Inhibition of the FKBP Family of Peptidyl Prolyl Isomerases Induces Abortive Translocation and Degradation of the Cellular Prion Protein

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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2015

Abstract

Prion disorders are a class of neurodegenerative diseases that feature a structural change of the prion protein from its cellular form (PrP\textsuperscript{C}) into its scrapie form (PrP\textsuperscript{Sc}). As these disorders are currently incurable, there is a crucial need for novel therapeutic agents. Here, the FDA-approved immunosuppressive drug FK506 was shown to cause an attenuation in the endoplasmic reticulum (ER) translocation of PrP\textsuperscript{C} by exacerbating an intrinsic inefficiency of PrP’s ER-targeting signal sequence, effectively causing the proteasomal degradation of PrP\textsuperscript{C}. Furthermore, the depletion of FKBP10 also caused the degradation of PrP\textsuperscript{C} but at a later stage following translocation into the ER. Additionally, novel FK506 analogues with reduced immunosuppressive properties were shown to be as efficacious as FK506 in downregulating PrP\textsuperscript{C}. Finally, both FK506 treatment and FKBP10 depletion were shown to reduce the levels of PrP\textsuperscript{Sc} in chronically infected cell models. These findings offer a new insight into the development of treatments against prion disorders.
Acknowledgments

The completion of the present thesis would not have been possible without the help and support of a number of people. First and foremost, I would like to thank my supervisor, Dr David Williams, for his constant guidance and expertise that allowed me to successfully complete my degree, as well as my committee members, Dr John Glover and Dr Gerold Schmitt-Ulms, for their invaluable advice and suggestions over the course of this project.

I am especially grateful to my fellow lab members, namely Dr Pawel Stocki, Dr Ronnie Lum, Daniel Chapman, Seo Jung Hong, and Samar Ahmad, for their support and company, through successful and unsuccessful experiments. I am also obliged to several faculty members of the Department of Biochemistry for their constructive criticism of my experimental work, most notably Dr Alex Palazzo, Dr Trevor Moraes, and especially Dr Angus McQuibban for his incredible… everything.

Last but not least, I am forever indebted to my parents for their invaluable support throughout the years.
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List of Abbreviations

aa    Amino Acid
AD    Alzheimer’s Disease
ADAM  A-Disintegrin-And-Metalloproteinase
bp    Base Pairs
BSE   Bovine Spongiform Encephalopathy
CJD   (classical) Creutzfeldt–Jakob Disease
      fCJD – familial Creutzfeldt–Jakob Disease
      vCJD – variant Creutzfeldt–Jakob Disease
CNS   Central Nervous System
CsA   Cyclosporine A
EEG   Electroencephalogram
ER    Endoplasmic Reticulum
ERAD  Endoplasmic Reticulum-Associated Degradation
FFI   Fatal Familial Insomnia
FKBP  FK506-Binding Protein
GPI   Glycophosphatidylinositol
GSS   Gerstmann–Sträussler–Scheinker syndrome
HD    Huntington’s Disease
MHC   Major Histocompatibility Complex
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>PBS</td>
<td>Phosphate Buffer Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s Disease</td>
</tr>
<tr>
<td>PK&lt;sup&gt;res&lt;/sup&gt;</td>
<td>Proteinase K resistant</td>
</tr>
<tr>
<td>PK&lt;sup&gt;sen&lt;/sup&gt;</td>
<td>Proteinase K sensitive</td>
</tr>
<tr>
<td>PNGase F</td>
<td>Peptidyl N-Glycanase F</td>
</tr>
<tr>
<td>Prnp</td>
<td>Prion Protein Gene</td>
</tr>
<tr>
<td>PPS</td>
<td>Pentosan Polysulphate</td>
</tr>
<tr>
<td>PrP</td>
<td>Prion Protein</td>
</tr>
<tr>
<td>PrP&lt;sup&gt;C&lt;/sup&gt;</td>
<td>Cellular Prion Protein</td>
</tr>
<tr>
<td>PrP&lt;sup&gt;Sc&lt;/sup&gt;</td>
<td>Scrapie Prion Protein</td>
</tr>
<tr>
<td>RNC</td>
<td>Ribosome Nascent Chain Complex</td>
</tr>
<tr>
<td>RML</td>
<td>Rocky Mountain Laboratory</td>
</tr>
<tr>
<td>SP</td>
<td>Signal Peptidase</td>
</tr>
<tr>
<td>TSE</td>
<td>Transmissible Spongiform Encephalopathy</td>
</tr>
<tr>
<td>UGGT</td>
<td>UDP-glucose:glycoprotein glucosyltransferase</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded Protein Response</td>
</tr>
<tr>
<td>UPS</td>
<td>Ubiquitin Proteasome System</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-Type</td>
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Chapter 1

1 Introduction

1.1 Overview

Prion-mediated disorders are a class of incurable neurodegenerative diseases. These disorders involve the misfolding of the cellular prion protein (PrPC) into an aberrant scrapie form (PrPSc), causing the accumulation of PrPSc in neurotoxic oligomers that eventually lead to patient death. There is intense interest in finding novel therapeutic approaches to prevent the spread of such diseases. It was discovered by a previous postdoctoral fellow in the Williams lab, Dr Pawel Stocki, that the inhibition of the FKBP family of peptidyl prolyl isomerases led to a marked decrease in the expression of PrPC in mouse and human cell lines. This is an exciting finding given the requirement for PrPC expression in prion infection and propagation. The objective of this thesis was to determine the underlying mechanism of the FKBP-dependent regulation of PrPC expression. In this chapter, the structure, functions, and biogenesis of PrP will be discussed, as well as the mechanism, symptoms, and treatments of prion-mediated neurodegenerative diseases. Additionally, protein folding in the ER will be covered, notably by focusing on the mechanism whereby proteins are translocated into this organelle as well as the ER folding machinery. Finally, the diverse nature of the family of peptidyl prolyl isomerases will be explored.

1.2 The Prion Protein

1.2.1 Structure

The cellular prion protein is a glycoprophosphatidylinositol (GPI)-anchored cell surface glycoprotein that is highly conserved in mammals. Paralogues can also be found in numerous organisms, including zebrafish, turtles, and other amphibian species [1-3]. In mammals, PrP is widely expressed in multiple tissues, but predominantly on the synaptic membrane of neurons and their associated cells (such as astrocytes and Schwann cells) as well as in heart, muscle, and hematopoietic cells [4, 5].
Mammalian prions (notably mouse, human, cattle, and hamster PrP) share common structural features and contain roughly 208 aa in their mature form (Figure 1-1). At the N-terminus of the protein, an ER-targeting signal sequence is cleaved following translocation under normal conditions and comprises the first 22 amino acids. Immediately downstream is a charged cluster composed of the first nine residues of the mature domain (aa 23 to 31, human numbering), followed by a long, flexible N-terminal tail (aa 32-128) that encompasses roughly half of the protein and is mostly disordered, except for two defined regions. The first region consists of five repeats of a conserved histidine-containing octameric sequence spanning residues 51 to 91. It has been shown to be responsible for PrP$^{C}$’s copper-binding properties, whereas PrP$^{Sc}$ does not bind copper [6, 7]. In addition, copper upregulates the expression of PrP in neurons [8]. Although the physiological role of PrP’s copper-binding abilities remains unknown, PrP was shown to be evolutionary descended from the ZIP family of metal ion transporters [9]. The second region is a hydrophobic domain located between residues 112 and 135 [10] that can under particular conditions act as a transmembrane domain (discussed in more detail in section 1.4.2). Further downstream are three $\alpha$-helices and a double-stranded $\beta$-sheet forming the globular C-terminal half of PrP$^{C}$. Additionally, a disulphide bond links helices two and three at positions 179 and 214, stabilising the C-terminus of the protein [11]. Two N-linked glycosylation sites (aa 181 and 197) are inefficiently used, resulting in a mixed PrP population with zero, one, or two glycan chains and causing a characteristic banding pattern as detected by western blot. Finally, the GPI signal peptide is found at the C-terminal end of the primary sequence (aa 231-254) and allows PrP to be bound by a GPI anchor [12].
a) Outline of the linear structure of human full-length PrP. The signal sequence, charged cluster (CC), octarepeats (OR), hydrophobic core (HC), and membrane anchor (MA) are highlighted. The disulphide bond and positions of glycan chains are indicated. In addition, the cleavage sites

Figure 1-1: Linear and three-dimensional structure of PrP^C.

a) Outline of the linear structure of human full-length PrP. The signal sequence, charged cluster (CC), octarepeats (OR), hydrophobic core (HC), and membrane anchor (MA) are highlighted. The disulphide bond and positions of glycan chains are indicated. In addition, the cleavage sites
described in section 1.2.3 are shown. Adapted from [13]. b) Three dimensional structure of the mouse cellular PrP based on NMR analysis. Starting at the N-terminus, the polybasic region immediately downstream of the signal sequence (cleaved in this model) is in green. The octapeptide repeats are shown in grey. A positively charged stretch appears in cyan and is immediately upstream of the hydrophobic domain (not highlighted), which includes the first β-sheet (yellow). The other β-sheet is coloured in purple, and the three α-helices are shown in blue, orange, and red. The disulphide bond linking helices two and three and the glycan chains are not visible. The conformation of the disordered region, corresponding to residues 23 to 121, was not determined by NMR and its shown structure is purely conjectural. Reprinted from Trends in Neurosciences, Vol 35 edition number 2, Emiliano Biasini, Jessie A. Turnbaugh, Ursula Unterberger, and David A. Harris, Prion protein at the crossroads of physiology and disease, pages 1-12, Copyright 2012, with permission from Elsevier.
1.2.2 Physiological Functions

Although a great number of putative PrP\textsuperscript{C} functions has been proposed, there is no consensus on its physiological role. Generally, PrP appears to be dispensable in mammals [5, 14]. PrP\textsuperscript{+/-} mice, goats, and cattle show only mild phenotypes, such as a slight increase in the locomotor activity of PrP\textsuperscript{+/-} mice during exploration of a new environment [15] or a minor decrease in the myelin sheath of neurons [16]. However, PrP\textsuperscript{+/-} zebrafish embryos are unable to progress past gastrulation due to loss of embryonic cell adhesion [3], which posits a possible role in development. This hypothesis is also supported by the discovery that the transcription of the Prnp gene in mouse embryos is triggered at different time points in different tissues [17, 18]. PrP has also been implicated in memory formation, notably in long-term potentiation and synaptic plasticity by controlling the activity of protein kinase A in synapses [19]. To further support this, PrP is abundantly expressed in the hippocampus, a brain region which plays an important role in both short- and long-term memory consolidation [5]. One of the other few differences between wild-type and PrP\textsuperscript{+/-} mice is that the knock out animals are more susceptible to strokes and ischemic damage [20]. This may be due to their lower levels of phosphorylated Akt, which suggests that PrP may be involved in the activation of cell survival pathways [21]. However, there is no dearth of contradicting evidence as PrP\textsuperscript{+/-} mice do not display any learning or cognitive impairment compared to wild-type control animals [15]. Moreover, crossing PrP\textsuperscript{+/-} mice with transgenic mouse models of several neurodegenerative disorders (Alzheimer’s, Huntington’s, and Parkinson’s diseases; AD, HD, and PD respectively) did not alter the phenotypes exhibited by the animals [5], showing the limitations of PrP\textsuperscript{C}’s putative neuroprotective effect.

In addition, a substantial body of evidence implicates a role for PrP in AD, but there is also an abundance of contradictory evidence. On the one hand, PrP\textsuperscript{C} has been identified as the receptor for Aß oligomers and may mediate the neurotoxic effects caused by these oligomers [22, 23], although this last point is controversial [24, 25]. On the other hand, PrP\textsuperscript{C} has been shown to downregulate the activity of ß-site APP-cleaving enzyme 1 (BACE1), which is responsible for the conversion of amyloid precursor protein (APP) into the aggregation-prone, neurotoxic Aß [26, 27]. Furthermore, certain PrP\textsuperscript{C} cleavage fragments (more on PrP cleavage events in section 1.2.3) have been shown to harbour neuroprotective properties. For instance, cleaved fragment N1 may inhibit the oligomerisation of Aß peptides, suppressing their neurotoxic effects [28, 29].
1.2.3 Biogenesis

As for most mammalian secretory proteins, PrP’s signal sequence is recognised by the signal recognition particle (SRP) as the nascent chain is leaving the ribosomal exit tunnel. The nascent chain is imported into the ER as translation occurs, where it undergoes several modifications. The signal peptide is rapidly cleaved off by signal peptidase (SP) and the C-terminal GPI anchor signal peptide is removed to allow for the GPI anchoring of PrP to the luminal leaflet of the ER membrane. As discussed previously, up to two N-linked oligosaccharides are added cotranslationally to the C-terminal half of the molecule and an intramolecular disulphide bond is formed [30].

It should be noted that PrP’s signal sequence is somewhat inefficient at establishing a tight seal between the ribosome nascent chain complex (RNC) and the translocon (a process termed “gating” and further described in section 1.4.2). Indeed, up to 20% of PrP synthesised under normal conditions fails to translocate and instead remains in the cytosol, to be degraded by the proteasome [31]. When overexpressed, cytosolic PrP may accumulate as aggregates which have been shown to impede cellular homoestasis and ultimately cause cell death [32]. Furthermore, certain proteins including PrP are subject to a translocational attenuation during ER stress in order to reduce the protein folding load in the ER lumen [31]. It has been shown that the levels of available BiP inversely correlate with this attenuation [33], implying that the chaperone content of the ER lumen is critical for the proper translocation of PrP, and especially so under stress conditions. This effect is likely due to the poor translocon gating efficiency of PrP’s signal sequence [31], which requires some help from the ER lumen for the nascent chain to be translocated. The list of helper proteins has not yet been fully populated, but the translocon accessory proteins TRAM [34] and TRAP [35] (described in more detail in section 1.4.2) are required for PrP’s successful ER translocation. Interestingly, chimeric PrP constructs equipped with the more efficient signal sequences from osteopontin and preprolactin (Opn-PrP and Prl-PrP, respectively) do not display such a dependence on ER chaperones, TRAM, or TRAP for their proper translocation [33].

