Calnexin and fatty acid binding protein 5 (Fabp5) complex in the blood-brain barrier

by

Tautvydas Paskevicius

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Department of Biochemistry
University of Alberta

Abstract

Calnexin is a type 1 integral endoplasmic reticulum (ER) membrane protein and molecular chaperone involved in the folding and quality control of membrane associated and secreted proteins. The ER luminal domain of calnexin is responsible for the chaperone function, whereas the C-terminal domain is oriented to the cytoplasm and provides the link between ER luminal events and cytoplasmic signaling. This unique C-terminal domain of the protein undergoes distinct post-translational modifications including palmitoylation, SUMOylation and phosphorylation. In addition to that, the calnexin C-terminal domain interacts with various proteins including fatty acid binding protein 5 (Fabp5), a small (~15-kDa) cytoplasmic lipid chaperone that binds long-chain fatty acids and other hydrophobic ligands.

We have discovered that multiple sclerosis (MS) patients have unusually high abundance of calnexin and Fabp5 in their blood-brain barrier (BBB) endothelial cells (1, 2). The loss of either calnexin or Fabp5 in mice confers resistance to experimental autoimmune encephalomyelitis (EAE) (1, 3), an animal model of MS. Moreover, calnexin-deficient (*Canx*$^{-/-}$) mice expressing a recombinant calnexin derivative that is missing the cytoplasmic C-terminal domain remain partially protected from EAE symptoms, indicating that deletion of the C-terminal domain alone, the site of the calnexin/Fabp5 complex formation, have protective effects against EAE symptoms (2).

We identified that resistance to EAE in *Canx*$^{-/-}$ mice was due to inhibition of trans-endothelial T-cell migration across the brain endothelial cell monolayer when calnexin and Fabp5 complex formation is prevented (2). These findings uncovered the existence of an unanticipated link between the calnexin C-terminal domain/Fabp5 complex and EAE/MS pathogenesis.

Preface

Chapter 1. The literature review on calnexin presented in the section 1.1 of Chapter 1 has been submitted for publication in *Cells* as a review article: Paskevicius T, Farraj RA, Agellon LB and Michalak M. 2022. Calnexin, not just another chaperone.

Farraj R.A, from the laboratory of Dr. Glover M, assisted in the creation and graphical design of the figures 1-3 and 1-4. I wrote the manuscript with editing help from Dr. Michalak M.

Chapter 2 has been published as Paskevicius T, Jung J, Pujol M, Eggleton P, Qin W, Robinson A, Gutowski N, Holley J, Smallwood M, Newcombe J, Zochodne D, Chen XZ, Tang J, Kraus A, Michalak M, Agellon LB. The Fabp5/calnexin complex is a prerequisite for sensitization of mice to experimental autoimmune encephalomyelitis. FASEB J. 2020 Dec;34(12):16662-16675

Dr. Bhagat Singh from the laboratory of Dr. Zochodne D. (presently at the University of Alberta) assisted with electro-physiological studies presented in the figures 2-3. Gutowski N, Holley J, Smallwood M, Newcombe J. from the laboratory of Dr. Eggleton P. (University of Exeter Medical School, UK) assisted in immunohistochemical analyses of human MS brain tissue presented in the figures 2-8B, 2-9, 2-10, 2-10. Qin W. from the laboratories of Drs. Tang J. and Chen XZ. at the Hubei University of Technology and University of Alberta, respectively, assisted in cell culture and immunoblot experiments. Dr. Jung J. assisted in bEND.3 cells immunostaining, EAE animal experiments and T-cell transmigration assays presented in the figures 2-1A, 2-4, 2-5, 2-7B. Robinson A. assisted in animal care, breeding and handling. Dr. Pujol M. assisted in splenocyte isolation and T-cell transmigration analysis using flow cytometry presented in the figures 2-7. Dr. Kraus A. assisted in initial generation of calnexin deficient mice. I was responsible for coordinating the work, experimentation, data collection and analysis. I wrote the manuscript with contributions and editing help from Dr. Jung. J., Dr. Agellon L.B. and Dr. Michalak M.

The approval for use of mice in research was granted by the Animal Care and Use Committee for Health Sciences, a University of Alberta ethics review committee (Permit AUP297).

Postmortem normal control or multiple sclerosis (MS) cerebral subventricular white matter samples used in this study were obtained from NeuroResource, UCL Queen Square Institute of

Neurology, London ethical approval (ID 18/LO/0721) from the National Health Service (NHS) London-Central Research Ethics Committee, or from the UK Multiple Sclerosis Society Tissue Bank (ethical approval reference number 08/MRE09/31). Approval for the study was from the Local Research Ethics Committee (ID04/Q2102/111).

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List of abbreviations

AJ – adherens junction

APC – antigen-presenting cell

ATZ – α1-antitrypsin Z

Bak – Bcl-2 antagonist/killer 1

Bap31 – B-cell receptor-associated protein 31

Bax – Bcl-2-associated X protein

BBB – blood-brain barrier

Bcl-2 – B-cell lymphoma-2

BCR – B-cell receptor

BiFC – bimolecular fluorescence complementation

BiP – immunoglobulin binding protein also known as GRP78

bps – base pairs

Canx – calnexin

CD3 – cluster of differentiation 3

CD4 – cluster of differentiation 4

CD8 – cluster of differentiation 8

CK2 – casein kinase 2

CN – calcineurin

CNS – central nervous system

CoIP – co-immunoprecipitation

CRISPR – clustered regularly interspaced short palindromic repeats

Cyp B – cyclophilin B

DAB – 3,3′-diaminobenzidine

DHA – docosahexaenoic acid

DHHC6 – palmitoyltransferase

DHSO – dihydrazide sulfoxide

DMT – disease modifying treatment

EAE – experimental autoimmune encephalomyelitis

EBNA1 – Epstein–Barr nuclear antigen 1

EBV – Epstein-Barr virus

EDEM – ERAD-enhancing α-mannosidase-like protein

EGF – epidermal growth factor

ER – endoplasmic reticulum

ERAD – endoplasmic reticulum-associated protein degradation

ERK-1 – extracellular signal-regulated kinase 1

ERLAD – ER-to-lysosome-associated degradation

Erp29 – endoplasmic reticulum protein 29 also known as PDIA9

Erp57 – endoplasmic reticulum protein 57 also known as PDIA3

FA – fatty acid

Fabp – fatty acid binding protein

Fabp5 – fatty acid binding protein 5

FAM134B – family with sequence similarity 134 member B (ER-phagy-regulating receptor)

FDA – food and drug administration

GI – glucosidase I

GII – glucosidase II

Glc – glucose

GlcNAc – N-Acetylglucosamine

HIV – human immunodeficiency virus

Hsp70 – 70 kilodalton heat shock protein

ICAM-1 – intercellular adhesion molecule 1

IFNγ – interferon γ

Il1β – interleukin 1β

IMAC – immobilized metal affinity chromatography

IP90 – 90-kDa intracellular protein

K_d – dissociation constant

kDa – kilodalton

LFA-1 – lymphocyte-associated antigen-1

MAFFT – multiple alignment using fast fourier transform

MAM – mitochondria-associated membrane

Man – mannose

MBP – myelin basic protein

MD simulation – molecular dynamics simulation

MEK1 – mitogen-activated protein kinase 1

MHC I – major histocompatibility complex class I

MHCII – major histocompatibility complex class II

MOG – myelin oligodendrocyte glycoprotein

MS – multiple sclerosis

MST – microscale thermophoresis

NAWM – normal-appearing white matter

Nef – negative regulatory factor

NMT – N-myristoyltransferase

OST – oligosaccharyltransferase

p88 – 88-kDa protein

PACS-2 – phosphofurin acidic cluster sorting protein 2

PDIA1 – protein disulfide isomerase A1

PDIA3 – protein disulfide isomerase A3

PDIA9 – protein disulfide isomerase A9

PECAM – platelet endothelial adhesion molecule-1

pI – isoelectric point

PIAS3 – protein inhibitor of activated STAT3

PLP – myelin proteolipid protein

pp90 – phosphoprotein 90 kilodalton

PPAR – peroxisome proliferation-activated receptor

PTM – post-translational modification

PTP1B – protein tyrosine phosphatase 1B

RER – rough endoplasmic reticulum

SDS-PAGE – sodium dodecyl sulfate–polyacrylamide gel electrophoresis

SERCA – sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase

SGIP1– SH3 domain GRB2 like endophilin interaction protein

SPR – surface plasmon resonance

STAT3 – signal transducer and activator of transcription 3

SUMO – small Ubiquitin-like Modifier

Th1 – T helper type 1

Th17 – T helper type 17

TJ – tight junction

TNFα – tumor necrosis factor α

UBC9 – SUMOylation E2 ligase

UDP – uridine diphosphate

UGGT – UDP-glucose:glycoprotein glucosyltransferase

UTR – untranslated region

VCAM – vascular cell adhesion molecule

VLA-4 – very late antigen 4

ZO – zonula occludens

CHAPTER 1

LITERATURE REVIEW

Part of this chapter will be published as a review article: Paskevicius T, Farraj RA, Agellon LB and Michalak M. Calnexin, not just another chaperone.

1. Introduction

The endoplasmic reticulum (ER) is a continuous membrane network composed of branching tubules and flattened sacs that surround a single lumen. The ER performs a plethora of functions in cells including lipid and steroid synthesis, Ca^{2+} storage and signalling, protein synthesis and folding, protein structural maturation and post-translational modification (4-6). Human cells express approximately 10,000 different proteins at any given moment (7). More than one third of these proteins are synthesized on the ER membrane-bound ribosomes where proteins are either destined for residence in the ER, plasma membrane, Golgi apparatus, lysosomes or secreted from the cell (8). Many of these newly synthesized proteins require assistance by molecular chaperones to reach their native fold in a biologically relevant time scale (9-10). To help facilitate proper folding and quality control, the ER employs two main folding systems: the general pathway - that is mediated by BiP (the ER homolog of 70-kDa heat shock protein Hsp70) together with protein disulfide isomerase PDIA1 and the N-linked glycoprotein pathway, which is governed by lectin chaperones calnexin and calreticulin, referred to as calnexin/calreticulin cycle (11). Both calnexin and calreticulin share high amino acid sequence homology, however, in contrast to calreticulin, which is a resident ER lumenal protein, calnexin is a type I integral ER membrane protein (12). Hence, the fine balance in proteostasis, lipidostasis, ER and cytoplasmic signalling, inter-organelle communication and metabolic crosstalk as a whole is crucial in maintaining the healthy state of a cell. This section of the Introduction is focussed on the structure and function of calnexin, an ER chaperone not only associated with ER folding pathways but also involved in the crosstalk between ER events and cytoplasmic signalling via its' unique C-terminal domain (13-18).

1.1. Calnexin – discovery

In 1982 an unknown 90-kDa ER-associated protein was described using polyclonal antibodies raised against membrane fractions from the rough endoplasmic reticulum (RER) (19). Cell culture immunofluorescence analysis using anti-RER sera revealed an extensive, reticular network spanning the entire cytoplasm and including the nuclear membrane (19). However, not until a decade later, a 90-kDa phosphoprotein (pp90) was identified associated with ER signal sequence receptor complexes in canine pancreatic microsomes (20). Simultaneously two other research groups reported the identification of an 88-kDa protein (p88) in transient association with partially assembled class I major histocompatibility molecules in murine lymphoma cell lines (21) and a 90-kDa intracellular protein (IP90) interacting with T-cell antigen and B-cell antigen receptor complexes (22). Molecular cloning of the canine cDNA encoding IP90 revealed that the 90-kDa protein was a type I ER integral membrane protein. Due to its' Ca^{2+} binding properties (20) and a high degree of amino acid sequence identity with calreticulin, a major Ca^{2+} binding ER resident protein, the pp90 (p88, IP90) was named calnexin (20, 23-25). Since then, calnexin has been shown to interact transiently with a wide array of nascent membrane or soluble N-linked glycoproteins, establishing its' widely known function as a key ER quality control protein. (26-33).

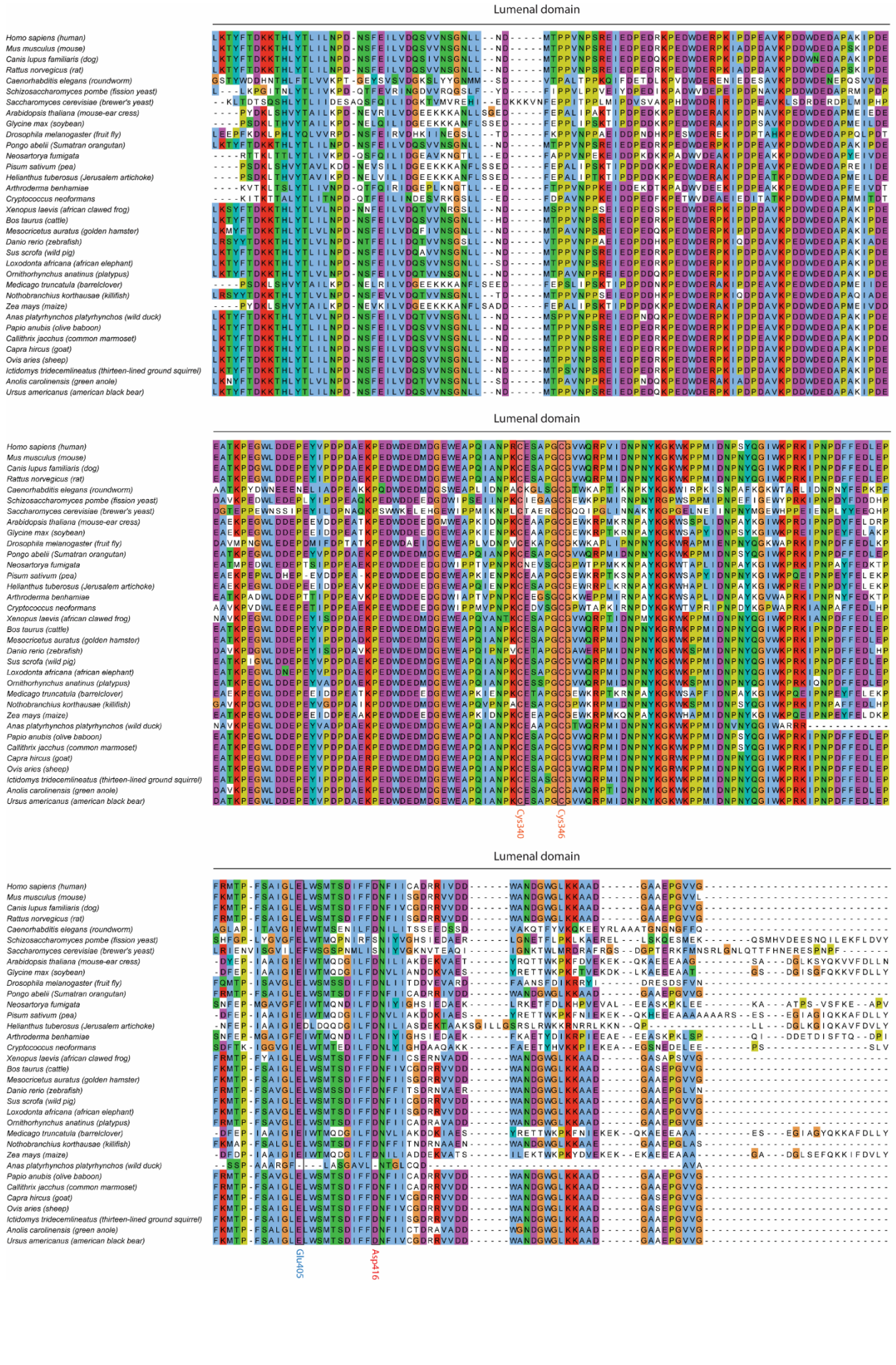
1.1.1. Calnexin

Calnexin as an integral ER membrane protein and molecular chaperone is ubiquitously expressed in all cells containing ER membrane. It is highly conserved between different species (Figure 1-1), with the ER lumenal domain responsible for chaperone function displaying the highest level of amino acid sequence conservation. This indicates evolutionary importance of the

chaperone domain of calnexin. Calnexin can be found distributed within different ER membrane subdomains, including perinuclear rough ER forming contacts with ribosome-translocon-complexes (34, 35) and in the smooth ER at mitochondria-associated membranes (MAMs) (16, 36). The selective distribution of calnexin molecules within the ER membrane is controlled by post-translational modifications including palmitoylation and phosphorylation at its C-terminal domain, which are discussed later. Moreover, it has also been reported that calnexin is expressed on the cell surface of immature thymocytes in complex with CD3 antigen due to incomplete ER retention (37, 38). Later studies detected a small fraction of calnexin on the cell surface of various cell types (39) and the distribution of calnexin between ER domains and plasma membrane was proposed to be controlled by the state of calnexin C-terminal domain phosphorylation and association with phosphofurin acidic cluster sorting protein 2 (PACS-2) (40). Also, plasma membrane localization of calnexin has been detected in cancer tumors including oral squamous cell carcinoma and melanoma (41). Moreover, another study reported calnexin being secreted in the serum of lung cancer patients, making calnexin a useful sero-diagnostic marker (42). However, cell surface localization of calnexin and its functional implications needs further study.

The calnexin family of molecular chaperones is highly conserved among fungi, plants and animals (44, 45). This family also includes calmegin, a type-I membrane protein spatiotemporally expressed in male germ cells during specific stages of spermatogenesis (46-48), and calreticulin, a soluble ER resident lectin like chaperone. Human calmegin has a similar structure as human calnexin, except its cytoplasmic C-terminal domain has an extra 28 amino acids (46, 48). Calnexin and calreticulin together with PDIA3 (ERp57) facilitate the folding and quality control of newly synthesized glycoproteins (49). In addition to these canonical functions, calnexin has also been implicated to affect Ca^{2+} signalling (31, 45, 46), endocytosis (56), sensitivity to apoptosis (77, 123-126), STAT3 signalling (76), HIV infection and cholesterol metabolism (58, 81, 127), ER-phagy (50, 51) and recently blood-brain barrier (BBB) permeability to immune cells (1, 2).


-20
N-terminal signal sequence
0
1
Lumenal domain
Homo sapiens (human)
Mus musculus (mouse)
Canis lupus familiaris (dog)
Rattus norvegicus (rat)
Caenorhabditis elegans (roundworm)
Schizosaccharomyces pombe (fission yeast)
Saccharomyces cerevisiae (brewer's yeast)
Arabidopsis thaliana (mouse-ear cress)
Glycine max (soybean)
Drosophila melanogaster (fruit fly)
Pongo abelii (Sumatran orangutan)
Neosartorya fumigata
Pisum sativum (pea)
Helianthus tuberosus (Jerusalem artichoke)
Arthroderma benhamiae
Cryptococcus neoformans
Xenopus laevis (african clawed frog)
Bos taurus (cattle)
Mesocricetus auratus (golden hamster)
Danio rerio (zebrafish)
Sus scrofa (wild pig)
Loxodonta africana (african elephant)
Ornithorhynchus anatinus (platypus)
Medicago truncatula (barrelclover)
Nothobranchius korthausae (killifish)
Zea mays (maize)
Anas platyrhynchos platyrhynchos (wild duck)
Papio anubis (olive baboon)
Callithrix jacchus (common marmoset)
Capra hircus (goat)
Ovis aries (sheep)
Ictidomys tridecemlineatus (thirteen-lined ground squirrel)
Anolis carolinensis (green anole)
Ursus americanus (american black bear)
Lumenal domain
Ser54
Asp97
Lumenal domain
Cys140
Tyr144
Lys146
Tyr165
Met168
Cys174
Glu196


Lumenal domain
Homo sapiens (human)
Mus musculus (mouse)
Canis lupus familiaris (dog)
Rattus norvegicus (rat)
Caenorhabditis elegans (roundworm)
Schizosaccharomyces pombe (fission yeast)
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Callithrix jacchus (common marmoset)
Capra hircus (goat)
Ovis aries (sheep)
Ictidomys tridecemlineatus (thirteen-lined ground squirrel)
Anolis carolinensis (green anole)
Ursus americanus (american black bear)
Lumenal domain
Cys340
Cys346
Lumenal domain
Glu405
Asp416

Figure 1-1. Amino acid sequence alignment of calnexin from different species

The figure 1-1 shows the alignment of calnexin amino acid sequences from different species. Clustal X default colour scheme was used to label similar/identical amino acid residues: hydrophobic – blue, positively charged – red, negatively charged – magenta, polar – green, aromatic – cyan, cysteine – pink, glycine – orange, proline – yellow, unconserved – white. N-terminal signal sequence, lumenal domain, transmembrane domain and C-terminal domain indicated based on human calnexin protein topology. Highly conserved amino acid residues include cysteines forming disulfide bond (Cys140-Cys174 and Cys340-Cys346), carbohydrate binding region (Tyr144, Lys146, Tyr165, Glu196, Glu405, Met168), Ca^{2+} binding site (Asp416, Asp97 and Ser54) indicated and labelled in orange, blue and red respectively. (The amino acid residue numbering coresponds to mature human calnexin protein and for the sake of consistency and clarity, this numbering will be used throughout the text unless otherwise indicated).

Alignment was performed using MAFFT (Multiple Alignment using Fast Fourier Transform) high speed multiple sequence alignment program (https://toolkit.tuebingen.mpg.de/tools/mafft). Alignment visualization performed using Jalview (Waterhouse, A.M., Procter, J.B., Martin, D.M.A, Clamp, M. and Barton, G. J. (2009))

1.1.2. Chaperone function of calnexin

In 1994, studies carried out by Ari Helenius and his colleagues described and proposed calnexin as a key molecular chaperone involved in folding and quality control of proteins with N-linked carbohydrate side chains (33, 52). Since then, this folding pathway has been widely studied and therefore well characterized and described in several excellent review articles (12, 53-57).

