis of PrP with human anti-3F4, Hrd1 and β-Actin in proteins extracted from CR7 cells treated with 0, 5 or 10 µg/mL TM, Hrd1-negative N2a cells, and PrP-negative MCF7 cells or recombinant PrP (R-PrP). Differentially glycosylated species of PrP are indicated: M-PrP; mature glycosylated PrP, i-PrP; immature glycosylated PrP and ug-PrP; full length, unglycosylated PrP. ¥ represents an unidentified splice variant or cleavage fragment of Hrd1 **C.** Relative amounts of PrP and Hrd1 in response to ER stress evaluated by densitometry, data represent the mean ± SEM of three independent experiments depicted as fold increase over β-Actin. *$p<0.05$ and ***$p<0.001$, statistically significant difference between untreated and cells treated with TM. **D**. Western blot analysis of PrP by 3F4, Hrd1 and β-Actin in 200 µg of proteins extracted from CR7 cells and treated without enzyme (-) or with 2 U of PNGase F (+) for 18 hours.

Figure 5.


A.
CR7 Cells
Plasmid Control
Untreated
5 µg/mL TM
10 µg/mL TM
650 bp
500 bp
100 bp
PRNP
SYVN1
HPRT1
B.
CR7 Cells
N2a
MCF7
R-PrP
Untreated
5 µg/mL TM
10 µg/mL TM
37 kDa
25 kDa
50 kDa
37 kDa
M-PrP
i-PrP
ug-PrP
Hrd1
¥
β-Actin
C.
PrP
Hrd1
Fold Increase Over β-Actin
0
1
2
3
4
*
***
Untreated
5 µg/mL TM
10 µg/mL TM
Untreated
5 µg/mL TM
10 µg/mL TM
D.
PNGase F
-
+
37 kDa
25 kDa
50 kDa
M-PrP
i-PrP
ug-PrP
β-Actin
PNGase F
-
+
84 kDa
50 kDa
37 kDa
Hrd1
¥
β-Actin

while treatment with lower doses resulted in a small but non-significant increase in expression. Overall, the trend indicates a dose-dependent increase in both PrP and Hrd1 proteins under increasing concentrations of ER-stress inducing tunicamycin (**Figures 5B and 5C**). Lastly we investigated if PrP and Hrd1 are normally trafficked within CR7 cells. Glycosylation occurs at Asn^{181} and Asn^{197} of PrP and it is transported through both the ER and Golgi to the cell surface (Endo *et al.*, 1989), while Hrd1 has two putative glycosylation sites in its sequence and is retained in the ER, purportedly as an unglycosylated protein (Kikkert *et al.*, 2004). In untreated CR7 protein extracts, PrP accumulates as mature and immature glycosylated species with a small amount of unglycosylated PrP detected (**Figure 5D**). The enzyme PNGase F is able to cleave both high mannose and modified high mannose sugars and resulted in the almost complete deglycosylation of PrP in CR7 protein extracts manifested as a migratory shift to 25 kDa (**Figure 5D**). The small amount of mature and immature glycosylated PrP is indicative of an enzymatic digestion that was not 100% efficient. Interestingly, treatment with PNGase F did not affect the migratory pattern of 68 kDa Hrd1 or the 45 kDa ¥-band (**Figure 5D**), indicating neither protein is glycoslyated *in vivo* and further confirming the ¥-band is not a glycosylated form of Hrd1, or vice versa. It is unclear why there is less Hrd1 in the PNGase F-digested sample, despite the fact that more protein was loaded overall, as evidenced by β-Actin. The possibility exists that Hd1 is being subjected to some limited proteolysis, perhaps by a protease either incompletely or not inhibited by the cocktail used (**Figure 5D**) Together these results show that both PrP and Hrd1 are endogenously expressed in CR7 cells, albeit Hrd1 levels are very low under normal conditions, are upregulated by ER-stress and are normally glycosylated. These findings allow us to utilize this model to study the ERAD of PrP in the absence of overexpression.

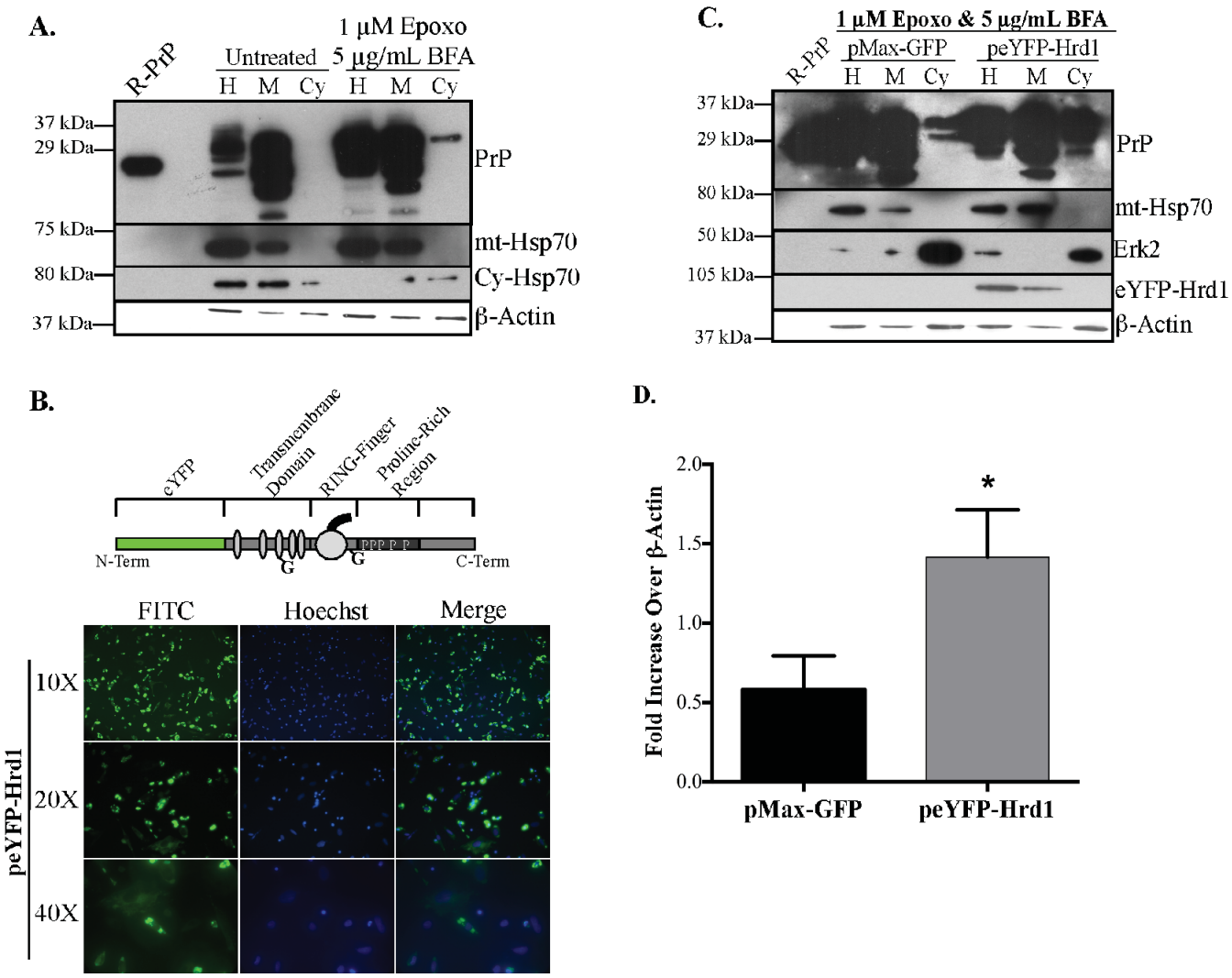
PrP is retrotranslocated and degraded by the proteasome in CR7 cells and overexpression of E3 ligase Hrd1 stimulates the ERAD of PrP

To assess PrP retrotranslocation, CR7 cells were treated for 18 hours with the potent irreversible proteasome inhibitor epoxomicin and the Golgi-disaggregating BFA. Generally the purpose of these drugs was to enhance the detection of CyPrP, specifically, epoxomicin was used to prevent degradation of ERAD substrates by the proteasome and BFA was utilized to limit trafficking of PrP to the cell surface, by redirecting it to the ERAD pathway and subsequent retrotranslocation. This is important to the experimental paradigm as normally less then 10% of PrP is retrotranslocated to the cytosol, and, as such, is likely below the detection limit by western blotting. Treated cells were submitted to subcellular fractionation and compared to their untreated counterparts. To ensure comparisons were as accurate as possible, equal amounts of each corresponding fraction were loaded. To accomplish this, proteins from all fractions were quantified using a BCA assay. Generally speaking, cells treated with epoxomicin resulted in some cell death and, as a result, had a lower protein concentration, therefore if 40 μg of total proteins were precipitated from the epoxomicin-treated cell's cytosolic fraction, an equal amount was precipitated from the cytosol of untreated cells, This was repeated for homogenate and membrane fractions. As expected, PrP was not detected in the cytosol of untreated cells, however, upon proteasomal inhibition, CyPrP was detected, migrating at approximately 30 kDa, consistent with immature glycosylated PrP originating form the ER (**Figure 6A**). While this seemed surprising, epoxomicin, in addition to inhibiting the proteasome, also inhibits endogenously expressed PNGase F and, therefore, CyPrP would be expected to remain glycosylated upon retrotranslocation from the ER (Karaivanova *et al.*, 2000). Mitochondrial heat shock protein 70 (mt-Hsp70) is a membrane-associated protein and its presence in only the

Figure 6. Over-expression of Hrd1 enhances PrP retrotranslocation in CR7 cells

A. Western blot analysis of endogenous PrP by 3F4, mitochondrial heat shock protein 70 (mt-Hsp70), and β-Actin proteins in CR7 cells treated for 18 hours with 1 μM epoxomicin (epoxo) and 5 μg/mL Brefedin A (BFA) and fractionated into homogenate (H), membrane (M) and cytosolic (Cy) fractions. Proteins were quantified and equal amounts of matched fraction pairs were loaded (Ex: Cy vs Cy). R-PrP; recombinant PrP_{1-253}. **B.** Schematic of eYFP-Hrd1 fusion protein with "G" depicting putative glycosylation sites. Fluorescent microscopy of CR7 cells transfected with peYFP-Hrd1 mammalian expression vector. Higher magnification (40X) images depict the intracellular localization of eYFP-Hrd1 fusion proteins. Nuclei are identified using Hoechst staining. **C.** Western blot analysis of endogenous PrP by 3F4, mt-Hsp70, eYFP-Hrd1 and β-Actin in CR7 cells transfected with either pMaxGFP or peYFP-Hrd1 and treated for 18 hours with 1 μM epoxo and 5 μg/mL BFA before being fractionated into (H), membrane (M) and cytosolic (Cy) fractions. Proteins were loaded as in 4A. **D**. Relative CyPrP levels evaluated by densitometry, data represent the mean ± SEM of four independent experiments depicted as fold increase over β-Actin. *$p<0.05$, statistically significant difference between control and Hrd1-transfected cells.

Figure 6.


A.
R-PrP
Untreated
1 µM Epoxo
5 µg/mL BFA
H M Cy H M Cy
37 kDa
29 kDa
75 kDa
80 kDa
37 kDa
PrP
mt-Hsp70
Cy-Hsp70
β-Actin
C.
1 µM Epoxo & 5 µg/mL BFA
R-PrP
pMax-GFP
peYFP-Hrd1
H M Cy H M Cy
37 kDa
29 kDa
80 kDa
50 kDa
105 kDa
37 kDa
PrP
mt-Hsp70
Erk2
eYFP-Hrd1
β-Actin
B.
eYFP
Transmembrane Domain
RING-Finger
Proline-Rich Region
N-Term
C-Term
FITC
Hoechst
Merge
peYFP-Hrd1
10X
20X
40X
D.
Fold Increase Over β-Actin
2.0
1.5
1.0
0.5
0.0
*
pMax-GFP
peYFP-Hrd1

homogenate and membrane fractions indicated that PrP detected in the cytosol was not due to cross-contamination from membrane proteins but represents bona fide CyPrP arising from retrotranslocation. Likewise, the presence of CyHsp70 in the cytosolic fractions, confirmed that the proteins are of cytosolic origin and that roughly equal amounts of proteins were loaded (**Figure 6A**). There was some degree of cross-contamination of the membrane fractions by cytosolic proteins, however, this does not change the interpretation of data as the primary focus was on CyPrP expression (**Figure 6A**). β-Actin loading controls indicated that, one, protein was loaded in all lanes, including the untreated cytosolic fraction, and two, that relatively equal amounts of protein were loaded between fractions of the same origin (i.e. untreated versus epoxomicin-treated cytosolic proteins) (**Figure 6A).** The exception to this would be the membrane fractions where less was loaded from untreated CR7 cells, however, most importantly, it appears that equal amounts of protein were loaded from the two cytosolic fractions. The smaller brands (below approximately 20 kDa) likely represent N-terminally truncated PrP fragments, resulting from proteolytic cleavage.

To determine if Hrd1 regulates PrP retrotranslocation, we obtained an expression vector encoding human Hrd1 with an enhanced yellow fluorescent protein (eYFP) moiety fused to the N-terminus. The location of the eYFP protein near the transmembrane domain but away from the catalytic RING-finger domain and putative substrate-binding proline rich region, increased the likelihood that the fusion protein would not interfere with the enzymatic activity of the E3 ligase (Schematic, **Figure 6B).** The fluorescent eYFP allowed for the control of transfection efficiency and the confirmation of correct subcellular expression. The peYFP-Hrd1 vector was transfected into CR7 cells with 70% efficiency and the eYFP-Hrd1 protein was localized using fluorescent microscopy. Nuclear DNA were simultaneously identified using Hoechst staining and visualized

in cells at higher magnification **(Figure 6B**). The non-uniform, perinuclear localization of eYFP-Hrd1 seen at higher magnification (**20 X**, **40 X, Figure 6B**) is consistent with ER-associated proteins (Ding *et al.*, 2001) and it is not detected in the processes or nuclei of cells. The patchy immunofluorescent signal observed has been reported for other ER-resident proteins following induction of ER stress (Preuss *et al.*, 1991). Following transfection with either peYFP-Hrd1 or control pMax-GFP vector, CR7 cells were treated with epoxomicin and BFA and subjected to subcellular fractionation, as described above. In treated, pMax-GFP-transfected cells, PrP was detected in the cytosol as immature (30 kDa) and mature glycosylated (35 kDa) CyPrP (**Figure 6C**). The presence of mature CyPrP indicates the Golgi was not completely disaggregated, or temporarily re-formed during BFA treatment and the resultant higher molecular weight band likely represents one of the carbohydrate remodeling stages occurring within this organelle (**Figure 6C**). Overexpression of eYFP-Hrd1 resulted in a marked increase in CyPrP. Corresponding increases of homogenate and membrane PrP in eYFP-Hrd1-transfected cells was not evident, due to high endogenous expression of PrP in this cell line (**Figure 6C**). As previously, mt-Hsp70 in the homogenate and membrane fractions but not the cytosol, confirmed the observed increase in CyPrP was not due to contamination from membrane proteins. Additionally, extracellular regulated kinase 2 (Erk2), a cytosolic protein, was present mostly in the homogenate and cytosolic fractions, while immunoblotting against Hrd1 confirmed expression of the fusion protein (at approximately 99 kDa, 68 kDa Hrd1 + 31kDa eYFP) in peYFP-Hrd1-tranfected cells (**Figure 6C**). β-Actin controls confirmed that equal amounts of corresponding subcellular fractions from pMax-GFP- and peYFP-Hrd1-transfected cells were loaded (**Figure 6C**). Analysis by densitometry revealed overexpression of Hrd1 resulted in a statistically significant, approximately three-fold increase in CyPrP as compared to pMax-GFP-

