INVESTIGATIONS INTO THE SIGNIFICANCE OF
THE EVOLUTIONARY DESCENT OF PRION GENES
FROM ANCESTRAL ZIP TRANSPORTERS

by

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A thesis submitted in conformity with the requirements
for the degree of Master of Science
Graduate Department of Laboratory Medicine and Pathobiology
University of Toronto

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Investigations into the Significance of the Evolutionary Descent of Prion Genes from Ancestral ZIP Transporters

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Master of Science

Department of Laboratory Medicine and Pathobiology

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Abstract

The conformational conversion of the cellular prion protein (PrP\textsuperscript{C}) to PrP scrapie (PrP\textsuperscript{Sc}) is a hallmark of prion diseases [1]. The cellular role of PrP and the mechanism of PrP\textsuperscript{Sc} neurotoxicity remain largely elusive. Therefore, the identification of new prion-like proteins can assist in revealing the function of PrP. A recent study identified a sub-branch of ZIP (Zrt-, Irt-like protein) metal transporters, including ZIP5, ZIP6 and ZIP10, to be evolutionarily related to PrP. This thesis attempts to understand the functional relevance of this relationship between PrP and ZIP transporters with regard to PrP pathobiology. Preliminary observations indicated that PrP, ZIP6 and ZIP10 underwent endoproteolysis in scrapie-infected mouse brains. PrP and ZIP10 processing mimicked the proteolysis which occurs in cell culture during zinc-deficient conditions, suggesting that scrapie infection may be associated with zinc deficiency. More work is needed to uncover whether ZIPS can contribute to the propagation of prion diseases.
Acknowledgements

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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Aβ</td>
<td>Amyloid β</td>
</tr>
<tr>
<td>ADAM</td>
<td>A disintegrin and metalloproteinase</td>
</tr>
<tr>
<td>ADAMTS</td>
<td>ADAM with thrombospondin motif</td>
</tr>
<tr>
<td>AE</td>
<td>Acrodermatitis enteropathica</td>
</tr>
<tr>
<td>Akt</td>
<td>RAC-alpha serine/threonine-protein kinase</td>
</tr>
<tr>
<td>ALLN</td>
<td>N-acetyl-leucinal-leucinal-norleucinal</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>AP-2</td>
<td>Activator protein 2</td>
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<tr>
<td>Apaf-1</td>
<td>Apoptotic protease-activating factor-1</td>
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<tr>
<td>ATM</td>
<td>Ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>Atox1</td>
<td>Antioxidant protein 1</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATP7A</td>
<td>ATPase, Cu$^{2+}$ transporting, alpha polypeptide</td>
</tr>
<tr>
<td>ATP7B</td>
<td>ATPase, Cu$^{2+}$ transporting, beta polypeptide</td>
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<tr>
<td>Bax</td>
<td>Bcl-2-associated X</td>
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<td>BCA</td>
<td>Bicinchoninic acid</td>
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<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>Bcl-xL</td>
<td>B-cell lymphoma-extra large</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BSE</td>
<td>Bovine spongiform encephalopathy</td>
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<tr>
<td>CC1</td>
<td>Charged cluster 1</td>
</tr>
<tr>
<td>CC2</td>
<td>Charged cluster 2</td>
</tr>
<tr>
<td>CDF</td>
<td>Cation diffusion facilitator</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CDP</td>
<td>Chronic demyelinating polyneuropathy</td>
</tr>
<tr>
<td>CFC</td>
<td>Cysteine-flanked core</td>
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<td>CHO</td>
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<td>CJD</td>
<td>Creutzfeldt–Jakob disease</td>
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<td>CNS</td>
<td>Central nervous system</td>
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<td>CWD</td>
<td>Chronic wasting disease</td>
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<tr>
<td>cytPrP</td>
<td>Cytosolic PrP</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle’s medium</td>
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<tr>
<td>DMT1</td>
<td>Divalent metal transporter 1</td>
</tr>
<tr>
<td>Dp1</td>
<td>Doppe1</td>
</tr>
<tr>
<td>dpi</td>
<td>Days post-inoculation</td>
</tr>
<tr>
<td>DTPA</td>
<td>Diethylene triamine pentaacetic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Epithelial-cadherin</td>
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<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-to-mesenchymal transition</td>
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</table>
ER  Endoplasmic reticulum
ERAD  ER-associated degradation
ERK  Extracellular signal-regulated kinase
Esg  Escargot
ESI-MS  Electrospray ionization mass spectrometry
FBS  Fetal bovine serum
fCJD  Familial Creutzfeldt–Jakob disease
FDC  Follicular dendritic cell
FFI  Fatal familial insomnia
FOI  Fear of intimacy
GALT  Gut-associated lymphoid tissue
GAPDH  Glyceraldehyde 3-phosphate dehydrogenase
gCJD  Genetic Creutzfeldt–Jakob disease
GFAP  Glial fibrillary acidic protein
GPI  Glycosylphosphatidylinositol
GSS  Gerstmann–Sträussler-Scheinker syndrome
HA  Hemagglutinin
HC  Hydrophobic core
H-E  Hematoxylin-eosin
HEK 293  Human embryonic 293
HRP  Horseradish peroxidase
iCJD  Iatrogenic Creutzfeldt–Jakob disease
ICP-AES  Inductively couples plasma atomic emission spectroscopy
Irt1  Iron-regulated transporter 1
Kb  Kilo base
kDa  Kilo Dalton
KLH  Keyhole limpet hemocyanin
LDS  Lithium dodecyl sulphate
LPS  Lipopolysaccharide
LTβR  Lymphotixin-β receptor
MAP  Mitogen-activated protein
M cell  Microfold cell
MDCK  Madin-Darby Canine kidney
MEM  Modified Eagle’s Medium
MES  2-(N-morpholino) ethanesulfonic acid
MHC  Major histocompatibility complex
MMP  Matrix metalloproteinase
MOPS  3-(N-morpholino) propanesulfonic acid
MRE  Metal response element
MT  Metallothionein
MTF-1  Metal regulatory transcription factor 1
MTT  3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
MyoD  Myogenic differentiation 1
N2a  Neuro 2a
N-CAM  Neural cell adhesion molecule
NGF  Nerve growth factor
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tr>
<td>NKX2-5</td>
<td>NK2 transcription factor related, locus 5</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NP-40</td>
<td>Nonyl phenoxypolyethoxylethanol</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>P53</td>
<td>Protein 53</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Purkinje cell</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PK</td>
<td>Proteinase K</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PL</td>
<td>Prion-like</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PNGaseF</td>
<td>Peptide N-glycosidase F</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>Prnd</td>
<td>Gene encoding the Doppe1 protein</td>
</tr>
<tr>
<td>Prnp</td>
<td>Gene encoding the prion protein</td>
</tr>
<tr>
<td>PrP</td>
<td>Prion</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>Prion scrapie</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>Rip</td>
<td>Regulated intramembrane proteolysis</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radio immunoprecipitation assay</td>
</tr>
<tr>
<td>RML</td>
<td>Rocky mountain laboratory</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>sAPP</td>
<td>α-secretase-cleaved soluble amyloid precursor protein</td>
</tr>
<tr>
<td>sCJD</td>
<td>Sporadic Creutzfeldt–Jakob disease</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Sho</td>
<td>Shadoon</td>
</tr>
<tr>
<td>SLC30A</td>
<td>Solute carrier family 30A</td>
</tr>
<tr>
<td>SLC39A</td>
<td>Solute carrier family 39A</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
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<tr>
<td>SP1</td>
<td>Specificity protein 1</td>
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<tr>
<td>Sprn</td>
<td>Gene encoding the Shadoon protein</td>
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<tr>
<td>STAT3</td>
<td>Signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>STI1</td>
<td>Stress-inducible protein 1</td>
</tr>
<tr>
<td>SUV</td>
<td>Small unilamellar vesicle</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>TBS containing 0.05% Tween-20</td>
</tr>
<tr>
<td>TNT</td>
<td>Tunnelling nanotubes</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TNFR1</td>
<td>Tumour necrosis factor receptor I</td>
</tr>
<tr>
<td>TPEN</td>
<td>N, N, N’-tetakis (2-pyridylmethyl) ethylenediamine</td>
</tr>
<tr>
<td>TSE</td>
<td>Transmissible spongiform encephalopathy</td>
</tr>
</tbody>
</table>
UV  Ultraviolet
vCJD  Variant Creutzfeldt–Jakob disease
YFP  Yellow fluorescent protein
ZIP  Zrt-, Irt-like protein
ZnT  Zinc transporter
ZnT1  Zinc-transporter 1
ZnT3  Zinc-transporter 3
Zrt1  Zinc-regulated transporter 1
Chapter 1

Introduction

Review of Prion and ZIP Biology, and Their Evolutionary Relevance

Portions of this section have been published in one review article:

1.1 Overview of Prion Biology

1.1.1 Cellular Prion Protein

Prion diseases or transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative diseases affecting both humans and animals. The agent causing TSEs is known as prion (PrP) and is defined as the proteinaceous infectious particle [1]. The cellular prion protein (PrP<sup>C</sup>) is a cell membrane glycoprotein located on the outer leaflet of the plasma membrane within caveolae domains or lipid rafts [1-4]. PrP<sup>C</sup> primarily contains both N-terminal and C-terminal signal sequences. The N-terminal signal sequence is cleaved upon entry of the protein into the endoplasmic reticulum (ER) lumen whereas the C-terminal signal peptide is cleaved upon the addition of a C-terminal glycosylphosphatidylinositol (GPI) anchor [1-4]. PrP<sup>C</sup> is expressed in a wide range of organisms such as mammals, birds, fish and frogs [3, 5-7]. The N-terminal region of PrP<sup>C</sup> spans through residues 23-124 and consists of a long flexible random-coil sequence (Figure 1.1A) [4, 8]. In addition, this N-terminal region contains a stretch of octapeptide repeats, five repeats of the sequence PHGGGWGQ (P: proline, H: histidine, G: glycine, W: tryptophan, Q: glutamine) in humans [9], which is flanked by charged cluster 1 (CC1), residues 23-27, and charged cluster 2 (CC2), residues 95-110 (Figure 1.1A) [3, 9]. The N-terminal and C-terminal regions are linked together by a region of hydrophobic amino acids known as the hydrophobic core (HC) [3, 4, 8]. The PrP molecule is found in non-, mono-, and di-glycosylated forms and it has been identified that asparagine residues 181 and 197 in human, and 180 and 196 in mouse are potential glycosylation sites for PrP<sup>C</sup> [3, 4, 9]. The globular C-terminal region contains three α-helices, which correspond to residues 144-154, 173-194, and 200-228 in human PrP (Figure 1.1) [3]. These α-helices are interspersed by two anti-parallel β-sheets formed by β-strands at residues 128-131 and 161-164 (Figure 1.1) [3]. A single disulfide bond is
found between cysteine residues 179 and 214 [3, 4]. Attachment of PrP\(^{C}\) to the outer leaflet of the plasma membrane is utilized by the addition of a GPI anchor on the serine residue 231 during PrP’s post-translational modification in the Golgi apparatus [3, 4]. Other than its regular location on the plasma membrane, PrP can also adopt two transmembrane topologies on the ER membrane. These transmembrane forms of PrP are called \(^{Ctm}\)PrP and \(^{Ntm}\)PrP, which have their C-terminus and N-terminus in the ER lumen respectively [10, 11]. Only a small portion of PrP exists in these transmembrane forms, less than 10% of the total cellular PrP [12], and it has been shown that the excess presence of \(^{Ctm}\)PrP, but not \(^{Ntm}\)PrP, induces neurodegeneration [3, 10, 12-14].

Some neurons located in the hippocampus, thalamus and neocortex also contain a cytosolic form of PrP known as cytPrP [12, 13, 15]. PrP can also accumulate in the nucleus of scrapie-infected cells and this localization requires a microtubule-dependent mechanism [16]. It is known that protein turn-over depends on the ER control system recognizing misfolded proteins and causing their transport to cytosol by a retrograde-transport mechanism [15, 17, 18]. These misfolded proteins are then poly-ubiquitinated and are degraded by proteasomes [15, 17, 18]. This degradation mechanism for misfolded proteins is known as ER-associated degradation (ERAD) [15, 17, 18]. This is relevant to PrP biology and physiology since both wild-type and misfolded forms of PrP\(^{C}\) also undergo ERAD [15, 17, 18], and abortion of PrP\(^{C}\) ERAD-degradation pathway through inhibition of the proteasomal complex leads to the cytosolic accumulation of PrP\(^{C}\) [15, 17, 18].
4

Figure 1.1 - Structure of the mammalian cellular prion protein. (A) Primary structure of the prion protein showing charged cluster 1 (CC1) and charges cluster 2 (CC2), which are rich in lysine residues. The octapeptide repeat region has five repeats in which histidine (His) residues facilitate binding to divalent cations, mainly copper. The C-terminal domain consists of two β-sheets and three α-helices. α-helices 2 and 3 can form a disulfide bridge that has implications in prion diseases. Asparagine residues 181 and 197 are potential glycosylation (CHO) sites. The prion protein is attached to the plasma membrane by means of its GPI anchor on serine residue 231. (B) The tertiary structure of the prion protein is shown with its disordered N-terminal domain and membrane attachment with the GPI anchor. There are two anti-parallel β-sheets and a loop connects the second β-sheet to the third α-helix (adapted by permission from Macmillan Publishers Ltd: [8], copyright © 2006).

1.1.2 Protein-Only Hypothesis and Models of Prion Propagation

Prions are different from other infectious pathogens in that they do not contain any nucleic acid component longer than 50 bases, and the only component of their disease-associated form is a misfolded conformation of PrPC which is known as prion scrapie or PrPSc [1]. Therefore, the underlying cause of prion diseases has been associated with the conversion of PrPC to PrPSc.

Although structure of PrPC has been resolved with high precision, no high-resolution structure is yet available for PrPSc. This can be explained by high level of scrapie agent’s
insolubility and its tendency to aggregate making it inaccessible for structural studies [19]. However, PrP$^{\text{Sc}}$ is known, based on primary structure of PrP$^{\text{C}}$, to have a high tendency for adopting a parallel left-handed $\beta$-helical conformation [19]. Nuclear magnetic resonance (NMR) studies have shown no difference between the primary structures of PrP$^{\text{C}}$ and PrP$^{\text{Sc}}$ [20]. In addition, their different characteristics are due to differences in their secondary and tertiary structures [20]. For instance, conversion of PrP$^{\text{C}}$ to PrP$^{\text{Sc}}$ is associated with higher development of $\beta$–sheets in the structure of PrP$^{\text{Sc}}$. While 45% of PrP$^{\text{C}}$ is comprised of $\alpha$-helices, only 30% of PrP$^{\text{Sc}}$ is composed of $\alpha$-helices and 45% of its conformation is comprised of $\beta$-sheets [20]. Further studies on PrP have indicated the importance of a core PrP fragment, PrP 106-126, in neurotoxicity as expression of this PrP fragment in primary rat hippocampal neurons leads to DNA fragmentation, apoptosis and neuronal death [21]. This peptide also has a high intrinsic ability to polymerize into amyloid-like fibrils \textit{in vitro} [21].

The earliest detection of scrapie goes back to the 18$^{\text{th}}$ century and later its devastating effects on the central nervous system (CNS) in the brains of scrapie-infected sheep were identified in the 19$^{\text{th}}$ century [1, 2]. First cases of prion diseases in humans were detected in 1920 as Creutzfeldt-Jakob disease (CJD) [1, 2]. Gajdusek & Zigas reported another type of prion diseases known as Kuru in 1955-1957 among the Fore people of Papua New Guinea [22]. The transmissibility of scrapie was demonstrated in early studies and led to the conclusion that prion diseases are caused by a viral-like particle [23]. This nucleoprotein hypothesis started by Sigurdsson who classified scrapie as a slow virus disease [23] and it was further confirmed by Gajdusek & Zigas [22]. In addition, Diringer suggested this virus to be unconventional in that it does not possess some of the characteristics of slow viruses [23]. It was also hypothesized that scrapie has an impermeable capsid protecting its nucleic acid from physical or chemical damage.
Although no group has yet succeeded in identifying this polynucleotide, despite the use of a wide variety of techniques including measurement of nucleic acids in purified preparations [1], categorization of prion into several different strains supports the nucleoprotein hypothesis [24, 25]. Prion strains are defined as infectious isolates that can cause distinct phenotypes after transmission to identical hosts [24, 25]. These phenotypes include distinct patterns of protein aggregation, incubation time, and specific target sites in the nervous system [25].

A study by Alper et al. in 1966 showed the resistance of PrP\textsuperscript{Sc} to both ultraviolet light (UV) and ionizing radiation causing doubts in the viral-like origin of PrP\textsuperscript{Sc} [26]. Furthermore, the unusual properties of PrP\textsuperscript{Sc}, such as resistance to UV light, partial resistance to proteinase K (PK) digestion, and resistance to both high-pressure and high-temperature treatments led to predications that PrP\textsuperscript{Sc} might solely consist of protein, or be devoid of both nucleic acid and protein, or be a polysaccharide or a membrane fragment [27, 28]. Nevertheless, observations by several groups have mainly supported the protein-only hypothesis proposed by Stanley Prusiner postulating the main or perhaps the only constituent of the infectious agent to be mammalian PrP\textsuperscript{Sc} [1]. Studies on \textit{Prnp}\textsuperscript{−/−} mice are in support of the protein-only hypothesis since \textit{Prnp}\textsuperscript{−/−} mice are resistant to prion diseases and introduction of PrP\textsuperscript{C} is necessary for the progression of disease and the onset of neurodegeneration in these animals [29]. PrP\textsuperscript{C} overexpression also leads to the higher aggregation of PrP\textsuperscript{Sc} in brains of these animals [29]. In addition, there are also inherited prion diseases only requiring mutations in the \textit{Prnp} gene [30]. Flechsig et al. showed that the octapeptide repeat region is not necessary for the onset of disease although its truncation leads to longer incubation times and a thirty-fold less PK-resistance of PrP\textsuperscript{Sc} [31]. Further studies indicated the GPI-anchorage of PrP\textsuperscript{C} to be essential in prion diseases as mice expressing PrP lacking the GPI anchor do not develop clinical signs of the disease [3, 32, 33].
Alternatively, another hypothesis, known as the unified theory of prion propagation and proposed by Weissmann, combines the protein-only hypothesis with the nucleoprotein hypothesis and suggests that the infectious agent, called holoprion, consists of two components; one component is PrP$^{Sc}$, apoprion, causing TSEs and the second component is a nucleic acid, coprion, which can exist in many variations [34]. PrP$^{Sc}$ can enter cells with or without coprion. In the absence of nucleic acid component, however, PrP$^{Sc}$ is able to recruit a nucleic acid to act as a coprion. This coprion is replicated by cellular enzymes and its replication is mediated by PrP$^{Sc}$. Therefore, in this theory PrP$^{Sc}$ is supposed to be a pathogen whose expression is encoded by both the host genome and a coprion [34].