It is predicted that more than 50% of all eukaryotic proteins are glycoproteins with more than 90% of these being N-glycosylated (58). The folding and maturation of newly synthesized glycoproteins in the ER is assisted by calnexin and its soluble ER resident homolog, calreticulin. Immediately after the nascent polypeptide exits the translocon and enters the ER lumen, oligosaccharyltransferase (OST) transfers a dolichol-pyrophosphate-bound branched core glycan to the side chain nitrogen of the asparagine residue (59). This modification occurs at an N-glycosylation consensus amino acid sequence motif of Asn-X-Ser/Thr (X is any amino acid except for proline). The branched core oligosaccharide is comprised of three terminal glucose, nine mannose, and two N-acetyl-glucosamine ($Glc_3Man_9GlcNAc_2$). After the glycosylation, N-linked glycans are then processed by the subsequent action of ER resident glucosidases I (GI) and II (GII) removing the outer and middle glucose residues, respectively. As a result, the processing intermediate containing the single terminal glucose moiety is recognised by calnexin and calreticulin (33, 60, 61). Additionally, using their extended arm-like P-domains, calnexin and calreticulin recruit PDIA3 (ERp57), Cyp B and ERp29 (62-66) to promote glycoprotein folding and maturation. Subsequently, the remaining innermost glucose moiety is removed by GII, releasing the glycoprotein substrate from the calnexin/calreticulin complex. At this point, if the protein is correctly folded, it is released from the ER and transported to the Golgi towards the

secretory pathway. However, if the protein hasn't reached its native three dimensional conformation, it is re-glucosylated by the ER-folding sensor uridine diphosphate (UDP)-glucose:glycoprotein glucosyl transferase (UGGT), facilitating the re-association with calnexin and calreticulin for an additional round of the folding cycle (67). Thus, the protein can cycle multiple times through the calnexin/calreticulin cycle until the native conformation is reached. However, if numerous folding cycles are not successful, terminally misfolded proteins are then targeted to the ER associated degradation (ERAD) pathway (57).

1.1.3. Calnexin in ERAD

Terminally misfolded glycoproteins and unassembled oligomers are retro-translocated to the cytoplasm and are degraded by the ubiquitin-proteasome system – a process known as ER-associated degradation (68). This process is regulated by ER α-mannosidase I (69) and ER degradation-enhancing α-mannosidase-like protein (EDEM) (70). Proteins undergoing multiple calnexin/calreticulin cycles are eventually subjected to mannose trimming by ER α-mannosidase I, converting $Man_9GlcNAc_2$-glycans to $Man_8GlcNAc_2$ (71). Slow kinetics of this enzyme essentially act as a timer for repeated glycoprotein folding cycles (72). As a result, $Man_8GlcNAc_2$ becomes a less efficient UGGT substrate (53) and instead it is recognized by EDEM, which acts as a signal triggering ERAD (73). It has been shown that EDEM directly interacts with calnexin and accepts terminally misfolded glycoproteins upon mannose trimming (74, 75). This is followed by retro-translocation into the cytoplasm where misfolded proteins are polyubiquitinated and degraded by the 26S proteasome (76).

1.1.4. Calnexin in ER-phagy

Due to extensive accumulation and aggregation of misfolded proteins, a selective form of autophagy named ER-phagy, is used to ensure timely removal of damaged ER (77). During ER-

phagy, excessive or damaged portions of the ER are fragmented and sequestered through ER-phagy receptors by double-membrane autophagic vesicles that eventually fuse with lysosomes for degradation (77). It has been shown that calnexin makes a stable complex with the ER-phagy receptor FAM134B (51). In collagen producing cells (mouse embryonic fibroblasts, human osteoblasts and rat chondrosarcoma cells), calnexin acts as a co-receptor recognizing ER luminal misfolded procollagen molecules, triggering FAM134B to recruit and bind the autophagosome membrane-associated protein LC3. In turn, the ER-phagy complex delivers a targeted portion of the ER that contains both misfolded procollagen and calnexin to the lysosome for degradation (51). Additionally, it has been shown that the calnexin-FAM134B complex can facilitate clearance of proteasome-resistant polymers of alpha1-antitrypsin Z (ATZ) in the ER through another vesicular transport pathway (50). This pathway was named ER-to-lysosome-associated degradation (ERLAD) since ER-derived vesicles containing misfolded proteins are not encapsulated by autophagosomes but instead fuse with endo-lysosomes for degradation. (50).

1.1.5. Calnexin – a key chaperone in the immune system?

Considering the importance of calnexin as an ER molecular chaperone in folding and quality control of secreted and membrane bound glycoproteins, the protein initially emerged as an important factor in the development and function of the immune system. For example, calnexin is involved in folding and assembly of major histocompatibility complex (MHC) class I (78-82). However, there is a discrepancy in the results from different studies describing the importance of calnexin in the maturation and proper folding of MHC class I molecules. While one study claims that calnexin is necessary for MHC class I biogenesis, the findings from a different study show that calnexin is not required for the efficient assembly of MHC class I molecules (82-84). Additionally, calnexin also participates in MHC class II (27), T-cell antigen receptor (TCR) (24,

28, 85-87) and B-cell antigen receptor (BCR) (88-90) assembly and maturation. Despite numerous reports on the role of calnexin in the development of the immune system, calnexin-deficient mice display normal immune function, showing that calnexin is not essential in the development of the immune system in mice (91). Instead, it has been identified that expression of calnexin affects T-cell trafficking across the BBB (1). It has also been reported that elevated levels of calnexin on the plasma membrane of multiple cancer tissues play an inhibitory role in supressing the T-cell infiltration in the tumor, promoting its growth (41). Therefore, calnexin may play a role in T-cell biology.

1.1.6. Calnexin, the gene

In the human genome, the calnexin gene is located on the forward strand of chromosome 5 long arm distal end within the 5q35.3 locus. The gene is composed of 15 exons, with the first one being a non-coding exon. The calnexin transcript is 4,915 bps in length and is translated into a 592 amino acid residue polypeptide (Figure 1-2). The mouse calnexin gene is located on the reverse strand of chromosome 11 long arm with a similar arrangement of 14 out of 15 coding exons that make up a 4,281 bps transcript, which translates into a 591 amino acid residue polypeptide (92).

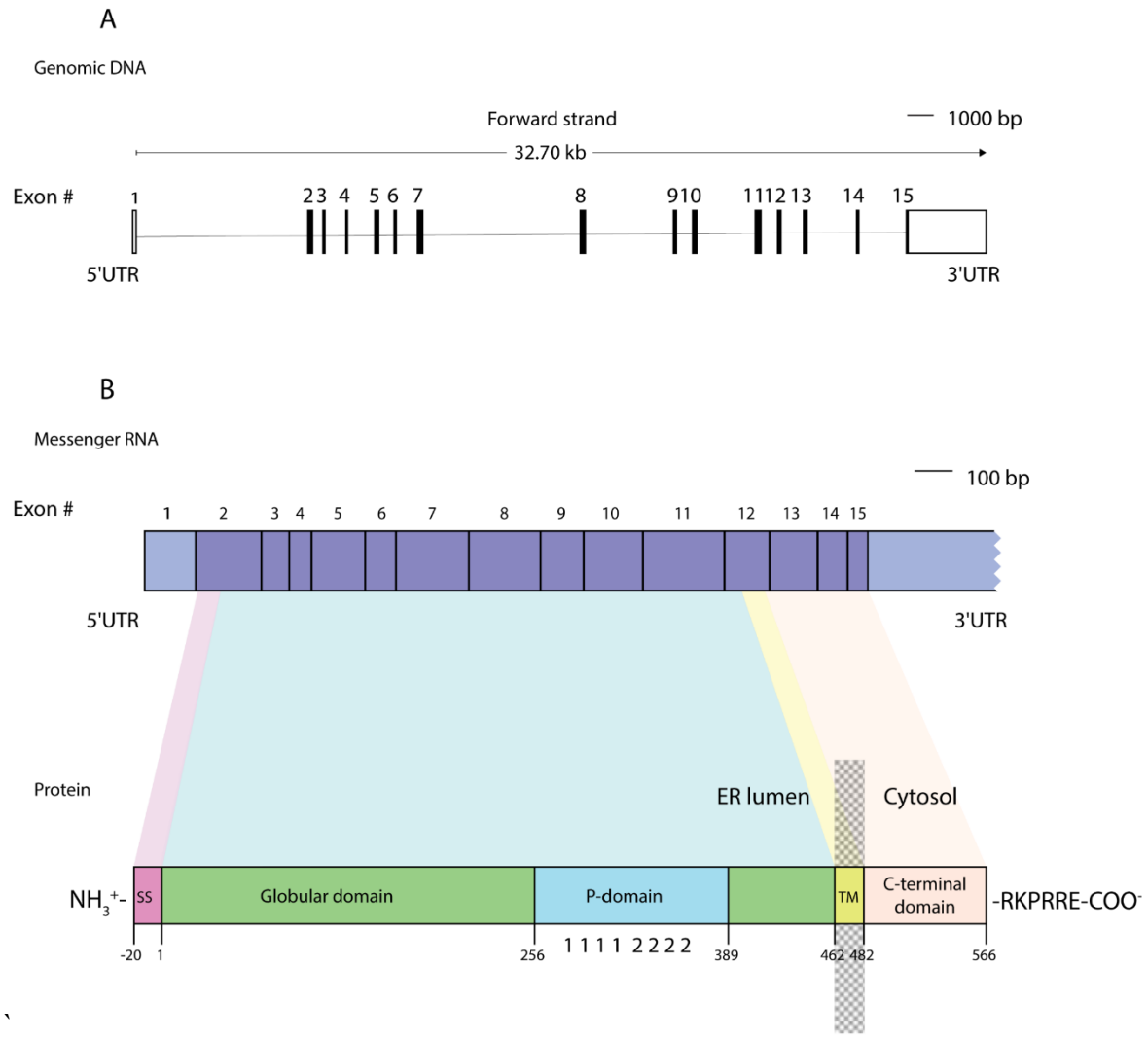


Figure 1-2. Schematic representation of the calnexin gene and protein

A. The calnexin gene is located on the forward strand of chromosome 5 long arm distal end. It contains 15 exons with 14 coding exons, making the transcript of 4,915 bps, which is translated into a 592 amino acid residue polypeptide.

B. Linear schematic representation of the calnexin protein and its corresponding mRNA transcript, indicating the exon region that codes for specific domains of the protein. Dark blue boxes in the mRNA diagram represent coding exons (2-15), while light blue indicates non-coding exon 1 together with 5' and 3'UTR (for visual simplicity 3'UTR region was partially cropped). Magenta, green, blue, yellow, and beige boxes represent the signal sequence, ER luminal globular domain and P-domain, transmembrane domain (TM), and C-terminal cytoplasmic domain respectively. In the proline rich amino acid P-domain, four amino acid repeats of motif 1 and four repeats of motif 2 are labelled as "11112222. –RKPRRE: distal C-terminal ER retention amino acid sequence.

1.1.7. Calnexin the protein

Calnexin is a 67-kDa type-I integral membrane protein. It is often mistakenly referred to as a 90-kDa protein due to its high content of acidic residues (theoretical pI of calnexin 4.46), which electrostatically repel SDS, resulting in insufficient electromotive incentive and lowered electrophoretic mobility on SDS-PAGE.

Calnexin is composed of three topological domains (Figure 1-3) – the ER intralumenal domain, the transmembrane segment and the cytosol facing C-terminal domain (20). The ER lumenal domain is responsible for calnexin chaperone function and is often labelled as the folding module (62, 93). The single spanning transmembrane segment anchors calnexin to the ER membrane and possibly contributes to its chaperone function (94). Additionally, a unique 90 amino acid long C-terminal domain is oriented to the cytoplasm where it undergoes distinct post-translational modifications (16, 34, 36, 95-100) and forms functional interactions with various proteins (18, 99, 101-104), facilitating crosstalk between ER and cytoplasmic events

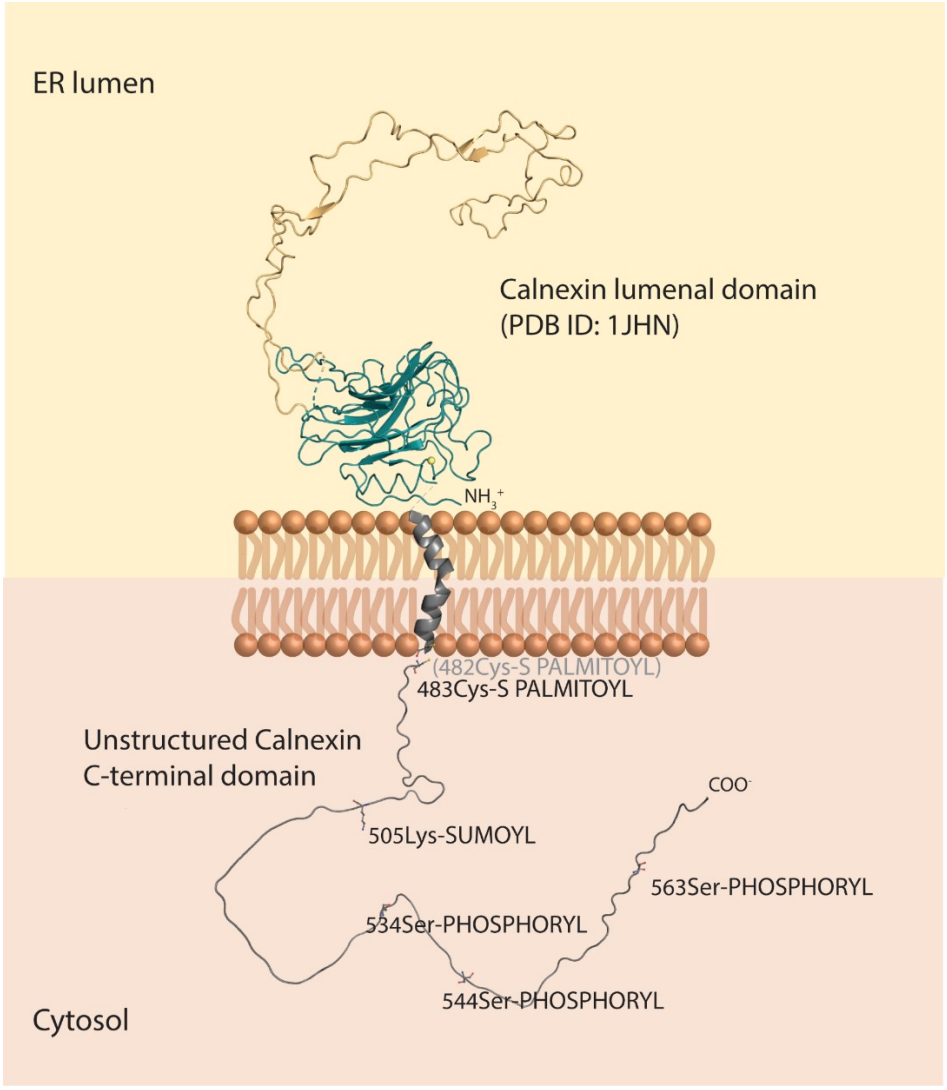


Figure 1-3. Schematic representation of a full length calnexin in the membrane

Calnexin has three structural and functional domains: luminal domain (PDB ID:1JHN), transmembrane domain and C-terminal domain (no experimental structural information). The transmembrane domain (TM) was modelled based on a MD simulation performed by Lakkaraju et al. (35) showing that the TM forms a kink at Pro-494. The calnexin C-terminal domain was modelled using alphafold (264). The calnexin C-terminal domain undergoes distinct posttranslational modifications including palmitoylation at Cys482 and Cys483 (36); SUMOylation at Lys506 (99) and phosphorylation at Ser534, Ser544, Ser563 (100).

1.1.8. Calnexin, the ER lumenal domain

The ER lumenal domain of calnexin is responsible for lectin-like chaperone function and is the site for PDIA3 (ERp57), Cyp B and ERp29 (62-66) interactions. Calnexin is synthesized with a 20 amino acid N-terminal signal sequence, which targets calnexin to the ER.

The crystal structure of the ER luminal portion of canine calnexin was solved by Schrag et al. (105) at 2.9 Å resolution, revealing its' unique asymmetry with two distinct structural components (Figure 1-4): a compact N-globular domain and an elongated arm-like proline rich P-domain (105). The N-globular domain is composed of concave and convex antiparallel β sheets that have six and seven β strands respectively, which together form the β-sandwich (105). Calnexin luminal domain co-crystallized with α-D-glucose revealed the putative site for the carbohydrate binding within the N-globular domain on the concave β sheet, where Tyr144, Lys146, Tyr165, Glu196, and Glu405 form hydrogen bonds to glucose hydroxyl groups, while the Met168 side chain interacts with the glucose ring via van der Waals' interactions (105).

The P-domain of calnexin (49) extends 140 Å away from the N-globular domain and forms a large hairpin loop (Fig. 1-4). This loop consists of two types of repeated motifs - motif 1 and motif 2 of proline rich sequence repeats (hence the name – P-domain) with consensus sequences of I-DP(D/E)A-KPEDWD(D/E) and G-W-P-I-N-P-Y, respectively. Each motif is repeated four times and arranged in a linear manner '11112222' where four repeats of motif 1 extend away from N-globular domain and then fold back onto the strand with four repeats of motif 2. Every copy of the motif 1 interacts with a copy of the motif 2 in a head-to-tail fashion (105). This hook-like arm is further stabilized via hydrophobic interactions of conserved isoleucine residues (12). In addition, there are three regions of high amino acid sequence similarity, flanking the repeat motifs. Both the

N-globular and P-domains harbour one disulfide bond each: Cys140-Cys174 and Cys340-Cys346, respectively (105).

Early studies have indicated that calnexin contains multiple low affinity Ca^{2+} binding sites in its N- and C-terminal regions (20, 43). However, the three-dimensional structure of the luminal domain of calnexin revealed only a single putative Ca^{2+} binding site with Ca^{2+} being coordinated by Asp416, Asp97 and Ser54 (105). This Ca^{2+} binding site is highly conserved between calnexin and calreticulin, likely playing a structural role in the P-domain. The calreticulin P-domain binds a single Ca^{2+} with high affinity (106) through Asp328 (Canx Asp416 equivalent), Gln26, Lys62, and Lys64, and two water molecules (105). Binding of Ca^{2+} to the ER luminal portion of calnexin plays a structural role by triggering Ca^{2+}-dependent conformational changes (107). In addition, the calnexin luminal domain binds Zn^{2+} and ATP where both regulate conformational changes in the protein (107). ATP alone enhances the aggregation suppression abilities *in vitro*, although no ATPase activity has been reported for calnexin (108). Zn^{2+} was shown to facilitate PDIA3 (ERp57) binding to the calnexin P-domain (109).

The structural insight into the lumenal domain of calnexin further established its role in the folding of N-glycosylated substrates.

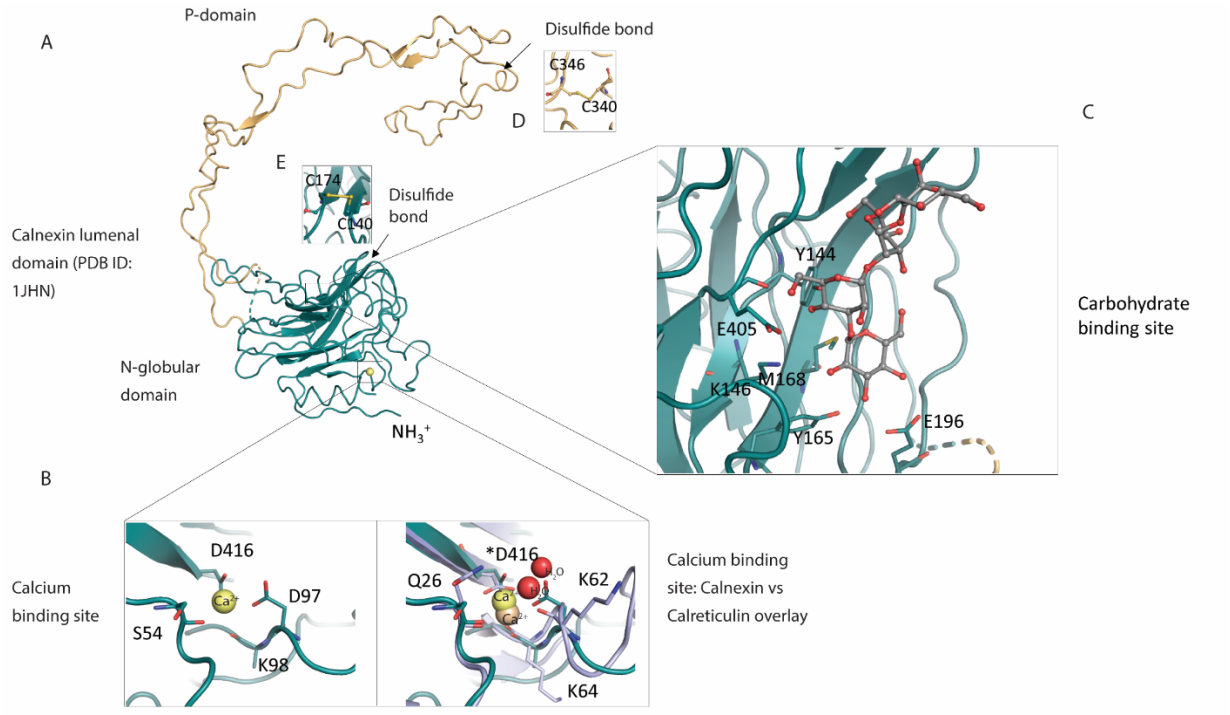


Figure 1-4. Crystal structure of calnexin lumenal domain and its characteristics

Crystal structure of calnexin lumenal domain. The extended arm P-domain is depicted in yellow, while the N-globular domain is in green. **A.** Calnexin putative Ca^{2+} binding site. The left panel indicates Ca^{2+} coordinated by Asp416, Asp97, Ser54 and potentially Lys98. The right panel represents superposition of calnexin PDB ID: 1JHN) (green) and calreticulin (PDB ID: 3POS) (magenta) Ca^{2+} binding sites. Labelled residues represent the calreticulin Ca^{2+} binding site where Ca^{2+} is coordinated by: Asp328 (*calnexin Asp416 equivalent), Gln26, Lys62, and Lys64, and two water molecules. **B.** Calnexin carbohydrate binding site. Amino acid residues involved in the binding of carbohydrate: Tyr144, Lys146, Tyr165, Glu196, Glu405 and Met168. (Carbohydrate molecule was positioned by structural alignment of calnexin PDB ID: 1JHN) and calreticulin (PDB ID: 3POS)). **D, E.** Two calnexin disulfide bonds. Cys340-Cys346 in P-domain; Cys140-Cys195 in the N-globular domain. Crystal structure superposition performed using PyMol (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.)