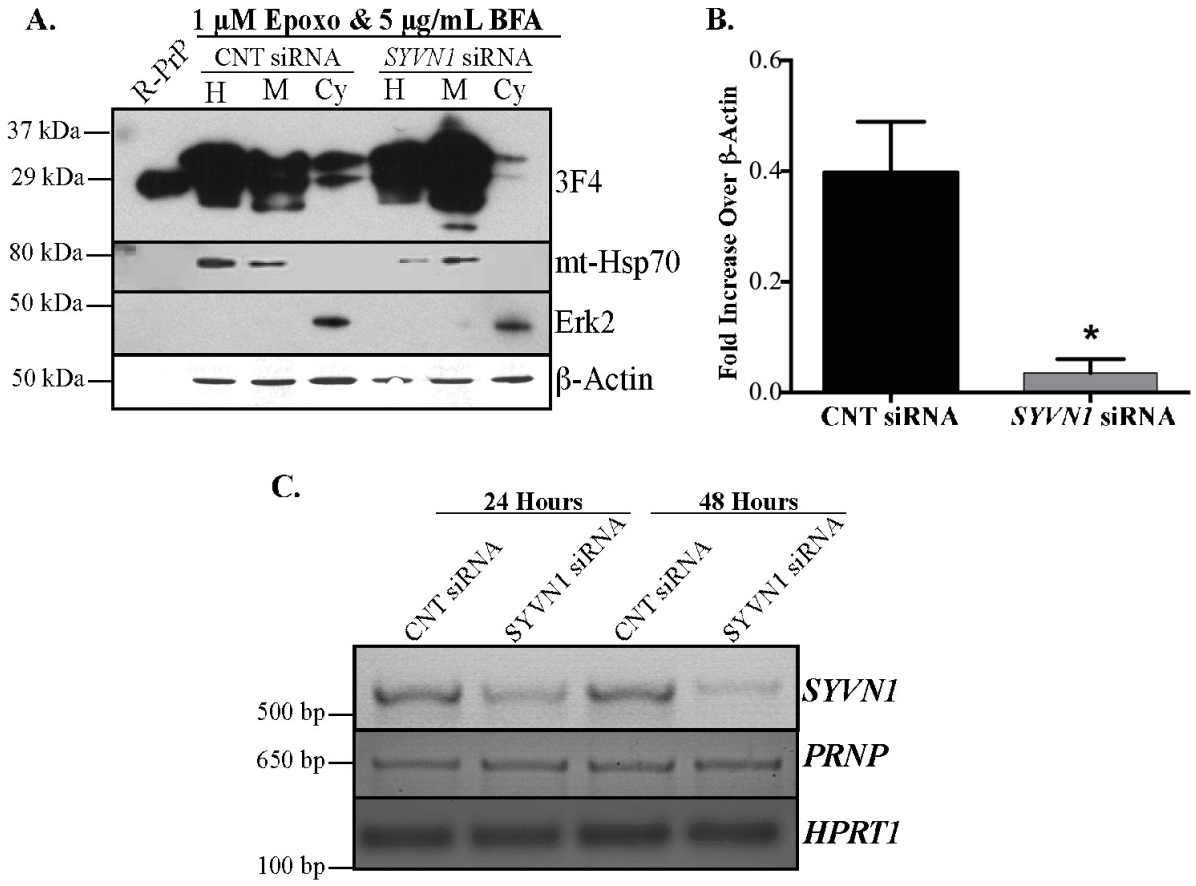
transfection (**Figure 6D**). These results indicate that Hrd1 likely plays a role in regulating the retrotranslocation PrP in human CNS cells.

Knockdown of Hrd1 by *SYVN1*-targeting siRNA abrogates PrP retrotranslocation

To complement the gain-of-function observed with Hrd1 overexpression, we examined the effect of targeted knockdown of Hrd1 mRNA by small interfering RNA (siRNA). Accordingly, CR7 cells were transfected with a *SYVN1*-targeting pool of four different siRNAs or a scrambled, non-targeting siRNA control pool and gene silencing was allowed over 24 hours. Cells were then treated with epoxomicin and BFA for 18 hours and subjected to subcellular fractionation, described previously. Transfection with scrambled siRNA did not impair PrP retrotranslocation into CyPrP. Conversely, a reduction in CyPrP was detected in cells transfected with *SYVN1*-targeting siRNA (**Figure 7A**). Membrane-associated mt-Hsp70 was not found in the cytosolic fractions, while Erk2 was found in equal amounts in both cytosolic fractions, indicating the lack of CyPrP was not the result of a loading error (**Figure 7A**). Lastly, β-Actin controls indicated equal amounts of proteins were loaded from both cytosolic fractions (**Figure 7A**). Both in Figure 7A and subsequent replications, an increase in membrane-associated PrP was found in *SYVN1*-targeting siRNA-transfected cells. This is what would be expected when cells are unable to target PrP to either the secretory or ERAD pathways, proteins would be retained in the ER, manifesting, in this case, as an increase in membrane-associated PrP (**Figure 7A**). In three independent replications of this experiment, transfection with *SYVN1*-targeting siRNA resulted in an approximately 20-fold decrease in CyPrP, with small amounts observed in the cytosol only when protein concentrations were doubled in western blot analysis (**Figure 7B**). As previously shown, endogenous levels of Hrd1 protein are too low in CR7 cells to be detected by western blotting when less then 100 μg of proteins are loaded, therefore knockdown of Hrd1

Figure 7. **Targeted knockdown of Hrd1 with siRNA against *SYVN1* abolishes CyPrP in CR7 cells. A.** Western blot analysis of PrP with 3F4, mitochondrial heat shock protein 70 (mt-Hsp70), Extracellular regulated kinase 2 (Erk2), and β-Actin in proteins extracted from the homogenate (H), membrane (M) and cytosolic (Cy) fractions of CR7 cells transfected with 250nm of either non-targeting scrambled or *SYVN1*-targeting siRNA followed by 18 hour treatment with 1 μM epoxomicin (Epoxo) and 5 μg/mL Brefeldin A (BFA). Proteins are loaded as in Figure 6**. B.** PrP in the cytosolic fraction of CR7 cells transfected with either non-targeting scrambled or *SYVN1*-targeting siRNA. Relative CyPrP levels evaluated by densitometry and is depicted as fold increase over β-Actin. Data represent the mean ± SEM of three independent experiments. *$p<0.05$, statistically significant difference between control and *SYVN1*-knockdown cells**. C.** Ethidium bromide agarose gel of *SYVN1* and *PRNP* amplicons obtained by RT-PCR from CR7 cells transfected for 24 or 48 hours with either non-targeting control or *SYVN1*-targeting siRNA.

Figure 7.


A.
1 µM Epoxo & 5 µg/mL BFA
R-PrP
CNT siRNA
SYVN1 siRNA
H M Cy H M Cy
37 kDa
29 kDa
80 kDa
50 kDa
50 kDa
3F4
mt-Hsp70
Erk2
β-Actin
B.
Fold Increase Over β-Actin
0.6
0.4
0.2
0.0
*
CNT siRNA
SYVN1 siRNA
C.
24 Hours
48 Hours
CNT siRNA
SYVN1 siRNA
CNT siRNA
SYVN1 siRNA
500 bp
650 bp
100 bp
SYVN1
PRNP
HPRT1

mRNA was confirmed by RT-PCR from cells transfected with either scrambled, non-targeting control siRNA or *SYVN1*-targeting siRNA and gene silencing was allowed for either 24- or 48-hours. This was followed by treatment with epoxomicin and BFA for 18 hours to mimic the experimental paradigm utilized above. Results show a clear reduction of *SYVN1* mRNA at both 24- and 48-hours post-transfection, as compared to control cells, with no concomitant reduction of *PRNP* mRNA expression (**Figure 7C**). With a concentration of 250 nM siRNA, there was the possibility of off-target effects, however, the concentration chosen was well within the recommended range used for targeted gene knockdown with Dharmacon siRNA Smartpools and using Amaxa nucleofection technology (50 nM – 1 μM) and was optimized to maximize gene knockdown, while limiting cytotoxicity. In addition, it is important to remember that each reaction included one million cells, as opposed to the much lower cell density typically used with lipofection. However, while we confirmed via RT-PCR that *SYVN1*-targeting siRNA had no effect on *PRNP* expression, we are currently testing the effect of custom designed *SYVN1*-targting siRNA pools on PrP retrotranslocation, in an effort to rule out off-target effects.

Results indicates that targeted knockdown of *SYVN1* mRNA by siRNA block PrP retrotranslocation to the cytosol and result in its retention within the ER and that this observed effect is not the due to off-target effects of the siRNA on *PRNP* mRNA expression. These, combined with results outlined in Figure 6, provide compelling and complementary gain-of-function, loss-of-function evidence for the mediation of PrP retrotranslocation by Hrd1.

PrP mutants inhibit retrotranslocation of endogenous PrP and model ERAD substrate TTR^{D18G} in CR7 cells

As previously shown by our group, PrP mutants associated with fCJD and GSS pathologies generate lower levels of CyPrP than WT PrP and the amount of

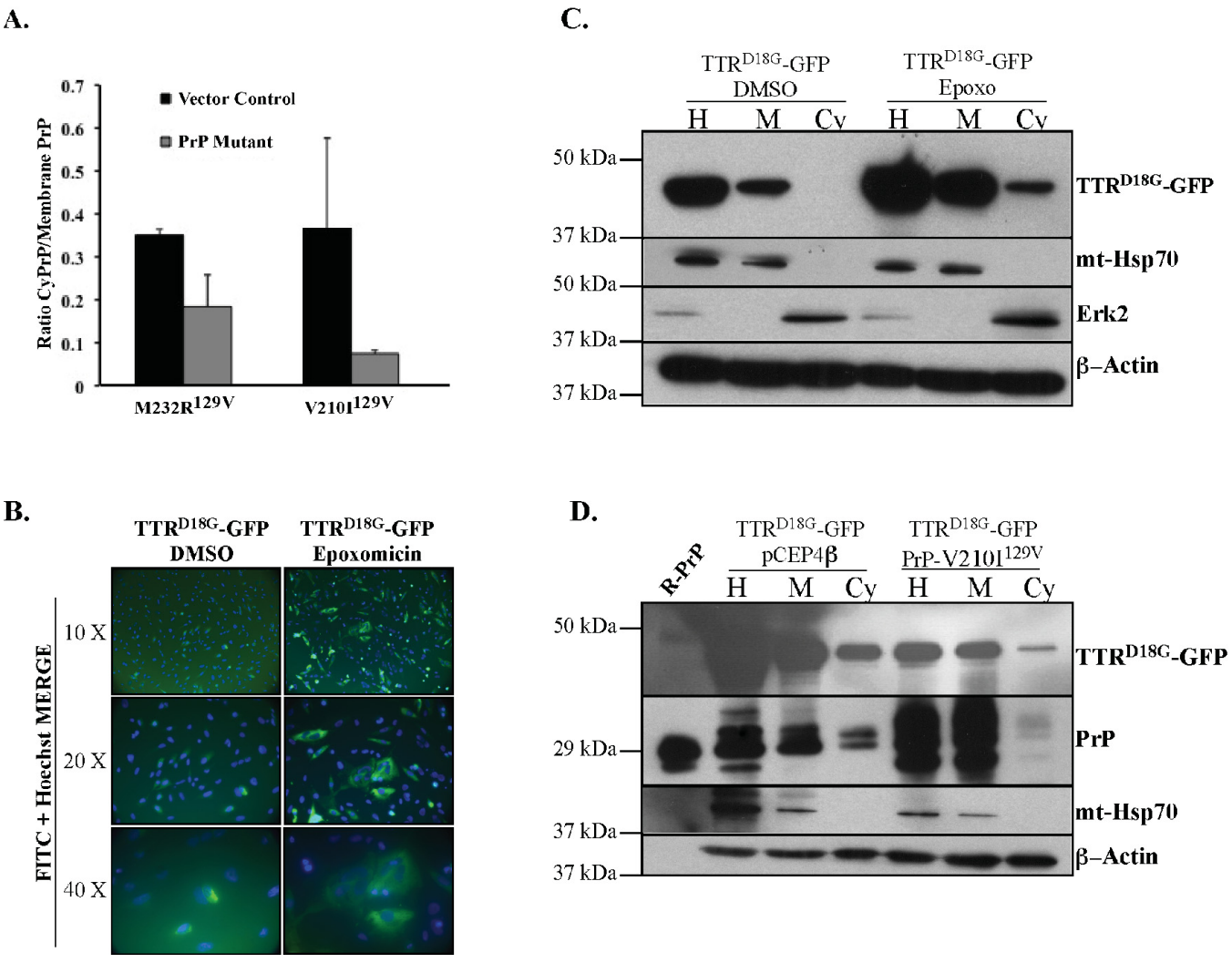
CyPrP was negatively correlated with the anti-Bax function (Jodoin *et al.*, 2007, Jodoin *et al.*, 2009). To further investigate this phenomenon, we selected two familial PrP mutants, $M232R^{129V}$ and $V210I^{129V}$, with mutations corresponding to the GPI anchor and ∝-helix III, respectively. Both mutants have been shown to result in a loss of Bax protection and reduced generation of CyPrP in N2a cells and PHNs (Jodoin *et al.*, 2007). PrP mutant cDNA was transfected into CR7 cells using the pCEP4β vector and allowed to express for 24 hours. Cells were treated with epoxomicin and BFA for 18 hours and submitted to subcellular fractionation, as previously described. In empty-vector transfected controls, the ratio of membrane to CyPrP is approximately equal between the two mutants, albeit with relatively high variability within the $V210I^{120V}$ condition, but nevertheless supporting the validity of the chosen measurement. Expression of both PrP mutants produced a substantial drop in levels of CyPrP, relative to membrane PrP (**Figure 8A**). Interestingly, expression of the $M232R^{129V}$ mutant in CR7 cells resulted in a 50% decline in the ability to generate CyPrP, while the $V210I^{129V}$ mutant inhibited CyPrP expression by approximately 75%, when compared to the vector control (**Figure 8A**). It would appear that disease-causing PrP mutants are inhibiting the ability of CR7 cells to generate CyPrP, likely by interfering with retrotranslocation machinery. To test this hypothesis we obtained the pcDNA3.1(-)-TTR^{D18G}-GFP expression construct, which results in the model ERAD substrate luminal, non-glycosylated mutant transthyretin with a GFP reporter fused to the C-terminal. To confirm that this mutant is, in fact, a substrate of the ERAD pathway in CR7 cells, as previously shown in HEK293 and HeLa cells as well as *in vitro* (Christianson *et al.*, 2011), pcDNA3.1(-)-TTR^{D18G}-GFP was transfected into CR7 cells. Cells were monitored by fluorescent microscopy before and after 18 hours of treatment with epoxomicin or DMSO (**Figure 8B)**, and submitted to subcellular fractionation and analysis of GFP by western blotting.

BFA was not used in this experimental paradigm because TTR^{D18G} is not a secreted protein, therefore there is no reason to disaggregate the Golgi. While cells treated with DMSO alone do show modest GFP reporter expression, inhibition of the proteasome results in a marked increase in GFP fluorescence, as compared controls (**Figure 8B).** At higher magnification (20 X and 40 X), it becomes clear that the expression of the GFP reporter is diffuse, localized throughout the cell body but not in the nucleus or cell processes (**Figure 8B**). This expression pattern is consistent with retrotranslocated TTR^{D18G}-GFP, accumulating in the cytosol of cells during proteasome inhibition. Interestingly, the protein appears to accumulate in perinuclear clusters in some, but not all cells both with, and, without epoxomicin. (**Figure 8B, bottom panel, DMSO-treated**). Hoechst staining of the nuclei confirm that GFP expression is, indeed, cytosolic. Immunoblotting against GFP, showed a small amount of TTR^{D18G}-GFP accumulating in the cytosol of DMSO-treated CR7 cells (41 kDa upper band, 14 kDa TTR^{D18G} + 27 kDa GFP), indicating the proteasome is not able to rapidly degrade all TTR^{D18G}-GFP under conditions of overexpression. However, upon proteasome inhibition, a substantial increase in GFP is detected in the cytosolic fraction, as expected of an ERAD substrate (**Figure 8C**). Lower bands co-migrate with control GFP-transfected MCF7 cells, representing possible cleavage of the GFP reporter from mutant TTR *in cellulo*. The presence of mt-Hsp70 in membrane and homogenate but not cytosolic fractions confirms the observed increase was not due to cross-contamination, while the presence of Erk1/2 only in cytosolic and homogenate fractions confirms purity of membrane proteins (**Figure 8C)**. For unknown reasons, GFP was not detected in the homogenate of epoxomicin-treated cells, despite the presence of mt-Hsp70 and β-Actin.