According to the protein-only hypothesis, conformational conversion of PrP$^{C}$ to PrP$^{Sc}$ can be explained by two theoretical models: 1- the refolding or template assistance model and 2- the seeding or nucleation-polymerization model [2, 3, 11]. In the refolding or template-assistance model, it is postulated that an exogenously introduced PrP$^{Sc}$ interacts with endogenous PrP$^{C}$. While an entry barrier might prevent the spontaneous conversion of PrP$^{C}$ to PrP$^{Sc}$, this conversion process can occur under certain circumstances, such as mutations in the Prnp gene, after induction by PrP$^{C}$-PrP$^{Sc}$ interaction [2, 3, 11]. Interaction of PrP$^{C}$ with PrP$^{Sc}$ may lower the activation energy barrier leading to further recruitment of PrP$^{Sc}$ (Figure 1.2A) [3, 11].

On the other hand, PrP$^{C}$ and PrP$^{Sc}$ are in reversible thermodynamic equilibrium according to the seeding or nucleation-polymerization model [2, 3, 11]. In this model, PrP$^{C}$ passes through an unfolded state before conversion to a PrP$^{Sc}$ monomer [2, 3, 11]. Certain circumstances such as mutations in the Prnp gene or exposure to exogenous PrP$^{Sc}$ can lead to irreversible conversion of PrP$^{C}$ to PrP$^{Sc}$ and this model points to the necessity for several monomeric PrP$^{Sc}$ molecules to mount on a highly ordered seed in order for further monomeric PrP$^{Sc}$ to be recruited on this seed.
and eventually aggregate into amyloid-like fibrils (Figure 1.2B) [3, 11, 35]. It has been shown that the conversion of PrP<sup>C</sup> to PrP<sup>Sc</sup> is also associated with significant fragmentation of PrP molecules and the seeding or nucleation-polymerization model proposes this fragmentation to assist in creating several nuclei or seeds that can remarkably utilize the recruitment of PrP<sup>Sc</sup> monomers on these seeds leading to significant aggregation of the protein [2, 3, 11].

![Figure 1.2- Two models for the conversion of PrP<sup>C</sup> to PrP<sup>Sc</sup>. (A) The refolding or template-directed model proposes the interaction between an exogenously introduced PrP<sup>Sc</sup> molecule and an endogenous PrP<sup>C</sup> molecule leading to PrP<sup>C</sup>-to-PrP<sup>Sc</sup> conversion, PrP<sup>Sc</sup> aggregation and formation of amyloid-like fibrils. A high-energy barrier may prevent from the spontaneous conversion of PrP<sup>C</sup> to PrP<sup>Sc</sup> (adapted by permission from Macmillan Publishers Ltd: [11], copyright © 2001). (B) The seeding or nucleation-polymerization model postulates that PrP<sup>C</sup> and PrP<sup>Sc</sup> are in thermodynamic equilibrium. In a condition in which several PrP<sup>Sc</sup> molecules can aggregate and form a small seed (the rate-limiting step), this equilibrium is shifted to the generation of more PrP<sup>Sc</sup> (fast), aggregation and formation of amyloid-like fibrils. The fragmentation of PrP<sup>Sc</sup> aggregates is essential to increase the number of nuclei on which further PrP<sup>Sc</sup> can be recruited (adapted by permission from Macmillan Publishers Ltd: [11], copyright © 2001).
1.1.3 Interacting Partners of Prion Protein

GPI-anchored proteins are in contact with several other proteins in lipid rafts and these interactions are necessary for their function in different signal transduction pathways [32]. Since PrP^C is also a GPI-anchored protein located in lipid rafts lots of efforts have been made to identify interacting partners of PrP and to show that other proteins may also be involved in conformational change and ultimately aggregation of PrP in TSEs [3, 12, 36]. Application of techniques such as microarray studies, co-immunoprecipitation and yeast two-hybrid system have led to identification of interacting proteins such as neural cell adhesion molecule (N-CAM) [37, 38], neuroglobin [39], synapsin 1b [40], tau [41], B-cell lymphoma 2 (Bcl-2) [42], Mahogunin [43] and ZIP transporters [44].

Interaction of neuroglobin with PrP enhances the aggregation properties of PrP [39]. Interaction of PrP with tubulin also results in the aggregation of PrP and inhibition of microtubule assembly [45]. Interaction of PrP with Bcl-2 leads to the loss of Bcl-2 function and results in PrP-aggregation and cellular apoptosis [42]. PrP^C has also been shown to be important in neurite outgrowth and synapse formation through its interactions with N-CAM and stress-inducible protein 1 (STI1) [37, 38, 46]. Interaction of PrP with N-CAM, laminin and laminin receptor mediates neuronal contact with extracellular matrix (ECM) and other neurons [37, 38, 47].

1.1.4 Mammalian Prion Protein Family Members

The Prnp gene is located on chromosomes 2 and 20 in mice and humans respectively [9]. It consists of three exons in both species and the entire open reading frame (ORF) is located on exon 3 (Figure 1.3A) [9]. The promoter region of the Prnp gene does not have any TATA (T:
thymine, A: adenine) box and is rich in G + C (G: guanine, C: cytosine), a likely site for binding of specificity protein 1 (SP1) as a transcription factor [1].

There is another gene, called Prnd, located 16 kilobase (kb) downstream of Prnp and it is responsible for expression of Doppe1 (Dp1), another member of the mammalian prion protein family [48]. Similar to Prnp, the Prnd gene also consists of three exons with its ORF located only on exon 3 (Figure 1.3A) [9]. The tertiary structure of Dp1 is similar to the C-terminal region of PrP with 25% homology [9]. The C-terminal region of Dp1 contains three α-helices but, unlike PrP, it has two disulfide bridges between second and third helices (Figure 1.3B) [3].

Dp1 lacks the hydrophobic core and octapeptide repeat regions found on the N-terminus of PrP, but Dp1 N-terminal region is disordered and positively charged like that of PrP [49, 50]. Dp1 N-terminal signal sequence is cleaved after transport into the ER lumen [3]. Its C-terminal signal sequence is cleaved upon the addition of a GPI-anchor and it becomes glycosylated at asparagine residues 99 and 111 in mice [3]. Dp1 mRNA is highly expressed in testis and heart, moderately in spleen and to a lower extent in the brain [48, 51, 52]. Dp1 is highly expressed in embryos and it has been suggested to play a crucial role in embryonic development [3]. Embryos from Dp1-deficient sperms cannot progress past the morula stage [48]. There exists a strain of Prnp−/− mice in which the splice acceptor of exon 3 for Prnp is removed [48, 51, 52]. This puts the Prnd gene under the control of Prnp promoter and leads to the generation of chimeric Prnp/Prnd mRNA [48, 51, 52]. As a consequence, Dp1 protein, regularly expressed in testis, is expressed in neurons and has neurotoxic effects that can be reversed by expression of wild-type PrP in the mouse brain [48, 51, 52]. However, Dp1 is not required for pathogenesis of prion diseases since Dp1-expressing mice do not show any alteration in their incubation time and disease symptoms.
when inoculated with PrP\textsuperscript{Sc} [53]. There is also no alteration in the expression of Dp1 in brains of human CJD patients and scrapie-infected mouse models [54].

\textit{Sprn} is another gene that is responsible for expression of Shadoo (Sho), the third member of mammalian prion protein family [55]. \textit{Sprn} is located on chromosomes 7 and 10 in mice and humans respectively [48, 51, 52]. \textit{Sprn} contains two exons and its ORF is located on exon 2 (Figure 1.3A) [48, 51, 52]. The expression of \textit{Sprn} is solely restricted to Purkinje cells of the cerebellum and dendritic processes of the hippocampal pyramidal cells in the CNS [56]. Sho is a ninety-eight-residue GPI-anchored glycoprotein with N- and C-terminal signal sequences, and it only contains one glycosylation site [48, 51, 52]. Although it lacks the N-terminal octapeptide repeat region, Sho contains a series of charged tetra-repeats on its N-terminus which are rich in glycine, serine, alanine and arginine (Figure 1.3B) [48, 51, 52]. Unlike Dp1, Sho contains the hydrophobic domain of PrP and most of its homology with PrP arises from this region of the protein [48, 51, 52]. Sho does not have any cysteine residues and it is not able to form any disulfide bridges in its C-terminal region [48, 51, 52]. Functional roles of Sho in the CNS have not been discovered yet. However, its expression in Dp1-expressing cerebellar granule cells, deficient in PrP expression, can reverse Dp1-induced neurotoxicity suggesting PrP and Sho to have overlapping functions in the CNS [9, 56].
1.1.5 Neuroprotective Functions of Prion Protein

Although the cellular function of prion protein is not yet uncovered, efforts to understand its role in both normal and scrapie-infected cells have revealed the importance of PrP in neuroprotection, neurite outgrowth, myelin-sheath maintenance and prevention against apoptosis. This section aims at reviewing these functions of PrP in more detail.
One neuroprotective function of PrP comes from the evidence indicating inhibition of Dp1-neurotoxicity upon overexpression of PrP in neurons of Prnp<sup>−/−</sup> mice as discussed previously [48, 51, 52].

Studies on the role of PrP in neurite outgrowth started by an observation from Prnp-null hippocampal cells which die within four days of incubation, beginning with cells grouping together, followed by condensation of cytoplasm and retraction of neurites [57]. In contrast, all Prnp wild-type cells maintained structural integrity under these culture conditions [57]. Observations by Caetano et al. presented interaction of PrP with STI1, a protein with roles in neuronal survival and differentiation, leading to activation of both protein kinase A (PKA) and extracellular signal-regulated kinase (ERK) 1/2 [57]. Activation of these proteins resulted in induction of signal transduction pathways for neuroprotection and neuritogenesis [58]. The incubation of Prnp wild-type and knockout hippocampal neurons in the presence of STI1 results in neurite outgrowth and neuritogenesis in wild-type neurons only [58]. Deletion mutation of residues 230-245 of STI1, which includes the PrP binding domain, did not lead to any PrP binding and neuritogenesis [58]. In addition, treatment of cells with an inhibitor of mitogen-activated protein (MAP) blocks ERK1/2 phosphorylation and results in decreased number of cells with neurites [58]. Another evidence for involvement of PrP in neurite outgrowth comes from the study by Mouillet-Richard et al. who showed cross-linking of PrP<sub>C</sub> to PrP-specific antibodies to increase dephosphorylation level of Fyn kinase, a tyrosine kinase, and to enhance its kinase activity accordingly in neurites of differentiated serotonergic and noradrenergic cells [58, 59]. Furthermore, interaction of PrP with laminin is also important for neuritogenesis in primary cultures of rat hippocampal neurons [47]. Neuritogenesis is more efficient when cells are pre-treated with nerve growth factor (NGF) [47]. On the other hand, Prnp-null neurons extended
neurites in response to laminin but this neuritogenesis was significantly lower than that observed for wild-type neurons [47].

Another line of evidence for the neuroprotective function of PrP comes from the study by Bounhar et al. showing that PrP prevents Bcl-2-associated X (Bax)-mediated apoptosis and cell death in human primary neuronal cell line by examining effect of PrP expression in the presence or absence of Bax [58]. In addition, it was also shown that octapeptide repeat region and not the GPI anchor is essential for this anti-apoptotic activity of PrP [60]. Another study by Heitz et al. showed the deletion of Bax from PrP knockout Purkinje cells (PCs), overexpressing Dp1, to rescue cells from Dp1-induced cell death [58]. Thus absence of BAX enables PCs to resist Dpl neurotoxicity and indicates that BAX plays a role in Dpl-mediated cell death [61].

Proteolysis of PrP<sub>C</sub> and the generation of C1 and C2 fragments are also essential for neuroprotective roles of PrP as they help maintaining neuronal myelin sheath in the periphery [58]. Bremer et al. observed the ablation of PrP<sub>C</sub> or its GPI-anchorage to the plasma membrane to result in chronic demyelinating polyneuropathy (CDP) [58]. All Prnp-knockout mice developed CDP after sixty weeks post-mortem as demyelination was observed in sciatic nerve, trigeminal nerve, and dorsal and ventral spinal roots [58]. Immunostaining showed macrophages are responsible for ingesting myelin sheaths of degenerating nerve fibres [58]. Quantification of axonal diameter to fibre diameter revealed a higher percentage of fibres with thinned myelin in Prnp-knockout brains in comparison to wild-type animal brains [58]. Expression and phosphorylation of RAC-alpha serine/threonine-protein kinase (Akt) and Erk kinases, which are crucially involved in myelination, were unaffected in neurons from ten- and thirty-day-old Prnp-knockout animals, and there was no change in the expression of peripheral myelin proteins [58]. Terminally sick Prnp<sup>−/−</sup> mice generated CNS white matter vacuolation and astrogliosis in the
cerebellum, brainstem and corpus collosum [58]. The octapeptide repeat region was not required for myelin maintenance, whereas mice expressing PrP that lacks the central domain, residues 94–134, developed CDP [58]. The hydrophobic core, but not CC2, of the central domain was also essential for peripheral myelin maintenance [58]. All transgenic mice with signs of CDP lacked the C1 fragment, whereas all PrP mutants in whom CDP was rescued produced abundant C1 [62]. Both wild-type and Prnp-null neurons developed neurite-like structures within an hour of incubation in serum-free medium. However, Prnp-null neurons started grouping together followed by retraction of neurites, DNA fragmentation and cell death within four days [57].

The role of PrP in reducing oxidative stress was observed by Brown et al. from studies on Prnp-null neurons [58] in which the authors showed that Prnp<sup>−/−</sup> mice are more susceptible to oxidative stress. It was later shown that both chicken and mouse PrP<sup>C</sup> have SOD-like activities [58]. SOD activity was measured by production of superoxide from xanthine oxidase [58]. This SOD-like activity was dependent on binding of copper to the octapeptide repeat region for proper refolding of the N-terminal region of PrP [63]. Examination of Prnp-null cerebellar granule neurons also showed cell viability to decrease, by up to 60%, due to exposure of these cells to hydrogen peroxide [64].

### 1.1.6 Endoproteolysis of Prion Protein

Transmembrane proteins can undergo proteolysis and cleavage to generate cytosolic fragments, which in some cases travel to the nucleus to regulate transcription. This process is known as regulated intramembrane proteolysis (Rip) and it affects processes such as cellular differentiation and lipid metabolism [65]. Notch is one example of these transmembrane proteins which is cleaved by a protein known as Presenilin-1, an intramembrane aspartyl protease [66], on the surface of Golgi and plasma membrane [65, 66]. Upon cleavage, the cytosolic fragment of
Notch plays an important role in cell differentiation [65, 66]. Amyloid precursor protein (APP) is another example cleaved by Presenilin-1 on the surface of Golgi and endosomes [65, 66]. But the function of its extracellular fragment is unknown and it is suspected to cause Alzheimer’s disease [65, 66].

Labeling PrP with membrane-impermeable iodination or biotinylation reagents showed that PrP cycles through cells with a transit time of around sixty minutes and 1-5% of the molecule undergoes proteolytic cleavage [67]. Cleavage of the prion protein has also been reported in brains of patients with CJD and Gerstmann-Sträussler-Scheinker syndrome (GSS) [68]. Type 1 CJD brains contain both full-length prion protein and a 2.5 kiloDalton (kDa) amino-terminally truncated fragment of this protein [68]. Type 2 CJD brains also contain full-length PrP and an amino-terminally truncated fragment (21-22kDa) [68].

Another study by Bremer et al. showed that prion fragments are not only important in prion diseases but they also play a role in the neuroprotective function of PrP in non-infected cells [62]. They observed sciatic nerves to contain higher levels of resultant prion fragments in response to cleavage in the hydrophobic domain [62]. They also showed that there is a correlation between CDP and PrP C1 fragment since CDP mouse brains had lower levels of this fragment. Therefore, the generation of PrP proteolytic fragments is important for both neuroprotective role of PrP in non-infected cells and for its neurodegenerative character in prion diseases.

The prion protein undergoes two main endoproteolytic cleavages known as \( \alpha \)-cleavage and \( \beta \)-cleavage (Figure 1.4A) [69]. The \( \alpha \)-cleavage occurs between residues 111 and 112 in the hydrophobic region of PrP, and it generates two fragments [69]. The C-terminal fragment is called C1 and it has a molecular weight of \(~17-18\text{kDa}\), while the N-terminal fragment is known
as N1 with the molecular weight of around 9.2kDa (Figure 1.4B) [69]. The β-cleavage is less common and it happens in the octapeptide repeat region near histidine 96 [69]. This cleavage generates a C-terminal fragment known as C2, 21kDa, and an N-terminal fragment known as N2 (Figure 1.4B) [69]. Neither the α-cleavage nor the β-cleavage of PrP requires the protein to be membrane-associated or delivered into lipid rafts [62, 70].

Figure 1.4- Endoproteolysis of PrP. (A) Schematic depicting positions of both α-cleavage and β-cleavage on the N-terminal region of PrP. The α-cleavage happens at position 111/112 and the β-cleavage occurs at the octapeptide repeat region near histidine residue 96 (adapted by permission from Elsevier Ltd: [69], copyright © 2004). (B) Schematic showing PrP proteolytic fragments generated in response to both α- and β-cleavages. α-cleavage of PrP leads to the generation of a 17-18kDa C-terminal fragment (C1) and a 9kDa N-terminal fragment (N1). PrP β-cleavage leads to the generation of a 21kDa C-terminal fragment (C2) and a 6kDa N-terminal fragment (N2) (adapted by permission from Elsevier Ltd: [69], copyright © 2004).

In an effort to identify domains that are important in cleavage of PrP, Oliviera-Martins et al. showed the α-cleavage of PrP to be strictly dependent on residues 106-119 [71]. Further analysis revealed that substitution of alanine residues to glycine residues in 112-119 region did not influence the generation of C1 fragment [71]. C1 generation is also independent of charge in the vicinity of the α-cleavage site [71]. Deletion of domains 100-110 and 121-129 in the vicinity of the α-cleavage site had no impact on cleavage whereas deletion of domains 105–110 and 111–120 resulted in about 50% impairment of α-cleavage [71]. Shorter deletions within domain 106–
119 centered in the α-cleavage site region only partially inhibited proteolysis of PrP [71]. Deletion of the hydrophobic region, from residue 114 to 121, causes reduction in α-cleavage of PrP and lower generation of the C1 fragment [71]. While deletion of residues 114 to 119 was essential for the cleavage, the last two residues, 120 and 121, in the hydrophobic domain had no significant effect on the cleavage [71]. Bremer et al. showed the CC2 domain of PrP$^C$ to be essential for both α- and β-cleavages as expression of PrP$^C$, lacking the CC2 region, in mouse brains resulted in lower level of C1 fragment and generation of no C2 fragment [71].

Several efforts have focused on identifying proteases necessary for both α- and β-cleavages of PrP$^C$. Proteases are enzymes that hydrolyze peptide bonds by addition of water to lead to breakdown of proteins [71]. They have high degree of specificity for binding to and processing their substrates [72]. Proteases are divided into five groups depending on active site residue or ion carrying catalysis [71]. These five groups include serine, threonine, cysteine, aspartic and metalloproteases [71].