1.1.9. Calnexin, Transmembrane domain

Molecular dynamics simulation analysis predicts the single span transmembrane domain of calnexin forms an α-helix with a kink at Pro494 that tilts the helix by ~30° with respect to the membrane surface (35). If Pro494 is replaced with leucine to remove the kink in the

transmembrane helix, the interaction between calnexin and ribosome-translocon complex was negatively affected (35). The transmembrane domain anchors calnexin to the ER membrane, localizing the protein within the plane of the ER membrane phospholipid bilayer. It has been proposed that the anchoring of calnexin to the ER membrane facilitates its association with membrane bound substrates, therefore contributing to its chaperone function (94).

1.1.10. Calnexin, C-terminal domain

While the function of the ER lumenal domain of calnexin is characterized as a lectin-like chaperone, and the transmembrane segment is implicated in facilitating this function, the C-terminal domain of calnexin has been largely ignored by many investigators, and consequently this domain is not functionally characterized. However, the calnexin C-terminal domain plays an important role in retention of the protein in the ER as it terminates with the –RKPRRE motif that acts as an ER retention signal (110). To date, no structural information is available about the calnexin C-terminal domain. The human calnexin C-terminal domain is 90 amino acids in length, flexible, unstructured and facing the cytoplasm (12, 20). It is highly acidic (theoretical pI of the C-terminal domain: 4.52). The acidic nature of the domain contributes to an unusual calnexin electrophoretic mobility on SDS-PAGE with mobility being dramatically decreased, making calnexin often mistakenly referred to as a 90-kDa protein (the actual protein molecular weight of calnexin is 67-kDa). Due to its negative charge, the calnexin C-terminal domain harbours multiple low affinity, high capacity Ca^{2+}-binding sites (43).

This unique cytoplasmic domain of calnexin undergoes numerous post-translational modifications (Table 1-1) including palmitoylation (95-97), phosphorylation and SUMOylation (16, 34, 36, 98-100), linking ER luminal events and cytoplasmic signalling. There is only limited amino acid sequence conservation of the calnexin C-terminal domain between different organisms (Figure 1-5). Interestingly, the sites for post-translational modifications, which include Cys502, Cys503, Ser563, Ser534, Ser544, Lys505, are moderately conserved (ranging from 44%-65%),

indicating potentially similar posttranslational modifications occurring in different organisms, driving calnexin C-terminal domain mediated cytoplasmic events.

Figure 1-5. Amino acid sequence alignment of calnexin C-terminal domain

The figure shows alignment of the calnexin C-terminal domain amino acid sequence from different species. Clustal X default colour scheme was used to label similar/identical amino acid residues: hydrophobic – blue, positively charged – red, negatively charged – magenta, polar – green, aromatic – cyan, cysteine – pink, glycine – orange, proline – yellow, unconserved – white. The location of post-translational modifications is indicated.

Alignment was performed using MAFFT (Multiple Alignment using Fast Fourier Transform) high speed multiple sequence alignment program (https://toolkit.tuebingen.mpg.de/tools/mafft). Alignment visualization was performed using Jalview (Waterhouse, A.M., Procter, J.B., Martin, D.M.A, Clamp, M. and Barton, G. J. (2009))

1.1.11. C-terminal domain post-translational modifications: palmitoylation

Calnexin is palmitoylated at both juxtamembranous cysteines Cys502 and Cys503 by an ER palmitoyltransferase DHHC6 (35). It has been shown that around 90% of calnexin molecules are S-acylated at steady state, suggesting that calnexin maintains a relatively static and robust pamitoylation-depalmitoylation cycle. Molecular dynamics (MD) simulation predicted that upon palmitoylation, the calnexin C-terminal domain acquires different conformations with respect to the transmembrane helix axis, suggesting that palmitoylation affects the capacity and/or selectivity of calnexin to interact with proteins in the cytoplasm via its cytoplasmic domain. Additionally, palmitoylation at Cys503 was predicted to induce a more prominent conformational shift of the C-terminal domain, indicating possible functional/regulatory differences between Cys502 and Cys503 states of palmitoylation (35). Also, using both computational and experimental approaches, it has been shown that the half-life of calnexin increased 9-fold upon palmitoylation (111). As a functional consequence, the palmitoylation of Cys502 and Cys503 targets calnexin to the perinuclear rough ER and facilitates the protein association with ribosome-translocon complexes. The latter is considered to be crucial for the chaperone to capture its substrates emerging from the translocon (35).

Other studies have shown that palmitoylated calnexin localized to the mitochondria-associated membranes (MAMs) – the domains of the ER that form close contacts with mitochondria (112, 113), where it interacts and controls sarco-endoplasmic reticulum (SR) Ca^{2+} transport ATPase (SERCA) 2b (17). Importantly, calnexin-SERCA interaction modulates ER–mitochondria Ca^{2+} signaling (15, 16, 36). It has been shown that upon short-term ER stress, the pool of palmitoylated calnexin is reduced, shifting non-palmitoylated chaperone localization to the

rough ER where it interacts with PDIA3 (ERp57) and facilitates protein folding and quality control (16).

Somewhat conflicting evidence from different studies on the effect of calnexin palmitoylation status presents the need for additional investigation to elucidate how palmitoylation of calnexin controls its distribution between rough ER and MAMs.

1.1.12. C-terminal domain post-translational modifications: phosphorylation

During one of the early studies, calnexin was identified as a 90-kDa phosphoprotein (pp90) associated with ER signal sequence receptor complexes (20). Since then, it has been elucidated that calnexin harbours three phosphorylation sites in its C-terminal domain: Ser534, Ser544 and Ser563. Several kinases were identified to phosphorylate calnexin: Casein kinase (CK2) was shown to phosphorylate Ser534 and Ser544 (100, 114), whereas extracellular signal-regulated kinase-1 (ERK-1) phosphorylated calnexin at Ser563 (34). It is known that ERK-1 is activated by mitogen-activated protein kinase 1 (MEK1) under protein misfolding conditions (115), therefore the phosphorylation state of Ser563 plays a role in ER quality control. Upon ER stress, accumulation of misfolded proteins leads to ERK-1 activation followed by enhanced calnexin phosphorylation at Ser563, which in turn, results in recruitment of calnexin to ER membrane bound ribosomes. This recruitment facilitates calnexin function as a chaperone, enhancing glycoprotein quality control at ER ribosome-translocon complexes by prolonging calnexin association with unfolded proteins (34, 98). Additionally, in synergy with phosphorylation of Ser563, phosphorylation of Ser534 and Ser544 by CK2 further promotes calnexin-ribosome interactions (34). Moreover, phosphorylation of Ser534 and Ser544 disrupts calnexin interaction with phosphofurin acidic cluster sorting protein 2 (PACS-2), shifting calnexin distribution from MAMs to rough ER (40).

It has been reported that calcineurin (CN), a Ca^{2+} dependent phosphatase, dephosphorylates Ser563 (117), controlling the phosphorylation status of Ser563 which was shown to modulate calnexin interaction with SERCA2b and regulate intracellular Ca^{2+} oscillations (17).

Taken together, phosphorylation of calnexin at its C-terminal domain unlocks another level of complexity in controlling calnexin distribution between different ER membrane subdomains and therefore mediating its functions.

Table 1-1. Calnexin C-terminal domain post-translational modifications

Post-translational modification	Amino acid residue (s)	Function	Enzymes
Palmitoylation	Cys502 Cys503	Preferentially localize calnexin to the perinuclear rough ER and facilitates the association with ribosome-translocon complexes (35); Localize calnexin to the mitochondria-associated membranes (MAMs) – where it interacts and controls the Ca^{2+} pump SERCA2b modulating ER–mitochondrial Ca^{2+} signaling (15, 16, 36).	ER palmitoyltransferase DHHC6 (35)

Post-translational modification	Amino acid residue (s)	Function	Enzymes
Phosphorylation	Ser563	ER stress induced phosphorylation at Ser563 recruits calnexin to ER membrane bound ribosomes, enhancing glycoprotein quality control by prolonging its association with unfolded proteins (34, 98); Phosphorylation status of Ser563 modulates calnexin interaction with SERCA2b which in turn regulates intracellular Ca^{2+} oscillations (17).	Extracellular signal-regulated kinase-1 ERK-1 (phosphorylates at Ser563 (34).
	Ser534 Ser544	In synergy with phosphorylation of Ser563, phosphorylation of Ser534 and Ser544 enhance calnexin-ribosome interactions (34); Disrupts calnexin interaction with PACS-2, shifting calnexin distribution from MAMs to rough ER (65).	Casein kinase CK2 (phosphorylates at Ser534 and Ser544) (100, 114).
SUMOylation	Lys505	Association with UBC9 via its C-terminal domain and subsequent SUMOylation mediates calnexin interaction with PTP1B at the ER membrane/cytosol interface (118).	SUMOylation E2 ligase UBC9 (118).
Proteolytic cleavage	At Asp519	Upon EGF stimulation, the cleaved calnexin C-terminal domain translocates to the nucleus and promotes STAT3-mediated transcription via inhibition of PIAS3 (14).	Caspase-8 mediated (14).

Post-translational modification	Amino acid residue (s)	Function	Enzymes
	DXXD	Potentially attenuates apoptosis (13).	Caspase-3 or caspase-4 (13).

1.1.13. C-terminal domain post-translational modifications: SUMOylation

SUMOylation is a type of post-translational modification which involves the covalent attachment of a small ~10-kDa SUMO (small ubiquitin-related modifier) protein to target proteins/substrates (119). Initial studies have identified calnexin as a potential SUMOylation substrate (120), therefore further investigation demonstrated that calnexin interacts with SUMOylation E2 ligase UBC9 via its C-terminal domain and undergoes SUMOylation at Lys505 (118). Additionally, the association of UBC9 and Lys505 SUMOylation mediated calnexin interaction with protein tyrosine phosphatase 1B (PTP1B) (118) links two divergent pathways: protein quality control and insulin and leptin signalling (121).

1.1.14. C-terminal domain post-translational modifications: proteolytic cleavage

It has been reported that apoptotic stimuli caused calnexin to undergo proteolytic cleavage at its C-terminal domain (13). It was suggested that the cleavage is carried out by caspase-3 or caspase-4 at the site DXXD and the cleavage product potentially attenuates apoptosis (13).

Another study showed that stimulation of A431 cells with epidermal growth factor (EGF) triggers caspase-8 dependent proteolytic cleavage of the calnexin C-terminal domain at Asp519 (14). The functional consequences of the aforementioned proteolytic cleavage will be discussed in chapter 1.1.20.

1.1.15. C-terminal domain: interacting partners

In addition to the post-translational modifications described above, which introduce another complexity level in controlling calnexin distribution within the ER membrane subdomains and link divergent signalling pathways, the calnexin C-terminal domain is also a site for multiple binding partners (Table 1-2), including SUMOylation E2 ligase (UBC9) (99), protein tyrosine phosphatase 1B (PTP1B) (99), SH3 domain GRB2 like endophilin interaction protein (SGIP1) (18), N-myristoyltransferase 1 (NMT1) (101), human immunodeficiency virus (HIV) protein Negative Regulatory Factor (Nef) (102), activity-regulated cytoskeleton-associated protein (Arc) (103) and fatty acid binding protein 5 (Fabp5) (104). Table 1-2 provides a summary of the functional consequences of calnexin C-terminal domain interaction with aforementioned proteins.

The ability of the calnexin C-terminal domain to form functional interactions with various cytoplasmic proteins and undergo distinct post-translational modifications suggests that the calnexin C-terminal domain is a highly dynamic and modifiable protein segment that can facilitate reciprocal crosstalk between cytoplasmic and ER luminal events, or even perform functions previously not attributed to calnexin.

Table 1-2. Calnexin C-terminal domain interacting partners

Calnexin C-terminal domain interacting protein	Functional consequence	Reference
SUMOylation E2 ligase (UBC9)	SUMOylates calnexin C-terminal domain and facilitates PTP1B interaction with calnexin.	(99)
Protein tyrosine phosphatase 1B (PTP1B)	Contributes to PTP1B association with the ER membrane, potentially affecting protein quality control and energy metabolism.	(99)

Calnexin C-terminal domain interacting protein	Functional consequence	Reference
SH3 domain GRB2 like endophilin interaction protein (SGIP1)	Affects clathrin-dependent endocytosis by modulating the internalization of the receptor-ligand complexes. Calnexin C-terminal domain acts as a potent inhibitor of clathrin-mediated endocytosis.	(18)
N-myristoyltransferase (NMT1)	Contributes to the retention of NMT1 at the ER membrane.	(101)
HIV protein Negative Regulatory Factor (Nef)	The interaction between Nef and calnexin disrupts the interaction of calnexin with ATP-binding cassette transporter A1 (ABCA1), leading to the retention of this transporter in the ER and its degradation, which as a result inhibits ABCA1-dependent cholesterol efflux. Also, Nef promotes calnexin-gp160 interactions.	(102, 122)
Activity-regulated cytoskeleton-associated protein (Arc)	Not defined.	(103)
Fatty acid binding protein 5 (Fabp5)	Affects immune cell trafficking across the blood-brain barrier.	(2, 104)

1.1.16. Other cellular processes attributed to the calnexin C-terminal domain

Calnexin has also been implicated in various cellular processes not directly linked to protein folding and quality control but rather mediated through its C-terminal domain. This includes Ca^{2+} signalling (15-17), endocytosis (18), sensitivity to apoptosis (13, 124, 126, 127, 129), STAT3 signalling (14), HIV infection and cholesterol metabolism (102, 122, 132) and BBB permeability to immune cells (1, 49) (Figure 1-6).

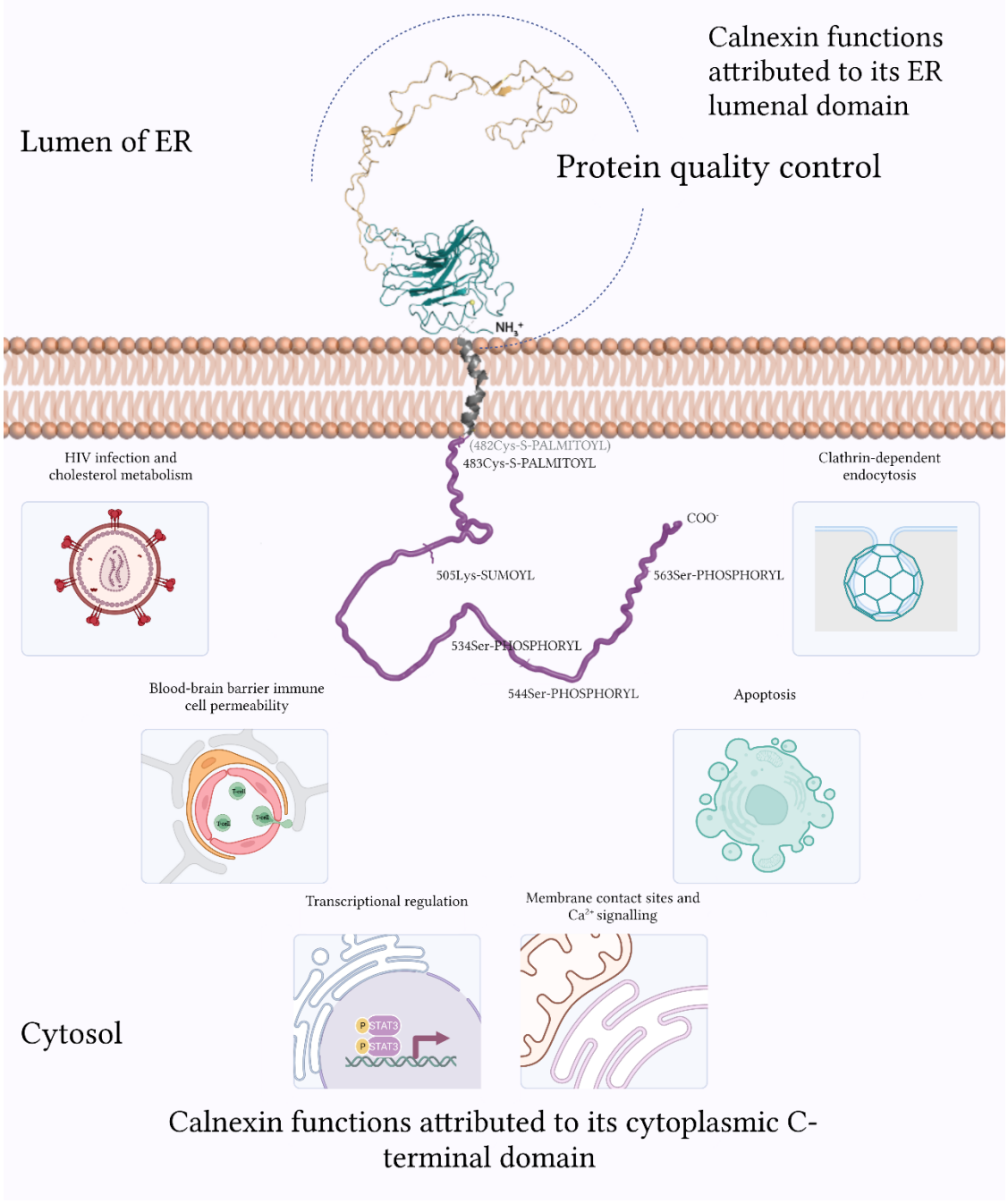


Figure 1-6. Calnexin ER lumenal and cytoplasmic domain-dependent functional roles in a cell

Calnexin ER lumenal domain is involved in the quality control of protein folding. The cytoplasmic C-terminal domain is implicated in various processes including Ca^{2+} signalling (15-17), endocytosis (18), sensitivity to apoptosis (13, 124, 126, 127, 129), STAT3 signalling (14), HIV infection and cholesterol metabolism (102, 122, 132), and BBB permeability to immune cells (1, 49). Created with BioRender.com

1.1.17. Calnexin C-terminal domain and Ca^{2+} signaling

Calnexin interacts with SERCA2b at MAMs and modulates its activity. It has been shown that both the phosphorylation (17) and palmitoylation (16) status of the calnexin C-terminal domain regulates the interaction between calnexin and the Ca^{2+} pump, SERCA2b. However, studies cannot agree if calnexin activates or inhibits SERCA2b (15, 17). Regardless, both studies implicate calnexin in Ca^{2+} signalling at or close to the MAM.

1.1.18. Calnexin C-terminal domain and clathrin-dependent endocytosis

It has been shown that calnexin regulates clathrin-dependent endocytosis via its interaction at the C-terminal domain with SGIP1 (18). The C-terminal domain of calnexin was identified as a potent inhibitor of endocytosis in neuronal cells (18). Moreover, the absence of calnexin in mice resulted in increased endocytosis in the nervous system (18), which might contribute to the phenotype of the calnexin-deficient mice (91, 123), which will be discussed later.

1.1.19. Calnexin C-terminal domain and apoptosis

Initial evidence of calnexin involvement in apoptotic pathways resulted from the study performed in yeast *S. pombe* showing that calnexin interacts with pro-apoptotic mammalian proteins Bcl-2 homologous antagonist/killer (Bak) and Bcl-2-associated X protein (Bax) (124). The overexpression of Bak is lethal in wild-type *S. pombe* cells, however, the cells that express C-terminally truncated calnexin display resistance to Bak mediated lethality, suggesting that the calnexin C-terminal domain mediates/propagates the lethal signal or recruits additional proteins at the calnexin-Bak complex (124).

Additionally, overexpression of full length or C-terminally truncated calnexin in S. *pombe* was shown to induce apoptosis (125). However, overexpression of the calnexin lumenal domain

only is protective against apoptotic death. This indicated that calnexin needs to be anchored to the ER membrane to elicit apoptotic death by overexpression (125).

Meanwhile, a deficiency in calnexin results in resistance to apoptosis induced by ER stress (126, 127). This could be attributed to the observed reduction in the level of B-cell receptor-associated protein 31 (Bap31) cleavage. Bap31 is an integral ER membrane protein which has a "death effector" cytosolic domain that is cleaved by caspase-8 into p20-Bap31 (128). Calnexin and Bap31 form a complex at the ER membrane and since both the calnexin C-terminal domain and the Bap31 "death effector" domain face cytosol, the calnexin C-terminal domain might act as a scaffold (129) to facilitate caspase-8 dependent cleavage of Bap31 (126). Moreover, another study reported that apoptotic cells underwent calnexin proteolytic cleavage by caspase-3 or caspase-7, resulting in cleaved calnexin C-terminal domain which in turn attenuated apoptosis (13).

1.1.20. Calnexin C-terminal domain and STAT3 signaling

Calnexin was also implicated in regulating the STAT3-mediated transcriptional response via its C-terminal domain (14). Cells stimulated with EGF triggered rapid caspase-8 dependent proteolytic cleavage of the calnexin C-terminal domain at Asp519 (14). The cleavage of calnexin at Asp519 released a 53 amino acid long fragment which was detected as a 16-kDa band on SDS PAGE. It was estimated that upon EGF treatment, only around 20% of the total calnexin population underwent proteolytic processing. Therefore, upon cleavage and release from the ER membrane to cytosol, the calnexin C-terminal domain was shown to translocate to the nucleus, where it made stable contacts with PIAS3, preventing it from inhibiting STAT3 (14). Additionally, palmitoylation of calnexin, which targets it to MAMs, was required for EGF-induced calnexin

cleavage. Interestingly, induction of ER stress prevented this calnexin C-terminal domain cleavage (14).

1.1.21. Calnexin C-terminal domain and HIV infection and cholesterol metabolism

Another study has demonstrated the role of calnexin in HIV infection (122). It was reported that the calnexin C-terminal domain is targeted by the HIV protein Nef, affecting ABCA1 main cellular cholesterol transporter maturation (122). The binding of Nef to the calnexin C-terminal domain disrupted the interaction of calnexin with ABCA1, leading to the retention of this transporter in the ER and its eventual degradation, which consequently inhibited ABCA1-dependent cholesterol efflux. As a result, HIV infected cells accumulate excessive cholesterol which leads to the formation of plasma membrane lipid rafts, which are sites of HIV entry, assembly, and budding (130). Additionally, this effect contributes to the development of atherosclerosis in HIV patients (131). Moreover, Nef promotes the calnexin-gp160 interaction, enhancing HIV envelope protein maturation (122). Through molecular modeling, mutagenesis and virtual compound screening, a small molecule was identified that blocked Nef-calnexin interactions, resulting in partial recovery of ABCA1 activity and restoring cholesterol efflux (102, 132). In addition, the treatment with the identified small molecule targeting the Nef-calnexin complex resulted in a 2-fold inhibition of viral replication (102, 132).