Figure 8. Familial PrP mutants inhibit retrotranslocation of both endogenous cellular PrP and other ERAD substrates

A. Quantification of western blot analysis for CyPrP in CR7 cells transfected with pCEP4β-empty or pCEP4β-PrP mutants followed by 18 hours treatment with 1 μM epoxomicin (epoxo) and 5 μg/mL Brefedin A (BFA) and fractionated into homogenate (H), membrane (M) and cytosolic (Cy) fractions. Data are depicted as a ratio of CyPrP relative to membrane PrP, following normalization to β-Actin **B**. Phase contrast and FITC microscopy of CR7 cells transfected with pcDNA3.1(-)-TTR^{D18G}-GFP. Micropictographs follow GFP reporter expression before and after 18 hours of treatment with epoxomicin. Higher magnification (40X) images depict the intracellular localization of TTR^{D18G}-GFP fusion proteins. Nuclei are identified using Hoechst staining. **C.** Western blot analysis of GFP, mt-Hsp70, Erk1/2 and β-Actin in CR7 cells transfected with pcDNA3.1(-)-TTR^{D18G}-GFP and treated for 18 hours with either DMSO or 1.0 μM epoxo before being fractionated into homogenate (H), membrane (M) and cytosolic (Cy) fractions. Proteins were loaded as in 6A. **D.** Western blot analysis of GFP, PrP, mt-Hsp70 and β-Actin in CR7 cells co-transfected with pcDNA3.1(-)-TTR^{D18G}-GFP and pCEP4β--PrP-$V210I^{129V}$, treated for 18 hours with either DMSO or 1.0 μM epoxo, followed by

Figure 8.


A.
Ratio CyPrP/Membrane PrP
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Vector Control
PrP Mutant
M232R129V
V210I129V
B.
TTRD18G-GFP DMSO
TTRD18G-GFP Epoxomicin
FITC + Hoechst MERGE
10 X
20 X
40 X
C.
TTRD18G-GFP DMSO
TTRD18G-GFP Epoxo
H M Cy H M Cy
50 kDa
37 kDa
50 kDa
37 kDa
37 kDa
TTRD18G-GFP
mt-Hsp70
Erk2
β–Actin
D.
R-PrP
TTRD18G-GFP pCEP4β
TTRD18G-GFP PrP-V210I129V
H M Cy H M Cy
50 kDa
29 kDa
37 kDa
37 kDa
TTRD18G-GFP
PrP
mt-Hsp70
β–Actin

Most interestingly, when TTR^{D18G}-GFP was co-transfected with familial PrP mutant $V210I^{129V}$ (representative example, **Figure 8D**), it not only resulted in an inability of the cells to generate CyPrP, as previously seen, but concurrently resulted in a drastic reduction in the cells ability to retrotranslocate Hrd1 model substrate TTR^{D18G}-GFP (**Figure 8D**). This reduction in both cytosolic TTR^{D18G}-GFP and CyPrP was not due to errors in protein loading, as evidenced by β-Actin controls or due to cross contamination from membrane proteins, as evidenced by the mt-Hsp70 marker (**Figure 8D**). In fact, it would appear, slightly less membrane and more cytosolic proteins were loaded in double transfected cells (**Figure 8D**: mt-Hsp70 and β-Actin), further supporting the notion that observed decreases in cytosolic TTR^{D18G}-GFP and CyPrP are a real effect. The relative increase in PrP expression in homogenate and membrane fractions extracted from co-transfected cells, appear to confirm the efficacy of the PrP mutant transfection, as there was not, overall, substantially more protein loaded in these conditions. This is particularly obvious when comparing the membrane fractions between the two conditions. However, double transfection with TTR^{D18G}-GFP and PrP mutants appeared to result in lower overall expression of the TTR^{D18G}-GFP protein compared to empty-vector-transfected controls, likely due to overloaded transcription machinery. To account for this effect, when quantification by densotometry is completed, data will be represented as in Figure 8A, relative to the respective membrane fraction and normalized to β-Actin.

DISCUSSION

The goal of the current work was to uncover the mechanism responsible for PrP retrotranslocation in human CNS cells. The ultimate purpose being to discover how disease-causing PrP mutants are blocking this process and, subsequently, losing CyPrP-mediated Bax protection. Here we show that both PrP and E3 ubiquitin ligase Hrd1 are endogenously expressed, undergo normal subcellular trafficking and are upregulated during ER stress in human CR7 glioblastoma cells. We found that overexpression of Hrd1 results in a marked increase in CyPrP under conditions of proteasome inhibition, while conversely, targeted knockdown of the Hrd1 gene, *SYVN1,* abrogates CyPrP detection. We confirmed that familial, disease-causing PrP mutants block the generation of CyPrP in human CNS and showed that retrotranslocation of model Hrd1 substrate TTR^{D18G} is also perturbed by familial PrP mutants. From these data we conclude that Hrd1 mediates the retrotranslocation of PrP in human CNS cells. Furthermore we can conclude that PrP mutants interfere with the generation of CyPrP by blocking the retrotranslocation machinery, while, simultaneously disrupting with the ERAD of other substrates, such as TTR^{D18G}, through blockade of the E3 ligase Hrd1.

While the retrotranslocation of human PrP has been studied previously, authors almost universally rely on the overexpression of PrP (Ma *et al.*, 2001, Yedidia *et al.*, 2001, Roucou *et al.*, 2003, Jodoin *et al.*, 2007, Ashok *et al.*, 2008, Jodoin *et al.*, 2009). While it is vital to acknowledge the importance of exogenously introduced protein as an experimental tool, there are inherent problems with studying PrP trafficking under conditions of overexpression, particularly when focusing on CyPrP generation via the ERAD pathway. When PrP is introduced under the regulation of a viral promoter, such as cytomegalovirus, mRNA copy number dramatically increases and the phenomenon is greatly exacerbated during proteasome inhibition

(Drisaldi *et al.*, 2003, Biasini *et al.*, 2004). The result is a saturation of cellular translation and translocation machinery and subsequent synthesis of PrP in the cytosol. As a consequence of the nascent chain's failure to enter the ER lumen, a form of CyPrP is generated, migrating as a single band at approximately 27 kDa, consistent with unglycosylated PrP retaining both its N- and C-terminal signal peptides (Drisaldi *et al.*, 2003, Fioriti *et al.*, 2005). This same effect was demonstrated when T-cell receptor α-chains were overexpressed, under cytomegalovirus regulation, in HEK293 cells and, therefore, is not exclusive to PrP (Yu *et al.*, 1997). Hegde, *et al*, claim that ER-stress alone causes the attenuation of translocation machinery as a form of "pre-emptive quality control", an attempt to reduce protein load within the ER lumen during UPR and the resultant accumulation of CyPrP leads to aggregation and neurodegeneration (Kang *et al.*, 2006, Rane *et al.*, 2008). This work has been repeated in mouse N2a cells, where authors showed that exogenously introduced human PrP, when expressed in the cytosol, is toxic, causing cellular apoptosis (Ma *et al.*, 2002a, Ma *et al.*, 2002b). However, it would appear that authors were examining SP-PrP, an artifact resulting from overexpression as opposed to retrotranslocated PrP because when *bona fide* ERAD-derived CyPrP is examined in N2a cells, it is not toxic (Fioriti *et al.*, 2005).

In addition, previous work generally takes place in either human cell lines derived from peripheral cell types such as breast (MCF7), prostate (PC3) or kidney (HEK293), yeast, or other mammalian cell lines such as mouse neuroblastoma (N2a) or Chinese Hamster Ovary (CHO) cells (Yedidia *et al.*, 2001, Fioriti *et al.*, 2005, Roucou *et al.*, 2005, Wang *et al.*, 2005a, Apodaca *et al.*, 2006, Bounhar *et al.*, 2006, Jodoin *et al.*, 2007, Jodoin *et al.*, 2009). Work in human cells tends to either stop at the point where retrotranslocation is demonstrated (Wang *et al.*, 2005a), or is limited to single cell analysis in PHNs (Bounhar *et al.*, 2001b, Roucou *et al.*, 2003, Jodoin *et*

al., 2007). Using a CNS-derived cell line is critical as PrP is most highly expressed in brain and prion disease pathology almost exclusively manifests in the CNS of patients. Using a peripheral cell type is, therefore, not a relevant model for examining the role of PrP in prion disease. The lone study to examine the mechanism underlying PrP retrotranslocation takes place in yeast, where authors show that human PrP interacts with endogenous yeast E3 ligase hrd1p (Apodaca *et al.*, 2006). Consequently, there is a hole in the existing literature resulting from a failure to examine the components of the ERAD pathway responsible for the retrotranslocation of endogenous PrP, in a human CNS cell line. This is important for a number of reasons, chief among them being that, while the Hrd1 retrotranslocation complex is highly conserved from yeast to mammals (Bays *et al.*, 2000), there are differences distinguishing Hrd1-mediated ERAD in mammalian systems. For example, there are two mammalian homologues to yeast hrd1p: Hrd1 and gp78, which have multiple and, often, overlapping substrates (Bernardi *et al.*, 2010, Christianson *et al.*, 2011). As such, establishing an interaction between human PrP and yeast hrd1p, while an important first step, does not necessarily mean that the same mechanism exists in the more complex, mammalian system.

Our choice of *in cellulo* model, directly addresses these issues. CR7 cells are a human glioblastoma cell line derived from type IV metastatic astrocytes. Not only is PrP^{C} known to be highly expressed in human astrocytes, but its interaction with STI1 has been shown to regulate proliferation and development in a PKA-dependent manner (Lima *et al.*, 2007, Arantes *et al.*, 2009, Hartmann *et al.*, 2012). Astroglial proliferation is well documented in TSEs and PrP^{Sc} deposits have been shown to co-localize with astrocytic markers (Sarasa *et al.*, 2012). So, not only is TSE pathology almost exclusively due to CNS degeneration but glial cells are intimately involved in the pathophysiology of the disease, making an astrocytic cell line, an appropriate and

physiologically relevant model in which to study both PrP^C trafficking and its involvement in prion disease.

Here we confirm baseline expression of PrP at both an mRNA and protein level in CR7 cells. We also confirm that *PRNP* is upregulated during tunicamycin-induced ER-stress, as previously shown (Déry *et al.*, 2013, Misiewicz *et al.*, 2013). Tunicamycin acts by inhibiting N-linked glycosylation, leading to a global increase in protein misfolding, prevention of normal intracellular trafficking and ER stress, followed by induction of the UPR (Heifetz et al., 1979). Genomic changes occur in metastatic cell types and can often lead to the differential regulation of genes as compared to WT counterparts (Ross *et al.*, 2000). The regulation of *PRNP* by ER stress is implicated in the pathophysiology of prion disease (Hetz *et al.*, 2003), it was, therefore, important to demonstrate normal gene regulation in our model. We showed that *PRNP* expression was upregulated upon induction of ER stress (**Figure 5A**). This was expected, as our group has previously demonstrated this phenomenon in MCF7 cells and PHNs. The *PRNP* gene contains three classical ERSE elements, regulated by ATF6 and XBP1 and one novel ERSE-like elements, regulated by XBP1 alone, and binding of these transcription factors during the UPR, appears to upregulate the gene (Déry *et al.*, 2013, Misiewicz *et al.*, 2013). While the physiological relevance of this is unclear, given the neuroprotective role of CyPrP, it would follow that increased expression under conditions of ER stress would be beneficial, perhaps by preventing the premature activation of apoptosis, via Bax inhibition, while the cell attempts to restore homeostasis. At the same time we showed upregulation of the Hrd1 gene, *SYVN1*, during tunicamycin-induced ER stress (**Figure 5A**). This has been demonstrated previously in HEK293T cells (Kaneko *et al.*, 2002). The *SYVN1* promoter contains both an ERSE and an UPRE element and is involved in the UPR via its central role in the ERAD pathway (Kaneko *et*

al., 2002). Upregulation of this gene, or associated protein has been shown to prevent cellular apoptosis by increasing the clearance of misfolded or excess proteins from the ER, thereby preventing ER stress (Kaneko *et al.*, 2002, Omura *et al.*, 2012). It would seem that under normal physiological conditions, both PrP and Hrd1 could work synergistically to protect the cell from ER stress and apoptosis.

During ER stress, there is a general inhibition of protein translation in order to reduce the load within the ER as aberrant proteins are degraded (Harding *et al.*, 2000). However, proteins vital to the health of the cell such as those involved in the UPR evade this process and continue to be translated. Transcription factor ATF4, important for the upregulation of genes involved in the remediation of cellular stress injury, fails to be degraded during the UPR (Harding *et al.*, 2000), and, if damage is beyond repair, powerful apoptosis inducer CHOP is upregulated (Zinszner *et al.*, 1998). Despite the general cessation of translation during ER-stress, a dose-dependent upregulation of both the PrP and Hrd1 proteins was still observed in CR7 cells (**Figure 5B and C),** hinting at a vital role in maintaining homeostasis and preventing apoptosis for both proteins. Interestingly, baseline Hrd1 expression was undetectable in CR7 cells by western blot unless upwards of 200 μg of proteins were loaded (**Figure 4B).** Initially it was reported that Hrd1 was only expressed in neurons but not glial cells (Omura *et al.*, 2006, Omura *et al.*, 2008b), however it has since been found in both neurons and reactive astrocytes in the human brain (Hou *et al.*, 2006). Maintaining the health of astrocytes during conditions of global stress, such as during prion disease, makes sense in that dysfunction of these supporting cells would drastically influence neuronal health. Indeed, in TSEs, it has been shown that accumulation of PrP^{Sc} precedes astrocytic gliosis, which could be an attempt to maintain

neuronal health as opposed to contributing to cellular toxicity (DeArmond *et al.*, 1997) and increased expression of Hrd1, as well as PrP^C, may be a part of this process.

When examining the regulation and glycosylation patterns of Hrd1, we made an interesting observation. A band was consistently detected by SDS-PAGE just below 50 kDa (**Figure 5B & D**), while endogenous Hrd1 runs at 68 kDa and eYFP-Hrd1 at 94 kDa. Originally it was thought to be non-specific, as reported by the manufacturer of the antibody (Abgent). However, with subsequent experiments and optimization procedures, we observed the expression pattern of this mystery band (denoted as the ¥-band), mimic that of both endogenous and overexpressed Hrd1. While this effect is clearly demonstrated in Figure 5B with tunicamycin treatment, we also saw a stepwise increase in the ¥-band when optimizing eYFP-Hrd1 transfections and a dose-dependent decrease when optimizing siRNA knockdown of endogenous *SYVN1* (data not shown). This led us to further investigate the identity of the ¥-band. Blasting the epitope of the antibody against all known proteins (NCBI Protein Blast) did not reveal any alignment with a protein of corresponding size, nor would any of the known proteolytic cleavage products produce an approximately 47 kDa band. The band could be due to cleavage at an unreported site, however, the protein sequence does not contain any *predicted* sites, which would produce a protein of the appropriate size. While not experimentally tested here, the sequence of Hrd1 does not contain any phosphorylation sites and this post-translational modification of Hrd1 has been empirically examined elsewhere (Kikkert *et al.*, 2004). It is possible within the metastatic environment of CR7 glioblastoma cells, Hrd1 is phosphorylated, which has been shown to affect the electrophoretic mobility of other proteins on SDS-PAGE gels (Wegener *et al.*, 1984). However, phosphorylation generally results in an decrease in migration rate, which manifests as an increase in molecular weight as opposed to a decrease, as seen with the ¥-band,

and can likely be ruled out as an explanation (Wegener *et al.*, 1984). Interestingly, the band does not represent an established alternatively spliced form of Hrd1 mRNA either. In addition to the canonical 617 amino acid, 68 kDa "long" isoform b, there is a "short" isoform a, lacking a singular codon encoding an alanine at position 413, and the 579 amino acid, 63 kDa KIAA1810 clone, which lacks two full exons (Kikkert *et al.*, 2004). Again, it could be the result of an unreported splicing event, specific to astrocytes, glioblastoma cells, or both, but no known variants corresponds to the band.