As mentioned in section 1.2.1, the hydrophobic segment of PrP can act as a transmembrane domain with two possible orientations: the N-terminus can be located in either the cytosol (Ctm-PrP) or the ER lumen/extracellular milieu (Ntm-PrP). Although Ntm-PrP only appears in cell-free
translational assays, Ctm-PrP has been observed in vivo at low levels in healthy organisms and may represent an incomplete translocation intermediate [36, 37]. Furthermore, certain disease-associated mutants cause an increase in the relative levels of Ctm-PrP compared to healthy organisms by increasing the hydrophobicity of the hydrophobic domain, e.g. mutants A117V and AV3 (triple Ala to Val mutations at positions 113, 115, and 118) [36, 38]. PrP<sup>C</sup> may be subject to cleavage events under physiological conditions, of which three variants are known: α-cleavage, β-cleavage, and shedding [39, 40]. All three events occur late in the secretory pathway, namely in the trans-Golgi or on the cell surface. In the case of α-cleavage, PrP<sup>C</sup> is cut immediately upstream of the hydrophobic core (between aa 110 and 111), leading to the creation of a soluble fragment (N1) and a GPI-anchored fragment (C1). The latter’s turnover rate is longer than that of full-length PrP<sup>C</sup>, and therefore C1 accumulates on the surface of the cell and makes up between 5 and 50% of total PrP<sup>C</sup> levels, depending on the cell type and brain region [41, 42]. Although the identity of the protease responsible for this cleavage remains controversial, the most widely suspected family is that of the A-disintegrin-and-metalloproteinase (ADAM) family, in particular ADAM10 and ADAM17 [43]. Regarding β-cleavage, which is less physiologically common than α-cleavage, PrP is cut immediately downstream of the octarepeat domain (between aa 90 and 91), and the resulting fragments are termed N2 and C2 [40]. β-cleavage occurs essentially as a response to an oxidative challenge to the cell and is dependent on the presence of copper ions. It has been suggested that PrP’s β-cleavage is directly caused by reactive oxygen species rather than a proteinacious complex [40]. Finally, shedding of PrP occurs when PrP is cleaved in close proximity to the GPI anchor, resulting in the release of an almost full-length PrP from the cell [40]. Just like α-cleavage, the protease responsible for this event is part of the ADAM family, likely ADAM10. Although shed PrP molecules have been identified in human cerebrospinal fluid and blood [44-46], indicative of a physiological role for this process, no clear function has been attributed to this cleavage [39]. It is possible that this shedding of PrP may be due to the non-specific targeting of PrP by proteases. Intact PrP<sup>C</sup> or truncated C1 and C2 forms of PrP<sup>C</sup> attached to the cell membrane are typically recycled by the endocytic pathway and ultimately degraded in the lysosome [47] with a half-life of roughly five to six hours for full-length PrP [48].
1.3 Neurodegenerative Prion Disorders

1.3.1 Overview

Transmissible spongiform encephalopathies (TSEs) are a group of invariably fatal neurodegenerative diseases that feature the irreversible misfolding of the cellular prion protein (PrP\(^\text{C}\)) into its scrapie form (PrP\(^\text{Sc}\)). They cause a severe loss of motor and cognitive skills in the victim and, often, behavioural changes. One of the pathohistological hallmarks of these diseases is neuronal death, eventually causing the brain’s appearance to be reminiscent of that of a sponge (hence spongiform). These disorders affect many mammals, including humans, sheep, cows, deer, and elk and can be classified in three distinct classes: sporadic, transmissible, and familial [49, 50].

Sporadic diseases represent 85% of the reported cases in humans, and are due to the spontaneous misfolding of PrP without prior exposure to exogenous PrP\(^\text{Sc}\) or inherited genetic mutations. The most common one is sporadic Creutzfeldt-Jakob disease (CJD), which is still rare with a worldwide lifetime incidence of 1 in 2 million people [49]. In all sporadic instances of prion disorders, the aberrant scrapie prions may be disease-causing in healthy individuals. The two main polymorphisms in PrP result in one of two amino acids at position 129, namely methionine or valine. In Caucasians, 51% of the population is heterozygous, 37% Met homozygous, and 12% Val homozygous [51]. Although no mechanistic link has been found between CJD and PrP polymorphisms, heterozyosity at codon 129 appears to diminish the risk associated with that disease [49].

Familial cases are less common, representing 10 to 15% of the cases of human TSEs. One such disease is fatal familial insomnia (FFI), which affects fewer than 40 families and 100 patients. Other than the usual TSE-associated loss of cognitive and motor skills, affected individuals suffer from a progressively worsening inability to attain sleep, and finally pass away an average of 18 months after onset [52, 53]. Another inheritable disease is the Gerstmann–Sträussler–Scheinker syndrome (GSS), which is very similar to CJD in its symptoms but distinct at the pathohistological level as it affects different regions of the brain [54]. On their own, neither methionine nor valine at codon 129 is disease-causing; however, they determine the pathology caused by the mutation D178N. Indeed, the 129Met D178N haplotype leads to FFI, whereas 129Val D178N causes familial Creutzfeldt-Jakob disease (fCJD) [55].
Finally, transmissible disorders are now extremely rare in humans and arise from the consumption of contaminated meat products (such as cows in the case of variant Creutzfeldt-Jakob disease, or vCJD), ritualistic cannibalism in the Fore tribe of Papua New Guinea (in the case of kuru), or through improperly sterilised surgical equipment (iatrogenic diseases, during corneal transplants or neurosurgery, for instance). Very recently, it has been reported that mice expressing human prion variant G127V (which was under positive evolutionary selection during the epidemic of kuru) were completely resistant to any and all TSEs. Heterozygotes were resistant to kuru and cCJD, but could still be infected with vCJD [56].

Following exposure to the infectious agent (for transmissible diseases) or an initial misfolding event (for familial and sporadic disorders), the latency period prior to the appearance of symptoms can last from a few months up to decades [49, 50]. Although each encephalopathy has slightly different features, hallmarks in human TSEs are dementia, ataxia, gait imbalance, and behavioural changes. For CJD, the classical diagnostic triad is progressive dementia, myoclonus, and a characteristic electroencephalogram. The mean duration of the illness is 8 months, and only 4% of patients survive more than two years following symptom onset. At the time of death, the patient is typically bed-ridden, mute, subject to hallucinations and delusions, and emaciated [50, 57].

1.3.2 Molecular Mechanism of Disease

When correctly folded, PrP is predominantly α-helical, proteinase-K sensitive (PK$^{\text{sen}}$), and referred to as a “cellular” prion (PrP$^{\text{C}}$). However, under certain circumstances, it can irreversibly refold into an aberrant, β-sheet rich, proteinase-K resistant (PK$^{\text{res}}$) species (“scrapie” prion, or PrP$^{\text{Sc}}$). PrP$^{\text{Sc}}$ is identical in its primary sequence to PrP$^{\text{C}}$ but is thermodynamically much more stable, although a high activation energy threshold prevents its spontaneous conversion at significant rates. As it is resistant to most degradation pathways, an infected individual accumulates over time high levels of PrP$^{\text{Sc}}$ in the nervous system until the onset of symptoms. It is however known that the cell is able to slowly degrade a proportion of PrP$^{\text{Sc}}$, and although the mechanism isn’t understood yet, the proteasome seems not to be involved [58]. PrP$^{\text{Sc}}$ acts as a nucleation seed to catalyse the conversion of more PrP$^{\text{C}}$ molecules into PrP$^{\text{Sc}}$, with the eventual appearance of fibrils, rods, and sometimes plaques, both extracellularly and in the endocytic
pathway (Figure 1-2). These PrPSc oligomers eventually lead to neuron death and brain spongiosis, although the exact molecular mechanism that ultimately triggers neurotoxicity remains unknown. It was observed in transmission studies that propagation of the disease requires a direct interaction between PrPC and PrPSc [59]. Certain cofactors may also be required, such as glucosaminoglycans, nucleic acids, or lipids [60]. Importantly, the susceptibility to infection by PrPSc and the development of disease depend on the level of PrPC expression. Indeed, PrP-/- mice are resistant to scrapie infection and propagation whereas heterozygous PrP +/- mice show enhanced resistance to scrapie compared to wild-type littermates [14]. It has recently been shown that cytosolic aggregates of untranslocated PrP may also be involved in neurodegeneration [61].

As mentioned above, the induced conversion of PrPC into PrPSc requires a physical contact, which is highly dependent on the sequence homology of the two conformers [59]. This constraint is responsible for the so-called “species barrier;” indeed, only in rare events is PrPSc from one species (e.g., cows) able to corrupt PrPC molecules from another species (e.g., humans) [62]. However, the conserved charged cluster located at the N-terminus of the flexible portion is critical for scrapie propagation and may be part of the interface between PrPC and PrPSc [63]. Disease transmission is further complicated by the existence of different PrPSc strains such as the Rocky Mountain Laboratory (RML) or Fukuoka-1 prions, named after the geographical location of their discovery. These strains are believed to adopt distinct disease-associated conformations and lead to slightly different phenotypes in infected organisms. They also may exhibit different biochemical properties, such as particular glycosylation patterns or altered resistance to denaturation by chaotrope agents or heat. It is also posited that different strains may require different cofactors which may influence the conformation of scrapie prions [60].

1.3.3 Treatments

As there is currently no treatment available to stop the course of TSEs, patient death is inevitable [57]. For a therapeutic agent to be efficacious, it must either cross the blood-brain barrier, which is no small feat, or be injected directly in the central nervous system (CNS), which is inconvenient at best. Most therapeutic approaches under development focus on preventing the misfolding of PrPC, and several compounds have proven efficacious in doing so in vitro.
Figure 1-2: The misfolding of PrPC occurs on the cell surface and in the endocytic pathway. PrPC typically encounters PrPSc after reaching the cell surface. The accumulating scrapie prions eventually form rods, fibrils, and plaques both extra- and intracellularly as both PrP species can be endocytosed. PrPC and PrPSc are shown in blue and red, respectively. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Drug Discovery, Prion diseases – close to effective therapy?, Neil R. Cashman and Byron Caughey, Vol 3 issue 10, copyright 2004. GAG, glycosaminoglycans.
Several classes of therapeutic agents have been studied over the years, with mixed results. Arguably, the most effective class of compounds currently known are polyanionic, polycyclic drugs such as pentosan polysulphate (PPS) [64]. PPS is a polyglycoside thought to act as a glycosaminoglycan mimic, competing with endogenous proteoglycans as a co-receptor for PrP on the cell surface [65]. Promising trials in human patients involving continuous infusion of PPS have shown an increase in the lifespan of the patients and, in a few cases, stabilisation of disease progression. Unfortunately, no improvements of clinical features were apparent in either sporadic, familial, or iatrogenic TSEs [66]. Another class of compounds is exemplified by the benzidine derivative Congo red, which is able to reduce the levels of PrPSc in a chronically infected neuroblastoma cell line by overstabilising the scrapie conformer to prevent the small conformational change required for its interaction with PrPc [67]. Furthermore, Congo red was shown to increase the mean survival of scrapie-infected hamsters from 89 to 125 days post-inoculation [68]. Certain antibodies have also been shown to prevent prion propagation and even to clear cultured cells of their prion infectivity properties [69, 70]. One example is the potent antibody D18 that binds to a key surface on PrPC that is thought to be responsible for direct contact with PrPSc, thereby preventing the misfolding of PrPc [71]. However, due to poor antibody influx into the brain and the high prion load characteristic of TSEs, passive transfer of anti-PrP antibodies only slightly slowed the onset of symptoms in infected mice [69]. Active immunization proved challenging as PrPc is expressed on a wide variety of mammalian cells, therefore causing the host’s immune system to be essentially tolerant to PrPc [72-74]. Moreover, another complication has been discovered: the antibody-mediated crosslinking of PrP molecules on the cell surface triggered neuronal apoptosis in the brain [75].

A few small molecules are able to reduce the amount of prions in certain animals, but not in others. For instance, valinomycin leads to an attenuation in the ER translocation of hamster PrP, thereby depleting the cell surface of PrPc by attrition, but it doesn’t affect other species [76]. Another example leads to an even more confusing result: in rodent cells, quinacrine reduced the levels of PrPSc, but had no impact on human patients and even increased the amount of PrPSc in elk and deer cells [77, 78]. Additionally, a given PrPSc strain may over time adopt a different conformation that may be less thermodynamically stable but resistant to a given drug; this is the case for two related 2-aminothiazole compounds, namely IND24 and IND81 [79].
Although a few compounds are able to extend the lifespan of scrapie-infected mice under certain specific experimental conditions [79], treatment with even the most potent of compounds must start prior to the appearance of symptoms. As the current lack of treatment for prion disorders condemns victims to certain death, there is a critical need for both novel therapeutic targets and agents. Given the requirement for PrP\textsuperscript{C} in PrP\textsuperscript{Sc} infection and propagation, an elegant approach could be to influence the translocation or folding of PrP at the level of the ER so that PrP would be degraded instead of reaching the cell surface.

1.4 Protein Folding in the Endoplasmic Reticulum

1.4.1 Overview

In mammalian cells, it has been estimated by open reading frame analysis that roughly a third of all proteins transit through or reside in the secretory pathway, the entry point of which is the ER [80]. However, as no protein synthesis occurs in the lumen of the ER, all the protein content of the secretory pathway must be imported. Three different translocation mechanisms have been discovered and will be described here. In addition, certain processing events take place in the ER such as N-linked glycosylation, disulphide bond formation and isomerisation, and GPI anchoring and these will be covered in this section. In addition, several quality control mechanisms ensure that only properly folded secretory proteins transit to the Golgi apparatus and these will be discussed as well.

1.4.2 Signal Peptide Processing and Translocation

In order to be targeted to the ER, proteins have been bestowed with signal sequences consisting of roughly 25 residues located at the N-terminus of the protein. Although a wide variety of signal sequences has been described, they can be divided into three regions. A basic N-domain at the N-terminus and a hydrophobic H-domain help position the signal peptide in a looped orientation inside the translocon with the N-terminus facing the cytosol during translocation. These are followed by a polar C-domain that contains the signal sequence’s cleavage site [81]. Indeed,
signal peptides are only present in the immature form of the protein as they are rapidly cleaved off in the ER by a protein called signal peptidase (SP) [82].