1.1.22. Calnexin and the blood-brain barrier

The calnexin C-terminal domain interacts with fatty acid binding protein 5 (Fabp5) both *in vitro* and *in vivo* (2, 104). Both calnexin- and Fabp5-deficient mice are resistant to experimental autoimmune encephalomyelitis (EAE) - inflammatory CNS demyelination (1, 3). Additionally, mice that express a calnexin derivative that is missing the cytoplasmic C-tail domain (the site for Fabp5 interaction) remain partially protected from EAE symptoms (2). This resistance was

observed to result from the inhibition of circulating T-cell infiltration across brain endothelial cells when the complex between the calnexin C-terminal domain and Fabp5 was prevented (1, 2).

1.1.23. Calnexin deficient animal models

Two calnexin-deficient mouse models have been independently generated (91, 123). While homozygous calreticulin gene knockout is embryonic lethal due to defects in heart development and function (133), calnexin-deficient mice are viable (91, 123). These animals develop a neurological disorder that includes severe ataxia (91, 123), while heterozygote mice are indistinguishable from wild-type (91). Initially it has been reported that mice with a disrupted calnexin gene displayed early postnatal death (123) and surviving mice exhibited ataxia due to significant loss of motor nerve fibers. However, a subsequent study by Kraus *et al*. (91) showed that calnexin knockout mice were fertile, 30-50 % smaller than wild-type, had a normal life span and no reduction in the numbers of neuronal fibers. Instead, these animals developed myelinopathy with apparent neurologic abnormalities including gait disturbance with instability, splaying of the hind limbs, tremors and a rolling walk (91). Electron micrographs of the spinal cord and sciatic nerve indicated that there was severe dysmyelination in the peripheral nervous system (PNS). While calnexin deficient spinal cords had thinner, decompacted and wavy myelin, sciatic nerve fibers, in addition to disorganized myelin, also had hypermyelinated sections. As a result, there was a significant reduction in the nerve conduction velocities in both motor and sensory neurons in calnexin deficient mice (91). Moreover, even though there was no significant change in the gross morphology of the brain of these mice, there were some white matter tracts variably affected with a patchy and loose myelination pattern (91). In addition to that, calnexin-deficient mice had a significantly elevated number of synaptic vesicles in the cerebellum (18). Interestingly, no other

histological abnormalities were observed in multiple examined tissues and importantly there was no aberration in immune system development and functionality in calnexin deficient mice (91).

Moreover, another mouse strain expressing a truncated form of calnexin was reported (123). The deleted region of calnexin includes the amino acid residues involved in disulfide bond formation (Cys141 and Cys175) and carbohydrate recognition (Tyr145 and Lys147) (123). These mice were phenotypically identical to the calnexin-deficient mice, suggesting that the chaperone function of calnexin is likely responsible for the observed neurological phenotype (123). In agreement with that, mice that express C-terminally truncated calnexin exhibited unaffected motor function with normal motor and sensory nerve conduction velocities (2).

Calnexin gene deletion in yeast *S. cerevisiae* had no affect on cell viability with no gross effects of the levels of secreted proteins (45). In contrast, calnexin deficiency is lethal in yeast *S. pombe* cells (134, 135). Interestingly, *S. pombe* calnexin mutants devoid of chaperone activity are viable, suggesting that the lethality does not result from the absence of calnexin chaperone function (136, 137).

D. melanogaster has three genes encoding calnexin among which calnexin99A has the highest similarity with mammalian calnexin (138). Mutations in calnexin99A affect the maturation and function of rhodopsin which in turn leads to age-dependent retinal degeneration (138). Additionally, calnexin99A mutants displayed impaired Ca^{2+} buffering, which contributed to the development of the retinal degeneration, possibly due to Ca^{2+} toxicity (138).

The deletion of the calnexin gene in *C. elegans* results in developmental and reproductive defects which are temperature sensitive (139). Additionally, calnexin deficient *C. elegans* exhibited growth impairment upon various stress conditions (139). Calnexin gene silencing using RNAi results in supressed necrotic-like cell death (140).

In *D. rerio* (zebrafish), calnexin is required for the development of the mechanosensory system called the lateral line (141). Upon knockdown of calnexin, zebrafish exhibited reduced posterior lateral line cell proliferation and elevated ER stress-dependent apoptosis (141).

1.2. Fatty acid binding proteins

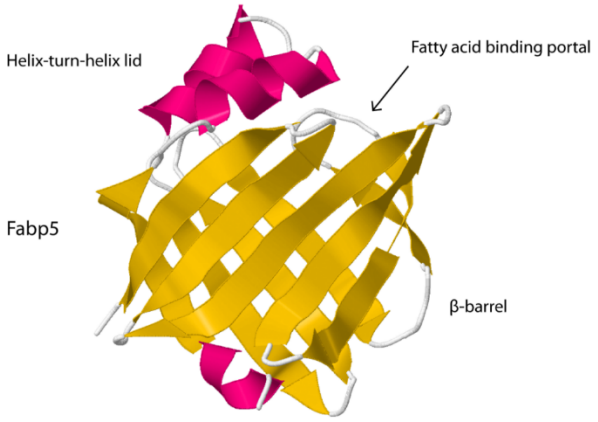
Fabps are a family of small (~15 kDa) conserved, cytoplasmic and abundantly expressed lipid chaperones that coordinate lipid responses in cells (142). Since the initial discovery in 1972 (150) at least nine FABP genes have been identified in the human genome, all encoding a protein product. This expansion of the Fabp family is thought to have occurred through gene duplication events (143). Initial discovery labelled each Fabp based on the tissue in which they were identified and isolated, however further studies showed that they are expressed with limited tissue specificity and showing substantial overlap in the expression pattern, therefore, numerical nomenclature for Fabps has been introduced. (151).

1.2.1. Fatty acid binding proteins: structure and ligand binding

The members of the Fabp family have an extremely wide range of amino acid sequence identity varying from 20% to 70% (142). However, the proteins display highly conserved tertiary structure: they fold into a β-barrel structure comprised of two orthogonal five-stranded β-sheets with a solvent accessible ligand binding cavity, capped by a helix-turn-helix motif which is thought to act as a portal for ligand entry and exit (144, 145) (Figure 1-7). The interior of the ligand binding cavity is aligned with the sidechains of both hydrophobic and polar amino acids which determines the cavity volume and the fatty acid binding specificity (146). The Fabps bind fatty acids with the fatty acid carboxylate group facing the inside of the cavity, interacting with Arg residues as well as other amino acid side chains (147). Binding of the ligand leads to a conformational change in

the portal region, suggesting conformational variability between apo- and holo-Fabps (148) (Figure 1-8). All Fabps bind saturated and unsaturated long chain fatty acids (C12-20) with different ligand selectivity and binding affinities (149). Generally, the binding affinity is high (nanomolar range) for more hydrophobic ligands, while shorter and less saturated fatty acids bind with lower affinity (150).

A



B

Homo sapiens	sequence identity (%)			
	FABP5	FABP7	FABP3	FABP4
FABP4	52.27	56.82	64.39	100
FABP3	48.87	66.67	100	
FABP7	44.7	100		
FABP5	100			

C

Fabp5 (PDB ID 4LKP) - yellow
Fabp4 (PDB ID 3Q6L) - magenta
Fabp3 (PDB ID 3RSW) - cyan
Fabp7 (PDB ID 1FDQ) - red

Figure 1-7. Structural and amino acid sequence identity of human brain expressed FABPs

A. Crystal structure of human FABP5 (PDB ID: 4LKP). The β-barrel structure is comprised of 10 anti-parallel β-strands (yellow) with a solvent accessible ligand binding pocket, capped by a helix-turn-helix motif (top pink).

B. Percentage of amino acid identity between human FABPs that are expressed in brain: FABP5, FABP3, FABP4, FABP7.

C. Superimposition of human FABP5, FABP4, FABP3, FABP7 crystal structures. Primary amino acid sequence alignment performed using Geneious Prime with inbuilt Clustal Omega. Crystal structure superimposition performed using PyMol (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.)

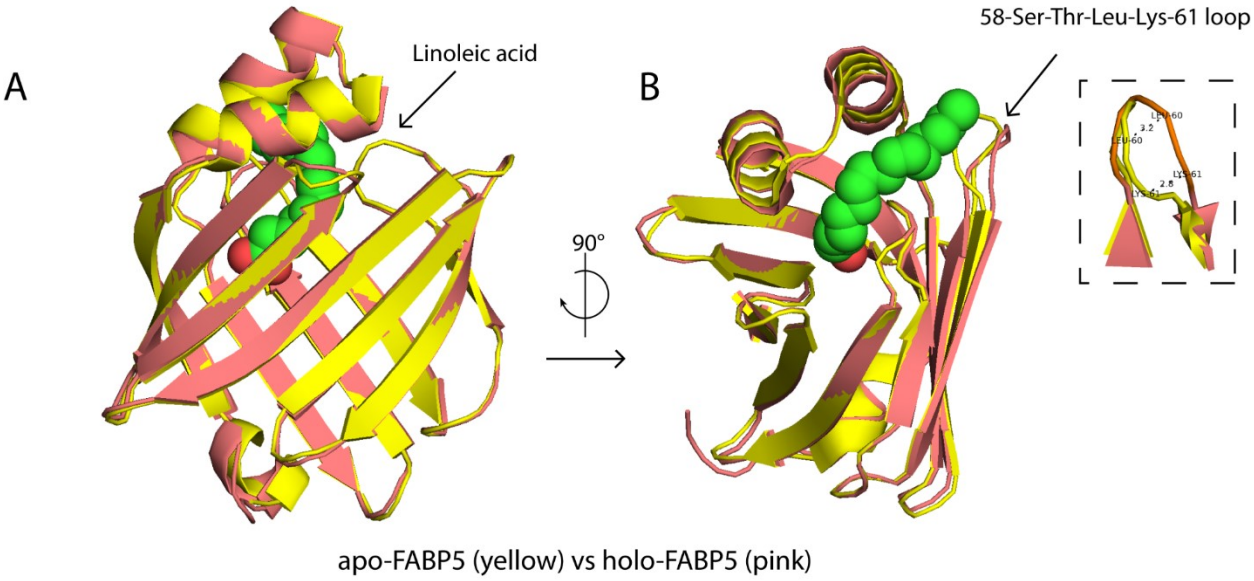


Figure 1-8. Superimposition of crystal structures of human apo- and holo-FABP5

A. Front view of superimposed apo-FABP5 (PDB ID: 4LKP) (yellow) and holo-FABP5 (PDB ID: 4LKT) (pink). Bound linoleic acid indicated by the arrow.

B. Side view of superimposed apo-FABP5 (yellow) and holo-FABP5 (pink). STLK loop, where the biggest shift is observed is marked by the arrow and depicted separately in the dashed square.

1.2.2. Fatty acid binding proteins: function

The insoluble nature of fatty acids in aqueous environments requires lipid chaperones to facilitate the solubilization, uptake and transport of fatty acids within different cellular compartments and organelles, including the plasma membrane, mitochondria, peroxisome, ER, lipid droplets and nucleus to support their function (151, 152). Therefore, a plethora of cellular processes are proposed to be influenced by Fabps and their fatty acid transport: lipid storage in lipid droplets, ER signaling, trafficking and membrane synthesis, fatty acid oxidation in

mitochondria and peroxisome, the activity of cytosolic and other enzymes, which is modulated by fatty acids, autocrine and paracrine signaling and lipid-mediated transcriptional regulation (151). The dysregulation of lipid metabolism plays a major role in the development and progression of various metabolic disorders, including obesity, non-alcoholic fatty liver disease, diabetes, atherosclerosis, and heart failure (153-156). Moreover, numerous Fabps are implicated in the development and progression of many cancers through fatty-acid signaling pathway affecting proliferation, angiogenesis and invasion of cancer cells. (157). Therefore, by modulating fatty acid concentration, Fabps can influence many aspects of cellular function including cell energetics, activity of enzymes, membranes, gene expression, cellular growth, and differentiation (150).

1.2.3. Fatty acid binding proteins: Fabp5

Fabp5, also often referred to as epidermal Fabp (E-Fabp5), was initially identified in human psoriatic keratinocytes, however later studies showed that Fabp5 has one of the broadest tissue expression patterns and can be found in epidermis, adipose, macrophages, mammary glands, brain, kidney, liver, lung, heart, skeletal muscle, and testis (158, 159). The gene encoding human *FABP5* is located on the chromosome 8 long arm and consists of four exons separated by three introns (146, 158, 160). Several *cis*-acting elements in the 5' promoter region along with trans-acting nuclear factors have been identified (160). Fabp5 is a small 135 amino acid in length, 15-kDa cytosolic protein with a three-dimensional structure similar to other Fabps. Unique to Fabp5 is six cysteine residues, two of which are redox active and form a disulfide bridge.

Fabp5 displays a non-selective ligand binding profile and interacts with long chain (C10-22) fatty acids of varying degrees of saturation as well as with the vitamin A metabolite all-trans-retinoic acid (145). However, *in vitro* studies using recombinant protein indicate that Fabp5 prefers stearic acid with a K_d of 0.2 μM (161). Structural studies using Fabp5 and linoleic acid complexes

reveal that the carboxylic headgroup of linoleic acid forms a salt bridge with Arg129 while it also forms a hydrogen bond with the hydroxyl moiety of Tyr131 (162). An additional hydrogen bond is formed between linoleic acid and Arg109 through an ordered water molecule (162). The alkyl chain of the fatty acid is stabilized largely by van der Waals interactions with hydrophobic side chains of the amino acids that line the binding pocket of Fabp5 (162).

In addition to the established roles of Fabps in solubilizing and shuttling fatty acids between different cellular compartments, the role of these lipid chaperones in lipid signaling is emerging. For example, Fabp5 participates in ligand-mediated signaling by binding certain ligands, relocating to the nucleus, and modulating the activity of a group of nuclear transcription factors known as the peroxisome proliferation-activated receptors (PPARs) (162, 163). Fabp5 undergoes ligand-specific nuclear translocation to stimulate PPARβ/δ transactivation (162), and its known gene targets are associated with glucose metabolism, lipid homeostasis, cell differentiation, proliferation, inflammation and apoptosis pathways (164-168). The nuclear translocation of Fabp5 is mediated by a subset of "activating" fatty acids such as arachidonic and linoleic acid that upon binding within the lipid binding pocket of Fabp5 induce a conformational change in the protein, exposing a tertiary nuclear localization signal (162). Fabp5 can also regulate PPARγ activity (169), which controls cell differentiation, glucose homeostasis and inflammatory responses (170). Therefore, Fabp5 regulates both PPARβ/δ and PPARγ and consequently influences cell metabolism. The protein has been implicated in various metabolic diseases including, but not limited to, atherosclerosis (171, 172), diabetes (173, 174), prostate and other types of cancer (175-179), as well as Alzheimer's disease (180), and Sjögren Syndrome (181).

1.2.4. Fabp5 in the nervous system

At the blood-brain barrier, Fabp5 facilitates brain uptake of docosahexaenoic acid (DHA), a blood-derived long chain (C22) highly unsaturated omega-3 fatty acid involved in brain development and cognition (182, 183). Additionally, Fabp5 facilitates endocannabinoid anandamide hydrolysis into arachidonic acid and delivers it to PPARβ/δ, enabling its activation, which in turn upregulates cognition-associated genes (184)

The mammalian brain expresses Fabp3, Fabp4, Fabp5, and Fabp7 (185), intriguingly, only Fabp5 was identified as a calnexin binding partner (104), and the interaction is implicated in mice in their susceptibility to experimental autoimmune encephalomyelitis (EAE) (2). Fabp5 expression is significantly increased in CNS tissue in mice with induced EAE and, therefore, not surprisingly genetic ablation of Fabp5 shows protection from EAE demyelinating phenotype (186). Fabp5-deficient mice exhibit reduced pro-inflammatory and increased production of anti-inflammatory cytokines with compromised T-cell priming by antigen presenting cells (APCs) (186). Pharmacological inhibition of Fabp5 by a small molecule that targets the fatty acid binding pocket suppressed differentiation of effector T-cells (Th1 and Th17) while inducing T regulatory (Treg) cells and their suppressive capacity (3, 187). Additionally, pharmacological inhibition of both Fabp5 and Fabp7 in mice with induced EAE results in decreased EAE symptoms and reduced myelin loss. There is also a decreased number of astrocytes in the spinal cord and reduced accumulation of pro-inflammatory cytokines (188).

Thus, Fabp5 emerges as a pivotal determinant in inflammatory responses, however the role of Fabp5 with respect to autoimmunity remains to be defined.

1.3. Multiple sclerosis (MS) pathogenesis

Multiple sclerosis (MS) is a heterogeneous chronic autoimmune disease of the CNS which presents with motor impairment, sensory and optic disturbances, fatigue, pain and cognitive deficits (189). It is the leading cause of non-traumatic neurological disability in young adults (190). It is estimated that around 2.8 million people live with MS worldwide with 90 000 MS patients in Canada. Moreover, Canada has one of the highest rates of MS in the world (190). People may be diagnosed with MS at any age, however in Canada the average age for diagnosis is 43 years with 75% of all the diagnosed patients being women (190).

The extensive heterogeneity of clinical manifestation is due to spatiotemporal dissemination of multiple lesions within different parts of the CNS. Demyelinating lesions within the white matter of brain or spinal cord, also knowns as plaques, are usually described as the pathological hallmark of MS (191) and are caused by the infiltration of auto-reactive immune cells into the CNS. This induces the breakdown of the BBB, multifocal neuroinflammation, loss of oligodendrocytes, and axonal degeneration (192). The key players in the pathogenesis of MS are the effector T-cells autoreactive against myelin proteins that breach the BBB, infiltrating the CNS and destroying oligodendrocytes, the cells which form and maintain the myelin sheath (189). This destructive inflammatory response is augmented by further reactivating local T-cells and triggering a cascade of inflammatory events, including glial activation, secretion of cytokines, chemokines and other neurotoxic molecules (193). It was thought for a long time that the primary driving force of MS were T-cells, however, emerging studies and clinical benefit from using B cell-depleting therapies with anti-CD20 monoclonal antibodies limiting new inflammatory lesions (194), reveals an important role for B cells in the pathogenesis of MS (195, 196).

The destruction of the myelin sheath, which normally insulates axons and facilitates effective electrical signalling, results in compromised action potentials and even axonal degradation coupled with loss of function (197). A repair mechanism – remyelination – is active during early stages of MS, where oligodendrocyte precursor cells remyelinate naked axons (198), however as the disease progresses through prolonged chronical demyelination, it becomes ineffective and unable to repair the tissue (199).

Even though susceptibility to MS has a genetic component with the strongest one being HLA class II alleles (200), it comprises only ~30% of the overall risk to develop the disease (201). Therefore, a larger contributing in factor in MS etiology is thought to be the environment (202) that acts upon individuals who harbour genetic risk variants. Those factors are thought to be nutrition, smoking, vitamin D levels and infection (203). One of the originally proposed associations between Epstein-Barr virus (EBV) infection and the risk to develop MS was recently reported in a longitudinal study showing a 32-fold increase in the risk of developing MS post-EBV infection (204). Further study has elucidated the potential mechanism that explains the aforementioned association, which involves molecular mimicry between virus and self-antigens. It was identified that approximately 20% to 25% of patients with MS have pathogenic antibodies directed against the EBV nuclear antigen EBNA1 that cross-react with a self-protein - glial cell adhesion protein (GlialCAM) found in the CNS (205).

However, there is still no defined cause and cure for the disease and the exact pathological mechanisms behind MS pathogenesis remain elusive. Nonetheless, the disruption of the BBB and enhanced transendothelial migration of autoreactive lymphocytes are among the earliest events seen in MS patients' brains (206).

1.4. Animal model of MS – Experimental Autoimmune Encephalomyelitis (EAE)

MS is unique to humans. There is no spontaneous disease in any other species that would resemble MS sufficiently enough to provide translatable insight into this demyelinating disorder (207). Therefore EAE, a family of disease models, is one of the most commonly used experimental animal models to study MS (208).

The discovery of EAE goes back to 1885 when Pasteur was developing the vaccine against rabies (209). His subjects were injected with the dried spinal cord of rabies-infected rabbits. As a result, neuroparalytic accidents, causing paralysis, occasionally occurred, which were not linked to the rabies infection itself (209). Further studies using rhesus monkeys and injecting them with normal rabbit brain extracts discovered that they developed a clumsy gait and muscle weakness with their brains revealing clear perivascular infiltrates and demyelination (210). This indicated that acute encephalitis could be induced by such immunizations (210, 211). Freund's adjuvant (212) containing killed *Mycobacterium tuberculosis* and paraffin oil together with pertussis toxin (213) were later added to potentiate the immune response (214) and improve reproducibility. Since these initial studies, EAE has been induced in multiple species including mice (215), rats (216), hamsters (217), guinea pigs (218), rabbits (219), goats (220), dogs (221), sheep (222), marmosets (223), chickens (224) and even zebrafish (225).

Over time, much of the research activity moved to a mouse animal model of the disease, taking advantage of abundant transgenic and knockout models (207). EAE in mice is characterized by CNS inflammation and demyelination manifested as an ascending paralysis, starting at the tail and progressing upward along the body (226). This is due to the inflammation, which starts at the lumbar region of the spinal cord, spreading to the entire spinal cord by the peak of disease. The severity of paralysis is evaluated using clinical observations and scored on a scale of 0 to 5 (227).