Lastly, as a final attempt to identify the ¥-band and, additionally, to confirm normal trafficking of both Hrd1 and PrP in CR7 cells, we looked at glycosylation patterns of both proteins. Two methods were utilized, firstly, tunicamycin, in addition to being a potent inducer of ER stress, allows determination of immature glycosylation. It acts by inhibiting the enzyme N-acetylglucosamine, thereby blocking the formation of N-glycosidic linkages in newly synthesized proteins (Varki, 2009). Proteins were also digested with PNGase F, an amidase capable of cleaving high mannose, hybrid and complex, N-linked sugars from the asparagine residues, indicative of proteins having traversed the secretory pathway (Varki, 2009). Interestingly, neither method effected Hrd1 nor the ¥-band. Generally speaking, if the two bands represent glycosylation states of the same protein, inhibiting addition of, or cleaving N-glycans from the proteins would result in the unification of the two bands, such as that seen with PrP (discussed below), which we did not observe for Hrd1. This indicates that, despite putative glycosylation sites within the sequence, Hrd1 is not glycosylated *in vivo*, as previously reported (Kikkert *et al.*, 2004). This is consistent with the fact that only two-thirds of proteins containing the consensus sequence Asn-X-Ser/Thr are actually glycosylated (Varki, 2009). In addition to a lack of immature glycosylation in the ER, it is quite clear that Hrd1 is not transported to the

distal Golgi for carbohydrate remodeling, rather it is retained in the ER as a non-glycosylated protein.

We also showed that PrP exists in a diverse glycosylated state and from this, inferred the protein is trafficked normally within our system (**Refer to Figure 5B & D).** The majority of PrP consists of mature glycosylated PrP, indicating it is fully processed, cell surface protein having passed through both the ER and the Golgi, as would be expected. Some immature PrP was detected, indicative of the addition of core, N-linked glycans in the ER, but with a lack of modification in the Golgi. This would make sense in a cell type with large amounts of PrP constantly being trafficked to the cell surface. Under normal physiological conditions, we found very little unglycosylated, full-length PrP in CR7 cells. This was interesting given that multiple groups report that unglycosylated PrP is more prone to aggregation and conversion to PrP^{Sc} (Ma *et al.*, 2001, Yedidia *et al.*, 2001). In addition, Lehmann and Harris claim that treatment with tunicamycin, as in the current work, or mutation of the glycosylation sites leads to a detergent insoluble, aggregated and PK^{Res} form of PrP (Lehmann *et al.*, 1997). However, as discussed previously, this phenomenon was observed under conditions of overexpression and was likely an artifact of the exogenously introduced protein. Indeed, while not directly tested, we did not observe any aggregation following treatment with tunicamycin or following deglycosylation with PNGase F, where PrP was found to migrate at 25 KDa, representing full-length, unglycosylated PrP. Our results are supported by work showing a lack of aggregation and associated toxicity of endogenous mouse PrP in N2a cells treated with tunicamycin (Taraboulos *et al.*, 1990).

Ultimately the goal of these experiments was to establish a system in which to study the generation of CyPrP in the absence of exogenously introduced protein. Normal gene regulation

and protein trafficking cannot be assumed in a cancer cell line, and it was important to establish normal trafficking before examining how it becomes disrupted in disease.

We went on to show that PrP is retrotranslocated in CR7 cells, manifesting as either a single 30 kDa band or as a doublet of 30 and 32 kDa bands. At first this was surprising, as proteins are generally deglycosylated prior to proteasomal degradation (Yoshida, 2003). However, proteasomal inhibitor epoxomicin has been shown to block endogenous cytosolically expressed PNGase F (Karaivanova *et al.*, 2000) and glycosylated CyPrP has been observed previously (Karaivanova *et al.*, 2000, Jodoin *et al.*, 2007). In addition, it seems that some mature PrP was detected in BFA-treated cells, which was initially concerning given that this treatment is meant to disaggregate the Golgi, thereby redirecting proteins accumulating in the ER to the ERAD pathway. Lippincott-Schwartz *et al*. (1989) examined this phenomenon in detail and proposed three possible reasons for the occurrence of complex glycosylated proteins in cells treated with BFA. One, proteins do leave the ER and are either modified in the cytosol or the *cis/*medial Golgi, two, newly synthesized Golgi enzymes such as mannosidase II and N-acetylglucosaminyltransferase are also retained within the ER and modify proteins prior to ERAD within the lumen. Lastly, they proposed, and supported with experimental evidence, that, following 30 minutes of treatment, BFA induces a redistribution of Golgi enzymes from the *cis*/medial compartment to the ER, where they are able to modify proteins (Lippincott-Schwartz *et al.*, 1989). This elegantly explains the existence of mature glycosylated PrP in BFA-treated CR7 cells. Newly synthesized PrP with core, N-linked glycans is unable to travel to the Golgi, rather they mature in the ER via enzymes redistributed from the *cis/*medial Golgi prior to ERAD. Epoxomicin inhibits endogenous PNGase F, and CyPrP remains glycosylated. The fact that the

CyPrP detected in the current work was glycosylated, albeit indirectly, supports our claim that it is *bona fide* ERAD-derived PrP originating from the ER, as opposed to SP-PrP.

Examining retrotranslocated CyPrP might seem pointless at first glance, despite its documented anti-Bax activity. If this isoform is normally degraded and only accumulates under artificial conditions, what possible physiological role could it serve? Retrotranslocated CyPrP has, in fact, been observed in the absence of proteasome inhibition in the CNS (Ma *et al.*, 2001), indicating under normal conditions, some PrP escapes degradation to remain in the cytosol. This applies to other ERAD substrates as well, some of which have been shown to evade proteasomal degradation following retrotranslocation (Schmitz *et al.*, 2004). Indeed, many proteins use the ERAD pathway as a mechanism to obtain cytosolic expression as part of their normal function. This is true for both epidermal growth factor and the anti-Bax protein clusterin, which transits to the nucleus following Sec61-mediated retrotranslocation to the cytoplasm (Jones *et al.*, 2002, Liao *et al.*, 2007). Lastly, because only 10% of total PrP is retrotranslocated, and a portion of this is degraded, detection of the small amount of neuroprotective CyPrP, under normal physiological conditions, would prove difficult.

To determine a mechanism for PrP retrotranslocation, we focused on a specific step of the ERAD pathway, namely the E3 ligase responsible for polyubiquitination. There were several reasons for this: one, earlier work showed that human PrP interacted with yeast Hrd1 homologue hrd1p (Apodaca *et al.*, 2006), and, more generally, while there is considerably overlap between substrates with regards to interaction with other ERAD proteins, interaction with the E3 ligase tends to be substrate-specific (Rubinsztein, 2006). This is because it is responsible for substrate recognition/binding and, therefore, comprises the rate-limiting-step within the ERAD pathway (Rubinsztein, 2006). We found that overexpression of human Hrd1 significantly enhanced

retrotranslocation of endogenous PrP while, conversely, silencing of endogenous Hrd1 using siRNA complexes, abrogated this effect (**Figure 6 and 7**). Initially there was concern the overexpressed Hrd1 was aggregating, manifesting as perinuclear "spots" when the eYFP-Hrd1 fusion protein was visualized via microscopy, as opposed to a more diffuse perinuclear expression pattern. Alternatively, the slightly abnormal expression pattern of eYFP-Hrd1 could potentially be a result of disrupted ER integrity. However, little to no cytotoxicity was observed in cells following transfection and based on the consistency of results, we conclude that eYFP-Hrd1 is localizing normally to the ER and is enzymatically active. This assumption does need to be tested empirically, and is discussed further below. In fact, the observed "spots" were likely the result of oligomerization of Hrd1, which has been previously reported due to the ability of molecules to bind one another (Schulze *et al.*, 2005)

Generally speaking, studies of this nature do not separate cytosolic from membrane proteins. The majority use overexpressed model ERAD substrates, often disease causing mutants not normally found in the cell, which are targeted exclusively for proteasomal degradation. This allows the utilization of a much simpler pulse-chase paradigm to examine the change in substrate level, in whole cell lysates with and without proteasome inhibition, as well as during overexpression and/or silencing of the requisite E3 ligase (Omura *et al.*, 2006, Cattaneo *et al.*, 2008, Ballar *et al.*, 2010, Kaneko *et al.*, 2010, Burr *et al.*, 2011). The result is an observed enhancement of substrate degradation when the ERAD component of interest is overexpressed and stabilization, when silenced, all in the absence of proteasome inhibition. This is very much in agreement with our own results. Under conditions of Hrd1 overexpression combined with proteasome inhibition, we see an increase in CyPrP, which would normally be degraded by the proteasome. Whereas when Hrd1 is silenced, we see an absence of CyPrP, as it is retained within

the ER, unable to retrotranslocate to the cytosol. Given that the E3 ligase represents the rate limiting step in ERAD (Rubinsztein, 2006) and taking into consideration that treatment with BFA is preventing trafficking to the cell surface and redirecting PrP towards ERAD-mediated degradation, more Hrd1 would translate to increased binding and retrotranslocation of PrP into the cytosol. As mentioned above, many studies use exogenously introduced ERAD substrates. A danger of this method is that increased degradation is not a result of experimental manipulation (i.e. overexpression of an E3), rather is the result of the stringent cellular quality control system monitoring, and attempting to alleviate, the rapid synthesis of excess mutant protein. We managed to elegantly avoid this problem by only manipulating the ERAD components of interest as opposed to the substrate. However, while the substrate of interest was endogenous, it is likely the use of BFA to enhance retrotranslocation, also resulted in the unintended ER-stress-mediated upregulation of both PrP and Hrd1 through ERSE and UPRE.

While at the time of thesis preparation, direct interaction between Hrd1 and PrP was not yet demonstrated (See Future Directions below), these experiments provide convincing gain-of-function and loss-of-function evidence for the involvement of Hrd1 in PrP retrotranslocation. It is important to acknowledge the possibility of the involvement of another E3 ligase in the degradation of PrP. In fact, substantial interplay has been demonstrated between E3 ligases within the ER. For example, the degradation of mutant cystic fibrosis conductance regulator (CFTRΔF508) is regulated by E3 ligase gp78. However, Hrd1 is intimately involved in that it, in turn, mediates the ERAD of gp78, and the two proteins are often found in complex (Ballar *et al.*, 2010). It can often be difficult to tease apart such an elegant system and examination of the potential contribution of other E3 ligases was, unfortunately, beyond the scope of the current work. Nevertheless, overexpression and silencing experiments provide the first evidence for the

involvement of human Hrd1 in the retrotranslocation of endogenous PrP within the CNS, even if the mechanism has not yet been precisely defined.

Combining our own data with previously published results, it is hypothesized the retrotranslocation of PrP occurs as follows (**partial model depicted in Figure 4**). Lectins OS9 and XTP3B recognize PrP designated for the ERAD pathway, likely through binding to trimmed mannose residues, and transport it to the retrotranslocation complex at the ER membrane (Christianson *et al.*, 2008). This high molecular mass complex is composed of Sel1L, Hrd1, Herp, Derlin 1, Vimp and p97. Hrd1 is stabilized in the ER membrane by Sel1L (Iida *et al.*, 2011), and binds to PrP, most likely, through its proline rich region, as has been demonstrated for other substrates (Omura *et al.*, 2008a). The putative retrotranslocation channel Derlin1, interacts closely with Hrd1 and likely allows passage of a portion of PrP into the cytosol where it can interact with the enzymatically active RING-finger domain of Hrd1 (Schulze *et al.*, 2005). Upon binding and partial translocation to the cytosol, PrP is polyubiquitinated in a RING-finger dependent manner by Hrd1, in cooperation with its corresponding E2 UBC7 (Kikkert *et al.*, 2004). Ubiquitin-like-domain containing Herp, binds to all retrotranslocation components and is thought to act as an initiating factor, as it is rapidly degraded post-binding (Kokame *et al.*, 2000). Meanwhile Vimp, recruits p97, while acting as a scaffold, connecting the AAA ATPase to the Hrd1-Derlin1 complex, although p97 has been shown to interact with both Hrd1 and Derlin1 directly (Schulze *et al.*, 2005, Ye *et al.*, 2005). p97 recognizes the post-ubiquitinated PrP , extracts it from the Derlin1 retrotranslocon in an ATP-depended manner and directs it to the proteasome for degradation (Ye *et al.*, 2005, Ballar *et al.*, 2006). It is important to note that the majority of this mechanism is merely hypothesized. However, because the E3 ligase determines the specificity of the pathway, and because each E3 is part of a well-defined retrotranslocation

complex, once the requisite ligase is identified, the remainder of the mechanism can be inferred with a reasonable degree of accuracy.

Elucidating the mechanism for PrP retrotranslocation is not simply important in the context of understanding the trafficking of cellular PrP. This mechanism also appears to be disrupted in familial prion disease and, therefore, represents a potential novel therapeutic target. Our group recently showed that a large proportion of PrP mutants associated with CJD, GSS and FFI, lost anti-Bax capability, and this loss was highly correlated with an inability to produce CyPrP (Jodoin *et al.*, 2007, Jodoin *et al.*, 2009). Expression of a cytosolically expressed PrP construct, lacking the ER-directing signal peptide, rescued Bax protection but full-length WT PrP could not. This indicates that PrP mutants are able to block the retrotranslocation of both mutant and WT PrP, thereby halting the generation of neuroprotective CyPrP. Authors insightfully noted this was consistent with the dominant-negative nature of familial prion disease (Jodoin *et al.*, 2007). An elegant mechanism for this phenomenon would involve an increased binding affinity of familial PrP mutants to Hrd1, thus blocking, or drastically impairing, the retrotranslocation of both mutant and WT PrP. We set out to test this hypothesis using the CR7 system and found that both the $M232R^{129V}$ and $V210I^{129V}$ familial PrP mutants caused a substantial reduction in the ability of CR7 cells to produce CyPrP and, simultaneously, blocked the retrotranslocation of Hrd1-mediated ERAD substrate TTR^{D18G} (**Figure 8**). The $M232R^{129V}$ mutation is located within the GPI anchor signal peptide, while the $V210I^{129V}$ mutation is found within α-helix III (Jodoin *et al.*, 2007). Loss of CyPrP was expected to coincide with $V210I^{129V}$ transfection as the α-helix III is critical for the anti-Bax function of PrP (Laroche-Pierre *et al.*, 2009), however, the GPI signal peptide is cleaved early on during post-translational modification in the ER lumen, and therefore it would be expected that the mutation would be removed and

M232R^{129V} would be trafficked normally. Interestingly, M232R^{129V} transfection resulted in a 50% reduction in the ability to generate CyPrP. This indicates that potential misfolding and subsequent redirection towards ERAD is a very early event, occurring before PrP is fully processed in the ER. It is important to note that the 3F4 antibody, detects total PrP, therefore, both endogenous PrP^{C} and exogenously introduced PrP mutants. While over-expression of PrP is demonstrated by western blotting in Figure 8D, it is not explicitly clear that the observed decrease in both CyPrP and cytosolic TTR^{D18G}-GFP is due to mutant PrP. It is possible that PrP mutants are able to inhibit retrotranslocation of proteins by competition. In preparation for publication experiments are being repeated with WT PrP used as a control, thereby determining if it is specifically mutant PrP blocking retrotranslocation versus transfection with PrP in general. In addition, experiments are currently underway to determine if PrP familial mutants have an increased binding affinity for Hrd1, as compared to WT PrP, however, they were not completed in time for the submission of the current work.