Secretory pathway proteins have two options to enter the ER: co- or post-translational translocation [83]. In mammals, co-translational translocation is the most common pathway. As the transcript is being translated by the ribosome, the nascent chain emerges from the ribosome’s exit tunnel. When the signal sequence is recognised and bound by the signal recognition particle (SRP), translation is paused and SRP brings the entire complex to the SRP receptor (SR). The latter delivers the RNC to the canonical Sec61 translocon complex, located in close proximity. When free of translating ribosomes, Sec61’s pore is kept closed by the channel’s plug domain [84]. Three distinct events, all dependent on the nature and strength of the signal sequence, subsequently occur [31, 85]. (1) The RNC binds to the translocon in a salt-resistant manner. The RNC-Sec61 interaction is initially salt-sensitive and becomes salt-resistant only after several additional amino acids (typically a few dozens) are added to the growing chain following translation resumption when SRP dissociates [85, 86]. (2) As the nascent chain is elongated, a tight seal is formed between the RNC and the translocon in order to maintain the permeability barrier of the ER during translocation. (3) The plug domain of Sec61 disengages from the aperture of the translocation channel to leave an open pore (a process termed “gating”). Once these three steps have been successfully completed, the nascent chain emerges into the ER lumen through the translocon pore [31, 85], at which point chaperones such as BiP bind exposed hydrophobic patches to prevent aggregation. The signal peptide remains bound in the translocon channel in a looped conformation until its cleavage by SP [82]. Additional factors are required for the translocation of certain substrates, such as the TRAP complex to control the topogenesis of membrane proteins [35] and TRAM to insert certain signal sequences in the translocon [34] or to regulate the integration of transmembrane domains into the membrane [87]. Moreover, the accessory protein Sec62 but not Sec63 was shown to affect co-translational translocation [88].

Post-translational translocation is comparatively poorly understood in the mammalian system. Two distinct post-translational translocation pathways exist [83, 89]: signal sequence-dependent (typically for short secreted proteins) and signal sequence-independent (typically for tail-anchored proteins). In the first case, the signal sequence is virtually indistinguishable from that present in co-translationally translocated proteins. Alone, it is not sufficient to allow a polypeptide to be post-translationally translocated, but the combination of particular signal sequences with
certain mature domains is [89]. Although post-translational translocation uses the same Sec61 translocon as co-translationally translocated substrates, additional factors are required, such as Sec62 [88, 90] and luminal chaperones such as BiP that provide the energy required for translocation by a ratchet mechanism [91, 92]. For the second case, no signal sequence exists as only the tail of the protein is anchored on the cytosolic side of the ER membrane. The substrate’s C-terminus is directly embedded in the membrane using the transmembrane recognition complex [93]. Additionally, both Sec62 and Sec63 are required, but Sec61 isn’t involved [89, 94]. Overall, post-translational translocation is fairly rare in mammals and will not be discussed further.

Despite all signal sequences having been shown to target and transfer the RNC to the translocon with a similar efficiency, a wide range of signal sequences with varying gating abilities can be found in mammals [31, 81]. This is likely due to differences in the three-dimensional structure adopted by the nascent mature domain in the translocon pore in order for the signal sequence to form a loop in the channel. Therefore, the mature domains of different proteins have different gating requirements, which are precisely matched by their natural signal sequences. For instance, a mature domain that doesn’t allow for a proper gating on its own will require a strong signal sequence for gating and translocation to occur. In contrast, a mature domain that can induce gating doesn’t need a particularly efficient signal sequence so long as the latter targets the RNC to the translocon. In addition, swapping the signal sequence of a secretory protein with that of another can modify the normal biogenesis of the substrate. Indeed, a signal sequence that doesn’t allow for an efficient gating to occur affixed on a mature domain that cannot trigger gating on its own may lead to an abortive translocation event. The substrate would therefore end up in the cytosol instead of the ER.

Two extreme examples of signal sequences are those from preprolactin (Prl) and PrP. As mentioned earlier, roughly 10 to 20% of PrP molecules do not translocate properly and end up in the cytosol [31]; in the case of Prl, that figure is much lower, between 2 and 4% [38]. This may be due in part to the fact that the signal sequence from Prl is much more efficient than that of PrP at translocon gating [31]. Although this might seem wasteful, it has been posited that this weakness of PrP’s signal sequence is in fact a way for the cell to modulate PrP’s translocation with trans-acting factors [33]. This ability to modulate translocation efficiency comes in handy for the cell given, for instance, an abundance of misfolded proteins in the ER due to various pathological conditions. In addition, it has been demonstrated that creating a chimeric Prl-PrP construct by
swapping PrP’s signal sequence with that of Prl allows Prl-PrP to be more efficiently translocated than wt-PrP. Furthermore, the translocation of Prl-PrP is unaffected by ER stress, in contrast with the attenuation seen with wt-PrP [31, 95].

1.4.3 Protein Folding and Quality Control Machinery

As the nascent chain is translocated into the ER lumen, it is acted upon by a variety of factors (Figure 1-3). Among the most important ones are molecular chaperones including members of heat shock protein (Hsp) families. Hsp70 family members such as BiP promiscuously bind exposed hydrophobic patches to prevent aggregation and promote the folding of their substrates by cycles of binding and release [96]. This cycling is dependent on the ATPase activity of Hsp70, which is regulated by the Hsp40 cochaperone family. The Hsp90 family, which in the ER is represented by Grp94, works as a part of a multichaperone complex that includes Hsp70 and Hsp40 and, unlike Hsp70, bind substrates at a nearly native state [97]. Two additional chaperones, calnexin and calreticulin (CNX/CRT), interact with many secretory proteins and will be described further below [98].

A common modification that secretory proteins often undergo is disulphide bond formation. These bonds link the thiol groups of two cysteine residues (on the same or two different proteins) and allow for the proper folding and function of proteins. In the case of PrP, the disulphide bond increases the stability of the globular domain [11]. For this redox mechanism to function properly, the ER environment is actively maintained in an oxidative state [99] by a protein relay starting with the FAD-binding oxidase Ero1 donating an electron to molecular oxygen [100]. Members of the mammalian family of protein disulphide isomerases (PDI) receive oxidising equivalents from Ero1 through their catalytic CXXC motif that is able to switch between disulphide and dithiol forms [101]. PDI members then transfer the oxidising equivalents to their substrates, allowing their oxidation. In their reduced state, PDI members can also catalyse substrate reduction and isomerisation [102]. Twenty members of the PDI family have been discovered in mammals, including the prototypical PDI, ERp57 for glycoproteins, ERp72 with a narrow substrate-specificity, and even P5 with no involvement in oxidative folding [101].
Figure 1-3: The extensive ER folding machinery.

The nascent chain is cotranslationally translocated into the ER, where it encounters a diverse array of chaperones, protein disulphide isomerases, peptidyl prolyl isomerases, and oligosaccharide processing enzymes. See text for details. *Figure courtesy of Dr David Williams.*
The vast majority of proteins that enter the ER, including PrP, are decorated with N-linked glycans at the consensus sequence Asn-Xaa-Ser/Thr [103]. First, a core oligosaccharide (Glc3Man9GlcNAc2, where Glc is glucose, Man is mannose, and GlcNAc is N-acetylglucosamine) is attached to the Asn residue by the enzyme oligosaccharyltransferase. Two terminal glucose residues are then successively removed by the ER-resident glucosidases I and II to yield GlcMan9GlcNAc2 (Figure 1-4), so that the nascent glycoprotein may interact with the ER-resident lectin chaperones CNX/CRT, which are respectively membrane-bound and luminal. CNX/CRT recognise the oligosaccharide GlcMan9GlcNAc2 to bind nascent glycoproteins and act as chaperones to prevent aggregation [98, 104]. They also associate with the protein disulphide isomerase ERp57 that oxidises, reduces, and isomerises its substrates’ disulphide bonds. Interestingly, ERp57 was recently shown to modulate the maturation and total levels of PrP, with ERp57−/− mice displaying a reduction in the levels of mono- and non-glycosylated forms of PrP in the brain [105].

In addition, CNX/CRT oversee a quality control mechanism ensuring that only properly folded glycoproteins exit the ER [106]. Both lectins retain their client proteins in the ER by binding GlcMan9GlcNAc2 oligosaccharides until glucosidase II cleaves the remaining glucose residue, disrupting the interaction. Two outcomes are then possible. If the client has adopted its native conformation, it is allowed to exit the ER and traffic to the Golgi apparatus. Otherwise, UDP-glucose:glycoprotein glucosyl-transferase (UGGT) catalyses the addition of a single glucose back on the glycan tree to renew the association with CNX/CRT for further chaperoning to occur. After a few cycles of glucose removal and addition, the client is considered terminally misfolded. At that point, a first mannose residue is cleaved by ER degradation-enhancing 1,2-mannosidase-like protein 2 (EDEM2) and a second one by EDEM1 or EDEM3 [107]. The resulting α1,6-linked mannose residue then serves as a signal for disposal of the misfolded glycoprotein by ER-associated degradation (ERAD) [108]. The exposed α1,6-linked mannose residues are recognised by the related ER-resident lectins OS-9 [109] and XTP3-B [110]. They also bind to the membrane-embedded Hrd1 ubiquitin ligase via the SEL1L adaptor protein, linking the ER lumen with the ubiquitination machinery in the cytosol [111]. Whereas it is clear that OS-9 recognises misfolded glycoproteins in coordination with the chaperone Grp94 [111], the mechanism of action of XTP3-B is not fully understood but may involve other cofactors [112].
Figure 1-4: The calnexin/calreticulin ER glycoprotein quality control mechanism.

Most secretory proteins are decorated with N-linked oligosaccharides before being processed by glucosidases I and II. Substrates may then enter the CNX/CRT cycle, and ERp57 catalyses the formation and isomerisation of disulphide bonds. A third glucose residue is subsequently removed by glucosidase II, at which point natively folded proteins exit the ER to proceed along the secretory pathway. Misfolded proteins return to the CNX/CRT cycle thanks to the addition of a glucose residue by UGGT, which senses non-native hydrophobic patches. Terminally misfolded clients are sent to ERAD by the family of EDEMs. Adapted from [103].
In addition, unglycosylated proteins are not left alone to mature and traffic along the secretory pathway. Indeed, both OS-9 and XTP3-B have been implicated in the recognition of terminally misfolded non-glycosylated proteins such as the glycosylation-deficient mutants of the ERAD substrates α1-antitrypsin variant NHK and sonic hedgehog. However, this mechanism requires the involvement of other factors such as BiP, EDEM2, or SEL1L to deliver their substrates to the ERAD machinery [112, 113].

Once recognised by one of the previously mentioned ERAD sensors, proteins that are terminally misfolded in the ER must be retrotranslocated into the cytosol for degradation by the ubiquitin proteasome system (UPS), a process that to date is not fully mapped [114]. Three different checkpoints have been discovered, depending on the localisation of the misfolded domain: cytosolic (ERAD-C), luminal (ERAD-L), and intramembrane (ERAD-M). In all three cases, the substrate must interact with valosin-containing protein (VCP, also known as p97), which delivers ubiquitinated substrates to the proteasome on the cytosolic side of the ER membrane [115]. However, the identity of the E3 ubiquitin ligase varies depending on the subtype of ERAD. ERAD-C recognises misfolding events in the cytoplasmic domain of membrane proteins [116]. In yeast, ERAD-C substrates require the integral membrane ubiquitin ligase Doa10 (homologous to the mammalian TEB4) [117]. Misfolded soluble proteins or transmembrane proteins with a lesion in their luminal domain are degraded by ERAD-L and require the ubiquitin ligase Hrd1 [118]. However, the existence and identity of a pore through which the substrate would exit the ER is hotly debated [119]. In yeast, Hrd1p appears to act both as an E3 ligase and retrotranslocation channel [120]. In the case of ERAD-M, integral membrane proteins are first ubiquitinated by the E3 ligases gp78 (as is the case of unanchored PrP) or Hrd1 and then simply stripped away from the membrane by VCP [121]. Interestingly, GPI-anchored proteins including PrP tend to be refractory to ERAD and are degraded by an alternate pathway involving a brief transit by the cell surface prior to lysosomal degradation.

An already described sensor of protein misfolding is the Hsp70 BiP, but it deserves to be mentioned again for one of its numerous roles. In an unstressed ER, BiP binds and maintains several transmembrane receptor proteins in an inactive state. Under stress conditions where the ER and the ERAD machinery are overwhelmed by misfolded proteins, the cell will try to reduce the protein load in the ER by various strategies. The first step is the dissociation of BiP from these receptors so that BiP may focus on preventing the aggregation of the misfolded proteins.
Oligomerisation of these receptors triggers a complex response: the unfolded protein response (UPR) [122], which will be briefly touched upon here and in Figure 1-5. The aim of the UPR is to minimise the misfolding and aggregation of proteins in the ER by decreasing general protein synthesis as well as upregulating chaperones and ERAD components to assist in the folding and disposal of secretory pathway proteins. If the stress cannot be resolved, the UPR induces apoptotic cell death to protect the organism by removing stressed cells. To accomplish these functions, the UPR acts by three branches. First, the dissociation of BiP from PERK induces the latter to phosphorylate eIF2α, thereby inhibiting general translation initiation and decreasing the volume of proteins to be folded [123]. The synthesis of particular proteins, however, is increased. This is the case for the transcription factor ATF4, involved in the activation of both pro-survival and pro-apoptotic components, such as amino acid synthesis and CHOP activation, respectively [124]. ATF4 is also responsible for inducing the dephosphorylation of eIF2α once the stress has cleared in order to allow the cell to recover [124]. Second, BiP releases the transcription factor ATF6, which migrates from the ER to the Golgi where it undergoes cleavage. The now-active ATF6 then upregulates a variety of chaperones (such as BiP and PDI) and ERAD players (such as EDEM). Additionally, ATF6 also induces the expression of XBP1, the activated version of which upregulates the synthesis of ER chaperones [125]. Third, the IRE1 receptor is activated as it is released by BiP in the ER and uses its endoribonuclease activity to splice 26 residues from the XBP1 mRNA. This leads to a frame shift and to the synthesis of a potent transcription activator, sXBP1, which upregulates the expression of ER chaperones and protein degradation-related genes and indirectly downregulates the activity of PERK [126]. Another function of activated IRE1 is to activate pro-apoptotic pathways. Overall, the precise balance between protein folding and degradation versus apoptotic signals will determine the fate of the cell: recovery or death [122].
Figure 1-5: The three arms of the unfolded protein response.