EAE serves as a powerful model to study the pathogenesis and immune regulation of predominantly $CD4^+$ T-cell mediated tissue damage (228). EAE in mice can be induced by immunization with spinal cord homogenate or myelin-derived antigens such as myelin oligodendrocyte glycoprotein (MOG), myelin proteolipid protein (PLP) or myelin basic protein (MBP) (228). One of the most commonly used models is chronic EAE where C57BL/6 mice are immunized with $MOG_{35–55}$ peptide representing residues 35-55 (although MOG_{1-125} can also be used) emulsified in Freund's adjuvant with concomitant administration of pertussis toxin (229). This model is highly robust and reproducible; however, it is monophasic without relapses, the T-cell component is predominantly $CD4^+$, and the spinal cord is predominantly affected when compared to brain (207). However, to address $CD4^+$ limitations, it has been shown that MOG_{37-46} is able to induce the $CD8^+$ T-cell responses (230). Another widely used type of EAE is relapsing-remitting EAE (RR-EE). In this model, SJL/J mice are injected with $PLP_{139-151}$ emulsified in complete freund's adjuvant (CFA), optionally followed by administration of pertussis toxin. As a result, mice develop severe and demyelinating encephalomyelitis with a relapsing-remitting disease course (231). Moreover, adoptive transfer can be used to transfer encephalitogenic T-cells from donor to recipient mice, separating the induction and the effector phase in EAE (232). In addition to actively induced models of EAE described above, spontaneous EAE was developed through the generation of transgenic mice expressing myelin-specific T-cell receptors (233-235).

None of the models described above give any clues about how MS is triggered in humans. Given the fact that there is a link between EBV past exposure and MS susceptibility (204, 205), another type of model has been developed to study the contribution of viruses in human MS pathogenesis. The two most widely used viral MS models include Theiler's virus (TMEV) or

Mouse Hepatitis Virus (MHV) induced chronic encephalomyelitis (236). These models allow for the examination of the transition between immune system viral recognition to autoimmunity (236).

Additionally, to study the mechanisms involved in de- and re-myelination, another set of models have been generated and these include toxic demyelination than most often involve administration of the copper chelator cuprizone, which triggers apoptosis in oligodendrocytes (237). Usually mice are fed cuprizone (2% in chow) for 4 weeks, which induces demyelination followed by rapid and extensive remyelination (237). Other demyelination models use the injection of lysolecithin (238) or ethidium bromide (239). These models provide some insight into the processes of myelin destruction and repair (240) (237).

In summary, all animal models of MS including EAE, viral and toxic demyelination have limitations and there is no single animal model which could display multiple factors involved in the MS pathogenesis. Therefore, the degree of applicability and translatability critically depends on using fitting models that target the specific scientific question being asked.

1.5. Blood-brain barrier (BBB) in MS

The BBB is a highly specialized structure that tightly controls cellular and molecular passage from the systemic blood circulation into the CNS. The barrier is formed by brain endothelial cells coated with basement membrane together with pericytes and astrocyte end-feet facing parenchyma (241). The barrier results from an extremely low transcytotic activity (242) and an elaborate network of tight junctions (TJ) and adherens junctions (AJ) that seal the gaps between endothelial cells (243). TJ are formed by the members of the claudin, occludin and junctional adhesion molecule (JAM) families together with intracellular scaffolding proteins ZO-1, ZO-2 and ZO-3. Moreover, TJ interact with VE-cadherin/catenin complexes of AJ which contribute to the

tightness of the barrier (244). In addition to TJ and AJ, platelet endothelial adhesion molecule-1 (PECAM-1; CD31) is concentrated at cell-to-cell junctions and contributes to vascular integrity (245). TJ integrity is regulated by various factors, including post-translational modifications, alterations of TJ protein expression and localization (246). The loss of the BBB integrity occurs early on in MS and can be visualized by a leakage of the magnetic resonance imaging (MRI) contrast reagent gadolinium into the CNS (247). The integrity of the BBB is compromised by various mechanisms, including TJ abnormalities (248), increased pinocytotic activity (249), and increased activity of the TNFα transporter system (250), all leading to a massive influx of leukocytes into the CNS, leading to demyelination and axonal damage.

In a healthy state, lymphocyte migration into the CNS is extremely low and strictly controlled by the BBB. However, under inflammatory conditions such as MS, circulating lymphocytes infiltrate the CNS in large numbers and induce widespread neuro-inflammation. The process by which lymphocytes cross the BBB is complex and requires intensive interactions of primed leukocytes with endothelial cells. It is initiated by pro-inflammatory stimuli such as tumor necrosis factor alpha (TNFα), which activates the BBB causing endothelial cells to upregulate the expression of adhesion molecules including P-selectin (251), E-selectin, vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 (252). The initial contact of lymphocytes and the BBB endothelium is mediated primarily by selectins and their ligands. During the initial step, lymphocytes are captured by selectins, which cause them to slow down and begin rolling along the inner surface of the blood vessel wall. During the rolling motion, the interactions mediated by integrins (lymphocyte-associated antigen-1 (LFA-1), very late antigen (VLA-4) and their ligands VCAM-1 and ICAM-1, facilitate leukocyte arrest, polarization and crawling to sites where diapedesis into CNS is permissive (253). Once in the CNS, T-cells are reactivated and the

neuroinflammation is further augmented via B cell, macrophage, microglia, and astrocyte activation that sustains secretion of various inflammatory mediators (193).

The mechanisms underlying BBB disruption and subsequent T-cell diapedesis into CNS parenchyma is highly complex, and therefore remains elusive and not fully understood. Nonetheless, this complexity provides a large avenue to explore for potential therapeutic targets for MS.

1.6. BBB drugs to treat MS

Nearly 30 years after the US Food and Drug Administration (FDA) approved the first drug to treat early stages of MS (254), the variety of treatments for MS has significantly expanded. Currently there are 18 FDA approved drugs for the treatment of MS (255) that modify the disease course (DMT – disease-modifying treatment) and manage the symptoms. Clinical trials of the 18 approved drugs showed a reduction of the number and the frequency of relapses, and delay progression of the disability (256). Unfortunately, there are still no efficient medications for progressive forms of MS and certainly there is no cure. In general, all DMTs exert their effect by immunomodulatory properties and only a few directly target lymphocyte trafficking into the CNS (256). One such a compound is natalizumab, which is a recombinant humanized monoclonal antibody that binds to VLA-4 on the surface of activated lymphocytes. This blocks lymphocyte adhesion to VCAM-1 on the cell surface of the activated brain endothelial cells (257). The other class of drugs that affects lymphocyte migration is sphingosine 1-phosphate receptor modulators, which include fingolimod (258), siponimod (259) and ozanimod (260, 261). All of them exert their action by inhibiting lymphocyte egress from lymph nodes into the blood and reduce their migration into the CNS (262). The increase of available DMTs has made it possible for MS patients to manage their disease and enhance the quality of life. However, the efficacy is limited from person

to person and is usually coupled with a risk of mild to severe adverse effects (263). Therefore, there is a constant need for safer, more efficient, personalized drugs that not only modulate the disease course but also target neurodegeneration and ultimately prevent the disability.

1.7. Objectives and hypothesis

Hypothesis:

Calnexin/Fabp5 complex modulates T-cell migration across the blood-brain barrier

Objectives:

i. Determine if calnexin and Fabp5 form a complex *in vivo*
ii. Evaluate EAE associated neurobehavioral features in transgenic mice that express a calnexin derivative which is missing the C-terminal domain – the site for Fabp5 interaction.
iii. Determine the effect on T-cell movement across the blood-brain barrier cell model when calnexin and Fabp5 complex formation is prevented in brain endothelial cells.

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CHAPTER 2

THE FABP5/CALNEXIN COMPLEX IS A PREREQUISITE FOR SENSITIZATION OF MICE TO EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

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2.1. Abstract

We previously showed that calnexin-deficient mice are desensitized to experimental autoimmune encephalomyelitis (EAE) induction, a model that is frequently used to study inflammatory demyelinating diseases, due to increased resistance of the blood-brain barrier to immune cell transmigration. We also discovered that Fabp5, an abundant cytoplasmic lipid binding protein found in brain endothelial cells, makes protein-protein contact with the cytoplasmic C-tail domain of calnexin. Remarkably, both calnexin-deficient and Fabp5-deficient mice commonly manifest resistance to EAE induction. Here, we evaluated the importance of Fabp5/calnexin interactions on EAE pathogenesis and on the patency of a model blood-brain barrier to T-cell transcellular migration. The results demonstrate that formation of a complex comprised of Fabp5 and the C-tail domain of calnexin dictates the permeability of the model blood-brain barrier to immune cells and is also a prerequisite for EAE pathogenesis.

2.2. Introduction

Fatty acid binding proteins (Fabps) are a family of small (~15 kDa) abundant cytoplasmic proteins that are distributed in various tissues (1, 2). All Fabps bind small hydrophobic molecules, particularly long chain fatty acids, but with different ligand selectivity and binding affinity (3, 4). As lipid chaperones, Fabps facilitate the solubilization of their ligands in the cytoplasm and transport them to the various membrane-enclosed compartments within the cell, and may participate in ligand-mediated signaling by binding to and transporting their ligands to the nucleus to modulate activity of specific members of the nuclear receptor family of transcription factors. Since their discovery, several unique Fabp genes expressed with distinct tissue specificity have been identified in the mammalian genome (2, 5). Fabp5 (also called eFABP, PA-FABP) was

initially described as an abundant cytoplasmic protein in skin (6) but was later also found to be present in brain tissue (1, 7). Fabp5-deficient mice are viable with no gross and histological abnormalities in the brain (8). Remarkably, these mice show protection from the clinical symptoms of EAE (9-12).

Calnexin is type-1 endoplasmic reticulum (ER) membrane protein and molecular chaperone that coordinates the folding and quality control of newly synthesized N-linked glycoproteins (13). Calnexin has three distinct functional domains: ER luminal domain, transmembrane domain and a unique flexible C-terminal domain that extends to the cytoplasm (14). The ER luminal domain is responsible for its lectin-like chaperone function whereas the C-terminal domain facing the cytoplasm is not functionally well characterized. Although initially suggested as important in the development of the immune system, mice with global deficiency of calnexin due to targeted ablation of the calnexin gene present with normal immune function (15). Instead, calnexin-deficient mice develop peripheral myelinopathy with reduced nerve conduction velocities (15). Surprisingly, these mice also show resistance to EAE induction which is due to inability of activated T-cells to cross calnexin-deficient blood-brain barrier endothelial cells (16).

During our search for proteins that interact with the cytoplasmic C-tail domain of calnexin, we identified Fabp5 as a brain-expressed protein showing high-affinity and stable binding (17). Because of the interaction of these two proteins and considering the noteworthy phenotypic similarity of mice lacking either Fabp5 or calnexin in showing resistance to EAE, we tested the hypothesis that interaction between these two proteins is central to EAE induction in mice. In this study, we report that Fabp5 and the cytoplasmic C-tail domain of calnexin can form intimate contacts at ER membranes. Mouse bEND.3 brain endothelial cells deficient in Fabp5 or expressing a calnexin mutant containing a large deletion in its cytoplasmic C-tail domain confer a defect in

brain endothelial cell function that prevented T-cell transcellular migration across monolayers of these cells and protected mice from the clinical symptoms of EAE.

2.3. Materials and methods

2.3.1. Ethics, human CNS tissue and grading

All methods were carried out in accordance with relevant guidelines and regulations and approved by Biosafety Officers at the Department of Environment, Health and Safety at the University of Alberta. All animal experiments were carried out according to the University of Alberta Animal Policy and Welfare Committee and the Canadian Council on Animal Care Guidelines. The approval for use of mice in research was granted by the Animal Care and Use Committee for Health Sciences, a University of Alberta ethics review committee (Permit AUP297).

Post-mortem normal control or multiple sclerosis (MS) cerebral subventricular white matter samples were obtained from NeuroResource, UCL Queen Square Institute of Neurology, London (ethical approval I.D. 18/LO/0721) from the NHS London - Central Research Ethics Committee), or from the Multiple Sclerosis Society Tissue Bank (ethical approval reference number 08/MRE09/31). Approval for the study was from the Local Research Ethics Committee (I.D.04/Q2102/111). A total of 12 MS patients (7 females, 5 males) who had been affected from secondary progressive (9), primary progressive (2) or relapsing progressive (1) MS were used for this part of the study. Control tissue was used from individuals without CNS disease (4) who had died of non-inflammatory conditions (cardiac failure, bladder cancer, tongue cancer, myelodysplastic syndrome).

Lesions were classified into acute (referring to tissue phenotype, see below), sub-acute, and chronic on the basis of the number and distribution of oil red-O-positive macrophages, the extent

of demyelination, cellularity in the borders and parenchyma of lesions, and perivascular cuffing as described previously (18). Briefly, acute lesions were identified via demyelination, invading macrophages, hypercellularity at the lesion border, and cuffing around the blood vessels. Sub-acute lesions showed a demyelinated plaque with fewer macrophages, mostly at the lesion border, and less perivascular cuffing. A chronic lesion consisted of a hypocellular demyelinated plaque completely lacking oil red-O-stained macrophages.

2.3.2. Human MS tissue immunohistochemistry

Examination of MS brain tissue was performed employing enzyme immunohistochemistry using a Vectastain ABC system® and 3,3′-*diaminobenzidine* (DAB) peroxidase substrate kit with nickel (Vector Laboratories, Peterborough, UK). Sections were fixed in acetone (10 min, 4°C), washed in phosphate buffered saline (PBS; pH 7.2) and blocked with serum diluted in PBS (30 min, room temperature [RT]). The first layer primary antibody diluted in PBS was applied (45 min, RT), sections washed then incubated with secondary biotin-labelled antibody (1:500, 30 min, RT). Following another wash sections were incubated with a peroxidase labelled avidin–biotin complex (Vector; 45 min, RT). Black staining was achieved using 0.05% (w/v) 3,3'-diaminobenzidine tetrahydrochloride (DAB) with 0.04% (w/v) $NiCl_2$. The sections were dehydrated and cleared in industrial methylated spirit–xylene and mounted in DPX (BDH, Lutterworth, UK).

For immunofluorescent double staining the sections were fixed in methanol (–20°C, 10 min) and blocked with 10% goat serum in PBS for 30 min. Sections were then incubated for 45 min with a mixture of two primary antibodies appropriately diluted in PBS, washed in PBS and incubated for 30 min using a combination of secondary antibodies (1:100, Sigma). Sections were washed in PBS followed by treatment with an autofluorescence eliminator reagent as instructed

by the manufacturer (Chemicon International, Harrow, UK) to suppress lipofuscin autofluorescence. Sections were then mounted in fluorescent mounting medium (Dako).

Primary antibodies used: rabbit anti-Fabp5 (Proteintech, 12348-1-AP), mouse anti-calnexin (Thermo Fisher Scientific, MA3-027), mouse anti-PECAM-1 (Abcam, Ab24590), mouse anti-GFAP (Abcam, Ab279289), mouse anti-NeuN (Abcam, Ab104224). All dilutions were 1:100.

Secondary antibodies used: goat anti-rabbit Alexa Fluor 488 (ThermoFisher, A11034), goat anti-rabbit Alexa Fluor 647 (ThermoFisher, A21244), goat anti-mouse Alexa Fluor 647 (ThermoFisher, A21235). All antibodies were used at the 1:100 dilutions.

2.3.3. Mouse models

Generation of calnexin-deficient mice. Gene trapping with the trap vector pGT1TMpfs was used to generate the calnexin gene disrupted embryonic stem cells, designated KST286. The cell line KST286 was from the Gene Trap Resource (BayGenomics, University of San Francisco, San Francisco, California). The KST286ES cell line was generated from the 129P2 (formerly 129/Ola) embryonic stem cell line, the E14Tg2A.4 subclone. Parental cell lines (CGR8 and E14Tg2A) were established from delayed blastocysts. Embryonic stem cells were microinjected into 3.5-day-old C57BL/6J blastocysts to generate chimeric mice (8). Chimeric males were analyzed for germ line transmission by mating with C57BL/6J females, and the progeny was identified by PCR analysis.

Generation of Canx$^{-/-}$ transgenic mice reconstituted with full length recombinant calnexin (designated Tg-calnexin-FL). An expression vector designed for ubiquitous expression under the ROSA26 promoter was used to generate transgenic mice expressing full-length calnexin protein on a CD1 wild-type mouse background, designated *Canx*$^{-/-}$-TG-calnexin-FL. Mice were bred by crossing CD1 WT mice expressing full-length calnexin transgene with WT C57BL/6 mice. To generate calnexin rescue mice, WT CD1/C57BL6 mice expressing the transgene were crossed with

calnexin-deficient mice, and the heterozygote progeny with the transgene were bred again with calnexin-deficient mice, producing animals with the transgene on a calnexin-deficient background. Transgene expression was determined by PCR using the following set of DNA primers:

First set: forward, 5′-GGAGGAGCGCTTCCGGCCGACG-3′;

reverse, 5′-CCACCCTGACAGAGACCCTCTGTC-3′;

Second set: forward, 5′-CGGAGAATGGGAGGCTCCTCAGATT-3′;

reverse, 5′-GCAAGAAAGCGAGCTTCTAGATGGCC-3′.

Generation of Canx$^{-/-}$ transgenic mice expressing cytoplasmic C-tail truncated form of calnexin protein (designated Tg-calnexin-ΔC). An expression vector designed for ubiquitous expression under the ROSA26 promoter was used to generate *Canx*$^{-/-}$ transgenic mice expressing cytoplasmic C-tail truncated form of calnexin. The vector encoded amino acids 1-505 of the full-length calnexin corresponding to ER lumenal and transmembrane domains of the protein followed by 10 amino acids C-terminal extension containing the ER retention signal (Fig. 2-2A). Mice were crossed with transgenic CD1 mice expressing a transgene encoding calnexin-ΔC with wild-type C57BL/6 mice and the offspring were bred with *Canx*$^{-/-}$ mice. Selected progeny of this cross (i.e., Tg-calnexin-ΔC$^{+/0}$; *Canx*$^{+/-}$ mice) were crossed again with *Canx*$^{-/-}$ mice to produce mice that carried the transgene encoding calnexin-ΔC in the calnexin-deficient background (i.e., Tg-calnexin-ΔC$^{+/0}$;*Canx*$^{-/-}$ mice). Transgene expression was determined by PCR using the following set of DNA primers:

First set: forward: 5’-GGAGGAGCGCTTCCGGCCGACG-3';

reverse: 5’-CCACCCTGACAGAGACCCTCTGTC-3'

Second set: forward: 5’-CGGAGAATGGGAGGCTCCTCAGAT T-3';

reverse: 5’-GCAAGAAAGCGAGCTTCTAGATGGCC-3'.

2.3.4. Multifiber motor and sensory conduction

Multifiber motor and sensory conduction studies were carried out in mice briefly anesthetized with isoflurane. In brief, sciatic-tibial motor fibers were supramaximally stimulated at the sciatic notch and knee, and a compound muscle action potential was recorded (base line-peak amplitude) from the motor end plate of tibial innervated dorsal interosseous foot muscles. Motor conduction velocity was calculated for the notch to knee segment. For sensory conduction, digital hind paw nerves were supramaximally stimulated, and the sciatic-tibial sensory nerve action potential (base line-peak amplitude) was recorded from the knee after averaging (5–10 times). Stimulation and recording were carried out using E2 subdermal platinum electrodes (Grass/Astromed). All of the recordings were carried out with near nerve temperatures maintained at 37.0 ± 0.5°C. This work was carried out in collaboration with Dr. D. Zochodne's laboratory.

2.3.5. EAE induction

Adult mice were subcutaneously injected with an emulsion of MOG_{35-55} peptide emulsified in complete Freund's adjuvant, followed by the intraperitoneal injection of pertussis toxin on Day 0 and Day 1. Control animals were immunized with Complete Freund's adjuvant (CFA) and PBS (1:1) and pertussis toxin, as above. All the reagents came from the EAE induction kit procured from Hooke Laboratories (Cat. No. EK-0114) (16). Disease score was monitored every day for up to 28 days. A scoring system from 0–5 for clinical assessment of EAE symptoms was used: 0, no sign of disease; 1, loss of tone in the tail; 2, partial hind limb paralysis; 3, complete hind limb paralysis; 4, front limb paralysis; and 5, moribund https://hookelabs.com/protocols/eaeAI_C57BL6.html#Introduction (16, 19). Disease incidence and disease onset day were recorded. Time course graphs represent daily mean maximal scores.

2.3.6. Immunoblot blot analysis

For immunoblot analysis cell or mice tissue lysates were prepared using RIPA lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.5 % Nonidet P-40, 1 % deoxycholate, 0.1 % sodium dodecyl sulfate and protease inhibitors (cOmplete™, Mini Protease Inhibitor Cocktail, Roche, 11836153001). Primary antibodies used as follows: goat anti-Fabp5 (1:1000) (R&D Systems, AF1476), rabbit anti-calnexin (1:1000) (Enzo Life Sciences, ADI-SPA-860-F), rabbit anti-calnexin (1:1000) (Enzo Life Sciences, ADI-SPA-865-F), mouse anti-γ-tubulin (1:1000) (Thermo Fisher Scientific, MA1-850). Secondary antibodies were used: Goat anti-Rabbit IgG (H+L) HRP conjugated (1:3000) (Thermo Fisher Scientific, 31460), Rabbit anti-Goat IgG (H+L) HRP conjugated (1:3000) (Thermo Fisher Scientific, 31402), Goat anti-Mouse IgG (H+L) HRP conjugated (1:3000) (Thermo Fisher Scientific, 31430).

2.3.7. Hematoxylin and Eosin (H&E) staining

Mice were euthanized with CO_2, then perfused with PBS followed by spinal cord fixation with 10% formalin neutral buffer solution, pH 7.4. Following fixation, the wet fixed tissues were dehydrated using a graded ethanol series (from 70% to 100%). After dehydration, the tissues were processed with clearing agent xylene and impregnated with paraffin wax. After infiltration, the tissues were embedded into a paraffin wax block to enable sectioning on a microtome. The paraffin-embedded tissues were sectioned into 5-µm thickness using a microtome. Following sectioning, the tissue samples were placed on glass slides. Next, the tissue slides were deparaffinized using absolute xylene (100%), then rehydrated with absolute ethanol, gradient ethanolic concentrations (70–95%), and, finally, with distilled water. (20). Following that, H&E staining kit (Abcam, ab245880) was used to stain the spinal cord section as per the manufacturer's instructions.