If mutant PrP binds to Hrd1 with an increased affinity, it would provide an interesting explanation for prion disease pathology. Hrd1 has multiple substrates, likely far more then have been currently identified, and high affinity binding of PrP mutants to the E3 ligase would block the retrotranslocation of more then just PrP, resulting in an accumulation of proteins within the ER, ER-stress and induction of the UPR. In a thought-provoking side note, ER stress resulting from mutant binding would result in an upregulation of PrP, as demonstrated above, forming an inescapable vicious cycle. To determine if this is indeed taking place, we chose to work with TTR^{D18G}, shown to be a model ERAD substrate whose retrotranslocation is Hrd1-dependant (Christianson *et al.*, 2011). The working hypothesis states that if mutant PrP is blocking the Hrd1 retrotranslocation machinery, then it should affect the ERAD of TTR^{D18G} in a manner similar to

that observed with WT PrP. The construct was created to generate a TTR^{D18G}-GFP fusion protein, allowing us to demonstrate retrotranslocation of this substrate using both fluorescent microscopy and western blot analysis. Fascinatingly, co-transfection with both the TTR^{D18G}-GFP and familial PrP mutants $M232R^{129V}$ and $V210I^{129V}$, resulted in a marked decrease in TTR^{D18G}-GFP expression in the cytosol, with a simultaneous reduction in CyPrP generation. This indicates that the effect of familial PrP mutants in the cell is not specific to WT PrP, in fact, it disrupts the ERAD of other, perhaps all, Hrd1-mediated substrates (**Figure 8**). While only partially demonstrated, this likely indicates that PrP mutants are blocking Hrd1-mediated retrotranslocation, resulting in the buildup of ERAD-substrates in the ER, induction of the UPR and subsequent ER-stress. If the current hypothesis holds true, it could indicate that neuronal apoptosis observed in familial prion disease is due to, at least in part, ER stress (Hetz *et al.*, 2003, Hetz *et al.*, 2005, Kristiansen *et al.*, 2005). Neuronal health would be additionally compromised by a concomitant inability to generate protective CyPrP, which, combined with PrP mutant-mediated ER-stress, would render neurons extremely susceptible to apoptosis. Take all of the above and place it within the context of the aging brain, already possessing an inability to effectively clear aberrant or misfolded proteins, and it becomes clear how a *PRNP* mutation can trigger such a rapidly progressing and devastating pathology, late in life. The proposed mechanism is not without precedent. Indeed, superoxide dismutase mutants have been shown to bind to Derlin1, blocking ERAD of both themselves, and other proteins degraded via this pathway, thereby inducing ER stress (Nishitoh *et al.*, 2008).

The last remaining question is how the ERAD dysfunction seen in familial prion disease could be targeted pharmacologically as part of a therapeutic approach. Global proteasome inhibition has been suggested and could, theoretically, stabilize the accumulation of

neuroprotective CyPrP, however, given that prion mutants block the generation of CyPrP in the first place, this would not be a particularly effective strategy in this case (Cohen *et al.*, 2003). Overexpression of ER chaperones has been shown to suppress neuronal apoptosis in PrP-transfected cells (Hetz *et al.*, 2005), implying the system is sensitive to potential modulation as a therapeutic strategy. Numerous drugs have been developed to target aspects of the ERAD pathway (Reviewed in Kim *et al.,* 2008), however, design and testing are still in infancy. Given that multiple parallel pathways exist and there is considerable interplay between ERAD components, modulating the UPR or ERAD itself would likely result in a host of acute and chronic side effects rendering the therapy useless, not to mention difficult to develop initially (Kim *et al.*, 2008). Small molecule chaperones could possible improve proper folding of mutant PrP within the ER (Cohen *et al.*, 2003), thus limiting the exposure of domains or residues responsible for increasing binding affinity to Hrd1, however it is critical to determine precisely which domains of the mutated PrP protein are responsible for the hypothesized heightened binding affinity to Hrd1, before effective therapy can be developed to counteract it.

CONCLUSION

In the current study we show that both PrP and E3 ubiquitin ligase Hrd1 are expressed, regulated by ER stress and normally trafficked within CR7 human glioblastoma cells. We show that PrP is normally retrotranslocated to generate glycosylated CyPrP and the ERAD of PrP is enhanced with Hrd1 overexpression and, conversely, blocked by siRNA silencing of endogenous Hrd1. We go on to show that familial PrP mutants $M232R^{129V}$ and $V210I^{129V}$ prevent the generation of CyPrP in CR7 cells. Lastly we demonstrated that model Hrd1-mediated ERAD substrate TTR^{D18G} is also retrotranslocated in our system, a process similarly disrupted by familial PrP mutants. These findings suggest that the retrotranslocation of PrP is mediated by E3

ubiquitin ligase Hrd1 in human CNS cells. Furthermore, preliminary evidence suggests that familial prion disease pathology could be due to both the loss of neuroprotective CyPrP and an increased retention of proteins within the ER due to ERAD dysfunction, leading to ER stress and subsequent neuronal apoptosis.

The role of PrP in normal cellular biology remains a subject of intense investigation and debate. However, the simple fact that PrP is highly evolutionarily conserved suggests it possesses an important, if not currently fully understood, function. This is particularly true within the CNS, where it is highly expressed. Converging evidence seems to pinpoint a role in neuronal survival, with our own group identifying an important anti-Bax function to CyPrP under native conditions. The current project went on to elucidate the mechanism by which this important PrP isoform is generated and provide preliminary evidence for how it becomes disrupted in familial prion disease.

Uncovering the mechanism responsible for CyPrP generation and subsequently, how this becomes disrupted represents a novel therapeutic target in treating familial prion disease. Irreversible or high affinity binding of PrP mutants to the Hrd1 complex block not only the retrotranslocation of mutant and WT PrP, but also lead to the accumulation of other Hrd1 substrates, such as TTR^{D18G}, within the ER lumen. This leads to an interesting and elegant gain-of-function/ loss of function theory to explain familial PrP pathology. Accumulated proteins due to PrP mutant-mediated dysfunction of ERAD leads to ER-stress and eventual neuronal apoptosis, while an inability to produce neuroprotective CyPrP would concomitantly compromise the cells ability to prevent Bax-mediated cell death.

Precisely how both WT and mutant PrP are interacting with Hrd1 remains to be addressed and further elucidating which domains and/or residues of the mutated PrP protein

mediate blockade of Hrd1 is critical for developing a comprehensive understanding of familial prion disease pathology and before effective therapy can be developed to target specific ERAD components.

FUTURE DIRECTIONS

Despite providing convincing evidence for the role of Hrd1 in modulating PrP retrotranslocation in human CNS cells, a number of issues remain to be addressed both to complete the existing story and prepare current data for publication. Firstly, it is important to confirm that the loss of CyPrP seen with siRNA knockdown of *SYVN1* is not due to off target effects. To determine this, fresh siRNA complexes targeting different regions of *SYVN1* mRNA have been ordered and are currently being tested. Next, using a co-immunoprecipitation approach, establish a direct interaction between Hrd1 and both mutant and WT PrP. Surface Plasmon Resonance will be performed to determine the relative strength of WT versus mutant binding to Hrd1 as well as to define the kinetics of these binding events. Lastly, an *in vitro* ubiquitination assay will be performed with both WT and mutant PrP to determine if they are ubiquitinated by Hrd1, repeating this assay using an Hrd1 RING mutant would allow us to confirm that the above ubiquitination was RING-finger dependent, however this is somewhat redundant and is likely beyond the scope of the current work.

REFERENCES

Alfaidy N, Chauvet S, Donadio-Andrei S, Salomon A, Saoudi Y, Richaud P, Aude-Garcia C, Hoffmann P, Andrieux A, Moulis J-M (2013) Prion Protein Expression and Functional Importance in Developmental Angiogenesis: Role in Oxidative Stress and Copper Homeostasis. Antioxidants & Redox Signaling 18:400-411.

Almer G, Hainfellner J, Brücke T, Jellinger K, Kleinert R, Bayer G, Windl O, Kretzschmar H, Hill A, Sidle K (1999) Fatal familial insomnia: a new Austrian family. Brain 122:5-16.

Alper T, Cramp W (1967) Does the agent of scrapie replicate without nucleic acid? Nature 214:764-766.

Amano T, Yamasaki S, Yagishita N, Tsuchimochi K, Shin H, Kawahara K, Aratani S, Fujita H, Zhang L, Ikeda R (2003) Synoviolin/Hrd1, an E3 ubiquitin ligase, as a novel pathogenic factor for arthropathy. Genes & development 17:2436-2449.

Apetri AC, Surewicz K, Surewicz WK (2004) The effect of disease-associated mutations on the folding pathway of human prion protein. Journal of Biological Chemistry 279:18008-18014.

Apodaca J, Kim I, Rao H (2006) Cellular tolerance of prion protein PrP in yeast involves proteolysis and the unfolded protein response. Biochemical and biophysical research communications 347:319-326.

Arantes C, Nomizo R, Lopes MH, Hajj GNM, Lima FRS, Martins VR (2009) Prion protein and its ligand stress inducible protein 1 regulate astrocyte development. Glia 57:1439-1449.

Ashok A, Hegde RS (2008) Retrotranslocation of prion proteins from the endoplasmic reticulum by preventing GPI signal transamidation. Molecular biology of the cell 19:3463-3476.

Baker H, Poulter M, Crow T, Frith C, Lofthouse R, Ridley R, Collinge J (1991) Aminoacid polymorphism in human prion protein and age at death in inherited prion disease. The Lancet 337:1286.

Baker H, Ridley R (1996) What went wrong in BSE? From prion disease to public disaster. Brain research bulletin 40:237-244.

Ballar P, Ors AU, Yang H, Fang S (2010) Differential regulation of CFTRΔF508 degradation by ubiquitin ligases gp78 and Hrd1. The international journal of biochemistry & cell biology 42:167-173.

Ballar P, Shen Y, Yang H, Fang S (2006) The role of a novel p97/valosin-containing protein-interacting motif of gp78 in endoplasmic reticulum-associated degradation. Journal of Biological Chemistry 281:35359-35368.

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

Bays NW, Gardner RG, Seelig LP, Joazeiro CA, Hampton RY (2000) Hrd1p/Der3p is a membrane-anchored ubiquitin ligase required for ER-associated degradation. Nature cell biology 3:24-29.

Bays NW, Gardner RG, Seelig LP, Joazeiro CA, Hampton RY (2001) Hrd1p/Der3p is a membrane-anchored ubiquitin ligase required for ER-associated degradation. Nature cell biology 3:24-29.

Bebök Z, Mazzochi C, King SA, Hong JS, Sorscher EJ (1998) The mechanism underlying cystic fibrosis transmembrane conductance regulator transport from the endoplasmic reticulum

to the proteasome includes Sec61β and a cytosolic, deglycosylated intermediary. Journal of Biological Chemistry 273:29873-29878.

Bell J, Ironside J (1993) Neuropathology of spongiform encephalopathies in humans. British medical bulletin 49:738-777.

Bernardi KM, Forster ML, Lencer WI, Tsai B (2008) Derlin-1 facilitates the retro-translocation of cholera toxin. Molecular biology of the cell 19:877-884.

Bernardi KM, Williams JM, Kikkert M, van Voorden S, Wiertz EJ, Ye Y, Tsai B (2010) The E3 ubiquitin ligases Hrd1 and gp78 bind to and promote cholera toxin retro-translocation. Molecular biology of the cell 21:140-151.

Bertuchi FR, Bourgeon DM, Landemberger MC, Martins VR, Cerchiaro G (2012) PrPC displays an essential protective role from oxidative stress in an astrocyte cell line derived from PrPC knockout mice. Biochemical and Biophysical Research Communications 418:27-32.

Biasini E, Fioriti L, Ceglia I, Invernizzi R, Bertoli A, Chiesa R, Forloni G (2004) Proteasome inhibition and aggregation in Parkinson's disease: a comparative study in untransfected and transfected cells. Journal of neurochemistry 88:545-553.

Bockman JM, Kingsbury DT, McKinley MP, Bendheim PE, Prusiner SB (1985) Creutzfeldt–Jakob disease prion proteins in human brains. New England Journal of Medicine 312:73-78.

Borchelt D, Taraboulos A, Prusiner S (1992) Evidence for synthesis of scrapie prion proteins in the endocytic pathway. Journal of Biological Chemistry 267:16188-16199.

Borchelt DR, Scott M, Taraboulos A, Stahl N, Prusiner SB (1990) Scrapie and cellular prion proteins differ in their kinetics of synthesis and topology in cultured cells. The Journal of cell biology 110:743-752.

Bounhar Y, Mann KK, Roucou X, LeBlanc AC (2006) Prion protein prevents Bax-mediated cell death in the absence of other Bcl-2 family members in Saccharomyces cerevisiae. FEMS yeast research 6:1204-1212.

Bounhar Y, Zhang Y, Goodyear CG, LeBlanc AC (2001a) Prion protein protects human neurons against Bax-induced apoptosis. The Journal of Biological Chemistry 276:39145-39149.

Bounhar Y, Zhang Y, Goodyer CG, LeBlanc A (2001b) Prion protein protects human neurons against Bax-mediated apoptosis. Science Signalling 276:39145.

Bratosiewicz-Wąsik J, Smoleń-Dzirba J, Watała C, Rozemuller A, Jansen C, Spliet W, Jansen G, Wąsik T, Liberski P (2012) Association of the PRNP regulatory region polymorphisms with the occurrence of sporadic Creutzfeldt-Jakob disease.

Brown D, Wong B, HAFIZ F, CLIVE C, Haswell S, Jones I (1999a) Normal prion protein has an activity like that of superoxide dismutase. Biochem J 344:1-5.

Brown DR (2002) Molecular advances in understanding inherited prion diseases. Molecular neurobiology 25:287-302.

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

Brown DR, Schmidt B, Kretzschmar HA (1997b) Effects of oxidative stress on prion protein expression in PC12 cells. International journal of developmental neuroscience 15:961-972.

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

Brown DR, Wong BS, Hafiz F, Clive C, Haswell SJ, Jones IM (1999b) Normal prion protein has an activity like that of superoxide dismutase. Biochemical Journal 344:1.

Brown P, Gajdusek DC (1991) Survival of scrapie virus after 3 years' interment. The Lancet 337:269-270.

Budka H (2003) Neuropathology of prion diseases. British medical bulletin 66:121-130.

Büeler H, Fischer M, Lang Y, Bluethmann H, Lipp H-P, DeArmond SJ, Prusiner SB, Aguet M, Weissmann C (1992) Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein.

Bugiani O, Giaccone G, Piccardo P, Morbin M, Tagliavini F, Ghetti B (2000) Neuropathology of Gerstmann-Sträussler-Scheinker disease. Microscopy research and technique 50:10-15.

Burr ML, Cano F, Svobodova S, Boyle LH, Boname JM, Lehner PJ (2011) HRD1 and UBE2J1 target misfolded MHC class I heavy chains for endoplasmic reticulum-associated degradation. Proceedings of the National Academy of Sciences 108:2034-2039.

Capellari S, Strammiello R, Saverioni D, Kretzschmar H, Parchi P (2011) Genetic Creutzfeldt–Jakob disease and fatal familial insomnia: insights into phenotypic variability and disease pathogenesis. Acta neuropathologica 121:21-37.

Carlton WW (1969) Spongiform encephalopathy induced in rats and guinea pigs by cuprizone. Experimental and molecular pathology 10:274-287.

Cattaneo M, Otsu M, Fagioli C, Martino S, Lotti LV, Sitia R, Biunno I (2008) SEL1L and HRD1 are involved in the degradation of unassembled secretory Ig-μ chains. Journal of cellular physiology 215:794-802.