This simplified model of the UPR shows the main players in the three branches. An increase in the synthesis of ER chaperones is triggered by both ATF6 and IRE1 and is required to help fold the accumulating misfolded proteins that caused activation of the UPR. PERK activation leads to a general inhibition of translation initiation and to growth arrest through phosphorylation of eIF2α, in order to reduce the protein folding load in the ER. It also activates ATF4, which will ultimately dephosphorylate eIF2α for protein synthesis to resume. See text for details. Reprinted from Gastroenterology, Vol 141 issue number 1, J. Alan Diehl, Serge Y. Fuchs, Costantinos Koumenis, The Cell Biology of the Unfolded Protein Response, pages 1-6, 2011, with permission from Elsevier.
Importantly, during ER stress, certain proteins such as PrP see virtually 100% of their newly synthesised molecules redirected to the cytosol instead of the ER [33]. Although the exact mechanism is not fully understood, this is likely due to the fact that such proteins require BiP for their successful translocation and shows that the wide variety of signal sequence strength found in nature may have a relevant physiological application. This rerouting allows for an immediate reduction in the amount of proteins to fold in the ER.

Given the importance of peptidyl prolyl isomerases for the foundations of the present thesis, an entire section of this chapter is dedicated to this class of proteins. Their varied physiological roles and involvement in neurodegeneration and immunosuppression will be described.
1.5 Peptidyl Prolyl Isomerases

1.5.1 Overview

In proteins, most amino acid side chains adopt a *trans* conformation a majority of the time (~99.9%) across a peptide bond due to steric hindrance between side chains. One exception is proline, due to its intrinsic conformational kink, with roughly 5 to 10% of Xaa-Pro occurring in *cis* in proteins [127, 128]. Given that the spontaneous isomerisation of such bonds occurs on the order of hundreds of seconds [129], evolution has graced the cell with catalytic tools to speed up the rate of this reaction. Collectively, they are termed peptidyl prolyl isomerases (PPIases, also known as rotamases), of which three structurally distinct subfamilies exist: the FK506-binding proteins (FKBPs), the cyclophilins (Cyp), and the parvulins [130]. The FKBPs and Cyps form the family of immunophilins due to their role in immunosuppression. In humans, there are 16 Cyps and 14 FKBPs that are present in various organelles including the cytosol, the ER, and the nucleus [131].

Although all three subfamilies possess PPIase activity, their structure and amino acid sequence is unrelated [131]. As shown in (Figure 1-6), cyclophilin A (CypA) adopts a closed right-handed β-barrel [132] whereas FKBP1A displays an overall conical shape containing a hydrophobic groove where the Pro-containing substrate can bind [133]. Both CypA and FKBP1A are the smallest members of their respective subfamilies and only consist of a PPIase domain. More elaborate members exist with an organelle-targeting signal peptide, EF-hands for calcium ion coordination, tetratricopeptide repeats to mediate protein-protein interactions, and more [131, 134]. Interestingly, FKBPs but not Cyps may contain several PPIase domains (up to four in the case of the large FKBP9 and FKBP10), although some differ heavily in sequence identity, ranging from 17 to 83% compared to the canonical FKBP1A, possibly due to their particular physiological roles [134]. Certain FKBPs even contain inactive PPIase domains, such as FKBP4’s C-terminal domain, suggesting that such domains may instead act as molecular chaperones [135, 136]. Finally, the prototypical parvulin Pin1 is composed of an N-terminal substrate-binding WW domain and a C-terminal PPIase domain that is structurally unrelated to that of FKBPs and Cyps [137]. The differences between the three subfamilies and between subfamily members is representative of the wide variety of functions these proteins perform.
Figure 1-6: The structures of FKBP1A and CypA display no homology.

The crystal structures of human a) FKBP1A [138] and b) CypA [139] were generated using Jmol: an open-source Java viewer for chemical structures in 3D. http://www.jmol.org/. Despite an identical catalytic function, neither the primary sequence nor the overall fold of the two proteins show any significant similarities.
1.5.2 Physiological Functions

Numerous roles have been attributed to the cis-trans isomerisation of Xaa-Pro peptide bonds. Perhaps most importantly, PPIases can function as signalling regulators. For instance, Pin1 is the only known PPIase to exclusively catalyse the isomerisation of phosphorylated Ser/Thr-Pro bonds, such as those found in the proteins Cdc25C and tau. As these proteins adopt two different conformations depending on the isomerisation state of the proline residue, the switching between an active and an inactive state depends on the phosphorylation of the substrate [140]. This allows the cell to control events such as cell cycle progression, microtubule formation, and stress response [141]. A similar regulatory function is performed by CypA on the protein tyrosine kinase Itk, which contains a proline residue in its SH2 domain. The isomerisation state of this residue regulates substrate recognition and ultimately affects signalling events leading to T cell activation [142].

PPIases may also perform distinct functions during protein folding. First, PPIases may simply use their catalytic activity on certain Xaa-Pro bonds to speed up the folding process, although this function is not essential for cell survival as the uncatalysed isomerisation occurs on a short enough time scale [131]. Second, certain PPIases are known to possess a chaperone function that is independent of their catalytic activity. For instance, FKBP10 mediates the appropriate folding of collagen and tropoelastin, two components of the extracellular matrix, without using its rotamase activity [141, 143]. FKBP10’s chaperone activity is significant and reportedly comparable with that of PDI, a well-characterised chaperone [144]. Yet other FKBP5 have been involved in regulating chaperone activity. This is the case for FKBP7 and FKBP14, which can interact with BiP in a Ca\textsuperscript{2+}-dependent manner to respectively activate and downregulate BiP’s ATPase activity. Surprisingly, FKBP14’s role on BiP activation is mediated by its PPIase domain yet is unaffected by the inhibitor FK506. In contrast, FKBP7 uses its PPIase domain to induce a conformational change in BiP, an event that is inhibited by FK506 [145, 146] (more on FK506 and other inhibitors in section 1.4.3).

Interestingly, the individual and collective deletion of all 12 immunophilins genes in *Saccharomyces cerevisiae* had no impact on the viability of the organism, with the dodecuplet mutant phenotype resulting in the addition of the subtle phenotypes of each individual mutations [147]. Of all the physiological parameters monitored, no major defects were observed for any of
the mutant yeasts, leading to the conclusion that immunophilins are non-essential to the healthy growth of *S. cerevisiae* in laboratory conditions. Moreover, the knockout of the two rotamases expressed in *Bacillus subtilis* significantly slowed cell proliferation under poor growth conditions such as amino acid starvation [148]. The previous two findings suggest that immunophilins may be required only in certain conditions in lower organisms. As for mammals, the depletion of a single PPIase may have severe consequences. For instance, CypA−/− mice are viable but spontaneously develop an allergic disease with elevated levels of IgE and tissue infiltration by mast cells and eosinophils [149]. FKBPIA−/− mice display repetitive behaviour and enhanced perseveration in several memory assays, mimicking phenotypes observed in autistic humans [150]. In addition, FKBPIA and its homologue FKBPIB are involved in the regulation of Ca^{2+} current through the ryanodine receptors, which are critical regulators of the heart rhythm [151]. Yet other immunophilins are involved in steroid hormone trafficking. Indeed, the highly related FKBPS4 and FKBPS5 act to respectively negatively and positively regulate the affinity of the steroid hormone receptor for its hormone [152]. Interestingly, this hormone receptor modulation relies on interactions through the PPIase domains of FKBPS4 and FKBPS5 but not their catalytic activity [153]. In contrast to humans, FKBPT10−/− mice do not survive birth and embryos present growth delays due to inefficient collagen and tropoelastin maturation [154, 155]. This difference between humans and mice may be due to a different expression profile of the FKBPs, perhaps with humans but not mice expressing enough of the highly similar FKBPS9 to partially compensate for the deletion of FKBPT10. However, human patients with mutations in FKBPT10 suffer from osteogenesis imperfecta and Bruck syndrome, two brittle bone diseases linked to collagen synthesis [156]. A list of immunophilins expressed in humans and a brief description of their characteristics and/or function, if known, can be found in Table 1-1.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Cellular localisation</th>
<th>Function [131, 157]</th>
</tr>
</thead>
<tbody>
<tr>
<td>FKBP1A  (FKBP12)</td>
<td>cytosol</td>
<td>immunosuppressive properties of FK506; calcium homeostasis</td>
</tr>
<tr>
<td>FKBP1B  (FKBP12.6)</td>
<td>cytosol</td>
<td>calcium homeostasis</td>
</tr>
<tr>
<td>FKBP2  (FKBP13)</td>
<td>ER/cell surface</td>
<td>chaperone</td>
</tr>
<tr>
<td>FKBP3  (FKBP25)</td>
<td>nucleus</td>
<td>nucleic acid processing (RNA splicing)</td>
</tr>
<tr>
<td>FKBP4  (FBK52)</td>
<td>cytosol/nucleus</td>
<td>intracellular trafficking of steroid hormone receptors</td>
</tr>
<tr>
<td>FKBP5  (FKBP51)</td>
<td>cytosol/nucleus</td>
<td>intracellular trafficking of steroid hormone receptors</td>
</tr>
<tr>
<td>FKBP6  (FKBP36)</td>
<td>nucleus</td>
<td>meiosis; transposon silencing</td>
</tr>
<tr>
<td>FKBP7  (FKBP23)</td>
<td>ER</td>
<td>chaperone; regulates BiP's ATPase activity</td>
</tr>
<tr>
<td>FKBP8  (FKBP38)</td>
<td>cytosol/mitochondria</td>
<td>involved in apoptotic signalling</td>
</tr>
</tbody>
</table>

**Table 1-1: Localisation and function of human immunophilins.**

All 14 FKBP s expressed in humans are listed here with their two alternative names. As there are upwards of twenty putative cyclophilin genes in mammals [158], only some of the best characterised cyclophilins were selected for this table. *Continued on the next page.*
<table>
<thead>
<tr>
<th>Immunophilin</th>
<th>Location</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>FKBP9 (FKBP60)</td>
<td>ER</td>
<td>chaperone; collagen processing [156]</td>
</tr>
<tr>
<td>FKBP10 (FKBP65)</td>
<td>ER</td>
<td>chaperone; collagen processing [156]</td>
</tr>
<tr>
<td>FKBP11 (FKBP19)</td>
<td>ER</td>
<td>transmembrane with a short cytosolic tail; function unknown</td>
</tr>
<tr>
<td>FKBP14 (FKBP22)</td>
<td>ER</td>
<td>chaperone; collagen processing</td>
</tr>
<tr>
<td>FKBP15 (FKBP133/135)</td>
<td>cytosol</td>
<td>involved in neuronal growth cones</td>
</tr>
<tr>
<td>CypA</td>
<td>cytosol/nucleus</td>
<td>cell signalling [159]</td>
</tr>
<tr>
<td>CypB</td>
<td>ER</td>
<td>chaperone; collagen processing; redox homeostasis [160-162]</td>
</tr>
<tr>
<td>CypC</td>
<td>ER</td>
<td>protein folding; redox homeostasis [160, 162]</td>
</tr>
<tr>
<td>CypD</td>
<td>mitochondria</td>
<td>mitochondrial permeability transition pore regulation [163]</td>
</tr>
<tr>
<td>CypE</td>
<td>nucleus</td>
<td>RNA-binding domain [164]</td>
</tr>
<tr>
<td>Cyp40</td>
<td>cytosol</td>
<td>glucocorticoid hormone trafficking; interacts with Hsp90 complexes [161]</td>
</tr>
</tbody>
</table>

*Table 1-1: Localisation and function of human immunophilins (continued).*
1.5.3 Inhibitors and Immunosuppression

The catalytic activities of FKBPs and cyclophilins are inhibited by small natural products, such as FK506 (Figure 1-7) and rapamycin for the FKBPs, and cyclosporine A (CsA) for the cyclophilins [165, 166]. These three compounds bind the PPIase domains of their respective targets, preventing the binding of the Pro-containing substrate [167, 168]. One of the most striking properties of the immunophilins is their ability to mediate immunosuppression in the presence of such inhibitors. Surprisingly, this is not caused by inhibiting their activities, but rather by the formation of a non-physiological interaction. When bound to FK506 or CsA, the rotamase FKBPIA or CypA (respectively) interacts with the phosphatase calcineurin, which prevents the activation of the latter. Calcineurin would otherwise dephosphorylate the transcription factor NFAT to allow it to translocate from the cytosol into the nucleus and trigger the transcription of T-cell cytokine genes. There also exists another immunosuppressor, rapamycin, which acts by a different but similar mechanism [169]. Like FK506, rapamycin binds to FKBPIA, although the rapamycin-FKBPIA complex inhibits the mechanistic target of rapamycin (mTOR) kinase rather than calcineurin. mTOR is then unable to perform one of its numerous physiological functions, namely triggering T-cell and B-cell growth, differentiation, and proliferation.

FK506, CsA, and rapamycin have been postulated to confer a certain protection against neurodegeneration. For instance, FK506 treatment of an AD mouse model led to a decrease in the Aβ burden and a restoration of synaptic proteins that are reduced in AD (such as synaptophysin and PSD-95) [170]. FK506 was also shown to reduce neuroinflammation and increase the survival of dopaminergic neurons in a rat model of PD [171], and to protect against necrosis and apoptosis under a mild cell death stimulus in a mouse model of HD [172]. Moreover, CsA was shown to prevent neurodegeneration and neurological dysfunction following a severe cortical impact in mice [173]. In addition, rapamycin is able to reduce the Aβ- and tau-mediated pathology in a mouse model of AD and decrease the levels of neuronal Aβ accumulation [174]. This effect of rapamycin may be caused by counteracting the Aβ-mediated hyperactivation of mTOR [175]. Finally, it has recently been discovered that FK506 is a potent antiprion agent and prolonged the survival time of prion-infected mice [176, 177]. However, the exact mechanism of action of all three drugs with regards to neuroprotection remains unclear and somewhat controversial [170, 172].
Figure 1-7: Structure of FK506.