Several groups have pointed out the importance of a disintegrin and metalloproteinase (ADAM) family of proteases in cleavage of PrP [73-76]. ADAMs belong to the metalloprotease group of protease family of proteins and they require binding of zinc ions for their catalytic activity [77]. The cysteine-rich pro-domain of ADAMs must be cleaved for activation since it blocks binding of zinc ions [77]. ADAM proteases are classified into two groups: membrane-anchored ADAM and ADAM with thrombospondin motif (ADAMTS) [77]. They are involved in cell adhesion, cell migration, membrane protein shedding or ectodomain shedding and proteolysis [77]. Membrane-anchored ADAM members are composed of common domains including propeptide, metalloproteinase, disintegrin, cysteine-rich, EGF-like, transmembrane, and cytoplasmic domains, whereas ADAMTS members contain thrombospondin motifs,
cysteine-rich and spacer domains in addition to propeptide, metalloproteinase and disintegrin domains [77]. Active sites in the metalloproteinase domain of proteinase-type ADAM molecules, such as ADAM9, ADAM10 and ADAM17 contain a common HEXGHXXGXXHD (H: histidine, E: glutamic acid, G: glycine, D: aspartic acid, X: any amino acid) sequence, which is also present in the catalytic metalloproteinase domain of matrix metalloproteinase (MMP) members [78].

Since ADAM9, ADAM10 and ADAM17 play roles in the Alzheimer’s disease by cleaving APP at the α-secretase processing site, leading to secretion of α-secretase-cleaved soluble amyloid precursor protein (sAPP) which exhibits neuroprotective and cytotrophic properties [75, 78], it was postulated that these members of ADAM proteases might also play a role in the proteolysis of PrP$^C$. In fact, Vincent et al. identified ADAM10 and ADAM17 as main proteases responsible for the α-cleavage of PrP [73]. Among a series of classical inhibitors that target distinct classes of proteases, only a zinc-metalloprotease-blocking agent was able to drastically reduce N1 production in human embryonic 293 (HEK 293) cells whereas serine, thiol, and acidic protease inhibitors were ineffective [73]. Overexpression of ADAM10 and ADAM17 in HEK 293 cells resulted in constitutive up-regulation of N1 fragment, which in the case of ADAM17 expression was dependent on the activation of protein kinase C (PKC) [73].

ADAM9 also participates together with previously discovered ADAM10 and ADAM17 in the proteolysis and shedding of PrP to generate the N1 fragment. Overexpression of ADAM9 in HEK 293 cells, neurons and fibroblasts increases N1 fragment, while its down-regulation reduces N1 generation [75]. ADAM9 acts upstream of ADAM10 since overexpression of ADMA9 in ADAM10 knockout background does not affect proteolysis while co-expression of both proteins increases N1 fragment [75].
However, mass spectrometric analysis showed that ADAM10 cleaves PrP at its C-terminus, three residues upstream of the GPI anchor [75]. Analysis of this fragment showed that it ends at glycine 228 [75]. Overexpression and knockdown studies showed that ADAM9 is also required for the catalytic activity of ADAM10, but it cannot cause the cleavage by itself [75]. Therefore, it was concluded that ADAM9, ADAM10 and ADAM17 are not involved in the α-cleavage of PrP between histidine residue 111 and methionine residue 112 in HEK 293 cells [75].

Endres et al. also concluded that ADAM10 and ADAM17 do not cleave PrP at the α-cleavage site [76]. However, they provided convincing evidence that the expression and activity of ADAM10 correlates with the level of prion proteolytic fragments, which might suggest an indirect role of ADAMs in the proteolysis of PrP [76]. In addition, moderate overexpression of ADAM10 also led to enhanced resistance to scrapie infection as it was suggested by a longer survival time of these mice in comparison to shorter survival time of wild-type animals [76]. Therefore, the authors proposed ADAM10 overexpression to prolong survival time in scrapie-infected mice by causing reductions in the levels of full-length PrP and a corresponding increase in PrP’s proteolytic fragment [76].

Several studies have also focused on the β-cleavage of the prion protein to generate the C2 fragment. It has been shown that the down-regulation of PrP α-cleavage and the up-regulation of PrP β-cleavage, and its resultant C2 fragment, are associated with prion diseases and scrapie infection [70, 79-81]. While the endocytosis of PrP seems to be required for its β-cleavage, there is a controversy regarding the specific proteases that are responsible for the endoproteolysis of PrP to generate the PrP C2 fragment. Yadavalli et al. proposed that calpain, a calcium-dependent protease, is responsible for the β-cleavage of PrP in the cytosol [79], whereas
Dron et al. postulated that cathepsins, lysosomal proteins, cleave PrP at the β-cleavage site to generate the C2 fragment [80].

Although a myriad of studies investigated PrP endoproteolysis, it can be concluded that no single protease has been identified to be directly involved in fragmentation of PrP and future studies are essential in revealing roles of proteases, other than ADAM family members, to cause α- and β-cleavages of the mammalian prion protein.

1.1.7 Role of Divalent Cations in Prion Biology and Pathogenesis of Prion Diseases

Investigations into the role of copper indicate that this element is extremely critical for optimal enzymatic activities in different cellular functions, such as electron transport and energy capture, and disruption of its homeostasis is associated with onset of diseases such as Wilson’s disease and Alzheimer’s disease with dramatic effects on the function of the CNS [82]. Wilson’s disease originates as a result of a mutation in the gene encoding ATPase, Cu²⁺ transporting, beta polypeptide (ATP7B), a copper transporter responsible for transporting copper to bile [82]. This disease affects different organ systems including the CNS and some neurological features of the disease include Parkinsonian and schizophrenia-like symptoms, and psychological abnormalities [83]. Depletion of copper in neuroblastoma cells also alters mitochondrial function by the formation of reactive oxygen species (ROS) triggering apoptosis [82]. Mammalian brain is one of the richest copper-containing organs in the body [82]. Human brain has the highest amount of copper in comparison to other mammals [82]. Copper transporters pump copper through physiological barriers such as blood-brain barrier [82]. Then copper transport in CNS is regulated by ATPase, Cu²⁺ transporting, alpha polypeptide (ATP7A), mutation of which is the cause of Menkes disease, in glia and neurons, and it becomes available for the synthesis of neurotransmitters such as dopamine [82]. Considering its concentration and distribution in the
brain, it is not surprising that copper is involved in neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), Alzheimer’s disease, and prion diseases [82]. For instance, Alzheimer patients have higher copper levels in the cerebrospinal fluid [82]. Furthermore, it has been shown that high β-sheet content of amyloid β (Aβ) in Alzheimer’s disease causes it to bind to copper with high affinity and to reduce copper from copper (II) to copper (I) with a catalytic generation of hydrogen peroxide and further Aβ aggregation [84].

In addition to copper, zinc also plays a significant role in cell physiology by acting as a co-factor for optimizing activities of enzymes and utilizing functions of zinc-finger transcription factors such as Snail [82]. Zinc-secreting cells are found in a wide variety of organs such as submandibular salivary gland, pancreatic β-cells, prostate epithelial cells, pituitary cells, and CNS neurons [85]. In neurons, cytosolic zinc concentration is in picomolar range, while it is in micromolar and millimolar ranges in the proximity of axon terminals and in synaptic vesicles respectively [82]. Zinc enters somata and dendrites of neurons through N-methyl-D-aspartic acid (NMDA) channels, voltage-gated calcium channels and calcium-permeable channels [82]. NMDA receptors contain several zinc binding sites which inhibit the effect of NMDA and they are important for triggering long-term changes in synaptic efficacy [82]. At the plasma membrane level, zinc-transporter 1 (ZnT1) controls Zn$^{2+}$ efflux, while Zn$^{2+}$-importing proteins facilitate Zn$^{2+}$ influx [82]. Zinc uptake in mitochondria induces mitochondrial depolarization and generation of ROS [82]. Zinc also promotes extra-mitochondrial oxidative stress by activating ROS-generating cytosolic enzymes such as neuronal nitric oxide synthase [86]. Similar to copper, disruptions in the homeostasis of zinc can also abrupt a variety of mechanisms with necessary implications for proper function of neurons. For instance, zinc is involved in
neurodegenerative diseases since it has been shown that Aβ aggregation is attenuated in the zinc transporter 3 (ZnT3)-knockout mouse models of the Alzheimer’s disease [86].

Since both copper and zinc are essential in the pathophysiology of neurodegenerative diseases such as the Alzheimer’s disease, several investigations also attempted to uncover roles of these metallic elements in pathology of prion diseases. In fact, a manuscript released by Brown et al. in 2007 revealed, for the first time, the N-terminal domain of PrP to have a high affinity for binding to copper [87]. The copper-binding ability of PrP is not highly specific to copper and other metals such as zinc and iron can also bind to PrP [87]. In general, there are two high affinity binding sites for divalent transition metals within the PrP molecule; the first binding site is located in the N-terminal octapeptide repeat region and the second site is between histidine residues 96 and 111, a region which is important for prion propagation [88]. Both of these sites have a higher affinity for copper than other transition metals [88]. Expression of the octapeptide repeat region of PrP in Prnp−/− mice prevents cell death induced by copper toxicity mimicking the effect of copper chelators [89]. Expression of the octapeptide repeat domain of PrP also increased the survival of cerebellar cells from Prnp−/− mice, but it did not increase the survival of astrocytes and microglia [89].

In addition, PrP may regulate concentrations of divalent cations by transporting them across cell membranes [90]. For instance, one study by Kralovicova et al. showed that PrP may directly regulate copper homeostasis [91]. The authors showed that knockout of PrP resulted in decrease in the uptake of copper and reduction in the expression of proteins involved in copper uptake and export such as divalent metal transporter 1 (DMT1) and antioxidant protein 1 (Atox1) [91].
The role of PrP in the regulation of divalent cations attracted further interest when it was revealed that PrP may possess some SOD-like activity facilitating response of cells to conditions of oxidative stress in the presence of metal ions. For instance, Wong et al. found Prnp−/− mouse brains to suffer from higher protein oxidation, lipid peroxidation and proteasomal activity in comparison to wild-type mouse brains [92]. Exposure of cerebellar granule neurons to hydrogen peroxide also reduced cell viability in Prnp−/− mice [92]. The authors attribute this to lower activity of glutathione reductase observed in these cells [92]. The same characteristic is also observed upon exposure of cells to the neurotoxic PrP peptide, PrP 106-126, suggesting that this fragment is essential for the SOD-like activity of PrP [64]. In addition, Prnp−/− mice had a slight decrease in SOD-1 activity [93] and knockout of Cu/Zn-SOD or Mn-SOD resulted in higher expression of PrP [93]. This may be explained by the fact that PrP is up-regulated to compensate for the activities of these SODs in neurons [93]. However, another study suggested that copper concentration in the synaptosomal preparations of Prnp-knockout mice is reduced by 50% and it does not significantly alter the activity of Cu/Zn-SOD and cytochrome c oxidase [94]. Therefore, the hypothesis that PrP compensates for the role of SODs in their absence in neurons may not be completely valid after all.

Copper also plays a role in PrP endoproteolysis since PrP cleavage in the octapeptide repeat region leads to the generation of C1 fragment in response to incubation of Chinese hamster ovary cells (CHO cells) in the presence of copper [95]. This cleavage is reversible by the addition of other divalent cations such as zinc, calcium and manganese, but not cobalt [95].

Another role for copper was discovered in endocytosis of the prion protein since exposure of PrPC to copper results in rapid internalization of the protein with the octapeptide repeat domain being essential for this endocytosis event [96]. Exposure of PrP to copper causes
PrP to undergo lateral movement on the plasma membrane from detergent-insoluble domains or lipid rafts to detergent-soluble domains containing clathrin-coated vesicles facilitating PrP endocytosis [96]. In addition, zinc can also promote endocytosis of PrP through clathrin-coated vesicles [96]. Another study by Brown & Harris confirmed roles of both copper and zinc in endocytosis of PrP [97]. According to this study, both copper and zinc can stimulate the distribution of PrP to transferrin-containing endosomes as well as Golgi [97]. However, one argument raised against these findings is the fact that endocytosis of PrP, a GPI-anchored protein, is different from endocytosis of other GPI-anchored proteins excluding clathrin-coated pits and involving rafts or caveolae domains [97].

Since divalent cations, specifically copper, are important for both SOD-like activity and endocytosis of PrP\(^\text{C}\), investigations continued to find a probable role of these cations in the pathogenesis of prion diseases. Choi et al. showed copper concentration to be reduced significantly in brains of sporadic CJD (sCJD) patients, while manganese and zinc concentrations have significant and slight increases respectively [64, 98, 99]. Cu/Zn-SOD activity was significantly reduced while Mn-SOD activity was increased by two-fold in brains of sCJD patients [99]. Copper also decreases in brains of scrapie-infected mice after ninety seven days post-inoculation (dpi), which is prior to onset of symptoms, while its concentration starts to increase in the liver after sixty dpi suggesting copper is displaced from the brain to the liver in response to disease [100]. Manganese concentration also shows an increase in brain correspondingly and the manganese to copper ratio reaches its peak in the brain at the time of onset of symptoms [100]. Incubation of mouse Neuro2A (N2a) and scrapie N2a (ScN2a) cells in the presence of increasing concentrations of copper shows no alteration in the amount of PrP’s mRNA level, while protein expression increases [101]. In addition, subjecting protein extracts
from these cells to PK digestion confirmed that quantity of resistant PrP species decreases [101]. Administration of copper for thirty days to scrapie-infected hamsters also delayed the onset of symptoms [101]. Thereupon, this may suggest that PrP is a regulator of copper in the brain and abruption of its metal-transporting function in scrapie-infected mouse models may be associated with the pathogenesis of disease although there is no evidence suggesting that the depletion of copper in the brain is a main factor and cause of prion diseases.

On the other hand, there are studies suggesting copper has a critical role in facilitating conversion of PrP and its aggregation. For instance, in one study Wong et al. showed the chelation of copper with different copper chelators to cause a decrease in the formation of PrPSc [99]. The investigation by Kenward et al. suggested not only copper but also zinc as potential divalent cations inducing PrP aggregation [102]. The authors determined both copper and zinc to promote PrP-PrP interactions. Copper and zinc increase interaction of PrP peptides by 117% and 300% respectively with both glutamine and tryptophan residues in the octapeptide repeat region to be important for this interaction [102]. In addition, copper and zinc are necessary for the β-sheet aggregation and formation of amyloidogenic fibrils by PrP 106-126 [103].

On the other hand, further analysis of other divalent cations confirmed previous findings that manganese and zinc have higher concentrations in brains of scrapie-infected mice while there is no change in the concentration of cobalt. Treatment of scrapie-infected mice with a manganese chelator also showed longer survival suggesting an important role for manganese in binding to PrP and facilitating PrP’s aggregation [104]. In addition, a manganese-rich diet for normal mice leads to replacement of copper with manganese in binding to PrP accompanied by gain of PK-resistant character of the prion protein [104]. Therefore, manganese may be essential
for the formation of a seed of PK-resistant PrP molecules with the potency to convert to prion scrapie [104].

PrP has also been indicated to regulate iron and the level of iron has also been shown to increase in scrapie-infected brains. For instance, Singh et al. looked at the effect of PrP overexpression on iron homeostasis in N2a cells [105]. PrP overexpression in N2a cells results in higher uptake of iron and leads to lower expression of iron uptake proteins such as transferrin and its receptor [105]. Both the octapeptide repeat region and the GPI-anchor of PrP were shown to be necessary for this effect in N2a cells [105]. Analysis of scrapie-infected and CJD mouse brains revealed a four to six-fold increase in the concentration of iron [106]. Incubation of neuroblastoma cells with iron causes an up-regulation of PrP expression followed by its proteolytic cleavage to generate two fragments with molecular weights of 20kDa and 12kDa [106]. Besides, incubation of PrP\text{C} with iron leads to conversion of PrP\text{C} to a PrP\text{Sc}-like molecule and its aggregation, which is ten-fold less PK-resistant in comparison to brain-derived PrP\text{Sc} [106]. Depletion of iron, from infected mouse and human brains, reduces the amount of PrP\text{Sc} indicating iron to be essential for generation and propagation of the scrapie agent [106]. Contrary to these results, Fernaeus et al. suggested that iron deficiency may be relevant in prion diseases since they recorded a two-fold decrease in iron concentration of ScN2a cells in comparison to N2a cells [107].

Finally, it can be concluded that divalent transition metals such as copper and zinc share common binding sites within the octapeptide repeat region of PrP, are involved in PrP-dependent responses to oxidative stress and are linked to PrP expression for their regulation and distribution in the brain [108]. For example, PrP may bind to excess extracellular zinc and copper after neuronal excitation and return them into the cell to prevent from their toxicity [108]. On the
other hand, PrP may also be responsible for the uptake and intracellular transport of these
divalent cations where they are endocytosed by endosomes and secondary lysosomes [108]. The
lower pH in these organelles causes dissociation of PrP and metals [108]. While PrP can then
return to the plasma membrane, copper and zinc transport to other organelles [108].
Furthermore, association of copper and zinc with prion diseases is also worthy of analysis since the regulation of both copper and zinc has been shown to abolish in response to scrapie infection and their binding to PrP has been indicated to promote PrP-PrP interaction and aggregation.

1.1.8 Neuropathology of Prion Diseases

Prion diseases or TSEs can occur in both humans and animals. Human prion diseases include Kuru, CJD, variant CJD (vCJD), iatrogenic CJD (iCJD), sCJD, familial CJD (fCJD), GSS and fatal familial insomnia (FFI) [1, 3, 109]. The most common prion diseases in animals are scrapie in sheep and goat, bovine spongiform encephalopathy (BSE) or mad cow disease, and chronic wasting disease (CWD) in deer and elk [1, 3, 109]. Prion diseases are associated with massive neurodegeneration and spongiosis of the CNS, astrocytic gliosis, and amyloidogenesis (Figure 1.5) [1, 3, 109].

![Image of neuropathology of prion diseases](Figure 1.5)

**Figure 1.5- Neuropathology of prion diseases.** Frontal cortex of a patient died of non-cerebral causes and a patient died of CJD were stained with Hematoxylin-eosin (H-E), with antibodies against glial fibrillary acidic protein (GFAP) and with an antibody against PrP. H-E staining shows neuronal loss or spongiosis. Probing with GFAP antibody shows high proliferation of astrocytes or gliosis and finally probing against PrP shows high accumulation of PrP aggregates in the cerebral cortex (adapted by permission from Macmillan Publishers Ltd: [11], copyright © 2001).
Human prion diseases have three different aetiologies: they can arise sporadically, or can be inherited in an autosomal dominant manner, or can be acquired due to exposure to the scrapie agent [35]. Almost 85% of prion diseases arise sporadically and they can affect one to two individuals per million worldwide [35]. On the other hand, 10-15% of all prion diseases are caused by autosomal dominant mutations in the \textit{Prnp} gene [35]. FFI and fCJD are two types of TSEs that can be inherited through highly variable mutations in \textit{Prnp} [35]. Exposure to scrapie agent can also happen through oral consumption of food contaminated with human prions due to cannibalism, such as the case of Kuru in Papua New Guinea, and also through consumption of cattle suffering from BSE, such as the case of vCJD [35].

1.1.9 Prion Disease Therapeutics

Prion infections can be transmitted orally or through food-borne contamination in cases of Kuru, BSE and vCJD [3, 110]. In addition, there are other routes for the transmission and initiation of disease such as intravenous and intraperitoneal injections [3, 110]. Eye provides another route for the transmission of scrapie by conjunctival instillation [3, 111] and intraocular injection [3, 112].