Caughey B, Race RE, Ernst D, Buchmeier MJ, Chesebro B (1989) Prion protein biosynthesis in scrapie-infected and uninfected neuroblastoma cells. Journal of virology 63:175-181.

Chen SG, Teplow DB, Parchi P, Teller JK, Gambetti P, Autilio-Gambetti L (1995) Truncated forms of the human prion protein in normal brain and in prion diseases. Journal of Biological Chemistry 270:19173-19180.

Chen X, Tukachinsky H, Huang CH, Jao C, Chu YR, Tang HY, Mueller B, Schulman S, Rapoport TA, Salic A (2011) Processing and turnover of the Hedgehog protein in the endoplasmic reticulum. The Journal of cell biology 192:825-838.

Chesebro B (2003) Introduction to the transmissible spongiform encephalopathies or prion diseases. British medical bulletin 66:1-20.

Chesebro B, Race R, Wehrly K, Nishio J, Bloom M, Lechner D, Bergstrom S, Robbins K, Mayer L, Keith JM (1985) Identification of scrapie prion protein-specific mRNA in scrapie-infected and uninfected brain.

Chiarini LB, Freitas ARO, Zanata SM, Brentani RR, Martins VR, Linden R (2002) Cellular prion protein transduces neuroprotective signals. The EMBO journal 21:3317-3326.

Christianson JC, Olzmann JA, Shaler TA, Sowa ME, Bennett EJ, Richter CM, Tyler RE, Greenblatt EJ, Harper JW, Kopito RR (2011) Defining human ERAD networks through an integrative mapping strategy. Nature cell biology 14:93-105.

Christianson JC, Shaler TA, Tyler RE, Kopito RR (2008) OS-9 and GRP94 deliver mutant α1-antitrypsin to the Hrd1–SEL1L ubiquitin ligase complex for ERAD. Nature cell biology 10:272-282.

Cohen FE, Kelly JW (2003) Therapeutic approaches to protein-misfolding diseases. Nature 426:905-909.

Coitinho AS, Freitas AR, Lopes MH, Hajj GN, Roesler R, Walz R, Rossato JI, Cammarota M, Izquierdo I, Martins VR (2006) The interaction between prion protein and laminin modulates memory consolidation. European Journal of Neuroscience 24:3255-3264.

Coitinho AS, Lopes MH, Hajj GN, Rossato JI, Freitas AR, Castro CC, Cammarota M, Brentani RR, Izquierdo I, Martins VR (2007) Short-term memory formation and long-term memory consolidation are enhanced by cellular prion association to stress-inducible protein 1. Neurobiology of disease 26:282-290.

Collinge J (1997) Human prion diseases and bovine spongiform encephalopathy (BSE). Human molecular genetics 6:1699-1705.

Collinge J (2001) Prion diseases of humans and animals: their causes and molecular basis. Annual review of neuroscience 24:519-550.

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

Collins S, McLean C, Masters C (2001) Gerstmann-Straussler-Scheinker syndrome, fatal familial insomnia, and kuru: a review of these less common human transmissible spongiform encephalopathies. Journal of clinical neuroscience 8:387-397.

Criado JR, Sánchez-Alavez M, Conti B, Giacchino JL, Wills DN, Henriksen SJ, Race R, Manson JC, Chesebro B, Oldstone M (2005) Mice devoid of prion protein have cognitive deficits that are rescued by reconstitution of PrP in neurons. Neurobiology of disease 19:255-265.

Crozet C, Vézilier J, Delfieu V, Nishimura T, Onodera T, Casanova D, Lehmann S, Béranger F (2006) The truncated 23-230 form of the prion protein localizes to the nuclei of inducible cell lines independently of its nuclear localization signals and is not cytotoxic. Molecular and Cellular Neuroscience 32:315-323.

Cui T, Holme A, Sassoon J, Brown DR (2003) Analysis of doppel protein toxicity. Molecular and Cellular Neuroscience 23:144-155.

de Pedro-Cuesta J, Glatzel M, Almazán J, Stoeck K, Mellina V, Puopolo M, Pocchiari M, Zerr I, Kretszchmar HA, Brandel JP (2006) Human transmissible spongiform encephalopathies in eleven countries: diagnostic pattern across time, 1993–2002. BMC Public Health 6:278.

Deak PM, Wolf DH (2001) Membrane topology and function of Der3/Hrd1p as a ubiquitin-protein ligase (E3) involved in endoplasmic reticulum degradation. Journal of Biological Chemistry 276:10663-10669.

DeArmond SJ, Prusiner SB (2008) Prion protein transgenes and the neuropathology in prion diseases. Brain Pathology 5:77-89.

DeArmond SJ, Sánchez H, Yehiely F, Qiu Y, Ninchak-Casey A, Daggett V, Camerino AP, Cayetano J, Rogers M, Groth D (1997) Selective neuronal targeting in prion disease. Neuron 19:1337-1348.

Déry MA, Jodoin J, Ursini-Siegel J, Aleynikova O, Ferrario C, Hassan S, Basik M, LeBlanc AC (2013) Endoplasmic reticulum stress induces PRNP prion protein gene expression in breast cancer. Breast Cancer Research 15:In Press.

Deshaies RJ, Joazeiro CAP (2009) RING domain E3 ubiquitin ligases. Annual review of biochemistry 78:399-434.

Ding W, Albrecht B, Luo R, Zhang W, Stanley JR, Newbound GC, Lairmore MD (2001) Endoplasmic reticulum and cis-Golgi localization of human T-lymphotropic virus type 1 p12I: association with calreticulin and calnexin. Journal of virology 75:7672-7682.

Dlouhy SR, Hsiao K, Farlow MR, Foroud T, Conneally PM, Johnson P, Prusiner SB, Hodes M, Ghetti B (1992) Linkage of the Indiana kindred of Gerstmann-Sträussler-Scheinker disease to the prion protein gene. Nature genetics 1:64-67.

Dorandeu A, Wingertsmann L, Chrétien F, Delisle MB, Vital C, Parchi P, Montagna P, Lugaresi E, Ironside JW, Budka H (1998) Neuronal apoptosis in fatal familial insomnia. Brain pathology 8:531-537.

Drisaldi B, Stewart RS, Adles C, Stewart LR, Quaglio E, Biasini E, Fioriti L, Chiesa R, Harris DA (2003) Mutant PrP is delayed in its exit from the endoplasmic reticulum, but neither wild-type nor mutant PrP undergoes retrotranslocation prior to proteasomal degradation. Journal of Biological Chemistry 278:21732-21743.

Endo T, Groth D, Prusiner SB, Kobata A (1989) Diversity of oligosaccharide structures linked to asparagines of the scrapie prion protein. Biochemistry 28:8380-8388.

Fioriti L, Dossena S, Stewart LR, Stewart RS, Harris DA, Forloni G, Chiesa R (2005) Cytosolic prion protein (PrP) is not toxic in N2a cells and primary neurons expressing pathogenic PrP mutations. Journal of Biological Chemistry 280:11320-11328.

Gabizon R, Rosenmann H, Meiner Z, Kahana I, Kahana E, Shugart Y, Ott J, Prusiner S (1993) Mutation and polymorphism of the prion protein gene in Libyan Jews with Creutzfeldt-Jakob disease (CJD). American journal of human genetics 53:828.

Gains MJ, LeBlanc AC (2007) Canadian Association of Neurosciences Review: prion protein and prion diseases: the good and the bad. The Canadian Journal of Neurological Sciences 34:126-145.

Gains MJ, Roth KA, LeBlanc AC (2006) Prion protein protects against ethanol-induced Bax-mediated cell death in vivo. Neuroreport 17:903-906.

Gambetti P, Kong Q, Zou W, Parchi P, Chen SG (2003) Sporadic and familial CJD: classification and characterisation. British medical bulletin 66:213-239.

Giannopoulos PN, Robertson C, Jodoin J, Paudel H, Booth SA, LeBlanc AC (2009) Phosphorylation of prion protein at serine 43 induces prion protein conformational change. The Journal of Neuroscience 29:8743-8751.

Gibbs Jr C, Gajdusek DC, Asher DM, Alpers M, Beck E, Daniel PM, Matthews W (1968) Creutzfeldt-Jakob disease (spongiform encephalopathy): transmission to the chimpanzee. Science (New York, NY) 161:388-389.

Goldfarb LG, Brown P, Vrbovska A, Baron H, McCombie WR, Cathala F, Gibbs CJ, Jr., Gajdusek DC (1992a) An insert mutation in the chromosome 20 amyloid precursor gene in a Gerstmann-Straussler-Scheinker family. Journal of the neurological sciences 111:189-194.

Goldfarb LG, Peteren RB, Tabaton M, Brown P, LeBanc AC, Montagna P, Cortelli P, Juliun J, Vital C, Pendelbury WW, Haltia M, Wills PR, Hauw JJ, McKeever PE, Monari L, Schrank B, Swergold GD, Autilio-Gambetti L, Gajdusek DC, Lugaresi E, Gambetti P

(1992b) Fatal familial insomnia and familial Creutzfeld-Jacob disease phenotype determined by a DNA polymorphism. Science 258:806-808.

Goldmann W (1993) PrP gene and its association with spongiform encephalopathies. British medical bulletin 49:839-859.

Graner E, Mercadante AF, Zanata SM, Forlenza OV, Cabral ALB, Veiga SS, Juliano MA, Roesler R, Walz R, Minetti A (2000) Cellular prion protein binds laminin and mediates neuritogenesis. Molecular brain research 76:85-92.

Gray F, Chrétien F, Adle-Biassette H, Dorandeu A, Ereau T, Delisle MB, Kopp N, Ironside JW, Vital C (1999) Neuronal apoptosis in Creutzfeldt-Jakob disease. Journal of neuropathology and experimental neurology 58:321.

Grenier C, Bissonnette C, Volkov L, Roucou X (2006) Molecular morphology and toxicity of cytoplasmic prion protein aggregates in neuronal and non-neuronal cells. Journal of neurochemistry 97:1456-1466.

Gu Y, Verghese S, Mishra RS, Xu X, Shi Y, Singh N (2003) Mutant prion protein-mediated aggregation of normal prion protein in the endoplasmic reticulum: implications for prion propagation and neurotoxicity. Journal of neurochemistry 84:10-22.

Hammarström P, Sekijima Y, White JT, Wiseman RL, Lim A, Costello CE, Altland K, Garzuly F, Budka H, Kelly JW (2003) D18G transthyretin is monomeric, aggregation prone, and not detectable in plasma and cerebrospinal fluid: a prescription for central nervous system amyloidosis? Biochemistry 42:6656-6663.

Harding HP, Novoa I, Zhang Y, Zeng H, Wek R, Schapira M, Ron D (2000) Regulated translation initiation controls stress-induced gene expression in mammalian cells. Molecular cell 6:1099-1108.

Harris DA (2003) Trafficking, turnover and membrane topology of PrP Protein function in prion disease. British medical bulletin 66:71-85.

Harris DA, Huber MT, Van Dijken P, Shyng SL, Chait BT, Wang R (1993) Processing of a cellular prion protein: identification of N-and C-terminal cleavage sites. Biochemistry 32:1009-1016.

Hartmann CA, Martins VR, Lima FRS (2012) High levels of Cellular Prion Protein improve astrocyte development. FEBS letters.

Hegde RS, Mastrianni JA, Scott MR, DeFea KA, Tremblay P, Torchia M, DeArmond SJ, Prusiner SB, Lingappa VR (1998) A transmembrane form of the prion protein in neurodegenerative disease. Science 279:827-834.

Heifetz A, Keenan RW, Elbein AD (1979) Mechanism of action of tunicamycin on the UDP-GlcNAc:dolichyl-phosphate Glc-NAc-1-phosphate transferase. Biochemistry 18:2186-2192.

Heitz S, Gautheron V, Lutz Y, Rodeau JL, Zanjani H, Sugihara I, Bombarde G, Richard F, Fuchs JP, Vogel M (2008) BCL-2 counteracts Doppel-induced apoptosis of prion-protein-deficient Purkinje cells in the Ngsk Prnp0/0 mouse. Developmental neurobiology 68:332-348.

Heitz S, Lutz Y, Rodeau JL, Zanjani H, Gautheron V, Bombarde G, Richard F, Fuchs JP, Vogel M, Mariani J (2007) BAX contributes to Doppel-induced apoptosis of prion-protein–deficient Purkinje cells. Developmental neurobiology 67:670-686.

Heller U, Winklhofer KF, Heske J, Reintjes A, Tatzelt J (2003) Post-translational import of the prion protein into the endoplasmic reticulum interferes with cell viability. Journal of Biological Chemistry 278:36139-36147.

Herms JW, Kretzschmar HA, Titz S, Keller BU (1995) Patch-clamp Analysis of Synaptic Transmission to Cerebellar Purkinje Cells of Prion Protein Knockout Mice. European Journal of Neuroscience 7:2508-2512.

Hetz C, Russelakis-Carneiro M, Maundrell K, Castilla J, Soto C (2003) Caspase-12 and endoplasmic reticulum stress mediate neurotoxicity of pathological prion protein. Science Signaling 22:5435.

Hetz C, Russelakis-Carneiro M, Wälchli S, Carboni S, Vial-Knecht E, Maundrell K, Castilla J, Soto C (2005) The disulfide isomerase Grp58 is a protective factor against prion neurotoxicity. The Journal of neuroscience 25:2793-2802.

Higy M, Junne T, Spiess M (2004) Topogenesis of membrane proteins at the endoplasmic reticulum. Biochemistry 43:12716-12722.

Hoshino S, Inoue K, Yokoyama T, Kobayashi S, Asakura T, Teramoto A (2004) Prions prevent brain damage after experimental brain injury: a preliminary report. In: Brain Edema XII: Proceedings of the 12th International Symposium: Hakone, Japan, November 10-13, 2002, vol. 86, pp 297-299: Springer Verlag.

Hou HL, Shen YX, Zhu HY, Sun H, Yan XB, Fang H, Zhou JN (2006) Alterations of hHrd1 expression are related to hyperphosphorylated tau in the hippocampus in Alzheimer's disease. Journal of neuroscience research 84:1862-1870.

Hu W, Kieseier B, Frohman E, Eagar TN, Rosenberg RN, Hartung HP, Stüve O (2008) Prion proteins: physiological functions and role in neurological disorders. Journal of the neurological sciences 264:1-8.

Iida Y, Fujimori T, Okawa K, Nagata K, Wada I, Hosokawa N, Iida Y, Fujimori T, Okawa K, Nagata K (2011) SEL1L protein critically determines the stability of the HRD1-SEL1L

endoplasmic reticulum-associated degradation (ERAD) complex to optimize the degradation kinetics of ERAD substrates. Journal of Biological Chemistry 286:16929-16939.

Imran M, Mahmood S (2011) An overview of animal prion diseases. Virology journal 8:493.

Jackson PK, Eldridge AG, Freed E, Furstenthal L, Hsu JY, Kaiser BK, Reimann JDR (2000) The lore of the RINGs: substrate recognition and catalysis by ubiquitin ligases. Trends in cell biology 10:429-439.

Jarosch E, Taxis C, Volkwein C, Bordallo J, Finley D, Wolf DH, Sommer T (2002) Protein dislocation from the ER requires polyubiquitination and the AAA-ATPase Cdc48. Nature cell biology 4:134-139.

Jobling MF, Huang X, Stewart LR, Barnham KJ, Curtain C, Volitakis I, Perugini M, White AR, Cherny RA, Masters CL (2001) Copper and zinc binding modulates the aggregation and neurotoxic properties of the prion peptide PrP106-126. Biochemistry 40:8073-8084.

Jodoin J, Laroche-Pierre S, Goodyer CG, LeBlanc AC (2007) Defective retrotranslocation causes loss of anti-Bax function in human familial prion protein mutants. The Journal of neuroscience 27:5081-5091.

Jodoin J, Misiewicz M, Makhijani P, Giannopoulos PN, Hammond J, Goodyer CG, LeBlanc AC (2009) Loss of Anti-Bax Function in Gerstmann-Sträussler-Scheinker Syndrome-Associated Prion Protein Mutants. PloS one 4:e6647.