FK506 is a natural product produced by the bacterium *Streptomyces tsukubaensis* and possesses two domains named after their roles in immunosuppression [178]. The binding domain binds the PPIase domain of the FKBPs whereas the effector domain interacts with calcineurin. The formation of this complex prevents the activation of calcineurin and therefore T cell activation. *Reprinted from Drug Discovery Research: New Frontiers in the Post-Genomic Era, Junhai Xiao, page 213, 2007, edited by Ziwei Huang, with permission from John Wiley and Sons.*
Although these three inhibitors display no specificity within the family they target, certain analogues or even completely novel compounds have been proven to specifically target a single family member. This is the case of N-(N’,N’-dimethylcarboxamidomethyl)cycloheximide (DM-CHX), which binds to and inactivates the PPIase domain of a single FKBP, the neurone-expressed FKBP8 [179]. Moreover, the minimal pharmacore of the FKBP family has recently been defined [180], opening a route for the design of novel, more specific inhibitors. In light of its effects on several pathologies such as cancer and metabolic disorders, rapamycin has been extensively modified to create a new class of compounds, the rapalogues, which bind different targets with different specificities to allow for a more tailored therapeutic approach [181].

1.6 Thesis Rationale and Objectives

Given the death sentence synonymous with a patient’s diagnosis of TSE, there is a critical need for new treatments and therapeutic agents. Most therapeutic approaches currently under development focus on preventing the misfolding of PrPC into PrPSc, which may not be the most effective method. A more elegant goal may be to prevent the delivery of PrP to the cell surface, where most of the misfolding occurs. This could be done at the level of the ER, either by inducing the ERAD machinery to degrade PrP or by foiling PrP’s translocation into the organelle. A previous postdoctoral fellow in the Williams lab, Dr Pawel Stocki, made the observation that FK506 treatment of mouse and human cell lines profoundly attenuated PrP expression, raising the possibility of its therapeutic potential in treating prion diseases. It was also discovered that FK506 triggered the rapid proteasomal degradation of PrP, leading to two possible mechanisms. First, FK506 may induce the misfolding and subsequent ERAD of PrP. Second, given the weak signal sequence of PrP, FK506 may affect the proper translocation of PrP into the ER. Consequently, the first objective was to elucidate the mechanism of action of the FK506-mediated degradation of PrP and is presented in chapter 3, with FK506 causing the abortive translocation of PrP. The second objective, described in chapter 4, was to identify the identity of the FKBP (or FKBP5s) whose inactivation leads to the abortive translocation of PrP.
Chapter 2

2 Material and Methods

2.1 Constructs, Antibodies, and Reagents

The pcDNA3.1 constructs PrP, Opn-PrP, and Prl-PrP encode hamster PrP with its wild-type signal sequence or that from rat osteopontin or bovine preprolactin, and were a kind gift from Dr Ramanujan Hegde (University of Cambridge, England) [31, 95]. Immunoblotting was performed using antibodies recognising mouse PrP (clone Sha31, Bertin Pharma, Montigny-le-Bretonneux, France, cat. number A03213), human and hamster PrP (clone 3F4, Cedarlane Labs, Burlington, ON, cat. number CLPN40020), FKBP10 (BD Transduction Laboratories, Franklin Lakes, NJ, cat. number 610648), GAPDH (clone 6C5, Millipore, Billerica, MA, cat. number MAB374), cyclophilin B (Abcam, Cambridge, England, cat. number ab16045), and horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, cat. number 115-035-003). Flow cytometry was performed using mouse anti-MHC Y3 (purified in-house, 1.6 mg/mL; described in [182]), anti-MHC W6/32 (purified in-house; described in [183]), and Alexa647 goat anti-mouse IgG (Life Technologies cat. number A21235, 2 mg/mL). Lactacycin was obtained from Cayman Chemicals (Ann Arbor, MI, cat. number 70890), FK506 and bortezomib were purchased from LC Laboratories (Woburn, MA; cat. numbers F-4900 and B-1408, respectively), and PNGase F was obtained from New England Biolabs (Ipswich, MA, cat. number P0704L).

2.2 Cell Culture and RNA Interference

Mouse neuroblastoma N2a cells, human hepatoma HepG2 cells, and human astrocytoma U373-MG cells were cultured in DMEM supplemented with 10% FBS, antibiotics, and 1 mM glutamine in 5% CO2 at 37°C. Transient transfections of the PrP-encoding plasmids were performed using 2.5 µg of the plasmid mixed with 10 µL of Lipofectamine 2000 (Life Technologies, Carlsbad, CA, cat. number 11668-019) and added to cells in 2 mL of DMEM without serum or antibiotics. After 4 h, the medium was supplemented to 10% serum. The cells were allowed to grow for 24 h post-transfection before analysis or further treatment.
Knockdowns in HepG2 cells by siRNA were performed using the previously described reverse transfection protocol [101, 184] and the selected siRNAs are listed in Table 2-1. Briefly, 4 μL of 20 μM Stealth siRNA or 5 μL of 2 μM Silencer Select siRNA stock were diluted in 490 μL of OptiMEM (Life Technologies, cat. number 31985-088) per well in 6-well plates and incubated at room temperature for 5 min. Oligofectamine (6 μL, Life Technologies, cat. number 12252-011) was then added to each well. After 20 min, 2.5x10^5 cells/mL in 1.5 mL of serum- and antibiotics-free DMEM were added on top of the siRNA complexes. After 4 h, each well was supplemented to 10% serum. In the case of double knockdowns, the protocol was repeated a second time the following day using an siRNA targeting the second transcript of interest.

Knockdowns in N2a cells by siRNA were performed as a direct transfection with 50 nM of Stealth Select siRNA (Life Technologies) and 7.5 μL of Lipofectamine RNAiMAX (Life Technologies, cat. number 13778150) in 3 mL of DMEM without serum or antibiotics. After 4 h, the medium was supplemented to 10% serum in a total of 5 mL. For both cell lines, transfections were performed on day 1 and again on day 4 followed by further treatment or analysis on day 6 or 7, as indicated. Controls were performed with the non-targeting negative control Stealth Select siRNA, medium GC content (Life Technologies, cat. number 12935-300).

Knockdowns using shRNAs were performed with GIPZ lentiviral shRNA plasmids (Thermo Scientific, Waltham, MA). The RNAi Consortium (www.broadinstitute.org/rnai/trc) protocol for lentiviral production was used. In brief, 293T cells growing in T25 filter cup flasks were used as packaging cells and were transfected using FuGENE 6 with 100 ng of envelope plasmid VSV-G/pMD2.G, 900 ng of packaging plasmid pCMV-dR8.74psPAX2 and 1 μg of targeting GIPZ plasmid. Virus was collected after 42 h and added to U373-MG cells in growth media with 8 μg/mL polybrene. Puromycin selection (1 μg/mL) was initiated 24 h post transfection and lasted for an additional three days.

For trypsinised FK506 time courses, N2a cells were plated and treated with the indicated drug immediately after being trypsinised. For untrypsinised FK506 time courses, cells were plated but allowed to recover for 24 h before drug treatment. The cells were treated with 20 μg/mL FK506 alone or in combination with 2.5 μM bortezomib, or DMSO as a vehicle control, and kept in culture before being harvested by scraping at various time points (as indicated) and lysed. For the “0 h” time point following trypsin treatment, cells were immediately lysed without being plated.
2.3 Cell Lysis and Immunoblotting

Following the above-indicated treatments, the cells were harvested in their growth medium without trypsin treatment, washed with 1 mL PBS, resuspended in 200 μL lysis buffer (100 mM Tris pH 7.4, 1% (w/v) SDS) containing protease inhibitors (Sigma-Aldrich, St Louis, MO, cat. number P8340), boiled 7 min, and vortexed for 30 s before clearing the lysates by centrifugation. Forty micrograms of total cell lysate were subjected to overnight digestion with 200 units of PNGase F (New England Biolabs, cat. number P0704L) at 37°C, after which proteins were precipitated by incubation with 5 volumes of methanol for 2 h at -70°C. The methanol-precipitated lysates were recovered by centrifugation, air dried at 37°C for 30 min, resuspended in 1X SDS-PAGE sample buffer containing 5 mM DTT, boiled, and analysed by SDS-PAGE (12.5%). The protein gel was transferred onto a PVDF membrane, which was blocked in 5% (w/v) milk in PBS for 1 h before overnight incubation at 4°C with the appropriate primary antibody, and subsequently for 1 h at room temperature with the appropriate HRP-conjugated secondary antibody. The membranes were incubated with homemade ECL buffer and the signal recorded on Denville HyBlot CL films (Metuchen, NJ, cat. number E3018), which were scanned using an Epson 1680 Professional scanner. The intensity of the bands was quantified using Image Studio Lite version 4.0 software (LI-COR Biotechnology, Lincoln, NE).

2.4 Flow Cytometry

Following drug treatment, the cells were harvested in their growth medium without trypptic digestion and washed with 2 mL phosphate buffer saline (PBS) with 3% FBS. The cell pellet was incubated with 0.5 μL mouse anti-PrP Sha31, 1 μL mouse anti-MHC Y3, or 1.5 μg mouse anti-MHC W6/32 for 15 min on ice following brief vortexing. The pellet was washed with 2 mL PBS with 3% FBS and incubated with 0.4 μL Alexa647 goat anti-mouse IgG for 15 min on ice following brief vortexing. The pellet was washed again and fixed in 300 μL 0.5% paraformaldehyde in PBS. The fluorescent signal was recorded on a BD FACSCalibur using CellQuest Pro software (Becton Dickinson, Franklin Lakes, NJ) and analysed using FlowJo software (Tree Star, Ashland, OR).
Table 2-1: List of the selected siRNAs targeting human FKBP transcripts.

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<thead>
<tr>
<th>Target</th>
<th>siRNA</th>
<th>Product code</th>
</tr>
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<td>Stealth Select</td>
<td>HSS176949</td>
</tr>
<tr>
<td>FKBP1B</td>
<td>Stealth Select</td>
<td>HSS176952</td>
</tr>
<tr>
<td>FKBP2</td>
<td>Stealth Select</td>
<td>HSS176989</td>
</tr>
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<td>FKBP4</td>
<td>Stealth Select</td>
<td>HSS177004</td>
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<td>FKBP5</td>
<td>Stealth Select</td>
<td>HSS103709</td>
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<td>FKBP7</td>
<td>Stealth Select</td>
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<td>Stealth Select</td>
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<td>FKBP15</td>
<td>Silencer Select</td>
<td>s23521</td>
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</table>
2.5 Cell Viability

N2a cells were plated in 96-well plates and treated with increasing concentrations of FK506, FK506 analogue, or DMSO and incubated 16 h at 37°C. A 0.1% resazurin dye solution in PBS was then added to each well and the plate was further incubated for 8 h at 37°C, after which fluorescence emission was monitored at 590 nm using an EnSpire® Multimode Plate Reader (Perkin Elmer, Waltham, MA, cat. number 2300-001M).

2.6 Metabolic Labelling and Immunoisolation

N2a cells were transfected with a hamster PrP-encoding plasmid as described previously and allowed to recover for 48 h to express the protein. The cells were then pretreated for 1 h with complete DMEM containing vehicle (DMSO), FK506, and/or bortezomib, as indicated, starved in methionine-free medium for 15 min, and labeled with 150 µCi/mL EasyTag™ L-[35S]-methionine (Perkin Elmer, Waltham, MA, cat. number NEG709A500UC; specific activity >1000 Ci/mmol) for 20 min. The cells were lysed in lysis buffer as described above. The lysates were diluted 5-fold in wash buffer (2% Triton X-100, 100 mM NaCl, 10 mM Hepes pH 7.4) prior to addition of 10 µg 3F4 antibody and rocking for 2 h at 4°C. Thirty µL of packed protein A beads were then added to the lysate-antibody mixture, which was rocked for 1 h at 4°C. The antibody-bead complexes were washed extensively in wash buffer. In the case of PNGase F-digested samples, 250 units of PNGase F were added to the beads and allowed to digest for 3 h at 37°C. All the samples were boiled in SDS-PAGE sample buffer containing 5 mM DTT and analysed by SDS-PAGE (large gel, 12.5%). Following exposure on X-ray film, the resulting autoradiograms were quantified as described previously for western blots.

2.7 FKBP Expression Screen

First, the expression profile of the FKBP in the N2a, HepG2, and U373-MG cell lines was analysed. Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen, Venlo, Netherlands, cat. number 74104) and used in a reverse transcription experiment to yield a cDNA library using the SuperScript VILO cDNA Synthesis Kit (Life Technologies, cat. number 11754-
The resulting cDNA library was used for the selective amplification of each FKBP transcript by PCR with two unique pairs of primers (synthesised by BioBasic, Markham, ON and listed in Table 2-2 and Table 2-3). The PCR program was as follows: 30 s at 95°C, 15 s at 56°C, and 1 min at 72°C using Taq polymerase and its supplied buffer (Life Technologies, cat. number 18038042). The final primer concentration was 0.1 µM each and that of dNTPs (Life technologies, cat. number 18427013) was 200 µM. The amplicons were resolved on 3% (w/v) agarose-TAE gels and detected under UV light.

Second, the levels of the FKBP transcripts were analysed for both steady-state and specific knock down conditions. HepG2 cells were transfected with an FKBP-specific siRNA or a non-targeting negative control as described previously. Total RNA was extracted using the RNeasy Mini Kit and used in a reverse transcription experiment to yield a cDNA library using the SuperScript VILO cDNA Synthesis Kit. The resulting cDNA library was used to selectively amplify by real-time quantitative PCR target transcripts using the TaqMan Gene Expression Master Mix (Life Technologies, cat. number 4369016) on an Applied Biosystems 7500 Standard thermocycler following the manufacturer’s standard protocol. A list of the primers and probes used can be found in Table 2-4. The relative levels of each mRNA were normalised to that of the reference transcript, β-actin, using the delta delta threshold cycle (ΔΔCt) method. Briefly, ΔΔCt = (Ct(target, untreated) – (Ct(reference, untreated)) – (Ct(target, treated) – Ct(reference, treated)). The ratio of the target transcript to the reference transcript is equivalent to 2−ΔΔCt.
Table 2-2: List of primers used for the FKBP expression screen in human cells.