There are three therapeutic approaches to prion diseases which are classified as curative, palliative and prophylactic therapies [113]. Clinical symptoms of prion diseases involve neuronal loss and vacuolation in the CNS; so, the curative therapy is looking for methods to replace these damaged neurons through regeneration and transplantation by using neuronal stem cells [113]. In addition, curative therapy is also concerned with the eradication of infection through elimination of scrapie prion and its aggregates [113]. On the other hand, palliative therapy attempts to decelerate the progression of disease by finding substances that can interfere with PrP$^\text{Sc}$ formation, lower PrP$^\text{C}$ expression, prevent from the activation of microglia and astrocytes, and
interfere with neuronal loss [113]. However, one major obstacle in this therapy is the lack of effectiveness of various drugs such as quinacrine [114], amphotericin B [115] and phorphyrins [116] in inhibition of PrPSc aggregation and infectivity in vivo [113] although they have proved effective in in vitro studies.

The third kind of therapy is called prophylaxis that looks for methods to intervene with the path through which PrPSc enters body and transports from lymphoreticular organs to the peripheral nervous system (PNS) and from there to the CNS [113]. The prophylactic therapy is divided into pre-exposure and post-exposure prophylaxes [113]. In order to understand how prophylactic therapy works, it is necessary to review the route of prion entrance and transport from the intestine to the CNS.

After consumption of scrapie-infected food, microfold cells (M cells) of intestine actively transport scrapie to the basolateral side of the intestine [109, 117]. PrPSc is then endocytosed by intestinal epithelial cells and can be transported to gut-associated lymphoid tissues (GALT), such as mesenteric lymph nodes, through a ferritin-dependent mechanism [118]. Since PrPC is widely expressed in different organs such as the brain, tonsils, spleen, GALT, and skeletal muscles, it induces tolerance in the body and therefore the introduction of scrapie agent to lymphoid tissues does not stimulate the body’s immune system to respond by generation of anti-PrPSc antibodies [109, 117]. PrPSc accumulates within the germinal B-cell follicles on follicular dendritic cell (FDCs) and also within macrophages while it is in the lymphoid tissue [119-121]. Dendritic cells can also directly obtain PrPSc from the intestine by opening tight junctions to join their dendrites to intestinal epithelial cells [122]. The expression of high levels of PrPC within FDCs helps to maintain PrPSc and induce its aggregation [109]. It is supposed that the spread of scrapie-agent from GALT to the CNS happens through the enteric nervous system [109, 117]. However, a
recent study by Gousset et al. showed that dendritic cells can directly transfer PrP\textsubscript{Sc} to the PNS through the formation of tunnelling nanotubes (TNTs) [123].

In the pre-exposure prophylaxis, specific prion antibodies are used to block the formation of PrP\textsubscript{Sc} and its aggregates [113]. For instance, pre-incubation of the scrapie agent with anti-PrP antibody has been shown to reduce prion titre in the hamster brain [124]. In addition, PrP antibodies can abolish PrP\textsubscript{Sc} formation in a cell-free system [125]. However, the widespread expression of PrP in the body and the tolerance of immune system to PrP make it difficult to stimulate B cells for the generation of these PrP-specific antibodies to test antibody efficiency in the inhibition of PrP\textsubscript{Sc} formation in \textit{in vivo} models [113, 126]. On the other hand, the post-exposure prophylaxis therapy mainly focuses on the inhibition of PrP\textsubscript{Sc} transmission through lymphoreticular organs and the PNS [113, 126]. For instance, it has been shown that both tumour necrosis factor (TNF) receptor I (TNFR1) and lymphotoxin-β receptor (LT\textbeta R) are required for the development and maintenance of FDCs [127, 128]. Inhibition of signalling pathways through TNFR1 and LT\textbeta R in FDCs has been shown to prolong scrapie development and aggregation after intraperitoneal injection of PrP\textsubscript{Sc} [127, 128]. Interruption of the function of the PNS or sympathectomy by using antibodies against the nerve growth factor also led to prolongation of scrapie development and aggregation [113, 126, 129].

1.2 Overview of Zrt-, Irt-Like Protein (ZIP) Biology

Zinc is an essential micronutrient for the body and it plays important roles in the cellular physiology and metabolism. Zinc acts as a catalytic cofactor for more than three hundred enzymes and it is associated with functions such as secretion, signal transduction and gene expression [130]. Zinc deficiency can lead to growth retardation, immune system deficiencies and neuronal disorders [131]. In the brain, zinc is mostly found in glutamate synaptic vesicles of
presynaptic nerve terminals [86]. During neurotransmission, zinc is released with glutamate and once its concentration reaches high amounts, it starts to inhibit postsynaptic glutamate receptors [86]. Zinc has also been associated with Alzheimer’s disease since its accumulation and interaction with Aβ leads to the aggregation of this protein [86]. The cellular regulation of zinc is mediated through zinc binding proteins and transporters. Metallothioneins (MTs) are cytosolic proteins and their expressions are regulated by metal regulatory transcription factor 1 (MTF-1) and concentration of metal ions [130]. MTs work by binding to and chelating the excess amount of extracellular zinc and other metals. Zinc transport into and out of cells is also mediated by facilitated diffusion through zinc transporters since zinc ions are positively charged and cannot cross the plasma membrane by simple diffusion [130].

Dietary zinc is first absorbed by epithelial cells on the apical surface of the intestine and it is transported by an unknown mechanism to the basolateral surface and from there to blood circulation where most of zinc concentration binds albumin and α2-macroglobulin [132, 133]. Blood circulation is responsible to provide cells with appropriate concentrations of zinc for four distinct applications. Cells may require zinc to bind to metalloproteins as a cofactor for the proper function of these enzymes [132, 133]. Another concern for cells is the buffering of zinc in the body to protect from zinc cytotoxicity [132, 133]. Therefore, MTs can bind to zinc and act as metal chelators for the buffering of this metal in the body. Once inside the cell, zinc can also be transported to different organelles for the proper function of zinc-dependent proteins [132, 133]. Finally, the sequestration of zinc in endosomes and lysosomes can lead to its storage and detoxification [132, 133].

There are two main groups of zinc transporters in mammalian cells. One group is called the solute carrier family 30A (SLC30A) and the second group is known as solute carrier family
39A (SLC39A) [130]. Other than transportation of zinc in and out of the cell, members of both families are able to contribute to transportation of other metal ions such as iron, copper, cobalt and nickel [132, 133].

Zinc transporters (ZnTs) or cation diffusion facilitators (CDFs) belong to the SLC30A family of zinc transporters and they are responsible for the reduction of intracellular zinc concentration by its transport to organelles and ECM (Figure 1.6B) [130]. ZnT family consist of eight members and they are divided into three subfamilies [130]. The members in Subfamily I are all prokaryotic zinc transporters, while members of subfamilies II and III contain both prokaryotic and eukaryotic zinc transporters [130].

ZnTs consist of six transmembrane domains with cytoplasmic N-terminal and C-terminal regions [132-134]. There is also a long histidine-rich motif between transmembrane domains IV and V proposed to be responsible for metal binding [132-134]. It has also been proposed that transmembrane domains I, II, V and VI form a channel through which zinc is transported out of the cell [132, 133].
Figure 1.6 - Comparison of ZIP and ZnT properties. (A) ZIP proteins are responsible for intracellular zinc import to increase the cytosolic zinc concentration. They may form ZIP dimers to facilitate zinc transport and the presence of an N-terminal ectodomain can prevent from the opening of the ion channel and zinc uptake. The histidine-rich region located between transmembrane domains IV and V may be involved in the formation of the ion channel and binding to zinc (adapted by permission from Elsevier Ltd: [135], copyright © 2011). (B) ZnTs or cation diffusion facilitators (CDFs) are involved in the intracellular zinc export to decrease cytosolic zinc concentration. They form ZnT homodimers to facilitate zinc export and studies on ZnT proteins from Escherichia coli (E. coli) shows ZnTs to be anti-port ion channels that can, concomitantly to zinc export, increase the uptake of hydrogen ions by facilitating their transport from ECM through the same channel (adapted by permission from Elsevier Ltd: [135], copyright © 2011).

On the other hand, Zrt-, Irt-like protein (ZIP) transporters, named after the yeast Saccharomyces cerevisiae zinc-regulated transporter 1 (Zrt1) and the Arabidopsis thaliana iron-regulated transporter 1 (Irt1) proteins, are members of the SLC39A family of zinc transporters and they consist of fourteen members [132-134]. ZIP transporters increase intracellular zinc concentration by its transportation from organelles and ECM to the cytosol (Figure 1.6A) [132-134]. During Zinc-replete conditions, the N-terminal ectodomain blocks the ion channel in ZIP proteins preventing from zinc import to the intracellular space [135]. On the other hand, ZIP transporters are expressed at a higher level during zinc-deplete conditions [135]. In addition, their ectodomain is cleaved by an unknown mechanism opening the ion channel and facilitating zinc transport (Figure 1.7) [135].
Figure 1.7- Adaptive cellular responses of ZIP proteins to changes in extracellular divalent cation levels. This model is based on biochemical data reported for ZIP4 and unpublished observations made for ZIP6 and ZIP10. (A) Zinc supplementation causes the lower expression of ZIP transporters and the inhibition of ZIP ion channel by the N-terminal ectodomain leading to the suppression of cytosolic zinc import (adapted by permission from Elsevier Ltd: [135], copyright © 2011). (B) During normal conditions ZIP proteins are expressed at a higher level. The ectodomain still partially blocks the ion channel and prevents from high zinc uptake by the transporter (adapted by permission from Elsevier Ltd: [135], copyright © 2011). (C) In zinc-deficient conditions ZIP proteins are highly expressed and the ectodomain of these proteins is cleaved by an unknown protease leading to the opening of zinc ion channel and zinc uptake (adapted by permission from Elsevier Ltd: [135], copyright © 2011).

ZIP transporters are predicted to have eight transmembrane domains and their N-terminal and C-terminal regions are located in the ECM (Figure 1.8C) [132-134]. There are conserved histidine residues between transmembrane domains IV and V that are involved in metal binding [134]. This is the most conserved region among ZIP family members predicted to play a role in the formation of a channel for zinc transport [132, 133]. ZIP zinc transporters also contain another histidine-rich region of the form HXHXH (H: histidine, X: any amino acid) between transmembrane domains III and IV proposed to be involved in zinc binding and transport [134]. ZIP transporters are divided into four subfamilies. Members of Subfamily I are fungal and plant ZIP transporters, while subfamily II consists of mammalian, nematode and insect genes [136].
Third subfamily is gufA subfamily which is related to the gufA gene of *Myxococcus xanthus* with unknown functions [136]. The fourth subfamily is called the LIV1 subfamily and is related to the oestrogen-regulated gene, LIV1 or ZIP6 [132, 133, 136]. LIV1 subfamily of zinc transporters consists of nine ZIP members [136]. These ZIP transporters are placed into a different subfamily based on the presence of a motif of the form HEXPHEXGD (H: histidine, E: glutamic acid, P: proline, G: glycine, D: aspartic acid, X: any amino acid) in the transmembrane domain V that fits the consensus sequence of the active site in metalloproteinases (Figure 1.8B) [136].

The most important subfamily of metalloproteinases is called matrix metalloproteinase (MMP) subfamily. MMPs are a family of zinc-dependent endopeptidases and they play crucial roles in tissue remodelling and organ development, and in diseases such as cancer [137, 138]. They consist of three domains: the pro-peptide, the catalytic domain and the hemopexin-like C-terminal domain [137, 138]. MMPs are initially in inactive state based on the interaction of a cysteine residue in the pro-peptide domain with a zinc ion located on the catalytic site [137, 138]. Disruption of this interaction by cysteine switch, proteolytic removal of the pro-peptide domain by convertases or the chemical modification of the cysteine, leads to the enzyme’s activation [137, 138]. Similarly, activation of the metalloproteinase domain of LIV1 subfamily of ZIP transporters can occur through the same mechanism and lead to proteolytic cleavage of membrane-located proteins. LIV1 members contain additional histidine-rich regions on their N-termini and also their extracellular loops between transmembrane domains II and III [132, 133, 139]. LIV1 members also contain a CPALLY (C: cysteine, P: proline, A: alanine, L: leucine, Y: tyrosine) motif between two conserved cysteine residues (Figure 1.8B) [132, 133, 139].

Among ZIP transporters, most investigations have focused on mammalian ZIP4 since mutations in this protein lead to the zinc-deficiency disease acrodermatitis enteropathica (AE)
In addition, several manuscripts have also focused on mammalian ZIP6 and ZIP10 since they are associated with breast-cancer metastasis [132, 133, 139].

In response to zinc deficiency, ZIP4 accumulates on the apical surface of enterocytes and endoderm cells for the absorption of zinc and its transport from the intestine to blood circulation [131, 140]. AE is a rare autosomal recessive disorder caused due to the loss of function of ZIP4 and thereby the inefficient absorption of zinc [132, 133, 139]. Analysis of ZIP4 in intestinal cells has shown that the active form of ZIP4 is a 37-40kDa protein suggesting that ZIP4 undergoes proteolytic cleavage to become activated [131, 141-143]. During zinc-replete conditions, ZIP4 is endocytosed and degraded through the ubiquitin-dependent pathway [144]. ZIP4 is also important during the development of an organism as homozygous ZIP4-knockout mice display severe malformations and die during embryogenesis on day ten, a phenotype that cannot be rescued by feeding pregnant mothers a zinc-enriched diet [145].

ZIP5 has high expressions in the liver, kidney, pancreas, and throughout the small intestine and colon [132, 133, 139]. Like ZIP4 transporter, ZIP5 is also involved in zinc absorption from the intestine, but, unlike ZIP4, ZIP5 is located on the basolateral surface of intestinal cells during periods of zinc supplementation [132, 133, 139]. However, during zinc-deplete conditions, the protein is internalized and degraded in enterocytes, acinar cells and endoderm cells [132, 133, 139]. This can suggest that ZIP5 opposes ZIP4 in function and it may be involved in the determination of the body’s zinc status and concentration in enterocytes [134, 142, 146, 147].

ZIP6 (LIV-1) was identified as a novel gene whose expression is stimulated by oestrogen treatment of breast cancer cells [132, 133, 139]. ZIP6 has higher expression levels in carcinoma cells and metastatic breast cancer cells suggesting an important role for ZIP6 in the progression
of cancer [134, 148, 149]. Studies on zebrafish have also shown that signal transducer and activator of transcription 3 (STAT3) can activate the expression of ZIP6 leading to facilitation of Snail translocation to the nucleus (Figure 1.9) [134, 150]. Snail is a zinc-finger transcription factor repressing the expression of adhesion molecules such as epithelial-cadherin (E-cadherin) and inducing epithelial-to-mesenchymal transition (EMT) [134, 150]. ZIP6 has also been shown to be important in the regulation of zinc concentration for the proper function of the host immune cells [134, 150]. Exposure of dendritic cells to lipopolysaccharide (LPS) leads to the down-regulation of ZIP6 that is further associated with decreased intracellular zinc and increased surface expression of major histocompatibility complex (MHC) class II molecules and therefore increased maturation of dendritic cells [134, 151].

The expression of ZIP10 is repressed by MTF-1 [134, 150]. Studies on zebrafish have shown that ZIP10 expression is supressed by the presence of zinc in both the gill and kidney of the fish [134]. Rat ZIP10 is located on the plasma membrane and it is involved in the transport of zinc [134, 150]. However, contrary to ZIP10 from the mouse and zebrafish, rat ZIP10 expression increases in the presence of zinc [134]. In addition, rat ZIP10 expression is increased in the presence of thyroid hormone [134]. Like ZIP6, ZIP10 is also involved in metastatic breast cancer due to its higher expression in cancerous cells in comparison to normal cells [134, 150]. Analysis of ZIP10 mRNA expression shows that ZIP10 is strongly involved in the metastasis of breast cancer to lymph nodes [134, 150]. It has also been indicated that ZIP10 may play a role in the migratory activity and invasiveness of breast cancer cells since knockdown of ZIP10 and the accompanied reduction in intracellular zinc concentration leads to lower migratory activity of breast cancer cell lines [134, 152].
1.3 Family Reunion – The ZIP/Prion Gene Family

One of the questions in prion biology has always been concerned with the evolution of prion gene from a known ancestor gene that may uncover mysteries about the cellular function of PrP or its pathogenesis in TSEs. Schmitt-Ulms et al. have recently proposed an evolutionary link between the prion gene and ZIP metal ion transporters merging the two protein families [44]. The authors analyzed the interactome of PrP from mouse N2a cells using electrospray ionization mass spectrometry (ESI-MS) and found that three members of the LIV1 family of ZIP transporters, including ZIP5, ZIP6 and ZIP10, are able to interact with PrP C [44]. Schmitt-Ulms et al. compared PrP and ZIP10 sequences from a wide range of species within the chordate lineage (Figure 1.8A). PrP and ZIP sequences are found among all mammals and most species of vertebrate lineage. However, ZIP transporters are also present in more primitive organisms and ZIP-related sequences can be found in all kingdoms, including bacteria and plants.

Sequence analysis and structural threading showed that the Prnp gene has evolved from the gene of a ZIP ancestor protein. It was indicated that a 111-amino acid fragment within the N-terminal region of ZIP10 transporter, residues 285-395, showed general alignment with the C-terminal globular region of PrP (16% identity, 42% similarity) (Figure 1.8A) [44]. PrP and ZIP10 sequence homologies show more divergence in mammals than in more primitive organisms such as turtle and fish (Figure 1.8A) [44]. Furthermore, conserved cysteine residues within the cysteine-flanked core (CFC) region of PrP, located on PrP’s C-terminal domain, are positioned in a similar region in comparison to cysteine residues in the ZIP10 conformation (Figure 1.8A) [44].
Figure 1.8 - Comparison of primary structures and membrane topologies of PrP and LIV-1 family of ZIP proteins. (A) Sequence analysis showing the common origin and divergent sequence evolution of ZIP and prion proteins. Multiple sequence alignments of PrP and ZIP10 from different species indicate that there is higher homology between PrP C-terminus and ZIP10 prion-like (PL) domain in earlier species such as turtle and fish, while there is greater divergence of sequences in humans and mice (adapted by permission from Schmitt-Ulms et al.: [44], copyright © 2009, open-access: creative commons attribution license, http://creativecommons.org/licenses/by/3.0/). (B) Depiction of the primary structures of PrP and ZIPs shows the N-terminal domains of ZIP5, ZIP6 and ZIP10 to be homologous to the C-terminal region of PrP in that they have the hydrophobic region and the PL domain in common. The PL domain is flanked by two cysteine residues, located within the cysteine-flanked core (CFC) region in both PrP and ZIP transporters. These cysteine residues may be able to form a disulfide bond in ZIP proteins, similar to PrP, and ZIP transporters are predicted to contain one glycosylation site in the PL domain. There is a conserved “CPALLY” motif in the PL domain of ZIP transporters. LIV1 proteins are predicted to consist of eight transmembrane domains on their C-terminus with a conserved “CHELHELGD” that is similar to the active site of metalloproteinases (adapted by permission from Elsevier Ltd: [135], copyright © 2011). (C) Schematic showing the tertiary structures and membrane topologies of PrP and ZIP transporter proteins. ZIP proteins are predicted to have a disordered N-terminal region followed by the PL domain that has striking resemblance to PrP with regard to relative position and order of secondary structure domains. Similar to PrP, ZIPs have three α-helices and two anti-parallel β-sheets in their PL domains (adapted by permission from Elsevier Ltd: [135], copyright © 2011).
Highly similar motifs were also found within the N-terminal regions of ZIP5 and ZIP6 [44]. High similarity of N-terminal motifs in ZIP5, ZIP6 and ZIP10 to PrP led Schmitt-Ulms et al. to name this region as the prion-like (PL) domain, which includes the CFC region [44].