Jones SE, Jomary C (2002) Clusterin. The international journal of biochemistry & cell biology 34:427-431.

Kaneko M, Ishiguro M, Niinuma Y, Uesugi M, Nomura Y (2002) Human HRD1 protects against ER stress-induced apoptosis through ER-associated degradation. FEBS letters 532:147-152.

Kaneko M, Koike H, Saito R, Kitamura Y, Okuma Y, Nomura Y (2010) Loss of HRD1-mediated protein degradation causes amyloid precursor protein accumulation and amyloid-β generation. The Journal of Neuroscience 30:3924-3932.

Kaneko M, Yasui S, Niinuma Y, Arai K, Omura T, Okuma Y, Nomura Y (2007) A different pathway in the endoplasmic reticulum stress-induced expression of human HRD1 and SEL1 genes. Febs letters 581:5355-5360.

Kang S-W, Rane NS, Kim SJ, Garrison JL, Taunton J, Hegde RS (2006) Substrate-specific translocational attenuation during ER stress defines a pre-emptive quality control pathway. Cell 127:999-1013.

Karaivanova VK, Spiro RG (2000) Effect of proteasome inhibitors on the release into the cytosol of free polymannose oligosaccharides from glycoproteins. Glycobiology 10:727-735.

Kerr JF, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. British journal of cancer 26:239.

Khosravani H, Zhang Y, Tsutsui S, Hameed S, Altier C, Hamid J, Chen L, Villemaire M, Ali Z, Jirik FR (2008) Prion protein attenuates excitotoxicity by inhibiting NMDA receptors. The Journal of cell biology 181:551-565.

Kikkert M, Doolman R, Dai M, Avner R, Hassink G, van Voorden S, Thanedar S, Roitelman J, Chau V, Wiertz E (2004) Human HRD1 is an E3 ubiquitin ligase involved in degradation of proteins from the endoplasmic reticulum. Journal of Biological Chemistry 279:3525-3534.

Kim I, Xu W, Reed JC (2008) Cell death and endoplasmic reticulum stress: disease relevance and therapeutic opportunities. Nature Reviews Drug Discovery 7:1013-1030.

Kim PS, Arvan P (1998) Endocrinopathies in the family of endoplasmic reticulum (ER) storage diseases: disorders of protein trafficking and the role of ER molecular chaperones. Endocrine reviews 19:173.

Kirschbaum WR (1968) Jacob-Creutzfeldt Disease. Amsterdam: Elsevier.

Kny M, Standera S, Hartmann-Petersen R, Kloetzel PM, Seeger M (2011) Herp regulates Hrd1-mediated ubiquitylation in a ubiquitin-like domain-dependent manner. Journal of Biological Chemistry 286:5151-5156.

Kokame K, Agarwala KL, Kato H, Miyata T (2000) Herp, a new ubiquitin-like membrane protein induced by endoplasmic reticulum stress. Journal of Biological Chemistry 275:32846-32853.

Kong Q, Surewicz WK, Petersen RB, Zou W, Chen SG, Gambetti P, Parchi P, Capellari S, Goldfarb L, Montagna P (2004) Inherited prion diseases. COLD SPRING HARBOR MONOGRAPH SERIES 41:673-776.

Kostova Z, Wolf DH (2003) For whom the bell tolls: protein quality control of the endoplasmic reticulum and the ubiquitin–proteasome connection. The EMBO journal 22:2309-2317.

Kovacs G, Kalev O, Budka H (2004) Contribution of neuropathology to the understanding of human prion disease. Folia Neuropathol 42:69-76.

Kramer ML, Kratzin HD, Schmidt B, Römer A, Windl O, Liemann S, Hornemann S, Kretzschmar H (2001) Prion protein binds copper within the physiological concentration range. Journal of Biological Chemistry 276:16711-16719.

Kretzschmar HA (1999) Molecular pathogenesis of prion diseases. European Archives of Psychiatry and Clinical Neuroscience 249:56-63.

Kristiansen M, Messenger MJ, Klöhn P-C, Brandner S, Wadsworth JD, Collinge J, Tabrizi SJ (2005) Disease-related prion protein forms aggresomes in neuronal cells leading to caspase activation and apoptosis. Journal of Biological Chemistry 280:38851-38861.

Kurschner C, Morgan JI (1996) Analysis of interaction sites in homo-and heteromeric complexes containing Bcl-2 family members and the cellular prion protein. Molecular brain research 37:249-258.

Kuwahara C, Takeuchi AM, Nishimura T, Haraguchi K, Kubosaki A, Matsumoto Y, Saeki K, Matsumoto Y, Yokoyama T, Itohara S (1999) Prions prevent neuronal cell-line death. Nature 400:225-226.

Ladogana A, Puopolo M, Croes E, Budka H, Jarius C, Collins S, Klug G, Sutcliffe T, Giulivi A, Alperovitch A (2005) Mortality from Creutzfeldt–Jakob disease and related disorders in Europe, Australia, and Canada. Neurology 64:1586-1591.

Laroche-Pierre S, Jodoin J, LeBlanc AC (2009) Helix 3 is necessary and sufficient for prion protein's anti-Bax function. Journal of neurochemistry 108:1019-1031.

Lawson VA, Collins SJ, Masters CL, Hill AF (2005) Prion protein glycosylation. Journal of neurochemistry 93:793-801.

LeBlanc A (1998) Unravelling the controversy of prion diseases. New York: Academic Press.

Lee K, Tirasophon W, Shen X, Michalak M, Prywes R, Okada T, Yoshida H, Mori K, Kaufman RJ (2002) IRE1-mediated unconventional mRNA splicing and S2P-mediated ATF6 cleavage merge to regulate XBP1 in signaling the unfolded protein response. Genes & development 16:452-466.

Lee KS, Magalhães AC, Zanata SM, Brentani RR, Martins VR, Prado MA (2001) Internalization of mammalian fluorescent cellular prion protein and N-terminal deletion mutants in living cells. Journal of neurochemistry 79:79-87.

Lehmann S, Harris DA (1997) Blockade of glycosylation promotes acquistion of scrapie-like properties by the prion protein in cultured cells. Journal of Biological Chemistry 272:21479-21487.

Liao H-J, Carpenter G (2007) Role of the Sec61 translocon in EGF receptor trafficking to the nucleus and gene expression. Molecular biology of the cell 18:1064-1072.

Liberski PP, Sikorska B, Bratosiewicz-Wasik J, Carleton Gajdusek D, Brown P (2004) Neuronal cell death in transmissible spongiform encephalopathies (prion diseases) revisited: from apoptosis to autophagy. The international journal of biochemistry & cell biology 36:2473-2490.

Lima FR, Arantes CP, Muras AG, Nomizo R, Brentani RR, Martins VR (2007) Cellular prion protein expression in astrocytes modulates neuronal survival and differentiation. Journal of neurochemistry 103:2164-2176.

Lin DT, Jodoin J, Baril M, Goodyer CG, LeBlanc AC (2008) Cytosolic prion protein is the predominant anti-Bax prion protein form: exclusion of transmembrane and secreted prion protein forms in the anti-Bax function. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research 1783:2001-2012.

Lippincott-Schwartz J, Yuan LC, Bonifacino JS, Klausner RD (1989) Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane cycling from Golgi to ER. Cell 56:801.

Lledo PM, Tremblay P, DeArmond SJ, Prusiner SB, Nicoll RA (1996) Mice deficient for prion protein exhibit normal neuronal excitability and synaptic transmission in the hippocampus. Proceedings of the National Academy of Sciences 93:2403-2407.

Lloyd S, Mead S, Collinge J (2011) Genetics of prion disease. Prion Proteins 1-22.

Lopes MH, Hajj GNM, Muras AG, Mancini GL, Castro RMPS, Ribeiro KCB, Brentani RR, Linden R, Martins VR (2005) Interaction of cellular prion and stress-inducible protein 1 promotes neuritogenesis and neuroprotection by distinct signaling pathways. The Journal of neuroscience 25:11330-11339.

Ma J, Lindquist S (1999) De novo generation of a PrPSc-like conformation in living cells. Nature cell biology 1:358-361.

Ma J, Lindquist S (2001) Wild-type PrP and a mutant associated with prion disease are subject to retrograde transport and proteasome degradation. Proceedings of the National Academy of Sciences 98:14955-14960.

Ma J, Lindquist S (2002a) Conversion of PrP to a self-perpetuating PrPSc-like conformation in the cytosol. Science 298:1785-1788.

Ma J, Wollmann R, Lindquist S (2002b) Neurotoxicity and neurodegeneration when PrP accumulates in the cytosol. Science 298:1781-1785.

Määttänen P, Gehring K, Bergeron JJM, Thomas DY (2010) Protein quality control in the ER: the recognition of misfolded proteins. In: Seminars in cell & developmental biology, vol. 21, pp 500-511: Elsevier.

Maglio LE, Martins VR, Izquierdo I, Ramirez OA (2006) Role of cellular prion protein on LTP expression in aged mice. Brain research 1097:11-18.

Mahal SP, Asante EA, Antoniou M, Collinge J (2001) Isolation and functional characterisation of the promoter region of the human prion protein gene. Gene 268:105-114.

Mahfoud R, Garmy N, Maresca M, Yahi N, Puigserver A, Fantini J (2002) Identification of a common sphingolipid-binding domain in Alzheimer, prion, and HIV-1 proteins. Journal of Biological Chemistry 277:11292-11296.

Makrinou E, Collinge J, Antoniou M (2002) Genomic characterization of the human prion protein (PrP) gene locus. Mammalian genome 13:696-703.

Mallucci GR (2009) Prion neurodegeneration: starts and stops at the synapse. Prion 3:195-201.

Masters CL, Gajdusek DC, GIBBS JR CJ (1981) Creutzfeldt-Jakob disease virus isolations from the Gerstmann-Sträussler syndrome with an analysis of the various forms of amyloid plaque deposition in the virus-induced spongiform encephalopathies. Brain 104:559-588.

Masters CL, Harris JO, Gajdusek DC, Gibbs CJ, Bernoulli C, Asher DM (1979) Creutzfeldt-Jakob disease: Patterns of worldwide occurrence and the significance of familial and sporadic clustering. Annals of neurology 5:177-188.

Mayor S, Riezman H (2004) Sorting GPI-anchored proteins. Nature Reviews Molecular Cell Biology 5:110-120.

Mead S (2006) Prion disease genetics. European Journal of Human Genetics 14:273-281.

Medori R, Tritschler HJ, LeBlanc A, Villare F, Manetto V, Chen HY, Xue R, Leal S, Montagna P, Cortelli P (1992) Fatal familial insomnia, a prion disease with a mutation at codon 178 of the prion protein gene. New England Journal of Medicine 326:444-449.

Mehrpour M, Codogno P (2010) Prion protein: From physiology to cancer biology. Cancer letters 290:1-23.

Meusser B, Hirsch C, Jarosch E, Sommer T (2005) ERAD: the long road to destruction. Nature cell biology 7:766-772.

Meyer HH, Wang Y, Warren G (2002) Direct binding of ubiquitin conjugates by the mammalian p97 adaptor complexes, p47 and Ufd1–Npl4. The EMBO journal 21:5645-5652.

Mironov A, Latawiec D, Wille H, Bouzamondo-Bernstein E, Legname G, Williamson RA, Burton D, DeArmond SJ, Prusiner SB, Peters PJ (2003) Cytosolic prion protein in neurons. The Journal of neuroscience 23:7183-7193.

Misiewicz M, Déry M-A, Foveau B, Jodoin J, Ruths D, LeBlanc AC (2013) Identification of a novel Endoplasmic Reticulum Stress Response Element regulated by XBP1. Journal of Biological Chemistry.

Monari L, Chen SG, Brown P, Parchi P, Petersen RB, Mikol J, Gray F, Cortelli P, Montagna P, Ghetti B (1994) Fatal familial insomnia and familial Creutzfeldt-Jakob disease: different prion proteins determined by a DNA polymorphism. Proceedings of the National Academy of Sciences 91:2839-2842.

Monnet C, Gavard J, Mège RM, Sobel A (2004) Clustering of cellular prion protein induces ERK1/2 and stathmin phosphorylation in GT1-7 neuronal cells. FEBS letters 576:114-118.

Moore RC, Lee IY, Silverman GL, Harrison PM, Strome R, Heinrich C, Karunaratne A, Pasternak SH, Chishti MA, Liang Y (1999) Ataxia in prion protein (PrP)-deficient mice is associated with upregulation of the novel PrP-like protein doppel. Journal of molecular biology 292:797-817.

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

Nadav E, Shmueli A, Barr H, Gonen H, Ciechanover A, Reiss Y (2003) A novel mammalian endoplasmic reticulum ubiquitin ligase homologous to the yeast Hrd1. Biochemical and biophysical research communications 303:91-97.

Narang H (1996) The nature of the scrapie agent: the virus theory. In: Proceedings of the Society for Experimental Biology and Medicine Society for Experimental Biology and Medicine (New York, NY), vol. 212, pp 208-224: Royal Society of Medicine.

Narang HK, Asher DM, Gajdusek DC (1988) Evidence that DNA is present in abnormal tubulofilamentous structures found in scrapie. Proceedings of the National Academy of Sciences 85:3575-3579.

Naslavsky N, Stein R, Yanai A, Friedlander G, Taraboulos A (1997) Characterization of detergent-insoluble complexes containing the cellular prion protein and its scrapie isoform. Journal of Biological Chemistry 272:6324-6331.

Nazor KE, Seward T, Telling GC (2007) Motor behavioral and neuropathological deficits in mice deficient for normal prion protein expression. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease 1772:645-653.

Negro A, Meggio F, Bertoli A, Battistutta R, Sorgato MC, Pinna LA (2000) Susceptibility of the prion protein to enzymic phosphorylation. Biochemical and biophysical research communications 271:337-341.

Nicolas O, Gavín R, Braun N, Ureña JM, Fontana X, Soriano E, Aguzzi A, del Río JA (2007) Bcl-2 overexpression delays caspase-3 activation and rescues cerebellar degeneration in prion-deficient mice that overexpress amino-terminally truncated prion. The FASEB Journal 21:3107-3117.

Nishitoh H, Kadowaki H, Nagai A, Maruyama T, Yokota T, Fukutomi H, Noguchi T, Matsuzawa A, Takeda K, Ichijo H (2008) ALS-linked mutant SOD1 induces ER stress- and ASK1-dependent motor neuron death by targeting Derlin-1. Genes & development 22:1451-1464.

Omura T, Asari M, Yamamoto J, Kamiyama N, Oka K, Hoshina C, Maseda C, Awaya T, Tasaki Y, Shiono H (2012) HRD1 levels increased by zonisamide prevented cell death and caspase-3 activation caused by endoplasmic reticulum stress in SH-SY5Y cells. Journal of Molecular Neuroscience 46:527-535.

Omura T, Kaneko M, Okuma Y, Orba Y, Nagashima K, Takahashi R, Fujitani N, Matsumura S, Hata A, Kubota K (2006) A ubiquitin ligase HRD1 promotes the degradation of Pael receptor, a substrate of Parkin. Journal of neurochemistry 99:1456-1469.

Omura T, Kaneko M, Onoguchi M, Koizumi S, Itami M, Ueyama M, Okuma Y, Nomura Y (2008a) Novel functions of ubiquitin ligase HRD1 with transmembrane and proline-rich domains. Journal of pharmacological sciences 106:512-519.

Omura T, Kaneko M, Tabei N, Okuma Y, Nomura Y (2008b) Immunohistochemical localization of a ubiquitin ligase HRD1 in murine brain. Journal of neuroscience research 86:1577-1587.

Ott CM, Lingappa VR (2002) Integral membrane protein biosynthesis: why topology is hard to predict. Journal of cell science 115:2003-2009.

Palmer MS, Dryden AJ, Hughes JT, Collinge J (1991) Homozygous prion protein genotype predisposes to sporadic Creutzfeldt–Jakob disease.