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<th>Target transcript</th>
<th>Pair 1 forward</th>
<th>Pair 1 reverse</th>
<th>Pair 2 forward</th>
<th>Pair 2 reverse</th>
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<td>ACAGGGGAATTTAAGGAAAAG</td>
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<td>AAGACGAGAGTGGCATGTGG</td>
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<td>ACATGAGGCTGAGCTGGTTTC</td>
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All primers are listed 5’ to 3’.
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Table 2-3: List of primers used for the FKBP expression screen in mouse cells.
All primers are listed 5’ to 3’.
Table 2-4: TaqMan primers and probe sets.
The primer pairs and probes used in the TaqMan quantitative PCR assays were obtained from Life Technologies.

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<tr>
<td>β-actin</td>
<td>Hs01060665_g1</td>
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</tbody>
</table>
Chapter 3

3 Inhibition of FKBP function attenuates PrP expression

3.1 Characteristics and kinetics of FK506 treatment

A previous postdoctoral fellow in the Williams group, Dr Pawel Stocki, made the observation that FK506 affects the levels of PrP. Indeed, following a 16 h treatment of the mouse neuroblastoma N2a cell line with 20 μg/mL FK506, a reduction of up to 80% in the levels of all three glycoforms of PrP was observed compared to the DMSO vehicle control (Figure 3-1). This reduction was specific to PrP as the levels of the ER-resident chaperone BiP and cytosolic protein GAPDH were unaffected. In addition, FK506 also caused a downregulation of PrP in the human HepG2 cell line (personal communication from Dr Pawel Stocki).

In order to investigate the possibility that FK506 may act by inhibiting the translation of PrP, a metabolic pulse-labelling experiment was performed on N2a cells (Figure 3-2). Although the total signal intensity of the resulting immunoisolated PrP was unaffected by FK506 treatment, a shift in the banding pattern can be observed. In the case of vehicle control, the predominant species is diglycosylated (2 CHO) with little unglycosylated (0 CHO) PrP, whereas in the FK506-treated sample are roughly equivalent amounts of diglycosylated and unglycosylated PrP. This suggests that the drug affects the maturation of PrP but not the transcription or translation of the Prnp gene.

To further ensure that the FK506-induced decrease in PrP levels was not due to a general cell stress response that may lead to an attenuation of PrP’s translocation into the ER [31, 37], activation of the UPR was assayed for a range of FK506 concentrations. Following overnight treatment with the positive control tunicamycin, a known ER stressor, the spliced Xbp1 mRNA migrated faster on an agarose gel than the unspliced mRNA from vehicle-treated cells (Figure 3-3). None of the concentrations of FK506 used led to the splicing of the Xbp1 mRNA, confirming that FK506 does not activate the UPR. Furthermore, the levels of the stress-inducible chaperone BiP were unaffected by FK506 treatment (Figure 3-1).
Figure 3-1: **FK506 selectively reduces the expression of PrP in cells.**

Mouse N2a cells were treated with FK506 for 16 hours, lysed, and selected proteins detected by immunoblot. *Performed by Dr Pawel Stocki.*

![Figure 3-1](image)

Figure 3-2: **FK506 does not affect the translation of PrP.**

N2a cells transfected with wild-type hamster PrP were labelled with $^{35}$S-methionine for 20 min in the presence of FK506 or vehicle. PrP was specifically immunoisolated from the lysates, resolved by SDS-PAGE, and detected by autoradiography.

![Figure 3-2](image)
Figure 3-3: FK506 does not activate the UPR.

N2a cells were treated for 16 h with DMSO (vehicle control), the indicated concentrations of FK506, or tunicamycin. The splicing state of the Xbp1 mRNA was monitored by PCR amplification of its cDNA with products resolved on a 3% agarose gel. *Performed by Dr Pawel Stocki.*
I then determined the kinetics of the FK506-mediated downregulation of PrP. N2a cells were treated for up to 22 h with FK506 or vehicle and the levels of PrP at various time points were analysed by immunoblot (Figure 3-4). Following quantification, the rate of disappearance of PrP following addition of FK506 to the cells’ growth medium was calculated to be 8.9 h.

As the synthetic rate of PrP was unaffected by FK506 treatment, it is possible that newly synthesised PrP might be unstable. To follow newly synthesised PrP molecules, the FK506 time course experiment was slightly modified. As PrPC is exquisitely sensitive to trypsin treatment, a brief tryptic digestion was performed to remove all cell surface PrP. The cells were then immediately treated with either vehicle or FK506. This experiment allows one to distinguish between de novo synthesised PrP molecules and cell surface material, the latter having been initially digested by trypsin. Samples were lysed at various time points, as indicated, and analysed by immunoblot (Figure 3-5). The initial tryptic digestion was successful as the levels of PrP at the 0 h time point were extremely low. In the case of vehicle-treated cells, PrP expression recovered over time and reached normal levels after roughly 10 h, with PrP’s typical banding pattern being observed. Treatment with FK506 prevented the significant accumulation of any PrP species for the entire time course of the experiment, suggesting that once PrP is synthesised, it is rapidly degraded. To determine the nature of the degradative process, the experiment was repeated in the presence of the proteasome inhibitor bortezomib. This led to the accumulation of a discrete PrP band migrating similarly to deglycosylated PrP (confirmed with PNGase F digestion, data not shown; see also Figure 3-8A, comparing lanes 2 and 4). This implies that although PrP was still being synthesised, it never reached the cell surface and instead was degraded by the proteasome, therefore excluding an increased surface turnover rate scenario. In addition, the FK506-mediated downregulation of PrP could not be prevented with chloroquine, therefore excluding a lysosomal process (personal communication from Dr Pawel Stocki).
**Figure 3-4: Time course of the FK506-mediated PrP downregulation.**

N2a cells were trypsin digested, plated at equal density, allowed to recover for 24 hours, and treated for up to 22 hours with FK506 or vehicle. A) Samples were lysed at different time points and analysed by immunoblot. B) Quantification of the PrP signal normalised to that of GAPDH.
Figure 3-5: FK506 treatment prevents PrP from reaching the cell surface.

N2a cells were digested with trypsin to remove all cell surface PrP at the 0 hour time point. The cells were then allowed to recover for up to 22 h in the presence of either vehicle, FK506, or FK506 and a proteasome inhibitor. At the indicated time points, samples were lysed for immunoblot analysis.
3.2 Mechanism of PrP downregulation

Two mechanisms could account for the degradation of PrP by the proteasome. For PrP to attain its native fold, it may require help from a particular FKBP. The inhibition of this FKBP’s rotamase activity by FK506 would eventually cause the ERAD of PrP (Figure 3-6A). Alternatively, the PPIase activity of an FKBP may be required for the translocation of PrP, for instance by regulating the activity of a translocon channel component through the isomerisation of a proline residue. FK506 treatment would then cause the abortive translocation of PrP (Figure 3-6B). In order to distinguish between these possibilities, two methods were used. First, if FK506 induces the ERAD of PrP, knock down of canonical ERAD components should prevent the degradation of PrP, some of which should then make it through the remainder of the secretory pathway and finally to the cell surface. Given their critical role in ERAD, SEL1L and VCP/p97 were individually knocked down by shRNA in the U373-MG cell line, which was selected for its excellent lentiviral infection properties. The levels of PrP at the cell surface was monitored by flow cytometry. The reduction induced by FK506 treatment was not prevented by depleting components of the ERAD machinery (Figure 3-7A), hinting at an ERAD-independent mechanism. As a positive control, I made use of the viral immunoevasion protein US11 that causes the constitutive ERAD of MHC class I molecules [185]. As shown in Figure 3-7B, the knock down of SEL1L and VCP in US11-expressing U373-MG cells prevented the degradation of MHC class I (Figure 3-7B).
Figure 3-6: Both ERAD and abortive translocation are proteasomal-dependent pathways.

Model showing the translation and translocation of PrP. Due to the inhibition of the PPIase activity of the FKBPs by FK506, two distinct mechanisms could lead to the proteasomal degradation of PrP, with the expected phenotypes described in boxes. A) The PPIase activity of an FKBP may be required for PrP to adopt its native conformation. FK506 would then cause PrP to misfold and be degraded by ERAD. B) The activity of an FKBP may be required for the translocation of PrP. FK506 would then prevent the translocation of PrP, leading to its synthesis and rapid degradation in the cytosol. Adapted from [30].
Figure 3-7: Depletion of the ERAD machinery has no impact on PrP’s FK506-mediated degradation.

A) U373-MG cells were stably transfected with a specific shRNA targeting luciferase (negative control), SEL1L, or VCP. The cells were then treated with FK506 or vehicle and the expression of cell surface PrP was measured by flow cytometry. B) The viral protein US11 causes the constitutive ERAD of MHC class I. U373-MG cells stably expressing US11 were depleted of VCP or SEL1L, and cell surface MHC class I expression was monitored by flow cytometry using the monoclonal antibody W6/32 which selectively recognises natively folded MHC class I. *Performed by Daniel Chapman.*
Second, the presence or absence of the N-terminal signal sequence on PrP accumulated in the presence of FK506 and a proteasome inhibitor can distinguish between ERAD and abortive translocation. In the case of an ERAD process, the signal sequence should be cleaved as PrP would have been translocated into the ER where SP rapidly cleaves off signal peptides from incoming substrates. If PrP fails to successfully translocate, however, the signal sequence would not be removed. The fate of the signal sequence under various conditions was investigated by treating N2a cells with the indicated drug and analysing the lysates by western blot (Figure 3-8A). Lane 1 shows a normal steady-state PrP banding pattern, which can be collapsed into a single band by removing the glycans from the protein with PNGase F as shown in lane 3. The species in lanes 1 and 3, being steady-state, fully-processed PrP, is expected to have had its signal sequence cleaved. This was confirmed by expressing a signal sequence-deleted mutant construct (lane 5, bottom band, which comigrated with the species in lane 3; note that the upper two bands in lane 5 are due to antibody crossreactivity with the endogenous PrP, which still contains its signal sequence and glycans). Treatment with FK506 and the proteasome inhibitor lactacystin led to the accumulation of an unglycosylated species (comparing lanes 2 and 4, untreated and PNGase F-digested respectively). This species migrated more slowly than the fully processed but deglycosylated species (lane 3), suggesting that the signal sequence was still present in the FK506-treated samples and making a strong case in favour of an abortive translocation mechanism.

Given that up to 20% of PrP molecules fail under normal conditions to translocate into the ER, PrP’s signal sequence can be considered weak. The signal sequence of wild-type hamster PrP (wt-PrP) was swapped with that of either osteopontin (Opn-PrP) or prolactin (Prl-PrP). Importantly, both Opn-PrP and Prl-PrP have been shown to be translocated with a higher efficiency than wt-PrP and their translocation is not attenuated by ER stress [31, 95]. A stronger signal may bypass the FK506-mediated degradation of PrP only if translocation itself is affected by the drug, but should have no impact in the case of an ERAD process. Therefore, the signal sequence’s influence on the efficiency of PrP translocation in the presence of FK506 was examined. N2a cells expressing one of these three constructs were treated with vehicle, FK506, or FK506 and the proteasome inhibitor bortezomib, and analysed by immunoblot (Figure 3-8B). As expected, wt-PrP was dramatically affected by FK506 treatment, and accumulation of a signal sequence-containing species can be seen with proteasome inhibition. However, both Opn- and Prl-PrP were resistant to FK506 treatment. This demonstrates that FK506 acts by exacerbating an intrinsic
weakness of PrP’s signal sequence, resulting in abortive translocation, and that this effect can be overcome by a stronger signal sequence.

3.3 Effects of FK506 on PrP\textsuperscript{Sc} propagation

PrP\textsuperscript{C} reduction is an important therapeutic candidate for the treatment of prion diseases. As FK506 attenuates the expression of PrP\textsuperscript{C}, its abilities to impede PrP\textsuperscript{Sc} propagation in cell models was examined by our collaborators Dr David Westaway and Dr Charles Mays III at the University of Alberta. Two mouse cell lines chronically infected with scrapie prions were treated with several concentrations of FK506. Lysates were either directly analysed by western blot (total PrP) or digested with proteinase K (PK\textsuperscript{res}), as PrP\textsuperscript{Sc} is protease resistant, prior to analysis. As shown in Figure 3-9, treatment with FK506 was able to induce a significant reduction in the amount of both total and PK\textsuperscript{res} PrP in only six days. In ScN2a cells, which are infected with the RML prion strain [186], the levels of PK\textsuperscript{res} were reduced to roughly a third of the control with 20 μg/mL FK506. In SMB cells, infected with the Chandler strain [187, 188], as little as 10% of PK\textsuperscript{res} prions remained at the end of the treatment. This result demonstrates the potential of FK506 as an anti-prion disorder agent.
Figure 3-8: The important role of the signal sequence in the fate of PrP.

Mouse N2a cells were treated with a combination of vehicle, FK506, and proteasome inhibitors prior to analysis by immunoblot. A) Cells expressing endogenous PrP (lanes 1 through 4) or a construct lacking its signal sequence (lane 5) were subject to various treatments. See text for details. 

Performed by Dr Pawel Stocki.

B) Hamster PrP constructs differing only in their signal sequence (either wild-type, osteopontin, or prolactin) were transfected in N2a cells subjected to various treatments and specifically detected by immunoblot. The chimeric constructs were resistant to FK506-mediated degradation.
Figure 3-9: FK506 decreases the amounts of total and proteinase K-resistant PrP.

A) ScN2a and SMB cells were treated for six days with up to 20 µg/mL FK506 or vehicle. The cell lysates were either directly analysed by immunoblot, or digested with proteinase K prior to analysis. B) Quantification of the results shown in A). Performed by Dr Charles Mays and Dr David Westaway, University of Alberta.
3.4 Novel FK506 analogues

Due to its immunosuppressive properties, FK506 is not an ideal candidate as a potential therapeutic agent for treatment of prion diseases. In order to investigate a possible cure for prion-mediated diseases, five novel FK506 analogues with reduced immunosuppressive properties were obtained from Biotica Technology Ltd for characterisation (namely BC345, BC354, BC358, BC368, and BC409). Their reduced immunosuppressive properties stem from modifications in the effector domain of the FK506 parent molecule (see Figure 1-7). By affecting the calcineurin-interacting moiety of FK506 but not its FKBP1A-binding capacity, the analogues are expected to retain their action on FKBP1A (and other FKBPs), but lose the inhibition of calcineurin that is responsible for immunosuppression. The immunosuppressive potential of each analogue was monitored in Jurkat cells by their inhibition of IL-2 production, which is upregulated by activation of the NFAT pathway. The measured IC$_{50}$ for each analogue is listed in Table 3-1, with each analogue’s IC$_{50}$ being roughly two- to tenfold higher than the IC$_{50}$ of FK506.