PL domains of ZIP5, ZIP6 and ZIP10 are predicted to contain three \(\alpha\)-helices and a small \(\beta\)-sheet composed of two short \(\beta\)-strands, similar to PrP conformation (Figure 1.8B and Figure 1.8C) [44]. The two C-terminal \(\alpha\)-helices of ZIP5, ZIP6, and ZIP10 PL domains are predicted to form a disulfide bridge (Figure 1.8B).

Comparison of ZIP and PrP expression shows that the expression profile of PrP may more closely resemble the expression of ZIP6 and ZIP10 than the expression profile of ZIP5 since the highest levels of PrP\(^C\) expression are observed in the mammalian CNS, substantial levels are also observed in cardiac muscle and lung, and lower levels can be found in other tissues including intestine, limb muscles and spleen, and on lymphocytes. However, cell specific characterizations of expression levels are needed to dissect whether levels of PrP and individual ZIP proteins are correlated. Like many ZIPs, expression of PrP appears to respond to the presence of metals in complex ways discussed previously [98, 108].

What can insights into signalling pathways operating upstream or downstream of ZIP5, ZIP6 or ZIP10 tell us about PrP? Very little is known about the regulation of expression of ZIP5 or ZIP10. Given the zinc-responsiveness of expression observed for many zinc transporters, it is not surprising that metal response elements (MREs) exist in their promoter regions [153]. This is similar to the prion gene for which it has been shown that copper-replete conditions can promote its expression in certain cell types, including human fibroblast cells, through the activity of MTF-1 [154]. Additional promoter elements may also come into play; in the case of PrP, for example, a well-known pathway that involves the ataxia telangiectasia mutated (ATM) protein kinase and...
components of the MAP kinase pathway culminates in the transcription factor SP1 occupying its corresponding binding sites on the PrP promoter and causing an up-regulation of PrP expression in response to an increase in intracellular copper [155]. The 5′-upstream region of the human Prnp gene contains putative binding sites for many additional transcription factors including activator protein 1 (AP-1) and activator protein 2 (AP-2), protein 53 (p53), myogenic differentiation 1 (MyoD) and NK2 transcription factor related, locus 5 (NKX2-5) [156, 157]. Comparably little is known about promoter elements within ZIP genes. While compelling data have established a role for STAT3 acting upstream of ZIP6 in zebrafish [150], it is currently not known whether STAT3 acts immediately upstream of ZIP6, for example as an activator of its transcription, or whether signalling mediators are positioned between STAT3 and ZIP6 (Figure 1.9). In the fruitfly, fear of intimacy (FOI) is transcriptionally activated by Tinman [158, 159], the Drosophila melanogaster ortholog to the mammalian NKX2-5 transcription factor [160] for which, as mentioned, consensus binding elements have also been described in the prion promoter (Figure 1.9).
Figure 1.9- ZIP-dependent signalling upstream of E-cadherin expression in three biological paradigms. Up-regulation of ZIP6 expression by STAT3 in zebrafish leads to the translocation of Snail to the nucleus and repression of E-cadherin expression that is essential for epithelial-to-mesenchymal transition (EMT) during zebrafish gastrulation. ZIP6-mediated nuclear translocation of Snail has also been indicated in human cancer metastasis. A parallel pathway also functions during Drosophila melanogaster tracheal development in which a homologue of ZIP transporter protein known as fear of intimacy (FOI) facilitates translocation of Escargot (Esg), homologue of mammalian Snail, to repress E-cadherin expression. Expression of FOI is induced by Tinman transcription factor, a homologue of mammalian NKKX2.5 transcription factor acting upstream of the mammalian prion protein. Moreover, zebrafish PrP-1 protein modulates cell adhesion by regulating the transport of E-cadherin to the plasma membrane. The effects of ZIP6 and PrP on E-cadherin regulation may simply be independent of each other and be based on the abilities of these proteins to influence cellular zinc and/or copper homeostasis. On the other hand, E-cadherin regulation by both ZIP6 and PrP during zebrafish gastrulation may happen through ZIP6-PrP interaction (adapted by permission from Elsevier Ltd; [135], copyright © 2011).

The similarity of gastrulation phenotypes linked to PrP deficiency and ZIP6 (LIV-1) inactivation in zebrafish may be a particularly rewarding angle from which to explore overlaps in signalling pathways downstream of ZIP6 and/or PrP [150, 161]. It is noteworthy that both publications tied PrP or ZIP6 functions to the regulation of cell adherence and the E-cadherin protein. How does ZIP6 expression influence E-cadherin levels? A first indication of what might be occurring is the observation of a ZIP6-dependent nuclear translocation of the transcriptional repressor Snail [150], a master regulator of EMT [162]. As a zinc finger protein, Snail requires zinc as a structural cofactor for binding to elements present within the E-cadherin promoter. Consequently, the possibility arises that a local increase in zinc levels, mediated by ZIP6-dependent zinc import, charges cytoplasmic Snail with zinc and thus triggers its translocation into the nucleus (Figure 1.9). Consistent with observations in zebrafish, the fruitfly ZIP ortholog
FOI acts upstream of the Snail family member Escargot (Esg) to exert tracheal branch fusion process [163]. Surprisingly, whereas both tracheal and gonadal FOI-mediated phenotypes in the fruitfly depend on E-cadherin expression, the gonad coalescence phenotype appears to be independent of Esg (Figure 1.9). In light of data reviewed herein, the link between ZIP6 and cancer metastasis may be explained by a body of literature that ties the molecular biology revolving around STAT3, EMT and Snail [164] to cancer malignancy [165, 166]. Cells with ectopic Snail expression adopt a fibroblastoid phenotype and acquire tumorigenic and invasive properties [162]. In line with this model, recent reports document that inhibition of ZIP6 in both cervical cancer-derived HeLa cell model and pancreatic cancer cells causes the deactivation of Snail and interferes with their metastatic features [167, 168]. However, it is already emerging that the underlying phenomena are considerably more complex than we currently understand and likely differ amongst experimental paradigms. Thus, additional stimuli, such as the exposure of cells to bacterial LPS, have been shown to feed into signalling pathways that control ZIP6 expression [151]. Furthermore, E-cadherin levels correlate inversely with ZIP6 levels in some paradigms but in others appear to follow ZIP6 levels directly as, for example, in breast tumour cells [169] or the previously mentioned FOI-dependent gonad formation phenotype in the fruitfly. Interestingly, ZIP6 also appears to have opposite effects when compared to PrP on E-cadherin levels in the zebrafish gastrulation paradigm. Whereas knockdown of PrP appears to cause a destabilization of mature E-cadherin pool, transcriptional inactivation of ZIP6 is linked to E-cadherin stabilization (Figure 1.9). The observed influence of PrP on cadherin may well be independent of ZIP zinc transport activities and instead be based on PrP's ability to influence cellular copper and zinc homeostasis [170]. Alternatively, PrP may exert its influence on EMT through binding to NCAM [38], an interaction known to facilitate the recruitment of NCAM into
lipid rafts and to promote activation of Fyn kinase [37]. Fyn has been shown to phosphorylate focal adhesion kinase and thus promote the assembly of integrin-mediated focal adhesions, cell spreading and EMT [171]. Ablation of NCAM inhibits EMT and overexpression of NCAM correlates with tumour invasion [172]. Finally, an intriguing possibility is that PrP may directly influence signalling events outlined above based on its capacity to bind to ZIP6, consistent with our data documenting co-affinity purification of PrP and ZIP6 following in vivo crosslinking with formaldehyde [173]. The latter explanation would suggest that the influence of PrP on ZIP6 is that of a negative regulator.

Currently, very little is known about the role of the PL domain in ZIP transporters and therefore one can only speculate about what such a function might be. The observation that both unicellular organisms and plant genomes appear to lack ZIP transporters containing a PL domain [44] indicates that this domain is not part of the catalytic core required for metal import. Mutations located in the N-terminal domain of ZIP4, including a subset of mutations mapping to the PL domain, have been linked to AE, indicating that this domain may be critical for a ZIP transporter responsible for dietary zinc uptake [141]. Significantly, the latter mutations have been shown to interfere with the aforementioned ZIP4 ectodomain shedding observed during prolonged zinc deficiency, a cellular response that led to an increase in the rate of zinc import by the truncated ZIP transporter [143]. Taken together, these data suggest that the entire N-terminal domain may serve a role as a negative regulator or buffer of metal import when zinc is replete, which can be shed when zinc is limiting [143]. The PL domain itself may represent a molecular module within the N-terminal domain which can integrate cues of metal sensing and translate them into the protection (replete metal status) or exposure (limiting metal status) of a nearby proteolytic cleavage site. Mechanistically, such a switch could, for example, rely on the PL
domain controlling access to a proteolytic cleavage site by engaging in protein–protein
interactions or steering the spatial distribution of ZIP transporters. Alternatively, the PL domain
may be able to undergo a metal-dependent conformational change that exposes a nearby scissile
bond. Due to their limited mobility or outright sessile nature, unicellular organisms and plants
are typically exposed to relatively stable metal levels in their environment. Metazoa, in contrast,
are exposed to relatively large fluctuations of metal levels either through dietary exposure or on
account of their mobility. Thus, the evolutionary success of the PL domain in this branch of life
might be the result of a natural adaptation that enabled metazoa to cope with complex changes to
metal levels.

Naturally, it is currently unclear whether present-day PrP\textsuperscript{C} molecules have retained
functional features of ZIP PL domains. It will, for example, be of interest to determine whether
PrP recapitulates zinc- or copper-dependent endoproteolytic cleavages observed in ZIPS. A hint
that endoproteolytic cleavages are critical for PrP to exert its biological role has come from the
previously mentioned myelin-maintenance phenotype observed in \textit{Prnp}-knockout mice. Interestingly, this phenotype could be rescued by reintroduction of full-length PrP or PrP
constructs that retained an amino-proximal cleavage site, but not by variants non-permissive to
cleavage [62].
Chapter 2

Rationale, Hypothesis, and Objectives
2.1 Rationale

Since its discovery and identification of its transmissibility from animals to humans by the consumption of BSE-infected cattle meat, vCJD was predicted to rise into an epidemic due to the exposure of the majority in the United Kingdom and other European countries to BSE-infected products [30, 35]. Although the number of vCJD cases reported has been small, one hundred and forty-three cases as of December 1, 2003 [30, 35], there are two concerns with regard to this disease. One concern is with the long incubation time for prion diseases that might take decades for the appearance of an epidemic in a significant number of individuals infected with PrP\textsuperscript{Sc} [30, 35]. In addition, there is a concern about the second peak of vCJD in response to human-human haematogenous transmission that have led to the increasing number of infected individuals since 2005 [30]. Therefore, it is important to continue research on the origin and function of PrP\textsuperscript{C}, the identification of PrP\textsuperscript{C} interacting partners and their contribution to prion diseases, and the mechanism of PrP\textsuperscript{C} conversion to the disease-associated form of the protein.

However, despite intensive studies on the mammalian prion protein, its physiological function remains elusive and several groups have attempted to better understand the cellular function of this protein by thorough analysis of its interacting partners on the plasma membrane. For instance, Sho was discovered as a PrP-interacting protein and sequence analysis led to its categorization as the third member of the prion protein family due to its homology to the N-terminal region of PrP [9, 56]. Furthermore, functional analysis revealed that Sho, similar to PrP, has neuroprotective functions in the CNS to play a compensatory role for PrP as PrP-deficient mice expressing Sho are rescued from the neurotoxic impacts of Dp1 in the CNS [56]. The expression level of endogenous Sho protein also decreases in response to scrapie infection in mouse brains [9, 56].
A recent interactome study by Schmitt-Ulms et al. not only introduced ZIP transporters to interact with PrP but also to be evolutionary linked to the prion protein [44]. Sequence analysis and structural threading further proposed PrP may have descended from an ancestor of LIV1 family of ZIP transporters [44]. The similarity between ZIP transporters and PrP is largely confined to hydrophobic and PL-like domains of the prion protein [44], two domains associated with prion diseases. The hydrophobic domain is important in both the PrP\textsuperscript{C}-PrP\textsuperscript{Sc} conversion [174] and the generation of C1 and C2 fragments through proteolytic cleavage of PrP [62]. In addition, C1 fragment plays a crucial role in neuroprotection by facilitating the myelination of axons in the PNS [62] during normal conditions, and PrP C1 and C2 fragments are also important in neurodegeneration which is associated with the down-regulation of the PrP C1 fragment and the up-regulation of the PrP C2 fragment [79]. The PL-like domain is also associated with prion diseases since the thiol groups, located in this region, are essential for PrP\textsuperscript{C} binding to PrP\textsuperscript{Sc} and PrP\textsuperscript{C}-PrP\textsuperscript{Sc} conversion process through a covalent thiol/disulfide exchange reaction [175, 176].

Since both the hydrophobic domain and the PL-like region are also present in ZIP transporters, these proteins may possess some functional and biochemical similarities to PrP. Therefore, it is important to study ZIP transporters in the context of the prion protein to identify their potential roles in the cellular function of PrP, the conversion of PrP\textsuperscript{C} to PrP\textsuperscript{Sc}, and the PrP pathogenesis in prion diseases.
2.2 Hypothesis

The study of the LIV1 sub-branch of ZIP proteins and their physiological crosstalk with the prion protein will uncover promising angles from which to understand the biology of the prion protein in health and disease.

2.3 Objectives

1. ZIP mRNA levels are identified in mouse N2a cells and CD1 brains by reverse transcriptase polymerase chain reaction (RT-PCR) to find differences in ZIP expression between N2a cells and CD1 brains and to decide whether N2a cell is a suitable model for studying ZIP transporters in vitro. High endogenous PrP expression, relative ease to infect with PrP$^{Sc}$ and the ability to retain PrP replication after several generations are characteristics that suggest N2a cells to be appropriate for studying PrP biology.

2. Antibodies are raised against the N-terminal domains of mouse ZIP6 and ZIP10 transporters to characterize these ZIPs biochemically by analyzing their protein expression levels and their post-translational modifications, such as endoproteolysis, in N2a cells and mouse brains. The N-terminal regions of both ZIP6 and ZIP10 have been selected to raise antibodies because of the relative flexibility of these N-terminal domains in comparison to C-terminal transmembrane domains predicted from structural threading of ZIP10.

3. Expression plasmids and knockdown reagents are constructed for ZIP transporters in order to observe any potential changes in PrP expression and/or post-translational modification in response to alterations in the expression of ZIP transporters in N2a cells.
4. The endogenous expression and post-translational modifications of ZIP6 and ZIP10 are compared between infected and non-infected N2a cells and FVB/NCr mouse brains to identify the involvement of ZIP transporters in scrapie infection and prion propagation.

5. Metal chelators with high affinity for zinc are used in the presence of N2a cells to develop assays for the assessment of post-translational modifications of PrP and ZIP transporters during zinc deficiency, and to draw similarities in the PrP/ZIP processing between periods of zinc deficiency and scrapie infection that may point to the importance of metal regulation in prion diseases.
Chapter 3

Investigations into the Significance of the Evolutionary Descent of Prion Genes from Ancestral ZIP Transporters
3.1 Introduction

The cellular prion protein (PrP\(^C\)) is a GPI-anchored glycoprotein [1-4]. According to the protein-only hypothesis, the conversion of PrP\(^C\) to the disease-associated form of the protein, known as PrP scrapie (PrP\(^Sc\)), is the cause of prion diseases [1]. PrP\(^Sc\) has more β-sheet content in comparison to PrP\(^C\) which makes it more potent for aggregation leading to neuronal loss and gliosis [1-4]. Mouse knockout models for the \(Prnp\) gene have shown that the expression and presence of PrP\(^C\) is absolutely required for the generation of PrP\(^Sc\) aggregates and neurodegeneration upon inoculation of mice with PrP\(^Sc\) [29].

The prion protein family has three members including PrP, Doppe1 (Dp1) and ShadoO (Sho) proteins. PrP is widely expressed in the human body with the highest expression being in the CNS [9]. On the other hand, expression of Dp1 is mainly restricted to the heart and testis [48, 51, 52]. The expression of Dp1 in brains of \(Prnp^{-/-}\) mice is associated with neurotoxic effects in the CNS not only suggesting that Dp1 has neurotoxicity but also that PrP has neuroprotective effects since its co-expression with Dp1 in \(Prnp^{-/-}\) mice prevents from the neurotoxic effects of Dp1 [48, 51, 52]. Sho is mainly expressed in the Purkinje cells of the cerebellum and dendritic processes of hippocampal pyramidal cells [56]. Sho also possesses the same neuroprotective characteristic as PrP when it is co-expressed with Dp1 in the brains of \(Prnp^{-/-}\) mice [9, 56].

Several efforts have been made to identify the cellular function of PrP\(^C\), but its function remains largely elusive. Although there are studies that suggest PrP to play roles in neurite outgrowth [57-59], myelin sheath maintenance [62], and prevention of apoptosis [60, 61], there is no definitive role dedicated to the prion protein. Several groups have attempted to better understand the cellular function of the prion protein by investigating the cellular environment in which PrP exists and by identifying PrP’s interacting partners on the plasma membrane. Proteins
such as NCAM [37, 38] and STI1 [46, 58] have been identified to interact with the prion protein and to cooperate with PrP in neurite outgrowth and synaptogenesis. However, interaction of PrP with none of these proteins has been indicated to be essential for the propagation of PrP\textsuperscript{Sc} and pathobiology of prion diseases.

A study by Schmitt-Ulms et al. identified ZIP transporters as interacting partners of the prion protein [44]. In addition, this study also used sequence analysis to show that three members of the LIV-1 family of ZIP transporters, including ZIP5, ZIP6 and ZIP10, have high homology in their N-terminal regions, so-called the prion-like (PL) domains, to the hydrophobic and the cysteine-flanked core (CFC) region of PrP that are both important for scrapie propagation [44]. Therefore, studying the biology of ZIP transporters, more specifically ZIP5, ZIP6 and ZIP10, may reveal important cellular roles for the prion protein, such as regulation and transport of metal ions, with significant implications in prion diseases. In addition, the interaction between ZIP transporters and PrP on the cell surface may influence PrP’s function and abolishment of this interaction may be involved in prion diseases.