2.3.8. Cell culture and generation of the *Fabp5*$^{-/-}$ bEND.3 cell line

The bEND.3 cell line was purchased from ATCC (ATCC CRL-2299) and cultured under in DMEM media supplemented with 10% (vol/vol) FBS at 37ºC and 5% CO_2 Disruption of the *Fabp5* gene in the bEND.3 cell line was achieved gene editing using two paired sgRNAs designed to excise a large part of the gene. sgRNAs flanking Exon 1 in *Fabp5* gene (Figure 6) were identified using the web-based tool: https://crispr.mit.edu/ : sgRNA1 5'-CACAACTCCGGGCGTGGCGA-3'; sgRNA2 5'-TTTATCGCCGAACCCCTCCC-3'. The sgRNA coding vector was created by ligating the sgRNA sequences into the SpCas9(BB)-2A-Puro (Px459) V2.0 plasmid [a gift from Feng Zhang (Addgene Plasmid #62988)] as previously described (16). The assembled plasmid was transfected into wild-type bEND.3 cells using TurboFect Transfection reagent (Thermo Fisher Scientific) following the manufacturer's recommended protocol designed for adherent cells. Individual clones were picked and assayed by both PCR and immunoblot analysis, to confirm successful inactivation of the *Fabp5* gene. For PCR analysis, genomic DNA from candidate clones were amplified using primers outside of the sgRNAs binding sites: F: 5'-CGCAGGCTGTCTTCAGATG -3'; R: 5'-GAAGGGCCGGAAGCAATG -3'. A PCR product amplified from transfected bEND.3 cells lacking 251 bp represented successful editing of the *Fabp5* gene using sgRNA pair. To confirm the deletion, the amplicon was sequenced to reveal the exact sites of the deletion (Figure 6). Immunoblot analysis was carried out with anti-mFabp5 antibodies (R&D Systems, AF1476) to verify the absence of the Fabp5 protein.

2.3.9. Bimolecular Fluorescence Complementation (BiFC) Analysis

YN-CNX expression vector encoding YN (amino acid 1–154 of YFP) appended to the calnexin C-terminal cytoplasmic domain (21) and YC-Fabp5 expression vector encoding YC

(amino acid 155–238 of YFP) appended to the N-terminus of Fabp5 were used. To generate YN-CNX plasmid the following primers were used: YN forward primer 5'-TATAAC CGGTCATGGTGAGCAAGG GCCAACGTCTATATCATGGCC-3' with AgeI restriction site and reverse primer 5'-TATATG TACAAGGCCATGATATAGACGTTG-3' with BsrGI restriction site. This allowed exchange of full-length EGFP in pEGFP-CNX with the YN fragments to generate YN-CNX plasmids. To generate YC-Fabp5 plasmid the following primers were used: YC forward primer 5-TATAACCGGTATGGACAAGCAGAAGAACGGC-3 with AgeI restriction site and reverse primer 5-TATATGTACAACTTGTACAGCTCGTCCATG-3 with BsrGI restriction site. This allowed the exchange of full-length EGFP in pEGFP-C1-FABP5 with the YC fragment to generate YC-FABP5 plasmid. 4,6-diamidino-2-phenylindole (DAPI) was used to stain the nucleus for fluorescence microscopy.

2.3.10. Trans-endothelial migration analysis

T-cell migration across endothelial bEND.3 monolayers was carried out as described previously (16). Briefly, wild-type or genetically modified bEND.3 cells were plated onto 12 well plates to form an intact monolayer. The endothelial cell monolayer was treated for 4 h with IL1ß (10 ng/ml, eBioscience) or 16 h with TNFα (3 ng/ml) (Invitrogen) and IFNɣ (60 ng/ml, eBioscience). Primary cultures of wild-type mouse splenocytes were isolated and activated with plate-bound CD3 antibodies (5 µg/ml, eBioscience) and soluble CD28 antibodies (2 µg/ml, eBioscience). T-cells were labelled using carboxyfluorescein succinimidyl ester (Invitrogen) according to manufacturer's protocol. The labelled cell suspension was added to the monolayer of bEND.3 cells. After a 3 h incubation period, the culture medium was removed from the lower well, and cells were collected, washed 3 times with PBS, with cells from the lower well collected and fluorescently-labelled leucocytes counted using a flow cytometer.

2.3.11. Real-time PCR

Total RNA from wild-type, *Canx*$^{-/-}$, and *Canx*$^{-/-}$;Tg-calnexin-ΔC mice tissue was isolated using Qiagen RNeasy mini kit (Qiagen, 74104) according to the manufacturer's instructions. A Rotor-Gene RG-3000 (Corbett Research) and iQ SYBR Green Supermix (Bio-Rad) were used for real-time PCR experiments. Five hundred ng RNA was used for reverse transcription by using iScript cDNA synthesis kit (Bio-Rad, 1708891), 20 ng cDNA was mixed with iQ SYBR Green Supermix (Bio-Rad, 170-8882) for real-time PCR reaction. The final quantitation of the amount of target (Ct value) in a real-time PCR reaction was converted to the amount of transcript and normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH). PCR primers used in this study were as follow:

calnexin: forward 5'-CAAGAGGGATGAAGAGGAAGAAG-3'

reverse 5'-CTCTGCTTTAGGCTTGCTATCT-3'

CD3 ε: forward 5'-TCTCGGAAGTCGAGGACAGT-3'

reverse 5'-TTGAGGCTGGTGTGTAGCAG-3'

CD69: forward 5'-CCCTTGGGCTGTGTTAATAGTG-3'

reverse 5'-AACTTCTCGTACAAGCCTGGG-3'

GAPDH: forward 5'-TTCACCACCATGGAGAAGGC-3'

reverse 5'-GGCATGGACTGTGGTCATGA-3'

2.3.12. Statistical analysis

Statistical analysis was performed using GraphPad Software v7. For flow cytometry analysis the normality of data distribution was determined using the Kolmogorov-Smirnov test. The differences between groups were determined by one-way ANOVA test and Tukey post-hoc test. For the rest of experiments requiring statistical analysis Student's t-tests were performed.

Differences were deemed statistically significant when $P < 0.05$.

2.4. Results

2.4.1. Fabp5 and calnexin interact at the ER membrane

Using a yeast two-hybrid screen, immunoprecipitation, microscale thermophoresis and surface plasmon resonance techniques we previously showed that Fabp5, a cytoplasmic lipid chaperone, forms a complex with calnexin via association with the cytoplasmic C-tail of calnexin (17). To establish if these proteins make contact in cells, we determined the sublocalization of Fabp5 and calnexin in the brain endothelial cell line bEND.3 using immunolocalization techniques. Immunofluorescence analysis of bEND.3 cells stained with anti-Fabp5 and anti-calnexin antibodies showed that the two proteins co-localized to ER-like network (Fig. 2-1A). Next, we used bimolecular fluorescence complementation (BiFC) analysis (22) to examine the interactions between Fabp5 and calnexin *in situ*. We co-expressed in bEND.3 cells a version of calnexin (CNX-YC) with the N-terminal fragment of YFP fused to the C-terminal end of calnexin (21) and a version of Fabp5 (YC-Fabp5) with the C-terminal fragment of YFP fused to the N-terminal end of Fabp5. *In situ* fluorescence analysis of bEND.3 cells expressing both fusion proteins showed robust BiFC signal (Fig. 2-1B), indicative of intimate interaction between Fabp5 and the cytoplasmic C-tail domain of calnexin in brain endothelial cells.

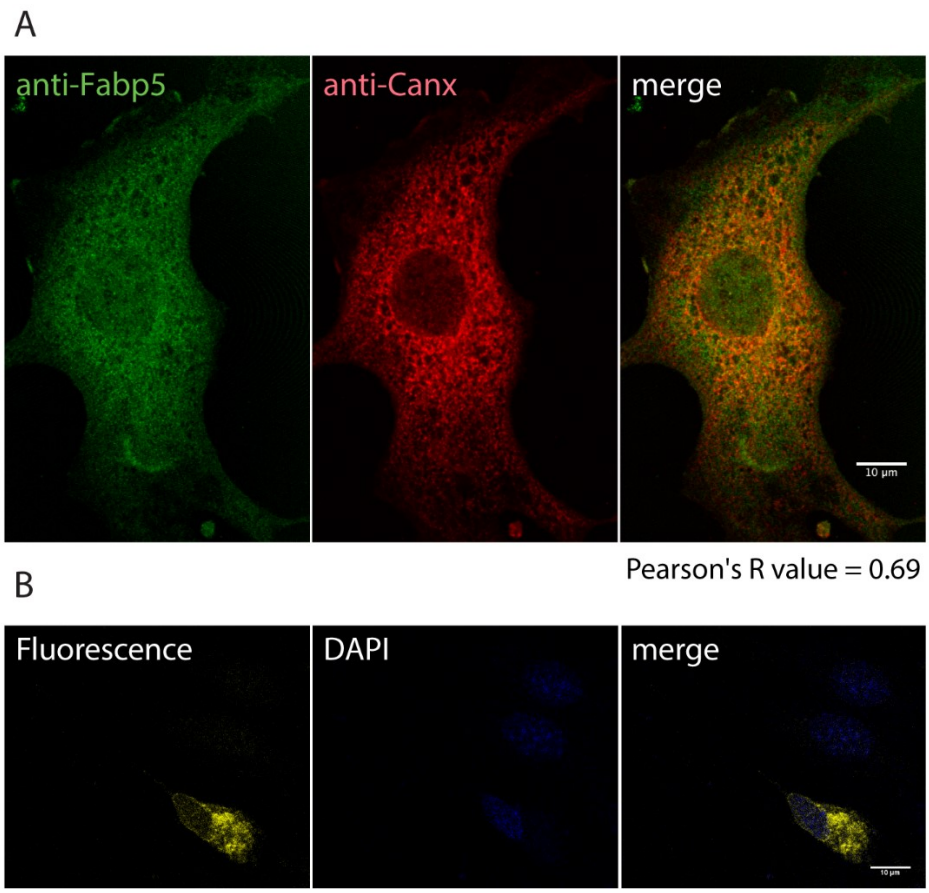

Figure 2-1. Calnexin and Fabp5 in brain endothelial cells

A. Pseudo-colored confocal images of bEND.3 cells stained with anti-Fabp5 (green) or anti-calnexin (red) antibodies. The yellow signal seen in the merged image represent colocalization of Fabp5 and calnexin. Canx, calnexin. (for visual simplicity calnexin abbreviation 'Canx' is used in the figures)

B. BiFC analysis of protein-protein interactions between Fabp5 and the cytoplasmic C-tail domain of calnexin in bEND.3 cells. The N-terminal half of the YFP protein was fused with the C-terminal portion of calnexin (YN-CNX) and the C-terminal half was fused with N-terminal portion of Fabp5 (YC-Fabp5). The yellow fluorescent signal is generated only as a result of direct interactions between the tagged proteins.

2.4.2. Mice deficient in calnexin C-terminal cytoplasmic domain are resistant to induction of EAE

We showed previously that calnexin-deficient mice (*Canx*$^{-/-}$), like Fabp5-deficient mice, are resistant to EAE induction and susceptibility of *Canx*$^{-/-}$ mice to EAE is restored in transgenic calnexin-FL mice expressing a transgene encoding the full-length calnexin protein (16). To evaluate the role of the cytoplasmic C-tail domain of calnexin in conferring sensitivity to EAE induction, we constructed a new derivative of *Canx*$^{-/-}$ mice that expresses a version of the membrane embedded calnexin protein (encoded by the Tg-calnexin-ΔC transgene) that lacks the cytoplasmic C-tail domain (Fig. 2-2A). Immunoblot analysis of cells isolated from the Tg-calnexin-ΔC mice showed that the abundance of calnexin-ΔC protein and its cognate mRNA was similar to the unmodified calnexin protein and its mRNA in wild-type mice (Fig. 2-2B,C). Furthermore, Tg-calnexin-ΔC mice exhibited normal motor function phenotype with respect to conduction velocity of peripheral motor and sensory axons (Fig. 2-3), indicating that only the ER luminal chaperone domain of calnexin was necessary and sufficient to restore the defective motor function exhibited by the parental *Canx*$^{-/-}$ mice (15)

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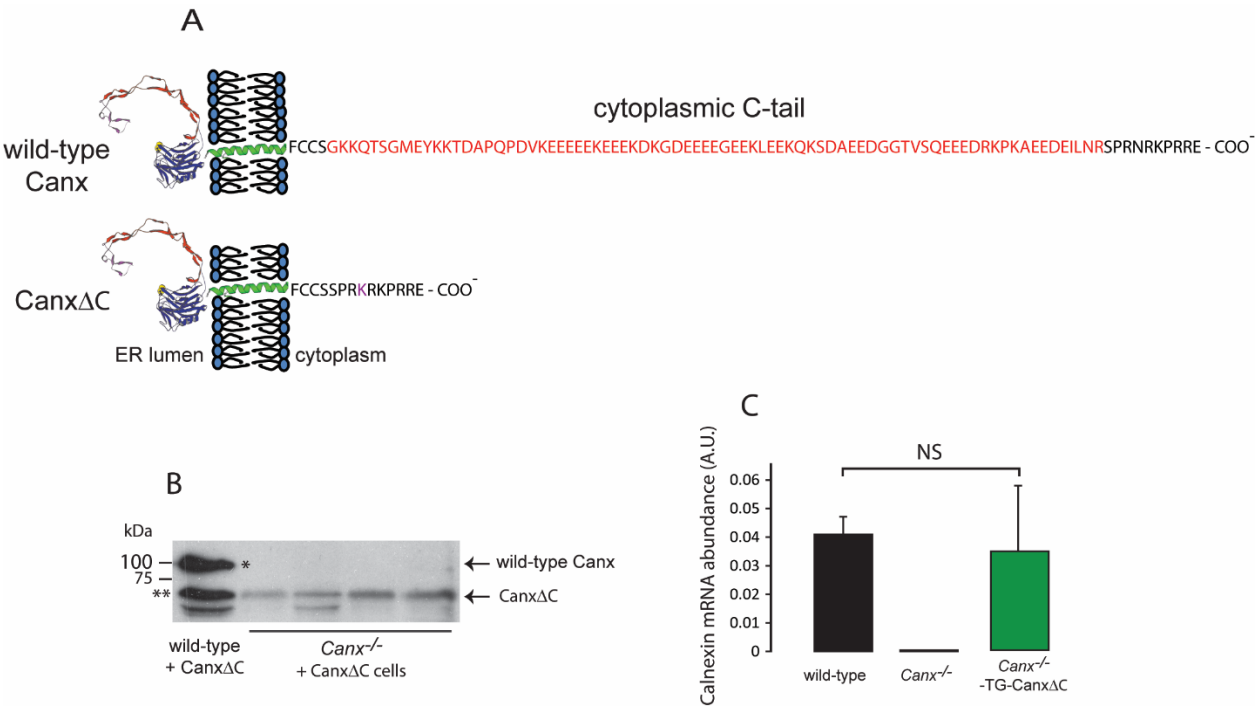

Figure 2-2. Transgenic *canx*$^{-/-}$ mice expressing truncated (ΔC) calnexin protein

A. A schematic representation of wild-type full-length calnexin protein (wild-type calnexin) and cytoplasmic C-tail deletion mutant of the truncated calnexin protein (calnexin-ΔC). Structure of the calnexin ER luminal domain is based on Protein Data Bank ID 1JHN (23) The construction of the calnexin-ΔC deletion mutant also resulted in the substitution of a basic amino acid residue (N) with another basic amino acid residue (K, shown in purple) in the remaining portion of the cytoplasmic C-tail domain of calnexin.

B. Immunoblot analysis of fibroblasts isolated from replicate calnexin-deficient mice expressing the calnexin-ΔC protein. Wild-type 293T cells transfected with the calnexin-ΔC expression vector were used as a positive control (wild-type+calnexinΔC). The locations of full-length calnexin protein (single asterisk) and truncated calnexin protein (calnexin-ΔC) (double asterisks) were visualized by immunoblotting with anti-calnexin antibodies.

C. Abundance of calnexin mRNA in fibroblasts obtained from wild-type mice, *Canx*$^{-/-}$ mice and *Canx*$^{-/-}$-Tg-calnexin-ΔC mice. Data are representative of three biological replicates. Statistical analysis by unpaired Student's t-test.

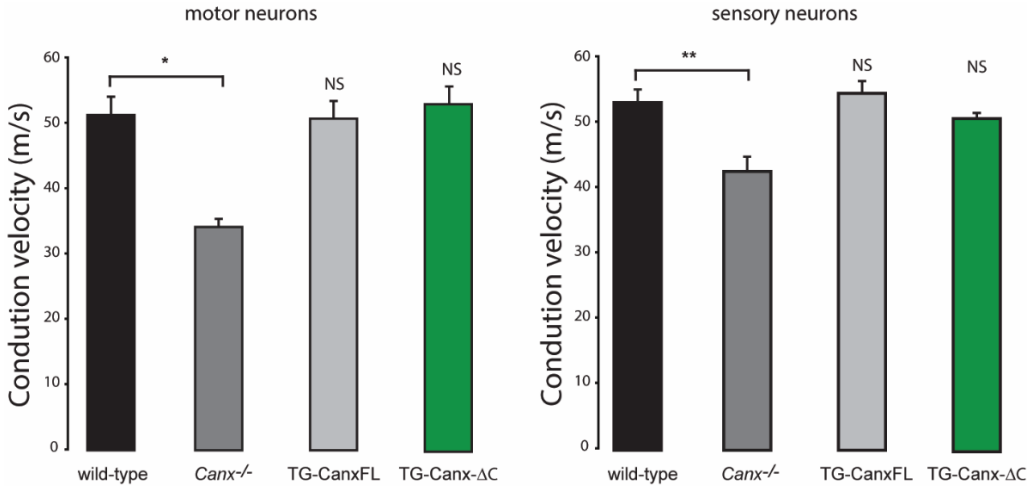

Figure 2-3. Nerve conduction velocity

Conduction velocity of motor (*left graph*) or sensory (*right graph*) neurons in wild-type, *canx*$^{-/-}$, Tg-calnexin-FL and Tg-calnexin-ΔC mice. **P*=0.013; ***P*=0.005; n=6. NS, not significant. Statistical analysis using unpaired Student's *t*-test. This work was carried out in collaboration with Dr. D. Zochodne's laboratory.

Next, we induced EAE in wild-type, *Canx*$^{-/-}$, *Canx*$^{-/-}$ mice expressing full-length calnexin protein (Tg-calnexin-FL) and Tg-calnexin-ΔC mice by inoculating the myelin oligodendrocyte glycoprotein MOG_{35-55} peptide. Consistent with what we reported previously (16), within the first two weeks of MOG_{35-55} injection both wild-type mice (16) and Tg-calnexin-FL mice (Fig. 2-4 and reference (16) developed neurobehavioral deficits typically associated with EAE induction. In contrast, Tg-calnexin-ΔC mice exhibited substantial resistance to EAE throughout the disease course (23 days) (Fig. 2-4) reminiscent of EAE resistance seen in *Canx*$^{-/-}$ mice (16). Consistent with these neurobehavioral features, histologic evaluation of CNS tissue at 14 days after immunization revealed only minimal infiltration of leukocytes/mononucleated cells into the white matter of the spinal cord in Tg-calnexin-ΔC mice while infiltration was readily apparent in wild-type littermates and Tg-calnexin-FL mice (Fig. 2-5A). In addition, the abundance of T-cell receptor CD3ε and CD69 mRNA, which are key early activation markers of inflammation, were

dramatically increased in the spinal cords of both wild-type relative to the *Canx*$^{-/-}$ and Tg-calnexin-ΔC mice (Fig. 2-5B). These results illustrated that the cytoplasmic C-tail domain of calnexin was a crucial component of the mechanism that determines the susceptibility of wild-type mice to EAE induction

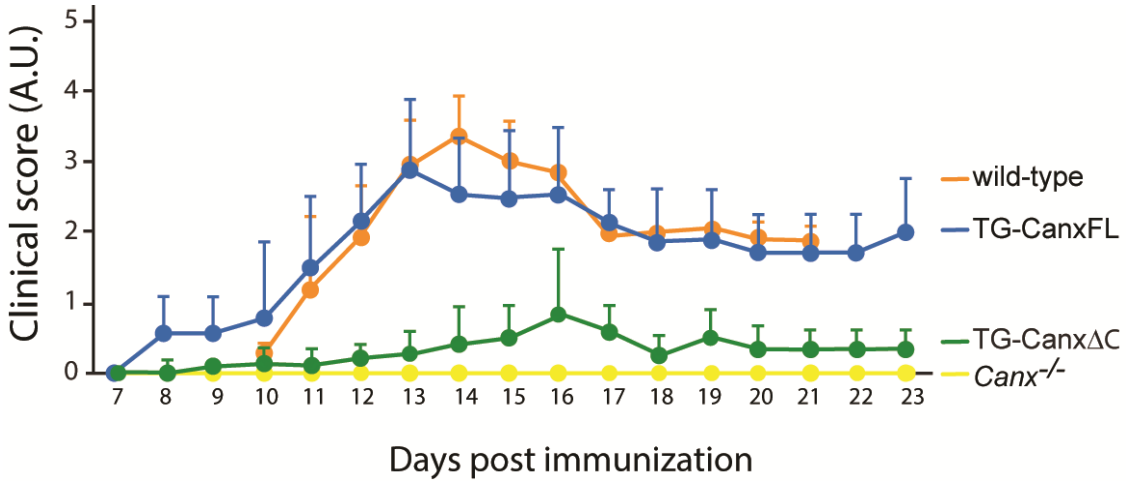

Figure 2-4. Induction of EAE in wild-type, calnexin-deficient, and calnexin-reconstituted mice

Time course of EAE induction. EAE was induced by MOG_{35-35} immunization of *Canx*$^{-/-}$ mice (yellow curve), and Tg-calnexin-FL mice (blue curve) (16) and Tg-calnexin-ΔC mice (green curve). Data presented are mean ± SD of 3 independent experiments (minimum of 5 mice per group). Statistical analysis using unpaired Student's *t*-test.