The toxicity of FK506 and its novel analogues was measured over a broad range of concentrations using a resazurin viability assay. Furthermore, the ability of the novel analogues to reduce PrP expression was measured by flow cytometry in comparison to MHC class I, a widely expressed membrane protein. An ideal compound would, at its optimal concentration, have no effect on cell viability and MHC class I surface levels while dramatically reducing the levels of PrP. With the exception of BC409, all the analogues were at least as efficacious as FK506 at their optimal concentration (empirically determined and listed in Table 3-1), with a large decrease in the levels of PrP but maintaining a high MHC class I expression and displaying low toxicity (Figure 3-10).

The effects of FK506 and analogues on PrP were also investigated by immunoblotting following a 16 h treatment at each compound’s optimal concentration (Figure 3-11). ER-resident proteins were used as controls to ensure specificity towards PrP. Most of the analogues induced a specific decrease in the steady-state levels of PrP. In this particular replicate, BC354 did not induce much of a decrease in the levels of PrP, although it did in others. The downregulation of PrP could be prevented by proteasome inhibition using lactacystin. Just as in the case of FK506 treatment, a small increase in the molecular weight of the PrP species targeted for degradation in proteasome-inhibited cells was seen with all the analogues; this species is likely the signal uncleaved form of
PrP. In addition, the levels of several control proteins were unaffected by FK506 or analogues, further validating the specificity of these compounds.

In order to ensure that the effect of the analogues was not due to activation of the UPR, XBP1 splicing assays were performed as for FK506. As shown in Figure 3-12, a doublet is present in the vehicle control as well as FK506 and analogues. However, this doublet was not due to the splicing of the XBP1 mRNA as it was previously shown that DMSO and FK506 do not activate the UPR (Figure 3-3). In addition, a triplet can be observed in the tunicamycin positive control, with the lower band representing the spliced XBP1 mRNA. Moreover, BiP levels were unaffected by the analogues (Figure 3-11). This demonstrates that the analogues do not trigger the UPR at their optimal concentration.
<table>
<thead>
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<th>Compound</th>
<th>IL-2 pathway IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
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Table 3-1: IC<sub>50</sub> and optimal concentration of FK506 and analogues

Serial dilutions of the compounds were used to determine the concentration at which IL-2 production was inhibited by 50% in Jurkat cells. Separately, the optimal concentration for each compound was determined by selecting the concentration that had the strongest effect on attenuating PrP levels while not impacting cell viability or MHC class I expression. The IC<sub>50</sub> values were provided by Dr Steven Moss, Director of Natural Products Chemistry, Biotica Technology Ltd.
Figure 3-10: Novel FK506 analogues behave similarly to FK506.

Following a 16 h treatment at each compound’s optimal concentration, N2a cell viability was determined using a resazurin assay, and the levels of PrP and MHC class I were determined by flow cytometry and normalised to the DMSO vehicle. Quantification of at least three replicates for each sample. Error bars represent the standard deviation.
Figure 3-11: The effects of FK506 and analogues on PrP and control proteins. FK506 and its novel analogues specifically reduce the levels of PrP in N2a cells compared to the vehicle control. To allow for an easier comparison between the samples, the lysates were PNGase F-digested.

Figure 3-12: FK506 analogues do not induce splicing of the XBP1 mRNA. N2a cells were treated for 16 h with vehicle, 10 µg/mL tunicamycin, or each analogue at its optimal concentration. The splicing state of the Xbp1 mRNA was monitored by PCR amplification of its cDNA and the resolving of the products on a 3% agarose gel.
Chapter 4

4 Identifying targets of FK506 involved in PrP biogenesis

4.1 Expression screen and knock down validation

As FK506 binds and inhibits the PPIase activity of all the FKBPs, its effect on PrP’s biogenesis may be due to the catalytic activity of one or more FKBPs being inhibited. It was hypothesised that the FKB (or FKBPs) whose inactivation triggers the abortive translocation of PrP would be the same in the three cell lines (HepG2, U373-MG, and N2a) in which the FK506 effect was validated. Therefore, only the FKBPs expressed in all three cell lines could be involved in this phenomenon. In order to identify whether or not each FKBP was expressed in each cell line, an expression screen was performed. Any gene that is expressed would necessarily have mRNA transcripts that can be detected by PCR following reverse transcription into cDNA. To reduce the false negative rate, two pairs of individual primers specific for each FKBP transcript were designed to span exon-intron boundaries (see Table 2-2) and used in PCR amplifications of reverse-transcribed cDNA from each cell line. The resulting amplicons were resolved on agarose gels and imaged under UV light (Figure 4-1). In all three cell lines, an amplicon of the expected size can be observed for each FKBP. Unfortunately, since every single FKBP is expressed in each of the cell lines, this approach failed to narrow the list of candidate FKBPs that may participate in the translocation of PrP.

It was then hypothesised that the FKB involved in the translocation of PrP may be expressed at higher levels than other FKBPs. The relative mRNA levels of the FKBPs was measured in HepG2 cells by quantitative PCR (qPCR) and normalised to the levels of the β-actin mRNA to allow for a rough comparison between samples (Figure 4-2). The HepG2 cell line was selected for its low cell lethality during knock down experiments. Two FKBPs stood out: the prototypical FKBP1A and the ER-resident chaperone FKBP10, which has been involved in the maturation of several secretory proteins.
Figure 4-1: FKBP expression screen.

Total RNA was extracted from each cell line and the mRNA reverse transcribed into a cDNA library, which was used to selectively amplify each target gene’s corresponding transcripts. The resulting amplicons were resolved on 3% agarose gels and detected under UV light. The panels were produced by the assembly of different gels or exposure times to ensure optimal visibility of the PCR products. The individual images were carefully aligned with the molecular weight markers in each case. A) N2a. B) HepG2. C) U373-MG.
Figure 4-2: Estimated relative mRNA levels of the cytosolic and ER FKBP5s.
The levels of specific transcripts were measured by qPCR and normalised to the β-actin transcript signal for comparison between samples. Quantification of at least two replicates. The error bars represent the standard error of the mean.
An elegant and thorough way to identify the FKBP whose inactivation is responsible for the FK506 effect on PrP was to perform RNAi experiments. Due to the high cost of siRNAs, two FKBPs were excluded from the remainder of this screen. FKBP3 and FKBP6, both nuclear proteins, were not expected to have a significant impact on the translocation of PrP into the ER. Sets of three unique Stealth siRNAs targeting each of the other twelve FKBPs were obtained from Life Technologies, and the knock down efficiency was validated by quantitative real-time PCR (qPCR). As none of the Stealth siRNAs targeting the transcripts of FKBP11 and FKBP15 resulted in a decrease of at least 60% (not shown), sets of different siRNAs (Silencer Select) were obtained for these two targets. Figure 4-3 shows the remaining amount of mRNA following knock down with the most potent siRNA in each case.

4.2 Depletion of certain FKBPs affects PrP expression

It was expected that depletion of the FKBP involved in PrP’s translocation should phenocopy the FK506 effect on PrP. Therefore, the siRNA giving the highest knock down for each target was selected to investigate the effect of a particular FKBP’s depletion on PrP expression. Individual knock down experiments were performed, and the levels of PrP assayed by immunoblot. Unfortunately, none of the individual knock downs consistently decreased the expression of PrP in a significant manner (Figure 4-4 and Figure 4-5). The knock down of FKBP7 was particularly toxic, which may explain the lower expression levels of PrP in these samples. The individual depletion of FKBP1A, 1B, 2, 4, 8, 11, and 14 had no significant impact on cell viability and PrP levels. Surprisingly, there was an increase in the expression of PrP in cells depleted of FKBP9 and FKBP15.
Figure 4-3: Efficiency of knock down of the cytosolic and ER-resident FKBPs.

Following two rounds of knock down over the course of six days, the total RNA was isolated from HepG2 cells in order to quantify by two-step qPCR the amount of specific mRNA remaining, normalised to the β-actin mRNA levels and compared to negative control cells. The one or two characters refer to the FKBP targeted. The siRNAs used were Stealth Select for all the FKBPs barring FKBP11 and FKBP15, for which Silencer Select siRNAs were used. NC, negative control. Quantification of at least three replicates. The error bars represent the standard error of the mean.
Figure 4-4: The individual depletion of FKBPs does not affect PrP levels in HepG2 cells.
HepG2 cells were depleted of the indicated FKBP by siRNA-mediated knock down. The lysates were PNGase F-digested to allow for an easier quantification and analysed by western blot.

Figure 4-5: Quantification of normalised PrP levels in FKBP-depleted cells.
Quantification of at least three western blot replicates, normalised to GAPDH for each sample. The error bars represent the standard error of the mean. See text for details. Quantification of at least three replicates. The error bars represent the standard error of the mean. The majority (13 out of 18) of the FKBP10 replicates were performed by Seo Jung Hong.
Given that two functionally or structurally similar FKBP\(s\) might have overlapping functions and therefore compensate for one another with regards to PrP, the knock down of a single FKBP may not be sufficient to obtain a phenotype. Therefore, several pairs of FKBP\(s\) were identified as having possible compensatory effects given their structure, function, and subcellular localisation. FKBP1A and FKBP1B are structurally very similar and interact with the ryanodine receptor for Ca\(^{2+}\) regulation. FKBP2 and FKBP11 both are secretory pathway proteins. FKBP4 and FKBP5 have a role in steroid hormone trafficking. FKBP7 and FKBP14 are ER-resident chaperones. FKBP8 and FKBP15 are cytosolic and neuron-expressed. FKBP9 and FKBP10 are ER-resident chaperones involved notably in collagen and tropoelastin folding. These pairs were knocked down in tandem and analysed by western blot (Figure 4-6). However, unlike most of the single knock downs, the double knock downs were generally very toxic to the cells, suggesting that there indeed are compensatory effects for each of the pairs. Gentler knock down conditions were used by increasing the initial cell density, removing the siRNA complexes after four hours, and increasing the proportion of serum in the medium. However, despite a noticeable improvement of cell viability, these conditions reduced the efficiency of knock down as assayed by FKBP10 western blots (not shown). Although the double knock downs of FKBP1A and 1B, 4 and 5, and 9 and 10 appeared to downregulate the expression of PrP as seen in Figure 4-6, this may be due to a decrease in the general metabolic activity of the cell due to the toxicity observed with these three pairs. The tandem depletions of FKBP2 and 11, 7 and 14, and 8 and 15 were less toxic, but had no impact on the levels of PrP. No conclusive evidence could therefore be obtained with regards to the levels of PrP in the case of double FKBP knockdowns in HepG2.
Figure 4-6: Double FKBP knock downs do not affect PrP levels in HepG2 cells.
HepG2 cells were depleted of the indicated FKBP or FKBP s and analysed by western blot. Most double knock downs were quite toxic (especially 1A and 1B, 4 and 5, and 9 and 10) and thus not reliable.
A different approach was used in the mouse N2a cell line. As FKBP10 has been involved in secretory protein folding in the ER lumen [154] and is abundantly expressed (Figure 4-2), it was posited that its depletion may influence the maturation of PrP. Following its siRNA-mediated depletion, the cells were analysed by western blot. Figure 4-7 shows that the depletion of FKBP10 in N2a cells dramatically decreased the expression of PrP\(^\text{C}\) (compare to FK506 treatment in lane 1). In addition, FKBP10 depletion specifically affected PrP as it did not influence the levels of BiP, GAPDH, and the GPI-anchored cell surface protein CD90.

In an attempt to elucidate the mechanism of action, N2a cells depleted of FKBP10 were treated with the proteasome inhibitor lactacystin and analysed by western blot. This led to the accumulation of an unglycosylated PrP species (Figure 4-8), confirming that PrP undergoes proteasomal degradation in the context of FKBP10 depletion. Interestingly, following PNGase F digestion, there was no difference in the mobility of the PrP species that accumulated in control or FKBP10-depleted, lactacystin-treated cells. This suggests that in the case of FKBP10 knock down, PrP’s signal sequence is cleaved, which implies that PrP’s translocation into the ER was not affected. In addition, the importance of the strength of the signal sequence with regards to FKBP10 depletion was assayed. As Opn-PrP and Prl-PrP contain stronger signal sequences than wt-PrP, they are more efficiently translocated and are not subject to translocation attenuation during ER stress. Following FKBP10 knock down, N2a cells were transfected with one of these three PrP constructs and analysed by immunoblot (Figure 4-9). None of the constructs resisted the degradation induced by the knock down of FKBP10, further supporting the view that the translocation of PrP was unaffected.

Despite the downregulation of PrP induced by FK506 treatment and FKBP10 depletion, the underlying mechanism appears to differ. Whereas FK506 causes the abortive translocation of PrP, FKBP10 depletion affects a later stage of PrP biogenesis, following translocation into the ER lumen.
Figure 4-7: The knock down of FKBP10 in N2a cells decreases PrP levels.
Following the siRNA-mediated depletion of FKBP10 in N2a cells, the lysates were analysed by western blot for the indicated proteins. *Performed by Dr Pawel Stocki.*

**Figure 4-8: FKBP10 depletion in N2a cells triggers the degradation of PrP.**
N2a cells knocked down for FKBP10 were PNGase F-digested and analysed by immunoblot. *Performed by Dr Pawel Stocki.*
Figure 4-9: FKBP10 depletion affects PrP independently of its signal sequence.
N2a cells depleted of FKBP10 were transfected with the indicated construct, digested with PNGase F, and analysed by immunoblot.
4.3 Effects of FKBP10 knock down on PrP$_{Sc}$ propagation

Given that the effects of FKBP10 depletion in N2a cells led to a dramatic decrease in the levels of PrP$^C$, a logical follow-up was to investigate whether there was an impact on PrP$_{Sc}$ propagation in chronically infected cell models. FKBP10 was knocked down in the chronically infected ScN2a and SMB cells. Lysates were then either directly analysed (total PrP) or digested with proteinase K (PK$_{res}$, or PrP$_{Sc}$), as shown in Figure 4-10. Despite a rather modest decrease in total PrP, the level of PrP$_{Sc}$ was strongly depressed. This is consistent with the role of PrP$^C$ in propagating the infectious prion. This finding demonstrates that FKBP10 is a potential new target in the treatment of prion diseases.
Figure 4-10: FKBP10 depletion decreases the amount of both total and PK-resistant PrP.
ScN2a cells were transfected with negative control or FKBP10 siRNA. The cell lysates were either directly analysed by immunoblot, or digested with proteinase K prior to analysis. The symbols # and ## refer to the C2 and C1 fragments, respectively. Performed by Dr Charles Mays and Dr David Westaway, University of Alberta.
Chapter 5

5 Discussion and Future Directions

5.1 Summary

In this thesis, I have shown that the immunosuppressive drug FK506 induces the downregulation of PrP\textsuperscript{C} expression. The effect of FK506 was not due to a generalised ER stress response that would have caused a rerouting of PrP into the cytosol for degradation. The PrP species that accumulated following FK506 treatment in proteasome-inhibited cells was found to contain its ER-targeting signal sequence and to be unglycosylated. Additionally, replacing the signal sequence of PrP with the more efficient signal sequences of prolactin and osteopontin was sufficient to abrogate the effect of FK506 on PrP. Therefore, FK506 induces the abortive translocation of PrP by exploiting the inefficiency of its signal sequence. Nonetheless, PrP is fully synthesised in the cytosol but rapidly degraded by the UPS. This eventually leads to the depletion of cell surface PrP\textsuperscript{C} molecules by attrition.