This study presents the biochemical analysis on ZIP transporters and PrP with the emphasis that the investigation into the function of ZIP transporters may draw parallel biology with regard to the prion protein and that the ZIP-PrP interaction may influence the cellular function of PrP or its pathobiology in prion diseases. The preliminary observations clearly showed that both overexpression and knockdown of ZIP transporters did not affect PrP expression or processing directly. However, scrapie infection in mouse N2a cells led to the lower expression of both ZIP6 and ZIP10 proteins. In addition, further analysis of ZIP transporters in 131dpi scrapie-infected mouse brain showed that ZIP6 and ZIP10 were proteolytically cleaved to generate 50-51kDa N-terminal fragments. PrP analysis revealed that the endoproteolysis of PrP
was also increased in response to scrapie infection in both N2a cells and FVB/NCr mouse brain. This may suggest that ZIP transporters and PrP undergo a similar proteolytic event with an involvement of the same protease in scrapie-infected cells. In addition, the sequence similarities between ZIP proteins and PrP may also indicate that ZIP fragments can incorporate into PrP aggregates during scrapie infection. Further observations in N2a cells also showed that both ZIP10 and PrP could undergo proteolytic cleavages during zinc deficient conditions. In addition, in vitro studies in N2a cells showed that ZIP10 cleavage during zinc deficiency also led to the generation of a 50-51kDa fragment, similar to scrapie infection in mouse brains, and another protein fragment at a lower molecular weight. However, further studies are required to find out whether zinc deficiency can cause the onset and/or propagation of the disease, or it is simply a by-product of prion diseases.

3.2 Materials and Methods

3.2.1 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from mouse N2a cells and CD1 brain tissue by using TRIzol Reagent from Invitrogen. The cDNAs were synthesized from 1mg of total RNA by using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). PCR was performed by adding 2μl of complementary DNA (cDNA) in 18μl of reaction mixture containing 10μl of 2×MasterMix (Applied Biosystems), 6μl water, 1μl of each of 10μM forward and reverse primers (Invitrogen) [177]. Amplification was achieved using a 9800 Fast Thermal Cycler (Applied Biosystems) with an initial denaturation at 95°C for 10 seconds, followed by 30 cycles of denaturation at 95°C for 0 seconds, and annealing at 66°C for 20 seconds [177]. PCR products were analyzed by electrophoresis on 1.5% (w/v) agarose/ethidium bromide gel [177]. The
forward and reverse primers used for amplifying ZIP4, ZIP5, ZIP6, ZIP8, ZIP10 and ZIP14 mRNAs are summarized in **Table 3.1**.

**Table 3.1- Summary of primers used for RT-PCR.**

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
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<tbody>
<tr>
<td>ZIP4</td>
<td>5´-AGACTTGGTGGCAGAGGAGA-3´</td>
<td>5´-GGGATGGGGATAGAGCTGCA-3´</td>
</tr>
<tr>
<td>ZIP5</td>
<td>5´-CTCCCTCTGCTGCTGTTG3-3´</td>
<td>5´-AGAAGCCGTCTGAGAAAGCA-3´</td>
</tr>
<tr>
<td>ZIP6</td>
<td>5´-GGATGTGGAGAGCAAGAAGC-3´</td>
<td>5´-CACAGCGACAGAGGTGCCTT-3´</td>
</tr>
<tr>
<td>ZIP8</td>
<td>5´-AGCAGTTGCTGTGGTTGGTG-3´</td>
<td>5´-GCCGAGAGGAAGTTGAACAG-3´</td>
</tr>
<tr>
<td>ZIP10</td>
<td>5´-TTACCTGGGTGGAGGAAG-3´</td>
<td>5´-TTTGCGAAGATCCAGAAGTG-3´</td>
</tr>
<tr>
<td>ZIP14</td>
<td>5´-TGCCTCCTTCACTGTGTCTG-3´</td>
<td>5´-AAGAGCTGCTTTTCCCATGA-3´</td>
</tr>
</tbody>
</table>

### 3.2.2 Generation of Expression Plasmids

C-terminally hemagglutinin (HA)-tagged mouse ZIP4, ZIP5 and ZIP6 overexpression constructs were purchased from Open Biosystems. In summary, ZIP ORFs were amplified from mouse genomic DNA. Mouse ZIP4 and ZIP5 ORFs were inserted between the *NotI* and *SalI* sites of pCMV-SPORT6.1 vector (**Figure 3.1A**). Mouse ZIP6 ORF was inserted between the *NotI* and *EcoRV* sites of pCMV-SPORT6.1 vector. Mouse ZIP10 construct, purchased from OriGene, was constructed by inserting the ZIP10 ORF in the pCMV6-AC-GFP vector (**Figure 3.1B**). HA-tag was later inserted on the C-terminal end of mouse ZIP10 by PCR based site-directed mutagenesis. For PCR, 2μl of DNA was placed into the reaction mixture containing 2μl of forward and reverse primers, 10μl of 5X buffer, 35μl water, and 1μl of HotStar HiFidelity DNA polymerase (Qiagen). Amplification was achieved using a 9800 Fast Thermal Cycler (Applied Biosystems) with an initial denaturation at 95°C for 5 minutes, followed by 15 cycles of denaturation at 95°C for 20 seconds, and annealing at 54°C for 20 seconds.
Figure 3.1- Expression vectors employed for ZIP proteins. (A) Depiction of pCMV.SPORT6.1 vector used to insert ORFs for ZIP6 protein by using restriction enzymes NotI and EcoRV. The pCMV.SPORT6 vector is also similar to the pCMV.SPORT6.1 vector and it was used to insert ORFs for ZIP4 and ZIP5 by using restriction enzymes NotI and SalI for both proteins (B) Schematic of ZIP10 expression construct with an HA-tag on the ZIP10 C-terminal domain.

3.2.3 Knockdown Reagents for ZIP6, ZIP10 and PrP

Small interfering RNAs (siRNAs) for mouse ZIP6 (SLC39A6), ZIP10 (SLC39A10) and PrP (Prnp) were purchased as ON-TARGETplus set of 4 siRNAs for each protein (Dharmacon). These sequences are summarized in Table 3.2.

Table 3.2- Summary of siRNA sequences used for ZIP6, ZIP10 and PrP knockdown studies.

<table>
<thead>
<tr>
<th>Protein</th>
<th>siRNA Sequences</th>
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<tbody>
<tr>
<td>ZIP6</td>
<td>5’-AGGGAUAUUCAUCGGGCAU-3’</td>
</tr>
<tr>
<td></td>
<td>5’-GGAAAGGUCUGACGGCUCU-3’</td>
</tr>
<tr>
<td></td>
<td>5’-GUAACAUAAAGGUACGUUU-3’</td>
</tr>
<tr>
<td></td>
<td>5’-GCUCUAAUAGCUCUGUCA-3’</td>
</tr>
<tr>
<td>ZIP10</td>
<td>5’-UCGACAUACUAGAAAGCGA-3’</td>
</tr>
<tr>
<td></td>
<td>5’-AGACUGAACUGACGGAUUU-3’</td>
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<tr>
<td></td>
<td>5’-GAAAGGACUUUGUGGCGCUA-3’</td>
</tr>
<tr>
<td></td>
<td>5’-CAUGAAUGUUUGAAGCUCA-3’</td>
</tr>
<tr>
<td>PrP</td>
<td>5’-GCAGGGCCCAUGAUCAUUU-3’</td>
</tr>
<tr>
<td></td>
<td>5’-GUGAAAACAUUGACCCGCUA-3’</td>
</tr>
<tr>
<td></td>
<td>5’-GUGCACGACUGCGUAAUA-3’</td>
</tr>
<tr>
<td></td>
<td>5’-GUGACUAUGUGGACUGAUG-3’</td>
</tr>
</tbody>
</table>

3.2.4 Generation of ZIP6 and ZIP10 Rabbit Polyclonal Antibodies

The peptides GTRFVETIETPK for mouse ZIP6 and NHDHSEQYEHNR for mouse ZIP10 were selected on the N-terminal domains of both proteins. The maleimide-activated
keyhole limpet hemocyanin (KLH)-conjugated peptides were purchased from Pierce and they were injected into New Zealand white rabbits. ZIP6 and ZIP10 polyclonal antibodies were purified from rabbit serum by precipitation with ammonium sulphate followed by affinity purification by using immunogenic mouse ZIP6 and ZIP10 peptides that were conjugated to a SulfoLink column (Pierce).

3.2.5 Treatment of Cells with Metals and Metal Chelators

N, N, N’, N’-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) and diethylene triamine pentaacetic acid (DTPA) were purchased from Sigma-Aldrich. TPEN was prepared at the original concentration of 234μM in 100% ethanol and added to mouse N2a cells at concentrations of 0.5μM, 1μM, 2μM, 2.5μM, 3μM, 3.5μM, 4μM and 5μM. In addition, DTPA was prepared at the original concentration of 1.2mM in modified eagle’s medium, MEM (Gibco), containing 1X penicillin/streptomycin (Gibco), 1X GlutaMax (Gibco) and 10% fetal bovine serum (FBS) from Gibco. DTPA was added to cells at final concentrations of 0.1mM, 0.2mM, 0.3mM, 0.4mM and 0.5mM. Cells were incubated in the presence of either TPEN or DTPA for 48 hours. Some cells were incubated in the presence of both DTPA (0.1mM) and metal supplements for 48 hours. The metal supplements included zinc at final concentrations of 5μM and 25μM (original concentration=1.2mM in MEM), copper at final concentrations of 5μM and 25μM (original concentration=1.2mM in MEM), and finally manganese at final concentrations of 5μM and 25μM (original concentration=1.2mM in MEM).

3.2.6 Preparation of Mouse Brain Homogenates

FVB/NCr Prnp+/+ mice were intracerebrally inoculated with 20μl of 1:10 dilution of brain homogenate in phosphate-buffered saline (PBS), from either clinically ill Rocky Mountain Laboratory (RML)-scrapie inoculated mice or from normal mice, in the left hemisphere for 84
and 131 days after which brains were harvested and the right hemispheres were used for analysis [178]. Mouse brains were weighed and sliced into small pieces. The extraction buffer (pH=7.5), containing 2% sodium dodecyl sulphate (SDS), 100mM dithiothreitol (DTT) and 60mM Tris-HCl, was added at 10X wet-weight of brain tissue. Brain tissues were incubated at 90°C water bath for 5 minutes in the presence of the extraction buffer and later they were homogenized by adding zirconia beads (BioSpec Products) and by beating 3 times with Mini Beadbeater-8 (BioSpec Products). Samples were spinned for 20 minutes at 38,000 x g to remove insoluble debris. Samples were then diluted 1:2 in dilution buffer (pH=6.5), containing 2% SDS, 100mM DTT, 30% glycerol, 60mM Tris-HCl and 0.001% Bromophenol Blue.

3.2.7 Proteinase K (PK) Digestion

Both N2a and ScN2a cells were lysed with radio immunoprecipitation assay (RIPA) buffer in the absence of protease inhibitors. Lysates were adjusted to 1mg/mL and 200μg total protein was digested with 20μg/mL PK for 30 minutes at 37°C. Digestion was terminated by the addition of 2mM phenylmethylsulfonyl fluoride (PMSF) and incubation on ice for 15 minutes. Protein digests were precipitated by the addition of 0.3% sodium phosphotungstic acid and incubation at 37°C for 30 minutes. Pellets were collected by centrifugation at 38,000 × g for 40 minutes at 4°C, were re-suspended in 1X lithium dodecyl sulphate (LDS) sample buffer and boiled for 10 minutes. Samples were then analyzed by Western blotting.

3.2.8 Cell Viability Assay

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was performed to measure cell viability in the presence of TPEN. N2a cells were plated on 96-well culture plates and they were incubated with 0.5μM, 1μM, 2μM, 2.5μM, 3μM, 3.5μM, 4μM and 5μM TPEN concentrations for 48 hours. After 48-hour incubation period, cells were washed
with PBS and incubated in the presence of 0.5mg/ml MTT at 37°C for 4 hours. The medium was then aspirated and the converted dye was solubilized with acidic isopropanol. Absorbance was measured at 570nm by using a microplate reader.

### 3.2.9 Cell Culture, Transfection, and Lysis

N2a and ScN2a cells were cultured in MEM (Gibco), containing 10% FBS (Gibco), 1X penicillin/streptomycin (Gibco) and 1X GlutaMax (Gibco), and maintained in a humidified incubator with 5% CO₂. Cells were transfected with DNA mixed with Lipofectamine-2000 (Invitrogen) in reduced serum medium, Opti-MEM (Gibco), according to the manufacturer’s instructions. The DNA/Lipofectamine-2000 ratio was 2μg/5μl. Sodium butyrate (Sigma-Aldrich), with an original concentration of 1.5mM, was also added to cells (final concentration=5μM) 24 hours after transfection of cells with DNA constructs.

For knockdown studies, cells were transfected with ON-TARGETplus siRNA (Dharmacon) at a final concentration of 100nM in Opti-MEM using Lipofectamine-2000. Cells were incubated with the transfection mixture for 24 hours and then incubated in MEM (Gibco), containing 10% FBS (Gibco), 1X penicillin/streptomycin (Gibco) and 1X GlutaMax (Gibco) for another 24 hours.

For cell lysis, cells were washed twice with PBS and lysed with RIPA lysis buffer (50mM Tris-HCl (pH=7.5), 150mM NaCl, 0.5% sodium deoxycholate and 1% nonyl phenoxypolyethoxylethanol (NP-40)) containing one Complete Mini Protease Inhibitor Cocktail tablets (Roche). Lysates were incubated on ice for 10 minutes after which DNA and other cell debris were removed by aspiration. Protein concentrations were determined using the bicinchoninic acid (BCA) assay (Pierce).
3.2.10 Bicinchoninic acid (BCA) Assay

BCA reagents A and B were purchased from Pierce. Reagents A and B were mixed in a 50:1 ratio and 150µl of the reagent mixture was added to 10µl of bovine serum albumin (BSA) dilutions at 0mM, 0.125mM, 0.25mM, 0.5mM, 1mM, 1.5mM and 2mM for the purpose of depicting a standard curve. 150µl of the BCA reagent mixture was also added to 5µl (1:2 dilution) of each protein extract from either N2a or ScN2a cells. Samples were incubated at 37°C in dark for 30 minutes and absorbance was measured at 550nm by using a microplate reader.

3.2.11 Western Blotting

For Western blotting, total protein level was adjusted to 1mg/ml by using RIPA lysis buffer. LDS sample buffer (1X) was added and samples were heated to 40°C. Samples were then separated on 4-12% NuPAGE gels (Invitrogen) with 1X 3-(N-morpholino) propanesulfonic acid (MOPS) or 1X 2-(N-morpholino) ethanesulfonic acid (MES) buffers from Invitrogen. Proteins were transferred to polyvinylidene fluoride (PVDF) membrane and blocked with 10% skim milk. Blots were incubated with primary antibodies in 5% skim milk at 4°C. The primary antibodies used for probing included mouse monoclonal anti-PrP antibody (Sha31) from SPI-Bio, rabbit polyclonal anti-ZIP6 antibody, rabbit polyclonal anti-ZIP10 antibody, mouse monoclonal anti-HA antibody (Covance) and mouse monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (Sigma-Aldrich). Following three washes with Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBST), blots were incubated with horseradish peroxidase (HRP)-conjugated mouse or rabbit secondary antibody (BioRad) for 2 hours at room temperature. Blots were then washed three times with TBST and developed by using enhanced chemiluminescence (ECL) reagent (Perkin-Elmer).
3.2.12 Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES)

MEM and MEM containing 10% FBS were injected into the ICP-AES device, AAS Perkin Elmer Analyst 100. A standard sample with known concentrations of zinc, copper and manganese was used to sketch a standard curve. This standard curve was used to identify the concentrations of zinc, copper and manganese at wavelengths 214nm, 325nm and 258nm respectively in MEM and MEM containing 10% FBS.

3.2.13 Quantifications and Statistics

Quantification of proteins was done by using the GS-800 calibrated densitometer (BioRad) and the amounts were compared to the quantity of GAPDH acting as a control. The Student’s t-test (two-tailed) was used to evaluate changes in protein levels in response to various treatments. A p-value of less than 0.05 indicated statistical significance.

3.3 Results

3.3.1 Identification of Endogenous ZIP Expression in Mouse N2a Cells and Brains

In order to determine which ZIP transporters are endogenously expressed in mouse N2a cells and brains, six members of the LIV1 family of ZIP proteins, including ZIP4, ZIP5, ZIP6, ZIP8, ZIP10 and ZIP14, were selected for analysis by using RT-PCR. ZIP6 and ZIP10 were selected since the previous PrP interactome study confirmed the interaction of these two ZIP transporters with mouse PrP in N2a cells [44]. The other ZIP transporters were selected to assess the complexity of LIV1 biology in mouse N2a cells, routinely used for studies on the prion protein, relative to the brain since the difference in ZIP expression between N2a cells and mouse brain may pose a confounder in biochemical analyses. In addition, the expressions of ZIP4, ZIP8 and ZIP14 were analyzed to investigate whether the absence of these ZIPs in the original interactome [44] was the result of them not being expressed in N2a cells or the consequence of
preferential affinity of PrP for ZIP6 and ZIP10. Finally, ZIP5 expression was also considered since the previous bioinformatics analyses had predicted ZIP5 to be closest in sequence to PrP and it was surprising why it had not co-purified with PrP from cross-linked N2a cells [44].

The primers for ZIP4, ZIP5, ZIP6, ZIP8, ZIP10 and ZIP14 were selected and the cDNAs corresponding to ZIP mRNAs were amplified in both mouse N2a cells and mouse CD1 brains. Analysis of expression levels showed that ZIP4, ZIP6, ZIP8, ZIP10 and ZIP14 are all expressed in mouse N2a cells and mouse CD1 brains (Figure 3.2). The only ZIP protein that was neither observed in N2a cells nor in CD1 brains was the mouse ZIP5 protein which explains why ZIP5 was not identified in the original PrP interactome study in N2a cells [44]. The larger PCR products at around 1500 base pairs (bps), seen only for ZIP5, represented amplification of genomic gene fragments and, thereby, validated the suitability of ZIP5 primers used for this analysis (Figure 3.2). It must be mentioned that amplifications of genomic DNA are not expected in the presence of an excess of amplifiable RNA templates. Therefore, the genomic DNA was not observed for other ZIP transporters. ZIP mRNAs must also be quantified by real-time PCR to confirm whether ZIP5 mRNA is subtly transcribed or it is not transcribed in N2a cells and CD1 brains at all.

Since the previous bioinformatics analysis indicated mouse ZIP5, ZIP6 and ZIP10 to be highly similar and homologous to mouse PrP sequence and since the RT-PCR analysis showed that mouse ZIP5 was not expressed in our N2a cell models, only ZIP6 and ZIP10 were selected for further biological investigations in future experiments except for overexpression studies in N2a cells.
RT-PCR confirmed the presence of ZIP4, ZIP6, ZIP8, ZIP10 and ZIP14 transcripts in N2a cells and mouse brains. ZIP5 transcript was not observed in N2a cells and CD1 brains. The larger PCR products seen only for ZIP5 represented amplifications of genomic gene fragments, thus validating the suitability of ZIP5 primers used for this analysis.