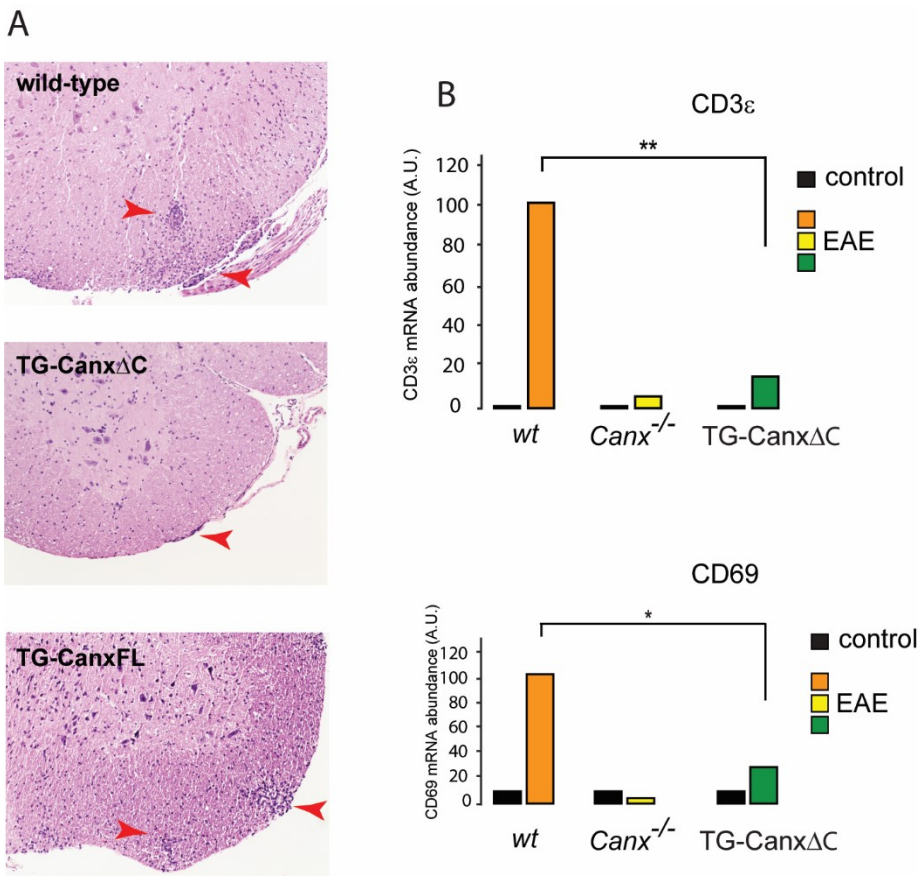

Figure 2-5. Histological and qPCR analysis of lumbar spinal cord from wild-type, calnexin-deficient, and calnexin-reconstituted mice

A. H&E staining of lumbar spinal cord sections of MOG_{35-35} immunized wild-type, *canx*$^{-/-}$, Tg-calnexin-ΔC and Tg-calnexin-FL mice.

B. Inflammatory response within central nervous system assessed by quantitative PCR of inflammation markers in spinal cord tissue of wild-type, *Canx*$^{-/-}$ and Tg-calnexin-ΔC mice. $^{**}P \leq 0.001$. Data presented are mean ± SD of three independent experiments, three mice per group. The means were compared using unpaired Student's t-test.

2.4.3. Loss of Fabp5 from brain endothelial cells arrests transcellular migration of T-cells

Using a monolayer of mouse brain endothelial cell line bEND.3 (24, 25) as a model of the blood brain barrier (25), we showed previously that the loss of calnexin from these cells prevents pro-inflammatory T-cells from traversing a monolayer of bEND.3 cells (16). This finding explains, in part, the protection of *Canx*$^{-/-}$ mice against EAE pathogenesis (16). Since Fabp5 and the calnexin cytoplasmic C-tail domain interact at the ER membrane, and mice lacking Fabp5 or calnexin show resistance to EAE induction (16, 26, 27), we examined whether Fabp5 deficiency affects T-cell transmigration across bEND.3 cells monolayer. First, we used an *in situ* gene editing technique (Fig. 2-6) to generate Fabp5-deficient bEND.3 cells (Fig. 2-7A). Next, we used lentiviral transduction method to reconstitute calnexin-deficient bEND.3 cells with full length calnexin (Tg-calnexin-FL) or with a cytoplasmic C-tail deletion mutant of calnexin (Tg-calnexin-ΔC). These cells were then used to create a monolayer of brain endothelial cells for T-cell trans-endothelial migration assay. As expected, cytokine-stimulated wild-type bEND.3 cells permitted trans-endothelial migration of T-cells (Fig. 2-7B). Fabp5-deficient bEND.3 cells arrested T-cell transmigration, similarly to that observed with calnexin-deficient bEND.3 cells (Fig. 2-7B). Restoration of full length calnexin, to calnexin-deficient bEND.3 cells by transgene reconstitution re-enables T-cell transmigration whereas calnexin lacking an intact cytoplasmic C-tail domain could not (Fig. 2-7B). These results demonstrated that Fabp5, a calnexin cytoplasmic C-tail domain binding partners, and calnexin C-tail domain were both necessary for the permeability of blood-brain barrier to the activated T-cells.

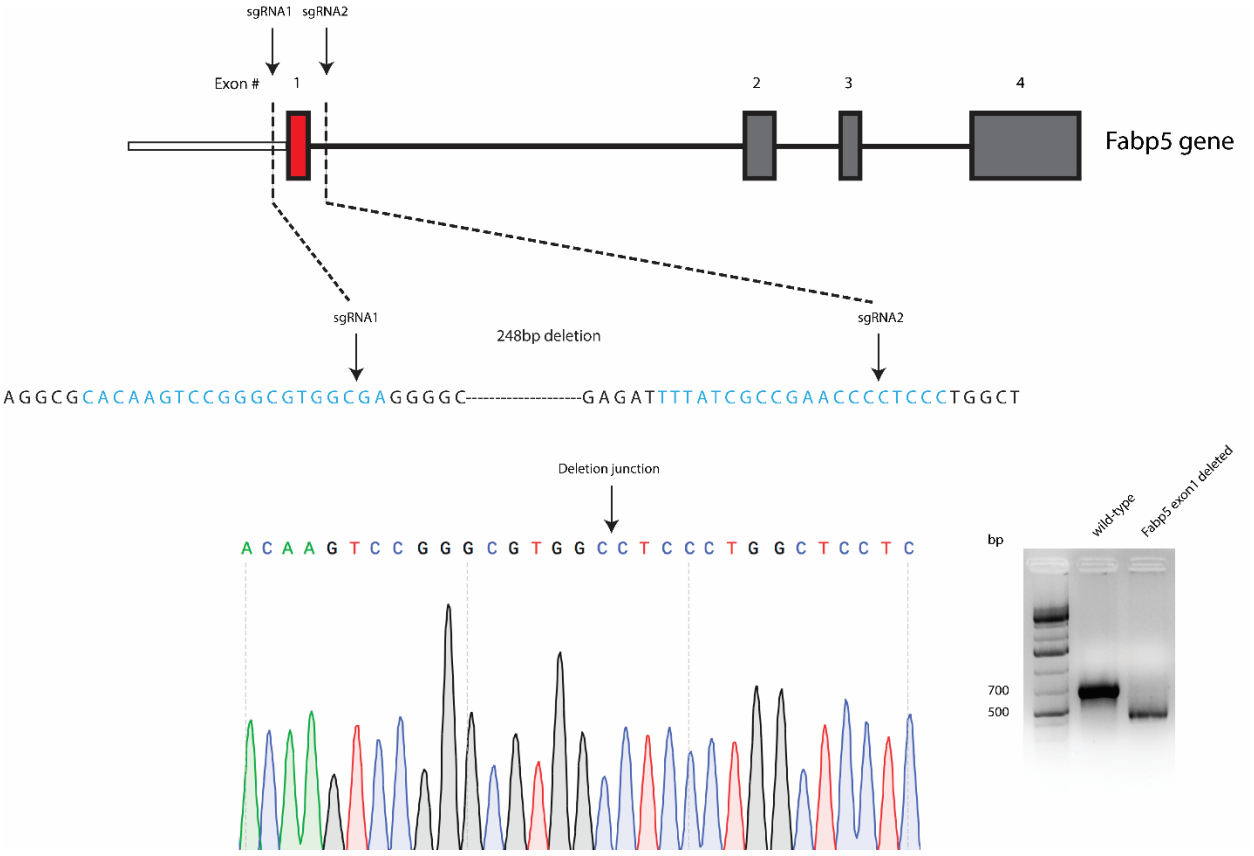

Figure 2-6. Inactivation of the Fabp5 gene in murine brain endothelial bEND.3 cells using CRISPR/Cas9-mediated in situ gene editing

Two paired sgRNAs flanking Exon 1 was designed to excise a large part of Fabp5 gene. This resulted in 251 bp deletion which was sequenced to confirm.

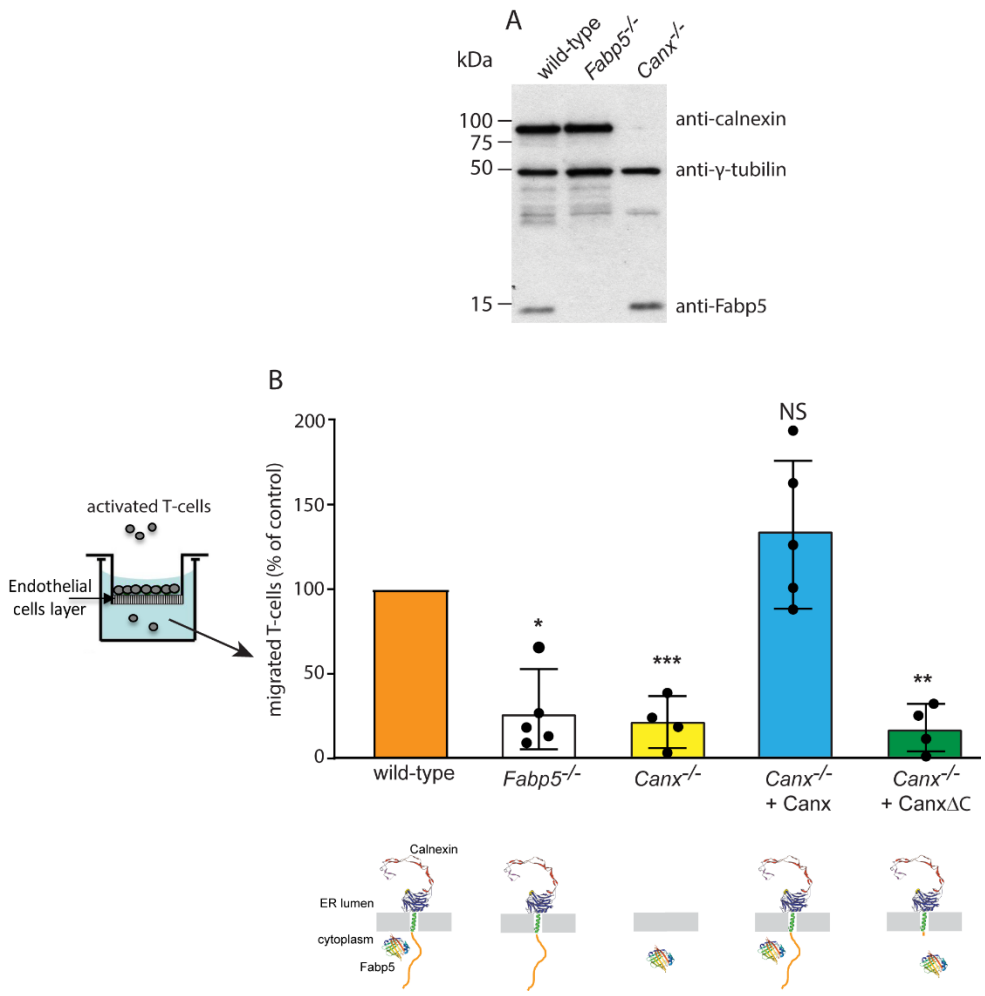

Figure 2-7. Trans-endothelial migration of activated T-cells

A. Immunoblot analysis of wild-type, Fabp5-deficient (*Fabp5*$^{-/-}$), calnexin-deficient (*Canx*$^{-/-}$) bEND.3 cells probed simultaneously with anti-calnexin, anti-Fabp5 and anti γ-tubulin antibodies.

B. Transmigration of activated wild-type splenocytes across wild-type, *Canx*$^{-/-}$, *Fabp5*$^{-/-}$, *Canx*$^{-/-}$ bEND.3 reconstituted with full-length calnexin or C-terminal deletion (calnexin-ΔC) calnexin mutant. *$P \leq 0.004$; **$P \leq 0.001$; ***$P \leq 0.002$. NS, no significant. Data presented are mean ± SD of three independent experiments with three replicates. The means were compared using unpaired Student's t-test. NS, not significant.

2.4.4. Fabp5 is upregulated in CNS samples from EAE mice and multiple sclerosis patients

Immunoblot analysis of spinal cord tissue isolated from wild-type mice with induced EAE showed increased abundance of Fabp5 (Fig. 2-8A). We previously reported that multiple sclerosis (MS) patients have an unusually high abundance of calnexin in CNS endothelial cells (16). Immunohistochemical staining of control non-MS and MS white matter for Fabp5 indicated high abundance in brain capillaries, neurons, and various glial cells of brain tissue sections of MS patients. Compared to non-MS white matter samples, diaminobenzidine (DAB)-nickel immunostaining with anti-Fabp5 antibody showed strong staining of capillary walls, and cells with the enlarged ramified morphology of reactive astrocytes in MS tissues (Fig. 2-8B).

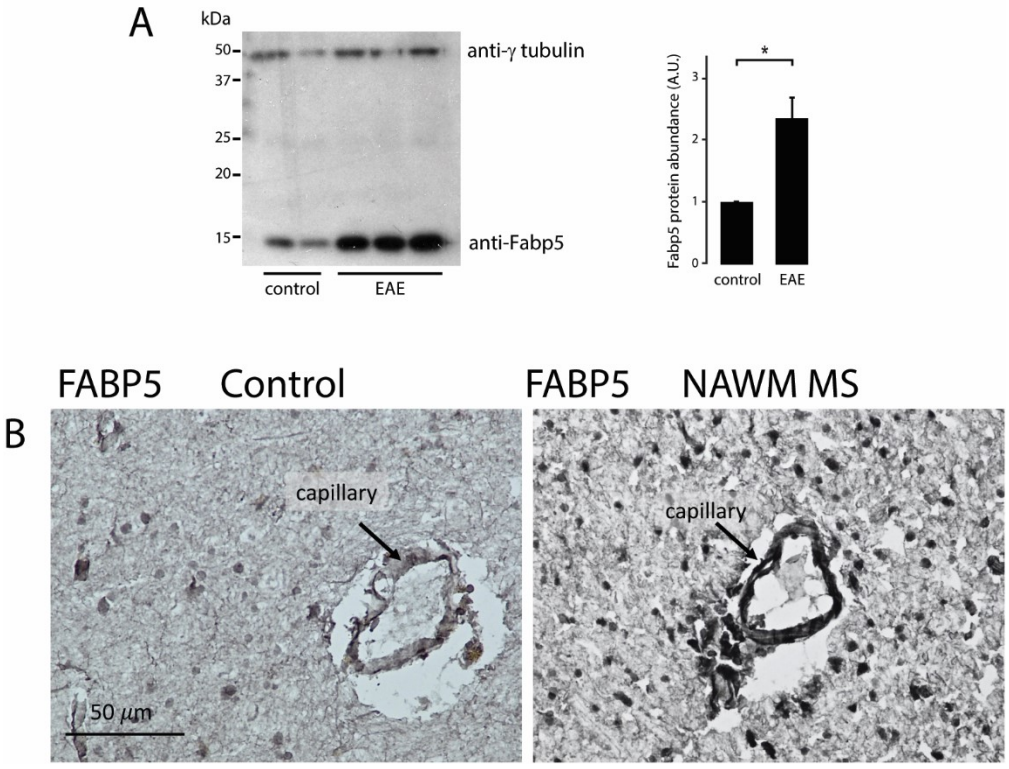

Figure 2-8. Distribution and abundance of Fabp5 protein in the spinal cord of EAE mice and brain capillaries of MS patients

A. The left panel shows an immunoblot of spinal cord tissue isolated from control and EAE mice probed with anti-Fabp5 antibodies and γ-tubulin antibodies. The right panel depicts the quantitative abundance of Fabp5 in control and EAE spinal cord tissue, **P*≤0.004; n=3.

B. DAB-nickel staining for FABP5 in non-MS control (left panel), and MS NAWM brain tissue; 20X magnification. Experiments presented in the Figure 2-8B were carried out with assistance from our collaborators at the University of Exeter.

Next, we performed a series of immunofluorescent double staining for FABP5 and cellular biomarkers for endothelial (PECAM), astrocytes (GFAP) or neurons (NeuN). Brain microvascular endothelial cells identified by PECAM-1 (CD31) showed high abundance of FABP5 in MS normal-appearing white matter (NAWM) relative to non-MS white matter (Fig.2-9A,B). FABP5 was enriched within acute lesion areas compared to NAWM within the same tissue sections.

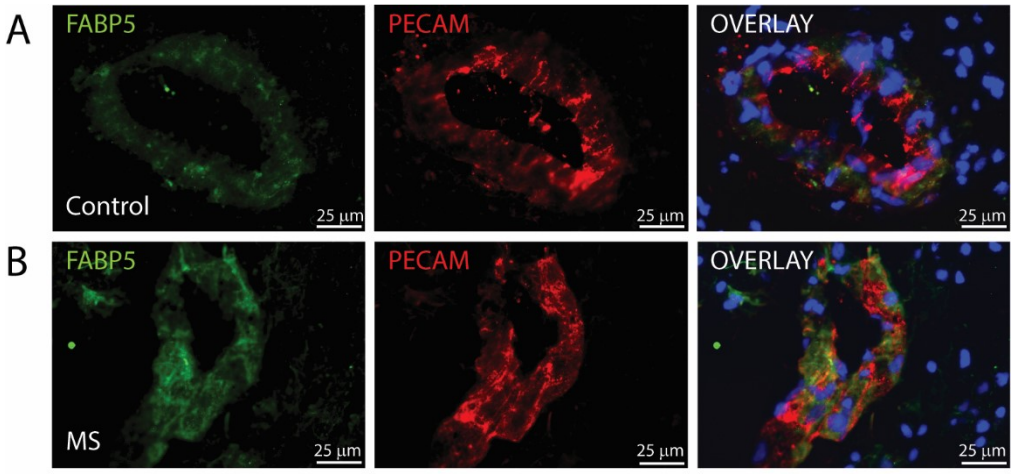

Figure 2-9. Distribution of FABP5 and PECAM protein in MS patients' brain tissue

Immuno-fluorescence staining for Fabp5 (green) in the PECAM positive (red) blood vessels of non-MS individual **(A)** and NAWM for an MS patient **(B)**. Overlay images of Fabp5 and PECAM and DAPI (blue) in non-MS and MS tissue are shown, respectively; 40X magnification. Data presented in the Figure were obtained in collaboration with the University of Exeter.

Figures 2-10A,B show increased calnexin abundance in MS NAWM. We detected FABP5^{+ve} activated astrocytes using double staining for the marker GFAP in acute MS lesions (Fig. 2-10C).

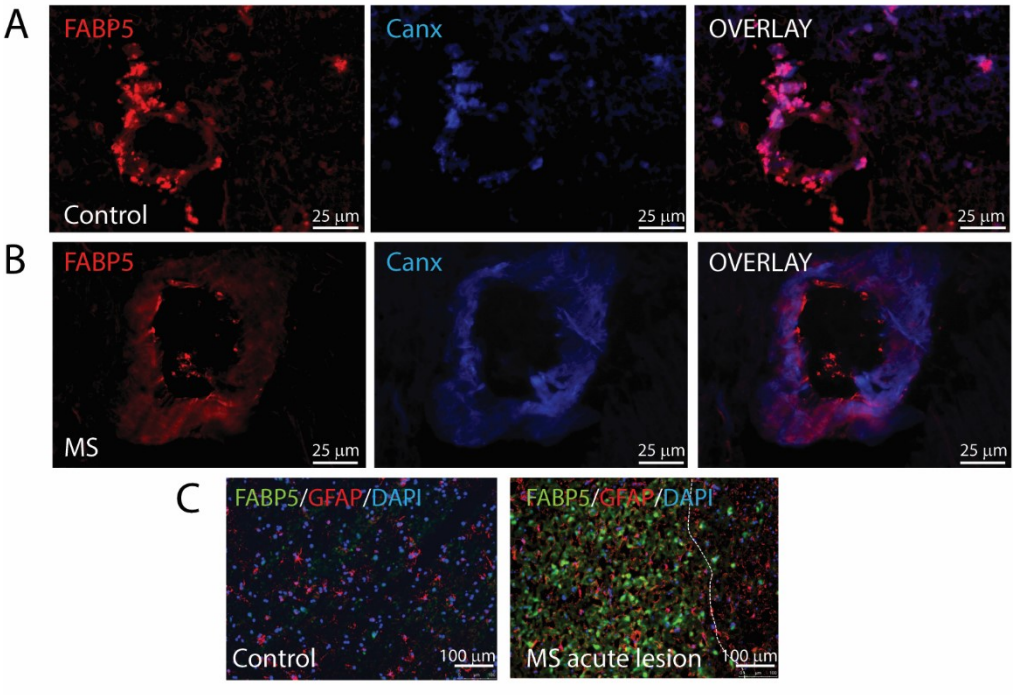

Figure 2-10. Distribution of FABP5, calnexin and GFAP in MS patients' brain tissue

Immunofluorescence staining for FABP5 (red) and calnexin (blue) in the blood vessels of (**A**) non-MS individual and (**B**) MS NAWM. 40X mag. **C.** GFAP+VE astrocytes (red) in non-MS control and MS acute lesion were co-stained for FABP5 (green) and DAPI (blue). Note abundant staining of FABP5 in astrocyte-rich acute lesion in a MS tissue section demarcated by a dotted white line. Data presented in the Figure were obtained in collaboration with the University of Exeter.

By DAB-nickel immunostaining with anti-Fabp5 antibodies we also identified neuron cell bodies at the grey matter/white matter border of biopsy material (Fig 2-11A). In both non-MS control and MS brain tissues with double-label immunofluorescence staining we observed neuron cell bodies co-stained with FABP5 but there was a greater abundance of FABP5 in the cell bodies of neurons in MS patient samples (Fig. 2-11B,C). Based on these findings, we concluded that both FABP5 and calnexin were upregulated in brain tissue of MS patients.