Unfortunately, the systematic depletion of the targets of FK506, the FKBP family, in the human HepG2 cell line did not reveal the identity of the FKBP involved in PrP’s translocation. However, in the mouse N2a cell line, the ER-resident FKBP10 was shown to be implicated in the maturation of PrP. Interestingly, FKBP10 depletion did not affect the translocation of PrP, which was processed in the ER by addition of glycan chains and cleavage of its signal sequence. In addition, the chimeric PrP constructs were all susceptible to the knock down of FKBP10, demonstrating that the nature of the signal sequence is irrelevant to FKBP10’s role in the biogenesis of PrP. In FKBP10-depleted cells, PrP was degraded by both the proteasomal and lysosomal pathways (personal communication from Seo Jung Hong). Although the exact mechanism of PrP degradation in the context of FKBP10 depletion is not known, FKBP10 may be involved in the folding and maturation of PrP in the ER. Its depletion may target at least a portion of the newly synthesised PrP pool for ERAD. Moreover, both FK506 treatment and FKBP10 depletion were shown to dramatically decrease the levels of PrP\textsuperscript{Sc} in two cell models of prion disorders. Finally, in an effort to study a more practical approach to treat prion-mediated diseases, five novel FK506 analogues with reduced immunosuppressive properties were tested for their effect on PrP\textsuperscript{C}, cell toxicity, and the control protein MHC class I. Four of these five analogues
were shown to be at least as efficacious as FK506, and a few at a lower concentration than their parent molecule.

5.2 Discussion

As scrapie infection and propagation are dependent on the expression of PrPC, even a partial reduction in the levels of PrPC can ameliorate the outcome of prion disorders [14]. However, the exact mechanism responsible for the FK506-mediated translational attenuation of PrP is still unknown. The signal sequence of PrP does not contain a single proline residue, and translation is paused by SRP shortly after the emergence of PrP’s signal sequence from the ribosome exit tunnel. Therefore, the effect of FK506 on PrP’s translocation cannot be due to inhibiting the isomerisation of a proline residue in PrP as it could only occur upon entering the ER lumen.

The translocation of PrP and other proteins with weak signal sequences relies on TRAM, TRAP, and luminal components of the ER, including BiP [33]. FK506 may inhibit the physiological function of a chaperone such as BiP, in light of the role of FKBP7’s rotamase activity in controlling BiP’s ATPase activity by isomerisation of a regulatory proline residue [146], therefore prohibiting BiP from helping PrP translocate. This involvement of BiP in the translocation of proteins with weak signal sequences might also explain the toxicity I observed upon FKBP7 depletion (BiP could be the chaperone represented in Figure 3-6A). Moreover, as Opn-PrP and Prl-PrP do not display such a dependence on ER components like BiP [31], they are not affected by FK506 treatment. Additionally, the exact role of proteins such as TRAM and TRAP in the translocation of secretory proteins with weak signal sequences remains unclear. A regulatory proline residue in TRAM or TRAP may require the activity of an FKBP to achieve an isomerisation state that would allow them to interact with their substrates. Interestingly, PDI contains a cis-proline close to its active site [102, 189]. The isomerisation state of this proline residue may be regulated by an ER-resident FKBP. The activity of PDI in disulphide bond formation or reduction may be required by a translocation accessory protein whose role would indirectly be inhibited by FK506, causing the abortive translocation of PrP.

In addition, an attempt was made to identify the FKBP(s) involved in the translocation of PrP. In the human HepG2 cell line, none of the individual knock downs resulted in a significant
reduction in the expression of PrP and double knock downs were quite toxic. However, depletion of FKBP10 in the mouse N2a cell line induced the downregulation of PrP. The differences between the human and the mouse cell lines regarding PrP downregulation in the context of FKBP10 depletion could be explained by a different expression profile of the FKBPs between mouse and humans. For instance, humans may express enough of the highly related FKBP9 to compensate for the depletion of FKBP10, whereas mice may not. This would further explain the neonatal lethality of FKBP10−/− mice [154]. In contrast, humans with a mutation in FKBP10 or not expressing FKBP10 at all are viable, although they suffer from bone diseases such as osteogenesis imperfecta [155].

Critical differences were observed between FK506 treatment in N2a, HepG2, and U373-MG cells and FKBP10 depletion in N2a cells. In the presence of a proteasome inhibitor, the PrP species that accumulated with FK506 treatment still contained its signal sequence and was not glycosylated, suggesting that PrP did not enter the ER lumen. However, in the case of FKBP10 depletion, the accumulating PrP species had its signal sequence cleaved. In addition, the degradation of PrP seemed to be caused by both the proteasomal and lysosomal pathways (personal communication from Seo Jung Hong). Moreover, the PrP species that accumulated in chloroquine-treated N2a cells was glycosylated, indicating that it must have first transited by the ER for glycosylation to occur. These disparities demonstrate that the depletion of FKBP10 acts on PrP by a different mechanism in N2a cells than FK506 treatment.

Several possibilities may explain the effect of FKBP10 depletion on PrP. First, it is important to appreciate that the degradation of PrP is not due to the lack of FKBP10’s PPIase activity. Otherwise, FK506 treatment would have caused the degradation of Opn-PrP and Prl-PrP, although without affecting their translocation. This implies that the chaperone activity or simply the physical presence of FKBP10 is required for the ER maturation of PrP. This scenario is supported by the FKBP10 chaperoning requirement of secretory proteins such as collagen and elastin [143] as well as glucocerebrosidase [190], interactions that are not disrupted by FK506 treatment. Alternatively, FKBP10 may be indirectly involved in PrP’s maturation as a part of a large protein network involved in general ER folding. For instance, several FKBPs such as FKBP2, FKBP7, and FKBP10 interact respectively with PDI members ERp57, ERp29, and ERp19 in the ER lumen. It is not inconceivable that, for instance, FKBP10 depletion would prevent ERp19 from mediating the formation of the appropriate disulphide bond in PrP, which would not be sufficiently
stable to allow for its export along the remainder of the secretory pathway and instead be degraded by ERAD. Moreover, ERp57 regulates the levels of PrP [105]. A PrP expression-regulating network could potentially consist of FKBP10 and PDI members, such as ERp19 and ERp57. The depletion (but not inhibition) of FKBP10 would disrupt this network, preventing the correct disulphide bond to be formed in PrP. The latter would likely misfold given the importance of the bond for the stability of PrP’s globular domain [11], and possibly be degraded by ERAD.

As most of our work focused on the downregulation of PrPC, the effects of FK506 treatment and FKBP10 depletion on PrPSc were verified in cell models of prion disorders by our collaborators in Dr David Westaway’s group at the University of Alberta. Following 6 d of FK506 treatment in the ScN2a and SMB cell lines, a profound reduction in the levels of PrPSc was observed by western blot (Figure 3-9). Interestingly, there was a stronger depression of PrPSc than total PrP, which highlights the importance of PrPC in prion infection and propagation. Similarly, after the depletion of FKBP10 in the same cell lines, the amount of total and PrPSc was dramatically reduced (Figure 4-10). The cause of the reduction in PrPSc is the sum of two phenomena. First, both FK506 treatment and FKBP10 depletion attenuate the delivery of new PrPC molecules to the cell surface, therefore greatly reducing the amounts of available substrate to be converted by PrPSc. Second, scrapie prions are slowly degraded in vivo by an unknown mechanism. In combination with a reduction in the substrate levels, this leads to a drastic decrease in the level of PrPSc over time.

Five novel FK506 analogues with reduced immunosuppressive properties were tested in cells for their effect on PrPC. Four of these showed promising results as they dramatically affected PrPC expression without any obvious adverse effects such as cell toxicity. Unfortunately, the amounts of these analogues made available to us were much too low to perform an animal study. In addition, since we obtained these analogues, Biotica Technology Ltd has been acquired by a new group that is not interested in further pursuing this study. Moreover, the modifications performed on the parent FK506 molecule to design these analogues are proprietary and unknown to us. However, it is possible to design other novel analogues with modification in the effector domain of the FK506 parent molecule. Extensive structure-activity relationship analysis may allow for the discovery of a non-immunosuppressive molecule that would retain FK506’s effect on PrP. Such a class of compounds would represent a better therapeutic avenue than FK506 for the treatment of prion disorders.
Although this is not the first report of the effect of FK506 on PrP, certain findings presented here contradict those of others. One group has shown that FK506 prolongs survival of scrapie-infected mice when administered 20 days post inoculation [177]. However, they concluded that the downregulation in PrP was due to autophagy as the levels of autophagy-related proteins such as LC3-II and ATG7 were increased in FK506-treated cells. It is possible that PrPSc may be slowly degraded by the autolysosome in infected cells, but it is not the main pathway causing the marked decrease in total PrP. Indeed, we have shown that FK506 does not increase the turnover rate of surface PrP (Figure 3-5) and that chloroquine, a lysosome inhibitor, could not rescue the FK506-mediated degradation of PrP (personal communication from Dr Pawel Stocki). Additionally, another group has validated in mice the effect of FK506 on prion disorders [191]. They have found that FK506 extends the survival of infected animals and reduces neurodegeneration, although they did not find a reduction in the levels of PrPSc in the brains of the animals. However, treatment was started only once symptoms appeared, which is likely too late to reap the full benefits of FK506. Furthermore, they determined the mechanism of action of FK506 to be caused by inhibition of the calcineurin pathway. As a negative control, they used rapamycin, which is an immunosuppressant that does not inhibit calcineurin but rather mTOR. Their conclusions would have been dramatically changed had they used CsA as a control, as CsA also inhibits calcineurin. In our hands, CsA did not affect the levels of PrPC (personal communication from Dr Pawel Stocki), demonstrating that the effect of FK506 is not caused by inhibiting calcineurin. Finally, a third group has validated FK506 as an anti-prion agent by high throughput screening [176]. However, they only concluded that FK506 caused a reduction in the expression of PrPC by a nontranscriptional mechanism and did not propose a more detailed mechanism.

5.3 Future Directions

The identity of the FKBP involved in the translocation of PrP has not yet been discovered. In order to elucidate this mystery, the effect of FK506 should first be validated in several other cell lines. It is likely that at least a few FKBP s would not be expressed in all the selected cell lines, reducing the number of candidate FKBP s. Additionally, it is known that the FK506-mediated reduction in PrP is due to the inhibition of the PPIase activity of an FKBP. Cells could be systematically depleted of each candidate FKBP and transfected with an siRNA-resistant,
catalytically inactive construct encoding the same FKBP. Western blot analysis of the lysates would reveal the identity of the FKBP involved in PrP’s translocation as at least one such experiment should phenocopy the effect of FK506. Given the minimal toxicity observed during FK506 treatment, this approach is expected to be less toxic than the individual depletion of the FKBP family members. However, compensatory effects between related FKBPVs may prevent a phenotype from being observed. These experiments would have to be performed either in numerous cell lines to reduce the likelihood of redundant effects, or in a cell line with less compensatory effects than HepG2 such as N2a.

To determine whether FKBP10 depletion in N2a cells causes the misfolding of PrP, the folding state of PrP in FKBP10-depleted cells may be monitored by detergent solubility assays. If misfolded, the solubility of PrP would be expected to be reduced as more hydrophobic patches would be exposed. In addition, the involvement of FKBP10 in the formation of PrP’s disulphide bond could be assayed. In control conditions, with the disulphide bond properly formed, PrP is expected to have a faster electrophoretic mobility in a reducing SDS-PAGE than in a non-reducing one. However, if FKBP10 has a role in the formation of PrP’s disulphide bond, the depletion of FKBP10 would prevent the formation of PrP’s disulphide. In this case, the mobility of PrP is not expected to change, whether the gel is reducing or not.

It would also be interesting to analyse the expression of PrP in patients receiving FK506, as in the case of organ transplants. Depending on the plasma concentrations required to induce immunosuppression and PrP downregulation, it is possible that these patients may express lower levels of PrP than the general population. Similarly, FKBP10−/− humans may also express lower levels of PrP, although FKBP9 may compensate in that regard.

The effect of FK506 on scrapie progression has already been tested in mouse studies [176, 177]. However, in both cases treatment was started weeks after scrapie infection, which may be too late for FK506 to prove efficacious. Initiating treatment earlier or even prior to infection may have a greater impact on the survival and degeneration of the animals. In addition, testing higher doses of FK506 as well as analogues with reduced immunosuppressive properties in animal models may yield more promising results.

Given the differences in the expression profile of the FKBPVs between different organisms, FKBP10 may be a more relevant target in the treatment of TSEs in cervids than in humans. Indeed,
chronic wasting disease is widespread among wild deer, elk, and other ruminants in the Canadian Prairies and American Midwest [192]. This is a concern as there is a risk of transmission to domesticated animals destined for human consumption. It is conceivable that the depletion of FKBP10 may prevent the spread of scrapie prion in cervids, as it does in mouse cell lines. Screening for small molecules capable of preventing FKBP10’s role in PrP biogenesis in cervid cell lines may reveal a profound effect on the expression of PrP.
Bibliography