3.3.2 Generation of Rabbit Polyclonal Antibodies against Mouse ZIP6 and ZIP10

In order to assess the existence and properties of ZIP6 and ZIP10, polyclonal antibodies were raised against the mouse peptide epitopes on the N-terminus of each of these proteins (Figure 3.3A). These antibodies were raised in New Zealand white rabbits and their specificities were evaluated in mouse N2a cells following their purification from rabbit serum. Upon overexpression of ZIP6 and ZIP10 constructs in mouse N2a cells, these ZIP antibodies recognized full-length ZIP6 at 86kDa and full-length ZIP10 at 94kDa (Figure 3.3B). In addition, these antibodies were also able to identify several N-terminally processed fragments of ZIP6 and ZIP10 (Figure 3.3B). Transfection of mouse N2a cells with ZIP6 and ZIP10 siRNA reagents also showed that these antibodies are capable of recognizing endogenously expressed full-length ZIP6 and ZIP10 at 86kDa and 94kDa respectively (Figure 3.3C). However, the anti-ZIP6 antibody recognized an unspecific protein slightly above 64kDa (Figure 3.3A and Figure 3.3B). ZIP10 antibody also recognized unspecific proteins and/or protein fragments at 64kDa (Figure 3.3A and Figure 3.3B). However, ZIP10 knockdown had low efficiency and other ZIP10 siRNA reagents are required for high knockdown efficiency to validate these results.
Figure 3.3 - Generation of antibody reagents for the detection of mouse ZIP6 and ZIP10. (A) Schematic depiction of antibody binding sites within mouse ZIP target proteins. (B) Overexpression of ZIP6 and ZIP10 in N2a cells showed the ability of polyclonal antibodies to recognize full-length ZIPs and their proteolytic fragments. (C) Knockdown of ZIP6 and ZIP10 in N2a cells indicated the ability of ZIP antibodies to detect endogenous ZIP6 and ZIP10 albeit with some unspecific binding.
3.3.3 ZIP Overexpression and Knockdown in N2a and ScN2a Cells

Mouse ZIP4, ZIP5, ZIP6 and ZIP10 constructs were transfected into N2a and ScN2a cells and cells were allowed to overexpress these proteins for 48 hours after which they were harvested and protein extracts were collected for analysis by Western blotting. Since ZIP5 and ZIP10 have the highest and second-highest homologies with PrP, they were expressed at increasing levels to observe any effect of their changing concentrations on the expression or maturation of PrP. Probing expression of ZIP4, ZIP5 and ZIP6 with anti-HA antibody for the HA-epitope, attached to the very C-terminus of these ZIP proteins, indicated successful overexpression of proteins (Figure 3.4A). ZIP5 levels were also successfully adjusted by changing the concentration of ZIP5 DNA construct for transfection (Figure 3.4A). Confirmation of ZIP10 overexpression was achieved by probing blots with both anti-HA and anti-ZIP10 antibodies (Figure 3.4A). Probing ZIP10 expression with the HA antibody also confirmed that the protein band below full-length ZIP10, which was also recognized by the ZIP10 polyclonal antibody, was specific for ZIP10 and may have suggested that this band constituted either a proteolytic fragment of ZIP10 or a conformer of ZIP10 that is not post-transnationally modified by the addition of heavy groups. Probing of overexpression samples with anti-PrP antibody, Sha31 antibody recognizing residues 145-152 of the human PrP [179], showed no alteration in the endogenous expression and maturation of the PrP protein in N2a cells since the intensity of full-length PrP, glycosylated or unglycosylated, and its proteolytic fragments did not change dramatically in response to ZIP overexpression (Figure 3.4A). However, further replicates of these overexpressions are required to confirm these results statistically and conclude whether the slight variations in the expression of PrP can be attributed to technical variations during the experiment or to alterations in the expression levels of ZIP transporters.
In order to assess the effects of ZIP on scrapie propagation, ZIP overexpression experiment was performed in ScN2a cells. Probing of protein extracts from ScN2a cells with anti-HA antibody also showed that these constructs could successfully overexpress their corresponding ZIP proteins in ScN2a cells (Figure 3.4B). However, neither PrP expression nor the resistance of PrP\textsubscript{Sc} to digestion by PK was altered dramatically in response to enhanced ZIP expression in ScN2a cells (Figure 3.4B) although these results require repetition and statistical validation.

Knockdown reagents for both ZIP6 and ZIP10 were transfected into both N2a and ScN2a cells and the knockdown process was allowed to continue for 48 hours. Knockdown of both ZIP proteins was successful in both N2a and ScN2a cells (Figure 3.5). The knockdown of one ZIP protein or both of them did not seem to influence the expression level of PrP in either N2a or ScN2a cells (Figure 3.5). In addition, PrP\textsubscript{Sc} propagation and its partial PK resistance were not affected by the knockdown of ZIP transporters. However, the knockdown of either ZIP10 or PrP might have modulated the expression of full-length ZIP6 (86kDa) in N2a cells as these knockdown samples showed a lower expression of mouse ZIP6 (Figure 3.5A). Further experiments with higher efficiency knockdown reagents are required to repeat this experiment and confirm the slight variations in the levels of ZIP6, ZIP10 and PrP, and to conclude whether these minute changes occur in response to knockdown or experimental variations.

It must be noted that the transfection efficiency in N2a and ScN2a cells was not ideal suggesting that effects on endogenous PrP\textsubscript{Sc} levels could have escaped detection. Zhang et al. estimated only 5% transfection efficiency for the transfection of a yellow fluorescent protein (YFP) expression vector in N2a cells [180]. Future experiments must concentrate on the stable
transfection of ZIP overexpression constructs and siRNAs into N2a cells to observe the potential effects on PrP expression with higher accuracy.

Figure 3.4 - Overexpression of ZIP proteins in N2a and ScN2a cells with no dramatic effect on PrP. (A) HA-tagged mouse ZIP4, ZIP5, ZIP6 and ZIP10 constructs were successfully overexpressed at different levels according to the amount of DNA added for transfection in N2a cells. ZIP4, ZIP5 and ZIP6 overexpressions were detected with anti-HA antibody (top left), whereas ZIP10 overexpression was detected with both anti-HA and anti-ZIP10 antibodies. Expression of ZIP proteins did not correlate with PrP expression or maturation in N2a cells. Increasing expressions of ZIP5 and ZIP10 were indicated by black triangles. (B) HA-tagged ZIP proteins were overexpressed in ScN2a cells as it is indicated by the anti-HA blot. Overexpression of ZIP transporters did not correlate with the expression level of PrP or the PK-resistance characteristic of PrPSc in ScN2a cells.
Figure 3.5- Knockdown of ZIP and PrP proteins in N2a and ScN2a cells with no dramatic effect on PrP. (A) Endogenous ZIP6, ZIP10 and PrP were knocked down in N2a cells as it is indicated by probing blots with their corresponding antibodies. Reduction of endogenous levels for ZIP6, ZIP10 or PrP did not lead to striking changes in the other two proteins suggesting that members of ZIP/PrP gene family may not influence each other’s expression and/or maturation although ZIP6 expression might have slightly decreased for both ZIP10 and PrP knockdowns. (B) Knockdown of endogenous ZIP6 and/or ZIP10 did not alter PrP expression and PK-resistant characteristic of PrPSc in ScN2a cells. Similar to N2a cells, full-length ZIP6 might have slightly down-regulated in response to ZIP10 knockdown in ScN2a cells.
3.3.4 ZIP Transporters in Prion-Infected N2a Cells and Mouse Brain Tissue

Since PrP\textsuperscript{C} and PrP\textsuperscript{Sc} have different characteristics, such as higher detergent insolubility and PK resistance for PrP\textsuperscript{Sc} in infected cells [1], it was considered that ZIP transporters may also possess some characteristic differences in response to scrapie infection of cells. Thus, N2a cells were selected and they were infected with brain homogenates from scrapie-infected mice. PK digestion assay confirmed that scrapie infection by the addition of brain homogenates has led to the generation of PrP\textsuperscript{Sc} since PrP from normal N2a cells was completely digested with PK while PrP from ScN2a cells was partially resistant to PK (Figure 3.6A). In addition, Figure 3.6A also showed that PrP cleavage pattern changed in response to infection. While PrP\textsuperscript{C} was abundant as full-length diglycosylated, monoglycosylated and unglycosylated forms, PrP\textsuperscript{Sc} was comprised of relatively lower quantities of full-length glycosylated and unglycosylated forms and, in addition, underwent more proteolytic processing resulting in the generation of lower molecular weight fragments at around 14-21kDa that might have corresponded to PrP C1 (17kDa) and C2 fragments (21kDa).

Following scrapie infection, expression levels and proteolytic processing of ZIP6 and ZIP10 were compared between N2a and ScN2a cells. Interestingly, the level of full-length ZIP6 (86kDa) decreased significantly after the infection of N2a cells with the scrapie agent (Figure 3.6B). However, ZIP6 proteolytic processing was not enhanced since there were no significant increases in the levels of lower molecular weight fragments of this protein. In addition, the stability of ZIP6 might have not been altered since there was no change in the degradation and processing of the protein. Similar to ZIP6, full-length ZIP10 (94kDa) level was also decreased in ScN2a cells albeit to a lower extent and there was no sign of increased ZIP10 proteolytic cleavage (Figure 3.6B). It is still plausible that both ZIP6 and ZIP10 underwent proteolytic
cleavage and degradation; however, the proteolytic fragments were not detected either due to the quick clearance and degradation of the proteins or due to the absence of the antibody binding sites on these fragments. Therefore, these results require substantiation and repetition in a distinct cell line and with different ZIP antibodies raised against other epitopes in these proteins.

Figure 3.6- Down-regulation of endogenous ZIP6 and ZIP10 expressions in ScN2a cells. (A) The level of prion infection could be estimated from the intensity of protease resistant bands following digestion with PK. (B) Probing both N2a and ScN2a cells with anti-ZIP6 antibody in three independent experiments showed a dramatic reduction of full-length ZIP6 in ScN2a cells. There was also a slight decrease in the expression level of endogenous ZIP10 in response to scrapie infection in N2a cells.
Since the levels of both full-length ZIP6 and ZIP10 were diminished in ScN2a cells, it was investigated whether or not the same phenomenon also occurs in prion-infected mouse brains. In order to assess the expression levels and proteolytic processing of ZIP6 and ZIP10 in prion-infected mice, wild-type mice (FVB/NCr) were inoculated intracerebrally with the RML strain of mouse-adapted scrapie prions [178]. Inoculated mice were allowed to progress to either 84 or 131 days post-inoculation (dpi) with the scrapie agent after which time the animals were sacrificed, half brains were homogenized and ZIP levels were assessed by Western blotting. Anti-ZIP polyclonal antibodies were used to examine ZIP6 and ZIP10 levels. The mouse brain harvested after 84dpi displayed no significant accumulation of PrP\textsuperscript{Sc} and spongiosis, whereas the mouse brain harvested after 131dpi had high accumulation of PrP\textsuperscript{Sc}, astrogliosis and spongiosis [178, 181, 182]. The Western blot analysis with anti-PrP antibody also confirmed the aggregation of PrP\textsuperscript{Sc} in 131dpi brain homogenate, but not in 84dpi brain homogenate, when they were compared with their corresponding controls (Figure 3.7).

Surprisingly, no difference was observed in expression levels of full-length ZIP6 and ZIP10 in 131dpi brain homogenates (Figure 3.7). However, antibodies directed against ZIP6 and ZIP10 detected additional bands of 50-51kDa only in diseased brains consistent with the interpretation that both ZIP6 and ZIP10 undergo proteolytic cleavages as the disease progresses (Figure 3.7). To examine the specificity of this phenomenon, levels of other proteins in prion-infected brains were examined. The expression levels and fragmentations of Munc18, a marker for exocytosis [183], S5a, a ubiquitin interacting protein important for the proteasomal degradation of proteins [184], MnSOD, a marker for oxidative stress [185], and finally Histone H4, a marker involved in the structure of chromatin and regulation of gene expression [186], were not affected in response to scrapie-infection and the onset of symptoms in prion disease.
after 131dpi (Figure 3.7). Drebrin, a protein responsible for the regulation of dendritic spine plasticity [187], was slightly decreased in response to scrapie-infection after 131dpi, but its expression stayed the same after 84dpi. This might be an indication of a small amount of neuronal loss and loss of synaptic density. In addition, Drebrin did not undergo endoproteolysis in 131dpi brain homogenate (Figure 3.7). These results might suggest that the endoproteolysis phenomenon was restricted to ZIP proteins. Studies to determine whether other metal regulating proteins, in addition to ZIPs, are affected will be needed to gauge the specificity of this observation. The selection of brains at different time points and a more thorough analysis of the brain regions which produce this result may be useful to determine whether these cleavages are likely secondary events or are more closely associated with the aetiology of the disease.

Figure 3.7- Endoproteolysis of ZIP6 and ZIP10 in RML-infected mouse brains. (A) Aggregation of PrP at 131dpi was indicative of the illness of mice and the appearance of disease phenotypes. Neither ZIP6 nor ZIP10 expression levels were altered in response to scrapie infection in mouse brains. However, both ZIP6 and ZIP10 underwent endoproteolysis after 131dpi in response to scrapie infection to generate a fragment at ~50-51kDa. In addition, probing protein extracts from both normal and RML-infected mouse brains with antibodies against drebrin (marker for the growth of dendritic spines), Munc18 (marker for cellular secretory pathway), S5a (marker for the activity of the ubiquitin-proteasomal complex), Mn-SOD (marker for oxidative stress) and Histone H4 (marker for chromatin integrity) showed no changes in the expression levels and/or proteolysis of these proteins although the level of drebrin expression slightly decreased due to RML infection at 131dpi.
3.3.5 ZIP and PrP Endoproteolysis in Response to Metal Chelation

Studies on ZIP4 transporter, a member of the LIV1 subfamily of ZIP proteins, shows that this protein undergoes endoproteolysis in response to zinc deficiency which can be experimentally mimicked by chelation of zinc in the growth medium of intestinal cells [143]. Kambe & Andrews showed that the N-terminal ectodomain of ZIP4 is endoproteolytically removed in response to prolonged zinc chelation [143].

Our previous observation from scrapie-infected mouse brains indicated that both ZIP6 and ZIP10 underwent proteolytic cleavages to generate 50-51kDa protein fragments in 131dpi mouse brain. In addition, studies by various groups have shown that PrP undergoes selective proteolytic cleavage in prion diseases which leads to the up-regulation of its C2 fragment and the down-regulation of PrP C1 fragment [68, 70, 79, 80]. We, therefore, wondered whether, similar to ZIP4, ZIP6 and ZIP10 can also become proteolytically cleaved in zinc-deficient conditions and whether PrP undergoes a similar proteolytic cleavage in response to prolonged zinc depletion. Finally, we aimed to address whether cleavages in ZIP transporters and PrP, assuming they can be observed in response to zinc chelation, mirror the aforementioned cleavages we had observed in RML-infected brains.

To find answers to these questions, the concentrations of zinc, copper and manganese, divalent cations that have been associated with prion diseases, in the cell culture medium were measured by ICP-AES. The concentration of zinc, 3.3μM, was the highest among the three metals followed by trace amount of copper and no manganese (Figure 3.8A). Since zinc had the highest concentration in MEM + 10% FBS cell culture medium, and since ZIPs have been shown to respond to concentrations of zinc, N2a cells were incubated in the presence of TPEN, a cell permeable metal chelator with high affinity for zinc (K_a=10^{15.58} M^{-1}) [188-191], for 48 hours
after which cells were harvested and protein extracts were evaluated by Western blotting. It must be noted that a range of TPEN concentrations were selected according to previous studies which indicated the excessive toxicity of this metal chelator leading to more than 50% cell death if it is used at above 5mM concentration in cell lines such as human neuroblastoma cells, human retinal pigment epithelial cells, and HEK 293, Madin-Darby Canine kidney (MDCK) and CaCo2 cells [143, 188-190].

The first analysis showed that ZIP10 underwent significant proteolytic cleavage after incubation with 3μM TPEN leading to the generation of a protein fragment which migrated with an apparent molecular weight of less than 51kDa (Figure 3.8A). Similarly, PrP was also processed in the presence of 3μM TPEN which could be observed from decreases in the diglycosylated and monoglycosylated forms of PrP and an increase in the generation of PrP proteolytic fragments at 14kDa to 19kDa (Figure 3.8A). The changes in the quantities of ZIP10 and PrP fragments during TPEN-dependent zinc chelation in N2a cells were measured from three different replicates (Figure 3.8B).

However, our analysis of cell viability with MTT assay at differing concentrations of TPEN showed that only 50% of cells were alive after 48 hours of incubation in the presence of 3μM TPEN indicating that TPEN is toxic for cells (Figure 3.8C). This is consistent with previous studies in which TPEN-dependent chelation of zinc led to the induction of apoptosis by activating caspase 11 and caspase 3 proteins [188-190]. Therefore, another metal chelator, DTPA, an extracellular chelator with high affinity for zinc (Kₐ=10¹⁴ M⁻¹) [190], was selected to determine whether the previous results with TPEN are reproducible and to clarify whether the ZIP10 and PrP endoproteolytic cleavages occur in response to metal chelation and/or cell death. According to a study by Nakatani et al. DTPA is less toxic than TPEN and DTPA-mediated
chelation of extracellular zinc in a culture of rat hepatocytes led to the lower activation of caspase proteins than TPEN-dependent zinc chelation in the same cells [190].

Treatment of N2a cells with 0.1mM, 0.2mM, 0.3mM, 0.4mM and 0.5mM DTPA concentrations for 48 hours showed that ZIP10 proteolytic cleavage was reproducible since a protein fragment was generated at lower than 51kDa (Figure 3.9A). This was associated with higher overall expression of ZIP10 transporter. It must be noted that the endoproteolysis of ZIP10 in response to zinc chelation by DTPA also resulted in the generation of a 51kDa protein fragment which was absent in TPEN-treated N2a cells. Similarly, PrP underwent processing in response to different DTPA concentrations. The amounts of diglycosylated and monoglycosylated forms of PrP decreased, whereas the quantity of PrP proteolytic fragments increased in response to zinc chelation by DTPA (Figure 3.9A).