Parchi P, Capellari S, Chin S, Schwarz HB, Schecter NP, Butts JD, Hudkins P, Burns DK, Powers JM, Gambetti P (1999) A subtype of sporadic prion disease mimicking fatal familial insomnia. Neurology 52:1757-1763.

Pastore A, Zagari A (2007) A structural overview of the vertebrate prion proteins. Prion 1:185-197.

Pastrana MA, Sajnani G, Onisko B, Castilla J, Morales R, Soto C, Requena JR (2006) Isolation and characterization of a proteinase K-sensitive PrPSc fraction. Biochemistry 45:15710-15717.

Pauly PC, Harris DA (1998) Copper stimulates endocytosis of the prion protein. Journal of Biological Chemistry 273:33107-33110.

Pickart CM (2001) Mechanisms underlying ubiquitination. Annual review of biochemistry 70:503-533.

Prestori F, Rossi P, Bearzatto B, Lainé J, Necchi D, Diwakar S, Schiffmann SN, Axelrad H, D'Angelo E (2008) Altered neuron excitability and synaptic plasticity in the cerebellar granular layer of juvenile prion protein knock-out mice with impaired motor control. The Journal of neuroscience 28:7091-7103.

Preuss D, Mulholland J, Kaiser CA, Orlean P, Albright C, Rose MD, Robbins PW, Botstein D (1991) Structure of the yeast endoplasmic reticulum: localization of ER proteins using immunofluorescence and immunoelectron microscopy. Yeast 7:891-911.

Prusiner SB (1982) Novel proteinaceous infectious particles cause scrapie. Science (New York, NY) 216:136.

Prusiner SB (1998) Prions. Proceedings of the National Academy of Sciences 95:13363-13383.

Puthalakath H, Strasser A (2002) Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins. Cell death and differentiation 9:505-512.

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

Rane NS, Kang S-W, Chakrabarti O, Feigenbaum L, Hegde RS (2008) Reduced translocation of nascent prion protein during ER stress contributes to neurodegeneration. Developmental cell 15:359-370.

Rane NS, Yonkovich JL, Hegde RS (2004) Protection from cytosolic prion protein toxicity by modulation of protein translocation. The EMBO journal 23:4550-4559.

Rao H, Sastry A (2002) Recognition of specific ubiquitin conjugates is important for the proteolytic functions of the ubiquitin-associated domain proteins Dsk2 and Rad23. Journal of Biological Chemistry 277:11691-11695.

Rapoport TA (2007) Protein translocation across the eukaryotic endoplasmic reticulum and bacterial plasma membranes. Nature 450:663-669.

Ribadeau-Dumas J, Escourolle R (1974) The Creutzfeldt-Jakob syndrome: a neuropathological and electron microscopic study. Neurology Amsterdam: Elsevier 316-329.

Riek R, Wider G, Billeter M, Hornemann S, Glockshuber R, Wüthrich K (1998) Prion protein NMR structure and familial human spongiform encephalopathies. Proceedings of the National Academy of Sciences 95:11667-11672.

Ross DT, Scherf U, Eisen MB, Perou CM, Rees C, Spellman P, Iyer V, Jeffrey SS, Van de Rijn M, Waltham M (2000) Systematic variation in gene expression patterns in human cancer cell lines. Nature genetics 24:227-235.

Roucou X, Gains M, LeBlanc AC (2004) Neuroprotective functions of prion protein. Journal of neuroscience research 75:153-161.

Roucou X, Giannopoulos P, Zhang Y, Jodoin J, Goodyer C, LeBlanc A (2005) Cellular prion protein inhibits proapoptotic Bax conformational change in human neurons and in breast carcinoma MCF-7 cells. Cell Death & Differentiation 12:783-795.

Roucou X, Guo Q, Zhang Y, Goodyer CG, LeBlanc AC (2003) Cytosolic prion protein is not toxic and protects against Bax-mediated cell death in human primary neurons. Journal of Biological Chemistry 278:40877-40881.

Rubinsztein DC (2006) The roles of intracellular protein-degradation pathways in neurodegeneration. Nature 443:780-786.

Safar J, Wille H, Itri V, Groth D, Serban H, Torchia M, Cohen FE, Prusiner SB (1998) Eight prion strains have PrPSc molecules with different conformations. Nature medicine 4:1157-1165.

Sajnani G, Silva CJ, Ramos A, Pastrana MA, Onisko BC, Erickson ML, Antaki EM, Dynin I, Vázquez-Fernández E, Sigurdson CJ (2012) PK-sensitive PrPSc is infectious and shares basic structural features with PK-resistant PrPSc. PLoS pathogens 8:e1002547.

Sakudo A, Lee D-c, Saeki K, Nakamura Y, Inoue K, Matsumoto Y, Itohara S, Onodera T (2003) Impairment of superoxide dismutase activation by N-terminally truncated prion protein (PrP) in PrP-deficient neuronal cell line. Biochemical and biophysical research communications 308:660-667.

Sarasa R, Martínez A, Monleón E, Bolea R, Vargas A, Badiola JJ, Monzón M (2012) Involvement of astrocytes in transmissible spongiform encephalopathies: a confocal microscopy study. Cell and Tissue Research 350:127-134.

Sato BK, Schulz D, Do PH, Hampton RY (2009) Misfolded membrane proteins are specifically recognized by the transmembrane domain of the Hrd1p ubiquitin ligase. Molecular cell 34:212-222.

Schäfer A, Wolf DH (2009) Sec61p is part of the endoplasmic reticulum-associated degradation machinery. The EMBO journal 28:2874-2884.

Schätzl HM, Costa MD, Taylor L, Cohen FE, Prusiner SB (1995) Prion protein gene variation among primates. Journal of molecular biology 245:362-374.

Schmitz A, Herzog V (2004) Endoplasmic reticulum-associated degradation: exceptions to the rule. European journal of cell biology 83:501-509.

Schröder M, Kaufman RJ (2005) ER stress and the unfolded protein response. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 569:29-63.

Schulze A, Standera S, Buerger E, Kikkert M, van Voorden S, Wiertz E, Koning F, Kloetzel P-M, Seeger M (2005) The ubiquitin-domain protein HERP forms a complex with components of the endoplasmic reticulum associated degradation pathway. Journal of molecular biology 354:1021-1027.

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

Shmueli A, Tsai YC, Yang M, Braun MA, Weissman AM (2009) Targeting of gp78 for ubiquitin-mediated proteasomal degradation by Hrd1: cross-talk between E3s in the

endoplasmic reticulum. Biochemical and biophysical research communications 390:758-762.

Shyng S-L, Heuser JE, Harris DA (1994) A glycolipid-anchored prion protein is endocytosed via clathrin-coated pits. The Journal of cell biology 125:1239-1250.

Silburn P, Cervenakova L, Varghese P, Tannenberg A, Brown P, Boyle R (1996) Fatal familial insomnia A seventh family. Neurology 47:1326-1328.

Spielhaupter C, Schätzl HM (2001) PrPC directly interacts with proteins involved in signaling pathways. Journal of Biological Chemistry 276:44604-44612.

Stahl N, Baldwin M, Hecker R, Pan KM, Burlingame A, Prusiner S (1992) Glycosylinositol phospholipid anchors of the scrapie and cellular prion proteins contain sialic acid. Biochemistry 31:5043-5053.

Stahl N, Borchelt DR, Hsiao K, Prusiner SB (1987) Scrapie prion protein contains a phosphatidylinositol glycolipid. Cell 51:229-240.

Stahl N, Prusiner SB (1991) Prions and prion proteins. The FASEB journal 5:2799-2807.

Steele AD, Lindquist S, Aguzzi A (2007) The prion protein knockout mouse: a phenotype under challenge. Prion 1:83-93.

Stewart RS, Drisaldi B, Harris DA (2001) A transmembrane form of the prion protein contains an uncleaved signal peptide and is retained in the endoplasmic reticululm. Molecular biology of the cell 12:881-889.

Sunyach C, Jen A, Deng J, Fitzgerald KT, Frobert Y, Grassi J, McCaffrey MW, Morris R (2003) The mechanism of internalization of glycosylphosphatidylinositol-anchored prion protein. The EMBO journal 22:3591-3601.

Tanaka M, Chien P, Naber N, Cooke R, Weissman JS (2004) Conformational variations in an infectious protein determine prion strain differences. Nature 428:323-328.

Taraboulos A, Rogers M, Borchelt DR, McKinley MP, Scott M, Serban D, Prusiner SB (1990) Acquisition of protease resistance by prion proteins in scrapie-infected cells does not require asparagine-linked glycosylation. Proceedings of the National Academy of Sciences 87:8262-8266.

Tateishi J, Sato Y, Koga M, Doi H, Ohta M (1980) Experimental transmission of human subacute spongiform encephalopathy to small rodents. Acta neuropathologica 51:127-134.

Tobler I, Deboer T, Fischer M (1997) Sleep and sleep regulation in normal and prion protein-deficient mice. The Journal of neuroscience 17:1869-1879.

Tobler I, Gaus S, Deboer T, Achermann P, Fischer M, Rülicke T, Moser M, Oesch B, McBride P, Manson J (1996) Altered circadian activity rhythms and sleep in mice devoid of prion protein.

Tsai YC, Weissman AM (2011) Ubiquitylation in ERAD: reversing to go forward? PLoS biology 9:e1001038.

Varki A, Cummings, RD, Esko, JD, Freeze, HH, Stanley, P, Bertozzi, CR, Hart, GW and Etzler, ME (2009) Essentials of Glycobiology, 2nd Edition. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Vassallo N, Herms J (2003) Cellular prion protein function in copper homeostasis and redox signalling at the synapse. Journal of neurochemistry 86:538-544.

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

Vembar SS, Brodsky JL (2008) One step at a time: endoplasmic reticulum-associated degradation. Nature Reviews Molecular Cell Biology 9:944-957.

Venneti S (2010) Prion diseases. Clinics in laboratory medicine 30:293.

Wadsworth JDF, Hill AF, Beck JA, Collinge J (2003) Molecular and clinical classification of human prion disease. British medical bulletin 66:241-254.

Walmsley AR, Zeng F, Hooper NM (2003) The N-terminal region of the prion protein ectodomain contains a lipid raft targeting determinant. Journal of Biological Chemistry 278:37241-37248.

Walz R, Amaral OB, Rockenbach IC, Roesler R, Izquierdo I, Cavalheiro EA, Martins VR, Brentani RR (2005) Increased sensitivity to seizures in mice lacking cellular prion protein. Epilepsia 40:1679-1682.

Wang X, Bowers SL, Wang F, Pu X-a, Nelson RJ, Ma J (2009) Cytoplasmic prion protein induces forebrain neurotoxicity. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease 1792:555-563.

Wang X, Wang F, Arterburn L, Wollmann R, Ma J (2006) The interaction between cytoplasmic prion protein and the hydrophobic lipid core of membrane correlates with neurotoxicity. Journal of Biological Chemistry 281:13559-13565.

Wang X, Wang F, Sy M-S, Ma J (2005a) Calpain and other cytosolic proteases can contribute to the degradation of retro-translocated prion protein in the cytosol. Journal of Biological Chemistry 280:317-325.

Wang X, Wang F, Sy MS, Ma J (2005b) Calpain and other cytosolic proteases can contribute to the degradation of retro-translocated prion protein in the cytosol. Journal of Biological Chemistry 280:317-325.

Watts JC, Drisaldi B, Ng V, Yang J, Strome B, Horne P, Sy M-S, Yoong L, Young R, Mastrangelo P (2007a) The CNS glycoprotein Shadoo has PrPC-like protective properties and displays reduced levels in prion infections. The EMBO journal 26:4038-4050.

Watts JC, Westaway D (2007b) The prion protein family: diversity, rivalry, and dysfunction. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease 1772:654-672.

Wegener A, Jones L (1984) Phosphorylation-induced mobility shift in phospholamban in sodium dodecyl sulfate-polyacrylamide gels. Evidence for a protein structure consisting of multiple identical phosphorylatable subunits. Journal of Biological Chemistry 259:1834-1841.

Weissmann C, Flechsig E (2003) PrP knock-out and PrP transgenic mice in prion research. British medical bulletin 66:43-60.

White MD, Farmer M, Mirabile I, Brandner S, Collinge J, Mallucci GR (2008) Single treatment with RNAi against prion protein rescues early neuronal dysfunction and prolongs survival in mice with prion disease. Proceedings of the National Academy of Sciences 105:10238-10243.

Whittal RM, Ball HL, Cohen FE, Burlingame AL, Prusiner SB, Baldwin MA (2000) Copper binding to octarepeat peptides of the prion protein monitored by mass spectrometry. Protein Science 9:332-343.

Wolter KG, Hsu Y-T, Smith CL, Nechushtan A, Xi X-G, Youle RJ (1997) Movement of Bax from the cytosol to mitochondria during apoptosis. The Journal of cell biology 139:1281-1292.

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

Yabal M, Brambillasca S, Soffientini P, Pedrazzini E, Borgese N, Makarow M (2003) Translocation of the C terminus of a tail-anchored protein across the endoplasmic reticulum membrane in yeast mutants defective in signal peptide-driven translocation. Journal of Biological Chemistry 278:3489-3496.

Yang H, Zhong X, Ballar P, Luo S, Shen Y, Rubinsztein DC, Monteiro MJ, Fang S (2007) Ubiquitin ligase Hrd1 enhances the degradation and suppresses the toxicity of polyglutamine-expanded huntingtin. Experimental cell research 313:538-550.

Ye Y, Shibata Y, Kikkert M, Van Voorden S, Wiertz E, Rapoport TA (2005) Recruitment of the p97 ATPase and ubiquitin ligases to the site of retrotranslocation at the endoplasmic reticulum membrane. Proceedings of the National Academy of Sciences of the United States of America 102:14132-14138.

Ye Y, Shibata Y, Yun C, Ron D, Rapoport TA (2004) A membrane protein complex mediates retro-translocation from the ER lumen into the cytosol. Nature 429:841-847.

Yedidia Y, Horonchik L, Tzaban S, Yanai A, Taraboulos A (2001) Proteasomes and ubiquitin are involved in the turnover of the wild-type prion protein. The EMBO journal 20:5383-5391.

Yin S YS, Li C, Wong P, Chang B, Xiao F, Kang SC, Yan H, Xiao G, Grassi J, Po T, Sy MS (2006) Prion proteins with insertional mutations have altered N-terminal conformation, increased ligand-binding activity and are more susceptible to oxidative attack. Journal of Biochemistry 281:10698-10705.

Yin X-M, Oltvai ZN, Korsmeyer SJ (1994) BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax. Nature 369:321-323.

Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K (2001) XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. Cell 107:881-892.

Yoshida Y (2003) A novel role for N-glycans in the ERAD system. Journal of biochemistry 134:183-190.

Yu H, Kaung G, Kobayashi S, Kopito RR (1997) Cytosolic degradation of T-cell receptor α chains by the proteasome. Journal of Biological Chemistry 272:20800-20804.

Zanata SM, Lopes MH, Mercadante AF, Hajj GNM, Chiarini LB, Nomizo R, Freitas ARO, Cabral ALB, Lee KS, Juliano MA (2002) Stress-inducible protein 1 is a cell surface ligand for cellular prion that triggers neuroprotection. The EMBO journal 21:3307-3316.

Zanusso G, Petersen RB, Jin T, Jing Y, Kanoush R, Ferrari S, Gambetti P, Singh N (1999) Proteasomal degradation and N-terminal protease resistance of the codon 145 mutant prion protein. Journal of Biological Chemistry 274:23396-23404.

Zimmermann K, Turecek, PL, Schwarz, HP (1999) Genotyping of the prion protein gene at codon 129. Acta neuropathologica 97:355-358.

Zinszner H, Kuroda M, Wang X, Batchvarova N, Lightfoot RT, Remotti H, Stevens JL, Ron D (1998) CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. Genes & development 12:982-995.