In order to find which metals can rescue ZIP10/PrP endoproteolysis, 0.1mM DTPA-treated N2a cells were selected for further studies since these cells were healthy in the presence of DTPA and they were growing to the extent that they retained their capability to form elongations and neurites. When these DTPA-treated N2a cells supplemented with zinc, copper and manganese at 5μM and 25μM concentrations, copper proved to be toxic for cells since cells started to die even in the presence of 5μM copper after 48 hours. On the other hand, zinc was able to decrease the endoproteolysis of ZIP10 and PrP at 5μM and it completely rescued the endoproteolysis event at 25μM concentration (Figure 3.9B). Manganese proved to be the most potent metal since it rescued endoproteolysis of ZIP10 and PrP at a concentration as low as 5μM (Figure 3.9B). These results showed that both ZIP10 and PrP could undergo proteolytic cleavages in response to metal chelation in N2a cells and this proteolysis event was not necessarily dependent on cell death.
Figure 3.8- PrP/ZIP endoproteolysis in response to zinc chelation in N2a cells. (A) Inductively coupled plasma atomic emission spectroscopy (ICP-AES) detected the concentrations of zinc, copper and manganese in MEM and MEM containing 10% FBS. Zinc had the highest concentration followed by copper. Neither solution contained manganese. Treatment of N2a cells with TPEN zinc chelator showed both ZIP10 and PrP undergoing proteolytic cleavage after 48 hours of incubation in the presence of 3μM TPEN. ZIP10 expression was up-regulated which was accompanied with higher proteolysis of the protein and the appearance of the ZIP10 fragment at <51kDa. PrP fragmentation increased after zinc chelation which was apparent by the lower amount of diglycosylated PrP and higher levels of PrP proteolytic fragments. (B) Quantification of fragmentation levels for both PrP and ZIP10 indicated the significance of the endoproteolysis event for these proteins in the presence of 3μM TPEN. However, the observed fragmentations might have simply been in response to apoptosis and cell death as half of the cells died in 3μM TPEN according to the MTT assay. (C) The MTT assay showed TPEN to be toxic for N2a cells as cell viability decreased by the increasing concentrations of TPEN from 1μM to 5μM. only 50% of cells were alive after 48 hours of incubation in the presence of 3μM TPEN.
Figure 3.9- DTPA-induced endoproteolysis of ZIP10 and PrP in response to metal chelation and the reversal of endoproteolysis by the supplementation of divalent cations. (A) Treatment of N2a cells with DTPA zinc chelator showed both ZIP10 and PrP undergoing proteolytic cleavages after 48 hours of incubation in the presence of increasing concentrations of DTPA. Similar to TPEN treatment, ZIP10 expression was up-regulated which was accompanied with higher proteolysis of the protein and the appearance of the ZIP10 fragment at <51kDa. PrP fragmentation increased after zinc chelation which was apparent by the lower amount of diglycosylated PrP and higher levels of PrP proteolytic fragments. PrP cleavage showed an increasing trend with the increasing concentration of DTPA. (B) N2a cells were healthy and viable in the presence of 0.1mM DTPA and this DTPA concentration was selected to indicate the significance of both PrP and ZIP10 cleavages during zinc deplete conditions. The DTPA effect on both PrP and ZIP10 fragmentation was reversible by the supplementation of increasing concentrations of zinc and manganese. Manganese seemed more effective than zinc in reversing this effect. On the other hand, copper was not effective in counteracting DTPA metal chelation. These results indicated a higher tendency of DTPA to chelate zinc and manganese than copper.
3.4 Discussion

Mouse N2a cells are a suitable *in vitro* cell model for studying prion biology and pathophysiology due to the high endogenous expression of the cellular prion protein in these cells, relative ease in infecting these cells with the scrapie agent extracted from brains of diseased animals, and the capacity of N2a cells to retain scrapie replication for several passages [192-194]. In order to determine whether N2a cells are also suitable for investigations into the biology of ZIP transporters with regard to the prion protein, expression levels of a few LIV1 members of ZIP transporters including ZIP4, ZIP5, ZIP6, ZIP8, ZIP10 and ZIP14 were compared between N2a cells and mouse CD1 brains. The RT-PCR study showed that only ZIP5 was not expressed in N2a cells and CD1 brains (Figure 3.2). Therefore, N2a cells were used to study ZIP transporters with regard to the prion protein pathophysiology since there was no significant difference in the expression of the LIV1 family of ZIP transporters between N2a cells and CD1 brains to pose a confounder in the study. In addition, ZIP5 was excluded from future studies, except for the *in vitro* overexpression experiment, since this protein is not endogenously expressed in N2a cells (Figure 3.2).

As a first step toward characterizing the significance of ZIP transporters in prion biology and pathogenesis, ZIP6 and ZIP10 antibodies were raised against the N-terminal regions of these proteins. Both overexpression and knockdown studies confirmed that these antibodies not only detect full-length ZIP proteins but also their proteolytic N-terminal fragments (Figure 3.3). In addition, the purification of antibodies resulted in the removal of some of the unspecific protein bands upon Western blotting.

Overexpression and knockdown of ZIP6 and ZIP10 did not result in a striking change in the expression or processing of PrP in both N2a and ScN2a cells suggesting that these proteins
might not be directly involved in the regulation of PrP (Figure 3.4 and Figure 3.5). However, it must be noted that the efficiency of transfection is not 100% at any time and that may affect the results of transfection studies. In fact, a study by Zhang et al. showed the transfection efficiency of a YFP expression vector to be around 5% in N2a cells [180]. The overexpression and knockdown studies were also performed in 48 hours in ScN2a cells and it might be required to prolong these experiments by performing stable transfections in ScN2a cells to observe any potential effects on the expression of PrP and/or scrapie propagation. In addition, RT-PCR studies suggested that there are several ZIP family members, in N2a cells, with homology to the PrP protein within their respective N-terminal ectodomains (Figure 3.2), albeit with less pronounced sequence similarity to PrP than ZIP5, ZIP6 and ZIP10, suggesting that there is an overall effect of the interaction of ZIP proteins with the PrP protein and only changing the expression of one ZIP protein at the time may not by itself have a dramatic effect on PrP regulation. On the other hand, knockdown of PrP and ZIP10 in N2a and ScN2a cells may be associated with lower expression of ZIP6 protein (Figure 3.5). However, both overexpression and knockdown studies must be repeated in N2a and ScN2a cells stably overexpressing and inhibiting the expression of ZIP transporters to find any potential effects on prion biology and/or scrapie replication.

The analysis of ZIP6 and ZIP10 in scrapie-infected N2a cells showed that full-length ZIP6 was significantly decreased in these cells whereas full-length ZIP10 was only slightly reduced (Figure 3.6). The fact that the reduction in full-length ZIP proteins was not associated with the generation of lower proteolytic fragments (Figure 3.6) might have implied that ZIP proteins are expressed at the same level in both N2a and ScN2a cells; in response to infection, however, ZIP proteins undergo proteolytic cleavage generating N-terminal fragments that are
secreted into the medium and are neither attached to the membrane nor endocytosed by the cell. Such a proteolysis of integral proteins and the secretion of their fragments to the medium have been reported for the $\alpha$-secretase cleavage of APP [195]. Therefore, the analysis of extracellular medium will be required to reveal whether endoproteolytic fragments are released into the medium. On the other hand, one study on ZIP4 protein under zinc-deficient conditions showed that the protein undergoes proteolytic processing to cleave its N-terminal ectodomain, a process required for intracellular zinc transport by this protein [143]. While, the ectodomain remains associated with the plasma membrane in MDCK and CaCo2 cells, it is released into the medium in HEK 293 cells [143]. Therefore, it is important to further investigate proteolytic fragments of ZIP6 and ZIP10 not only in scrapie-infected N2a cells but also in scrapie-infected GT1 cells and other prion cell models to conclude whether the lower expression of these ZIP proteins under scrapie-infection condition is because of the higher proteolytic processing. It must also be noted that ZIP6 and ZIP10 may undergo proteolysis to either generate functionally relevant protein fragments or to become degraded by the lysosomal system [196]. In any case, the detection of these proteolytic fragments may be limited due to the fact that these fragments do not retain the ZIP6 and ZIP10 antibody epitopes on the N-terminal regions of these proteins. Therefore, application of other antibodies directed against different regions of ZIP6 and ZIP10 may be necessary to detect these fragments.

In order to identify whether ZIP proteins endoproteolytically processed in prion diseases, scrapie-infected mouse brains were investigated for the regulation of ZIP6 and ZIP10 (Figure 3.7). Two time-points were selected for analysis. In one time-point, scrapie-infected animals were sacrificed after 84dpi. Animals at this stage do not show any significant progression of disease since they behave normally and there is a slight accumulation of PrP$^\text{Sc}$ [178, 181, 182].
The second time-point was 131dpi at which time animals have uncoordinated motor function and there is profound neurodegeneration and PrP^Sc aggregation [178, 181, 182]. Interestingly, analysis of ZIP6 and ZIP10 showed that these proteins are proteolytically cleaved to generate a 50-51kDa protein fragment in 131dpi scrapie-infected brain homogenate (Figure 3.7). This may clarify the previous observation in ScN2a cells since it suggests that both ZIP6 and ZIP10 undergo endoproteolysis during scrapie infection and the reduction in the levels of full-length ZIP6 and ZIP10 in ScN2a cells may be in response to degradation and/or processing of the proteins for which the proteolytic fragments are undetectable because of the lack of antibody epitopes on protein fragments or the release of fragments into the extracellular medium or degradation of fragments by the lysosomal system.

If the endoproteolytic cleavages of ZIP6 and ZIP10, similar to ZIP4, happen in the N-terminal regions of these proteins, containing the PL domain with homology to PrP, this can suggest that ZIP fragments may be generated with a mechanism similar to that for the generation of PrP C1 and/or C2 fragments. During prion diseases the PrP endoproteolysis is favoured toward the β-cleavage and generation of more C2 fragment than C1 fragment, with C2 being prone to aggregate on the plasma membrane and cytosol following its uptake by endocytosis [68, 79-81]. In addition, PrP endoproteolysis to generate C1 and C2 fragments does not need the membrane-anchorage of the protein and its insertion into lipid rafts [62, 70]. Walmsley et al. showed that PrP C1 fragment is generated mainly in the Golgi apparatus during or after the post-translational modification of PrP [70]. Walmsley et al. further proposed that the down-regulation of α-cleavage in prion diseases may lead to the higher quantities of full-length PrP reaching the plasma membrane which leads to higher conversion of PrP^C to PrP^Sc [70]. Once PrP^Sc molecules are endocytosed, they can undergo endoproteolysis through PrP β-cleavage to synthesize the PrP
C2 fragment. In support of this hypothesis, two groups have shown that C2 fragment is generated in the intracellular space. However, these two groups contradict on the responsible proteases and the potential sites of cleavage [79, 80]. Yadavalli et al. concluded that calpain, located in the cytosol, is responsible for PrP β-cleavage [79], whereas Dron et al. showed that PrP cleavage by cathepsins, lysosomal enzymes, leads to the generation of C2 fragment [79, 80]. In addition, ZIP4 is endoproteolytically cleaved under zinc deficiency to generate a 37kDa fragment attached to the plasma membrane and an N-terminal ectodomain fragment that has the potency to accumulate in the cytosol [143]. Kambe & Andrews showed that the endoproteolysis of ZIP4 during zinc deficiency requires the endocytosis of the protein after which its C-terminal fragment is recycled back and is inserted into the plasma membrane [143]. Therefore, it is plausible that both the β-cleavage of PrP and endoproteolysis of ZIPs happen through the same mechanism of endocytosis. Furthermore, since ZIP4 has been shown to undergo proteolytic cleavage during zinc deficiency [143], the endoproteolysis of ZIP6 and ZIP10 may also be due to the fact that scrapie-infection is associated with the down-regulation of the concentration of divalent cations, mainly zinc, leading to the fragmentation of proteins. However, it remains to be investigated whether other metal transporters are also subject to endoproteolysis in scrapie-infected mouse brains or whether this observation is specific to ZIP transporters. Even the possibility that these ZIP fragments may themselves get incorporated into PrP aggregates in prion diseases can be considered given the structural homology of these ZIP ectodomains to PrP [44]. In addition, some ZIP fragments can also be released into the extracellular space with soluble PrP [197]. It also remains to be elucidated whether endoproteolysis of ZIP transporters is responsible in the onset and/or progression of the disease or solely happens as a by-product of scrapie infection, PrP Sc accumulation and the neurodegenerative characteristics associated with prion diseases. It is
also essential to observe ZIP regulation from mouse brains infected with other strains of PrP\textsuperscript{Sc} to confirm whether ZIP processing is only the result of RML infection or occurs widely in other prion diseases, such as CJD and GSS, which suggests that insights to be learned from the study of ZIP transporters in prion diseases may have broader implications.

To analyze the possibility that there may be similarities in the mechanism of endoproteolysis observed for ZIP6 and ZIP10 with PrP in scrapie infection and that, similar to ZIPs, the PrP endoproteolysis may depend on the concentration of divalent cations, an experiment was designed to observe whether PrP fragmentation and processing occurs in zinc-deficient conditions, similar to ZIP proteins. N2a cells were treated with TPEN, an intracellular and extracellular zinc chelator, and after 48 hours of treatment in the presence of 3\(\mu\)M TPEN, ZIP10 was slightly more expressed accompanied by its proteolytic cleavage leading to the generation of a protein fragment at less than 51kDa (Figure 3.8A and Figure 3.8B). Similarly, PrP also underwent endoproteolysis resulting in the lower amount of full-length PrP molecules and generation of higher levels of PrP fragments (Figure 3.8A and Figure 3.8B). Since the MTT assay showed that only 50\% of cells were alive after the 3\(\mu\)M TPEN treatment (Figure 3.8C) and the previous studies also indicated that TPEN-dependent chelation of zinc leads to apoptosis by the activation of caspase 11 and caspase 3 proteins [188-190], another metal chelator, DTPA, an exclusively extracellular zinc chelator with less toxicity than TPEN [190], was selected to reproduce the results with TPEN and to show endoproteolysis is not a mere by-product of cell death. Treatment of cells with DTPA after 48 hours showed similar proteolysis events for both ZIP10 and PrP although the analysis of ZIP10 blot revealed an extra protein fragment (51kDa). This observation parallels the observation from scrapie-infected brain homogenates (Figure 3.9A). It must be noted that there have been controversial studies on the importance of different
divalent cations, including zinc, copper, iron and manganese, on prion propagation and function of PrP in oxidative stress, and also the metal-rescue experiment in the presence of DTPA indicated the specificity of DTPA not only for zinc chelation but also for manganese chelation (Figure 3.9B). Therefore, it is necessary to repeat the experiment with other zinc and manganese chelators to find out whether the aforementioned observations are due to zinc and/or manganese chelation, and determine the relative efficacy with which individual divalent metal ions can rescue the endoproteolysis event we observed in N2a cells.

Cumulatively, these results point at a model whereby scrapie infection and prion aggregation are associated with metal deficiency. Entirely unclear, is whether zinc deficiency is important in the onset or propagation of the disease. Involvement of zinc regulation in prion diseases is plausible since PrPC may bind to and sequester extracellular zinc ions based on its well-characterized divalent cation coordination chemistry associated with the presence of the octapeptide repeat region. PrP may further regulate the uptake of zinc through endocytosis or by acting as a zinc sensor to activate a signal transduction pathway leading to zinc sequestration [170].

Several studies have concentrated on identifying proteases involved in both α- and β-cleavages of the prion protein. With regard to the α-cleavage of PrP, controversial studies have introduced three ADAM family members, including ADAM9, ADAM10, and ADAM17, to be crucial for endoproteolysis of PrP [73, 75]. However, it remains to be elucidated whether these ADAM proteins can directly cleave the prion protein and lead to the generation of C1 and N1 fragments [76]. In addition, a few studies focused on the β-cleavage of the prion protein and generation of the C2 fragment that is up-regulated in prion diseases, especially CJD. A study by Yadavalli et al. proposed calpain proteases to be important for the cleavage of PrP [79], whereas
a contradictory study by Dron et al. showed that cathepsins, a group of lysosomal proteins, are necessary for the β-cleavage of PrP [80]. Whether it is calpain or cathepsin or any other protease, it must be revealed how PrP is endocytosed and in which cellular compartment PrP is cleaved. It may be that the interaction of PrP with another protein located on the plasma membrane is necessary to activate signal transduction cascades to facilitate the endocytosis and intracellular transport of the prion protein for endoproteolysis [198]. With regard to this hypothesis, concentrating on ZIP biology may be a promising direction since the interaction of ZIP transporter with PrP may cause endocytosis and endoproteolysis of these proteins by similar mechanisms. In support of this hypothesis, the interaction of PrP with an integral protein or a PrP receptor, located on the plasma membrane and termed L_{PrP} or Tr, has been postulated to play a role in the neuroprotective function of PrP [2, 9, 199-201] and it is possible that this transmembrane protein is also involved in the endocytosis and endoproteolytic cleavage of the prion protein. However, further experiments are required to define the relationship between ZIP proteins and PrP^{Sc}, to clarify the mechanism of ZIP down-regulation and endoproteolysis in ScN2a cells and mouse scrapie-infected brains respectively, and to assess any direct effect of ZIP transporters on prion replication.
Chapter 4

Conclusions and Future Directions
The similarities in PrP and ZIP10 endoproteolysis events occurring in both mouse brains in response to scrapie infection and mouse N2a cells during zinc depletion can implicate that prion diseases are associated with deficiencies in the regulation of metal ions, mainly a deficiency in zinc homeostasis. This is not unlikely since PrP$^C$ has been postulated to be important in zinc regulation by binding to and sequestering excess extracellular zinc ions [170], or by sensing excess zinc and signalling to activate a signal transduction pathway leading to zinc sequestration [59, 170, 202]. PrP may also be involved in zinc regulation by binding to zinc which is followed by the endocytosis of zinc-bound PrP into the cell, the separation of zinc from PrP, and zinc accumulation in intracellular compartments [97, 170, 203, 204]. PrP can then be transported back on the plasma membrane [170, 204].

Considering the role of PrP in zinc regulation and the observations reported in this study, one can propose that the dysfunction of the prion protein due to its conversion to PrP$^{Sc}$ and aggregation may lead to deficiencies in zinc homeostasis that can facilitate further PrP aggregation. However, future studies must concentrate on analyzing the endoproteolysis of other metal transporters such as copper channels to confirm whether this endoproteolysis is restricted to ZIP transporters or it occurs as a general event for the degradation of several metal-regulating proteins. The observations reported in RML-infected mouse brains with regard to the endoproteolysis of ZIP6 and ZIP10 must also be confirmed in other prion diseases such as CJD and GSS cases to evaluate whether ZIP cleavage is associated with all prion diseases or it is only limited to RML-infected mouse brains. It is also worthy of analysis to compare brain homogenates from mice fed with zinc-deficient diet and zinc supplements for the duration of scrapie propagation to observe whether zinc deficiency is associated with the higher propagation of the disease and also higher PrP/ZIP10 endoproteolysis.
In addition, one can consider the fact that ZIPs and PrP may undergo similar endoproteolytic events, with the involvement of either similar or identical proteases, due to their homologies in the PL domain [44]. Therefore, it is essential to investigate the mechanisms of endoproteolysis for both proteins by incubating ScN2a cells and zinc-chelated N2a cells in the presence of several protease inhibitors to find clues regarding the location of the endoproteolysis events and the involvement of potential proteases. It is also worthy to generate stable N2a cell lines expressing ZIP6 and ZIP10 constructs with an attached C-terminal HA-tag. This would allow one to analyze the interactome of both ZIP6 and ZIP10 proteins to identify their interactions with newly found proteases that have not been detected for PrP.

Finally, it is important to perform immunoprecipitation studies in N2a cells to confirm that ZIP5, ZIP6 and ZIP10 can in fact interact with the prion protein in vitro. ZIP constructs can be transiently overexpressed in N2a cells and ZIP proteins can be immunoprecipitated by either anti-HA or anti-ZIP antibodies. Probing with the anti-PrP antibody will reveal any potential interaction between ZIP proteins and the prion protein. This will allow further investigations to map the PrP-ZIP interaction site on ZIP transporters by designing DNA constructs that can overexpress N-terminally truncated ZIP transporters in N2a cells. Confirmation of PrP’s interaction with the N-terminal ectodomains of ZIP6 and ZIP10 proteins is essential since it may suggest that this interaction is important in the activation of signal transduction pathways by signalling of PrP through ZIP transporters leading to the endocytosis of PrP protein and its endoproteolysis [2, 9, 199-201]. The potential role of ZIP transporters in PrP cleavage can be further uncovered by stably overexpressing both full-length and truncated ZIP proteins in N2a cells under zinc-deficient conditions to observe any differences in the level of PrP proteolytic fragments.
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