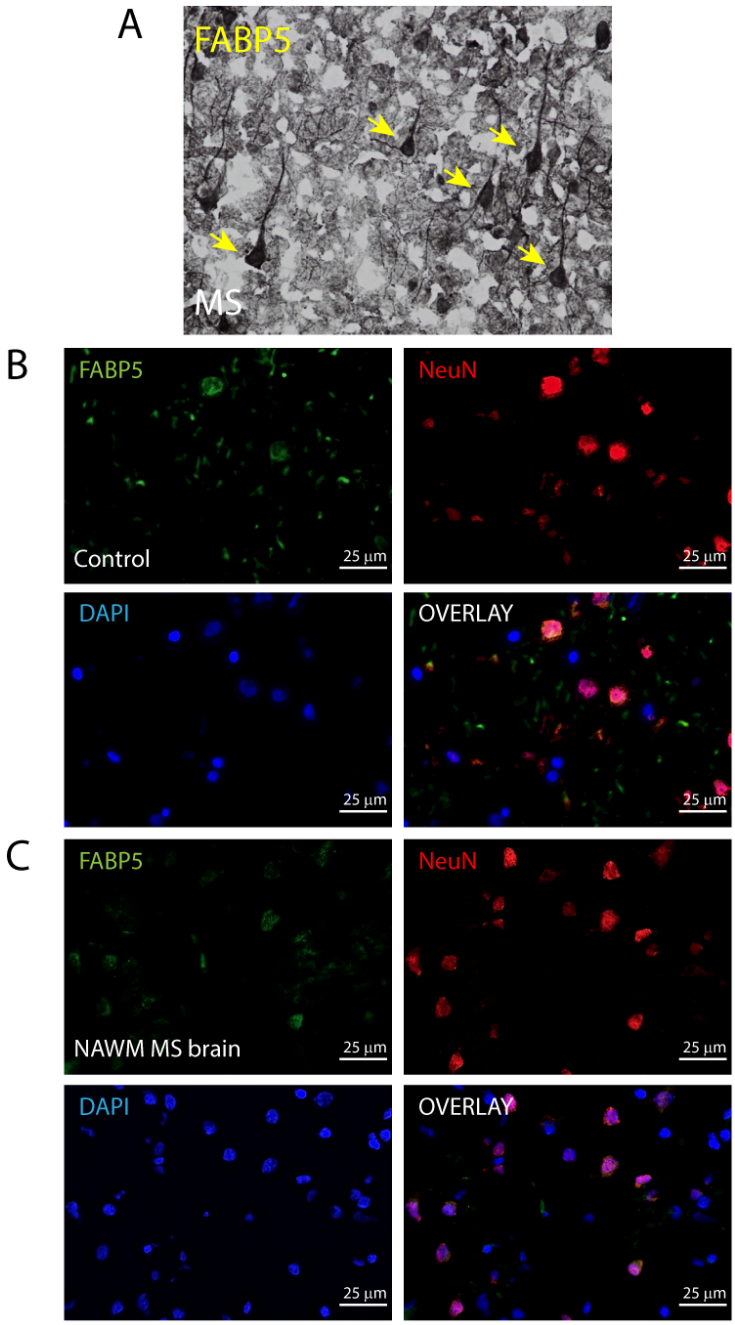

Figure 2-11. Detection of FABP5 in neurons of non-MS and MS brains.

A, DAB-nickel staining for FABP5 at the white/grey matter border in an acute lesion, showing a high abundance of FABP5 in neurons with axonal dendrites (yellow arrowheads). **B, C.** Immunofluorescence staining for FABP5 (green) in the cell bodies of neurons co-stained with neuron-specific anti-NeuN antibodies (red) in the brain tissue of **(B)** non-MS and **(C)** MS tissue donors. Data presented in the Figure were obtained in collaboration with the University of Exeter.

2.5. Discussion

Fabp5 is a member of the fatty acid binding protein family of small and soluble cytoplasmic proteins that are known to bind small hydrophobic molecules (2). In mice and humans, the gene encoding Fabp5 shows widespread expression, including skin, immune cells, endothelium, and CNS (6, 8, 28, 29). The expression of the *Fabp5* gene in endothelial cells and macrophages likely accounts for its apparent detection in a wide variety of tissues, albeit at much lower abundance than other tissue specific Fabp species. The exact function of Fabp5 is not clear, but it is thought that this protein fulfills in a variety of roles including intracellular buffering and transport of its ligands. In humans, elevated levels of Fabp5 are observed in psoriasis-affected skin tissue (6). In mice, inactivation of the Fabp5 gene is not lethal (30) although Fabp5-deficient mice do display impairment of cognitive function (31). Remarkably, however, mice lacking Fabp5 are highly resistant to EAE induction (26, 27, 32).

EAE in mice is a commonly used animal model of human inflammatory demyelinating diseases, and involves a combination of immunopathological and neuropathological mechanisms that result in the development of clinical features that approximates the key pathological features of MS which include neuroinflammation, demyelination, axonal loss and gliosis (9-12). The formation and accumulation of EAE or MS lesions is traditionally thought of as a consequence of the disruption of the blood-brain barrier allowing the passage of pro-inflammatory T-cells into the brain where they become sensitized to proteins specific to mature myelinating oligodendrocytes within the CNS thereby resulting in loss of oligodendrocytes, and axonal demyelination and neurodegeneration (33). The blood-brain barrier is formed by the brain endothelial cells coated with basement membrane together with pericytes and astrocyte end-feet facing parenchyma (34). This barrier is maintained by an elaborate network of tight junctions and adherens junctions that

seal gaps between adjacent endothelial cells (35), resulting in extremely low transcytotic activity (36).

We previously discovered that, like Fabp5-deficient mice, calnexin-deficient mice also show resistance to EAE induction (16). Calnexin is a membrane embedded ER-resident molecular chaperone with its C-terminal region extending into the cytoplasm (13, 14). As calnexin is expressed by virtually all cells in the body (13), it was unexpected to observe peripheral myelinopathy as the predominant phenotype with global inactivation of the *calnexin* gene in mice (15, 37). In a subsequent study, we discovered through yeast two-hybrid screening that the C-terminal cytoplasmic tail of calnexin had a high affinity interaction with Fabp5, which was then confirmed through microscale thermophoresis and surface plasmon resonance analyses (17). Based on the phenotype of resistance to EAE induction common to both *Fabp5*$^{-/-}$ and *Canx*$^{-/-}$ mice, we posited that the formation of a complex comprising Fabp5 and the C-terminal cytoplasmic tail of calnexin is a key component that determines the susceptibility of mice to EAE pathogenesis.

Since the interaction between Fabp5 and calnexin was discovered in yeast and validated *in vitro* using bacterially produced recombinant proteins (17), we sought to determine if protein-protein interactions of these two proteins occurred within the cell at the ER membrane. Double staining of mouse bEND.3 brain endothelial cells with anti-Fabp5 and anti-calnexin antibodies did reveal a reticular image pattern that was consistent with co-localization at ER membranes. To exclude possible artifacts contributed by immunolocalization procedure, we employed the BiFC technique (22, 38, 39) which does not rely on permeabilization and fixation of cells to report subcellular localization of the protein targets; moreover, the image signal of the YFP reporter is only visible when the targets are in very close proximity (22). We found that chimeric proteins of

Fabp5 and calnexin each specifically bearing one of the two domains of holo-YPF could reconstitute the YPF fluorophore, indicating that these two proteins make stable complexes within cells.

To determine if sensitization of *Canx*$^{-/-}$ mice to EAE induction was attributable to defective interaction of the cytoplasmic C-tail domain of calnexin with Fabp5 rather than loss of calnexin itself, we compared EAE pathogenesis in *Canx*$^{-/-}$ mice reconstituted with holo-calnexin or C-tail deletion mutant of calnexin by transgene expression. We found that adding back the full length calnexin, but not the C-tail deletion mutant of calnexin, to *Canx*$^{-/-}$ mice resensitized mice to EAE induction, indicating that the resistance to EAE pathogenesis exhibited by both *Fabp5*$^{-/-}$ and *Canx*$^{-/-}$ mice was attributable to the collaboration of Fabp5 and the cytoplasmic C-tail domain of calnexin rather than the loss of calnexin *per se*. Furthermore, the normalization of conduction velocity in both motor and sensory neurons in *Canx*$^{-/-}$ mice reconstituted with *both* holo-calnexin and C-tail deletion mutant of calnexin supports the notion that myelinopathy and EAE resistance phenotypes displayed by calnexin-deficient mice involve distinct calnexin functions. Coincidentally, a model of the blood-brain barrier based on a monolayer of Fabp5-deficient bEND.3 cells was resistant to activated T-cell transmigration in the same manner as calnexin-deficient bEND.3 cells that could be made permissive by reconstitution with holo-calnexin, but not with the C-tail deletion mutant of calnexin.

The normalization of conduction velocity in both motor and sensory neurons in *Canx*$^{-/-}$ mice reconstituted with both holo-calnexin and C-tail deletion mutant of calnexin supports the notion that myelinopathy and EAE resistance phenotypes (15, 16) displayed by calnexin-deficient mice involve distinct calnexin functions. In this regard, the observed myelination defect displayed by *Canx*$^{-/-}$ mice may reflect the loss of calnexin chaperone function whereas resistance

to EAE induction stems from faulty regulation of calnexin function via its cytoplasmic C-tail domain. Numerous calnexin C-terminal post-translational modifications including palmitoylation, phosphorylation, and SUMOylation have been reported (21, 40-45). Moreover, in addition to Fabp5, there is growing list of cytoplasmic proteins featuring SH3 domain GRB2 like endophilin interaction protein (SGIP1) (46), HIV-1 protein Nef (47), SUMOylation E2 ligase (UBC9) (21), protein tyrosine phosphatase 1B (PTP1B) (21), N-myristoyltransferase (NMT) (48) and activity-regulated cytoskeleton-associated protein (ARC) (49) that interact with the cytoplasmic C-tail domain of calnexin, suggesting that calnexin function is sensitive to events occurring in the cytoplasm, or even at membrane contact sites (50) and are conveyed to the ER luminal domain of calnexin and likely result in either potentiation or inhibition of its function within the ER lumen. Given the multiplicity of cytoplasmic proteins that interact with the cytoplasmic C-tail domain of calnexin, there is a high probability of overlapping interaction sites and thus result in competition for binding. Additional studies are necessary to determine which of these possibilities account for the impermeability of the model blood-brain barrier to T-cell transcellular migration. It is also not known at this time whether specific Fabp5 ligands affect the stability or longevity of the Fabp5/calnexin complex.

We previously reported increased calnexin abundance in the brains of MS patients (16). MS is a heterogeneous chronic inflammatory disease of the CNS which presents itself with motor impairment, sensory, optic disturbances, fatigue, and cognitive deficits (51). This heterogeneity of clinical manifestation is due to spatiotemporal dissemination of multiple lesions within different parts of the CNS. Demyelinating lesions, also knowns as plaques, are usually described as the pathological hallmark of MS (52) and are caused by the infiltration of auto-reactive immune cells into the CNS (53). In the present study, we also discovered that Fabp5 is increased in the brains of

MS patients which opens up the possibility that the persistence of the Fabp5/calnexin complex is a feature of MS pathology. However, since both Fabp5 and calnexin are normally present in brain endothelial cells and both proteins can form intimate contacts, additional circumstances are required to elaborate the disease. The increased abundance of both proteins in MS pathology suggests long lived Fabp5/calnexin complex, which could be due to stabilization of the complex by certain Fabp5 ligands or genetic variations in the genes that encode Fabp5 and calnexin. Insights into the factors that determine the stability of the Fabp5/calnexin complex will be provided by future studies.

In summary, the findings of the study presented in the Chapter 2 provide evidence for the idea that Fabp5, a cytoplasmic protein, makes stable interactions with the cytoplasmic C-tail domain of calnexin, and that the complex formed by these two proteins is crucial for sensitization of mice to EAE and possibly the pathogenesis of MS in humans.

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CHAPTER 3

GENERAL CONCLUSIONS

3.1. General conclusions

Calnexin is an ER membrane bound molecular chaperone that facilitates the folding and assembly of secreted or transmembrane glycoproteins (1). However, recent studies have shown that the conventional widely known role of calnexin extends far beyond just being a chaperone. The unique properties of calnexin stem from its structure and molecular topology (2). In addition to its ER luminal domain, which is extensively studied and described as a 'folding module', calnexin harbors a single transmembrane segment and a long C-terminal domain oriented to the cytoplasm. This unique C-terminal domain was shown to undergo distinct post-translational modifications (3-5) (6-11), linking the status of modifications with different functional outcomes affecting various cellular processes. Additionally, to date seven proteins were shown to interact with calnexin at its C-terminal domain (8, 12-16). This includes Fabp5, which is a member of the fatty acid binding protein family of small and soluble cytoplasmic proteins that coordinate lipid responses in cells (16, 17, 18). In addition to our previous *in vitro* study that reported calnexin C-terminal domain and Fabp5 interaction (16), to further validate the interaction *in vivo,* we used immunohistochemical staining and BiFC techniques and showed that calnexin and Fabp5 co-localize in close proximity in the blood-brain barrier endothelial cells (18). As a functional consequence, we propose that the complex between the calnexin C-terminal domain and Fabp5 controls the movement of inflammatory immune cells across the blood-brain barrier, preventing MS symptoms in a pre-clinical animal model of MS (Figure 3-1) (18). Additionally, we discovered that MS patients have unusually high abundance of both calnexin and FABP5 in their vessel endothelial cells (18, 19).

Figure 3-1 illustrates and summarizes my discoveries and contributions to this field. The study described in this work advanced and shifted the conceptual understanding of the role of calnexin, which was and still is widely regarded as just a molecular chaperone, to a more broad and expanded view as a regulatory molecule that contributes to many cellular processes via its C-terminal domain. Early studies implicated calnexin as an essential molecule in the development of the immune system which was long upheld as a general notion. However, the creation of calnexin-deficient mice challenged that idea and showed that that immune system generally was not affected in the absence of calnexin, which is evident due to the fact that calnexin-deficient mice exhibit motor dysfunctions with severe peripheral myelin abnormalities due to calnexin function as a molecular chaperone (25). Mice expressing the ER luminal chaperone domain of calnexin with the transmembrane domain but lacking the cytoplasmic C-terminal domain display normal motor properties (18). Therefore, this study sheds new light on the calnexin C-terminal domain, which was largely dismissed and rarely considered as functional. We show that the calnexin C-terminal domain is spatially separated from the ER lumenal domain and is functionally distinct from the rest of the calnexin protein and acts in divergent cytoplasmic signaling pathways.

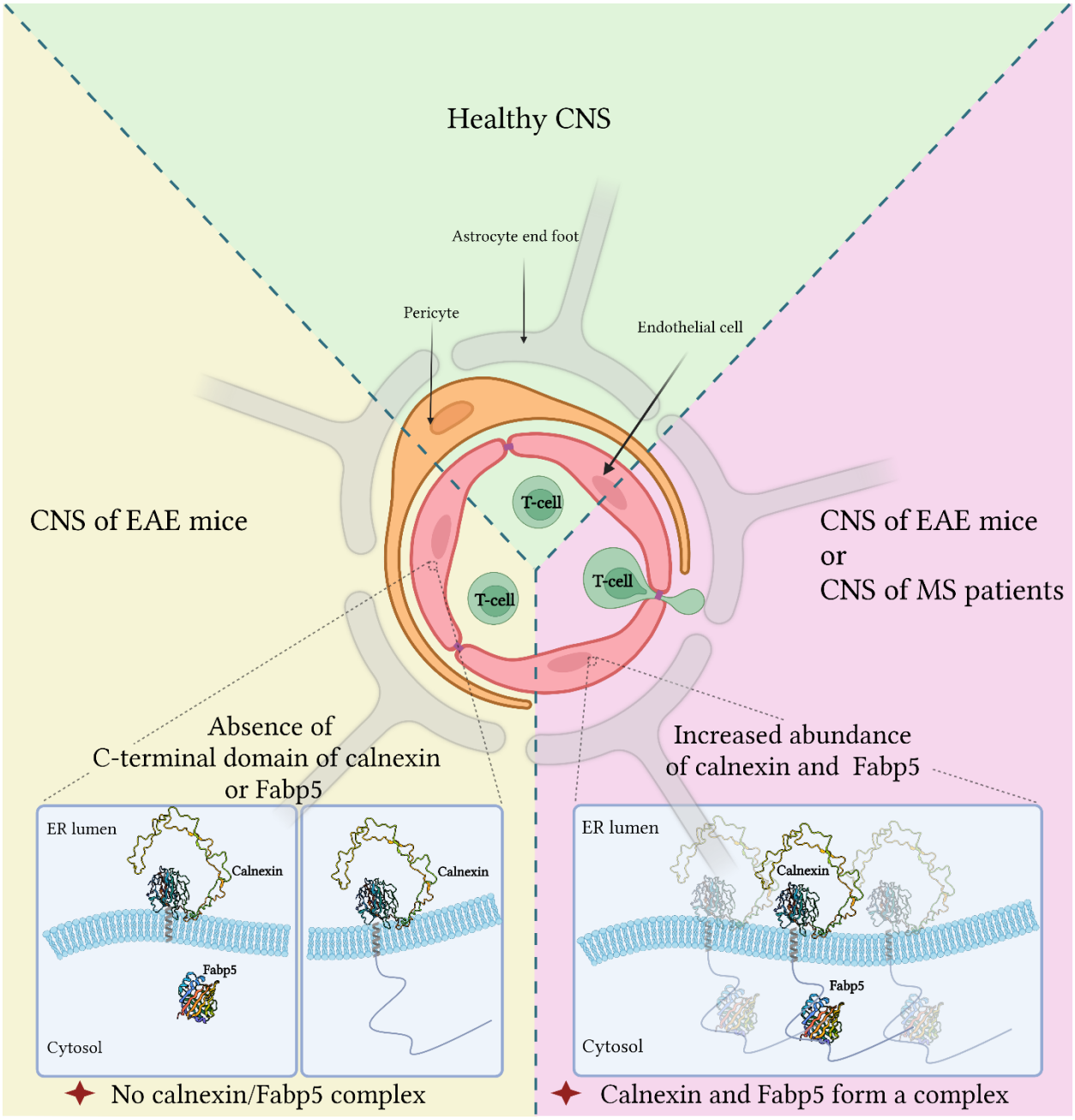

Figure 3-1. Schematic representation of the link between the presence and abundance of calnexin C-terminal domain/Fabp5 complex and the permeability of the blood-brain barrier to immune cells in the neuroinflammatory environment. (Created with BioRender.com)

Under normal healthy conditions (Figure 3-1, top section, green), the immune responses in the CNS are tightly controlled. One of the main factors that restricts immune cell entry into the CNS is the presence of the blood-brain barrier which is formed out of brain endothelial cells coated with basement membrane, together with pericytes and astrocyte end-feet facing parenchyma (20). This barrier results from an extremely low transcytotic activity (21) and an elaborate network of tight junctions (TJ) and adherens junctions (AJ) that seal the gaps between endothelial cells (22). This protects the CNS from neurotoxic plasma components and immune response mediated injury. However, during inflammatory diseases such as MS (Figure 3-1, right section, red), autoreactive immune cells manage to traverse the BBB and trigger a cascade of immune responses leading to demyelination, axonal loss and eventually neurodegeneration that results in irreversible neurological impairment (23). We have discovered that both calnexin and Fabp5 are upregulated in the endothelial cells of the BBB in both the brain tissue of MS patients and EAE mice spinal cord samples (18, 19). Thus, the presence and abundance of both calnexin and Fabp5 might potentiate the immune cell movement across the BBB. Previously, we have shown that the loss of calnexin in mice confers resistance to EAE and this resistance results from inhibited T-cell movement across the BBB (19, 24). Coincidentally, Fabp5-deficient mice displayed a similar phenotype in terms of resistance to EAE (24). Importantly, this work explored the role of calnexin in EAE and delineated that the resistance of *Canx*$^{-/-}$ mice to EAE is attributed to the cytoplasmic C-terminal domain of calnexin, which is the site for the Fabp5 binding (Figure 3-1, left section, yellow) (18). We discovered that resistance to EAE is due to inhibition of trans-endothelial T-cell migration across brain endothelial cells when the calnexin C-terminal domain and Fabp5 interaction is not occurring, either by silencing of genes encoding calnexin or Fabp5, or by deletion of the C-tail of calnexin, the site for calnexin-Fabp5 interactions (18, 19). The striking

commonality in the phenotype of the *Canx*$^{-/-}$ (19) and *Fabp5*$^{-/-}$ (24) mice with respect to resistance to EAE induction, coupled with the ability of Fabp5 to interact with the C-terminal cytoplasmic tail of calnexin (16), provides very strong support for the idea that the calnexin C-terminal domain and Fabp5 complex is a key feature of MS pathology.

Even though MS is defined as an autoimmune disease triggered by environmental factors that act on a genetically susceptible host, there is still no defined cause(s) or cure(s) for the disease, and the development of therapies to slow down the progression of the disease remain challenging. Therefore, the unanticipated discovery of a role for the calnexin C-tail/Fabp5 complex and a new, not previously described function of the calnexin C-terminal domain in the EAE animal model provides a promising target for novel protein-protein interaction drug design. Disrupting the calnexin and Fabp5 interaction could be an effective way to decrease inflammation and T-cell transmigration and thus possibly prevent or reverse MS progression.

While this study provides some first insights into the calnexin C-tail/Fabp5 complex and its role in modulating immune cell trafficking across blood-brain barrier, it is not without its limitations. First of all, the observed resistance to EAE in both calnexin deficient mice and mice that express C-terminally truncated calnexin was attributed to the impermeability of the blood-brain barrier to T-cell transcellular migration. However, it has not been addressed weather calnexin deficiency or the expression of C-terminally truncated calnexin in mice may affect the early events in EAE including generation of MOG-autoreactive T-cells which could lead to resistance to EAE. This could be addressed by the adoptive transfer where MOG-autoreactive T-cells generated in wild-type mice are transferred into both calnexin deficient mice and mice that express C-terminally truncated calnexin. Also, since our study was mostly focused on blood-brain barrier endothelial cells, the immunohistological staining of human brain MS lesions for FABP5 were evaluated

mostly looking at brain endothelial cells. Therefore, a more comprehensive analysis is needed to identify other FABP5 positive cells and describe their role in the active MS lesion. Also, the use of the t-test for the analysis of the five groups in Figure 2.7 was not appropriate since t-test is used to compare the means of two groups therefore ANOVA test should have been used instead.

3.2. Future challenges

The discovery presented in this study provides novel insight into a direct phenotypic relationship between the presence and abundance of calnexin C-terminal domain/Fabp5 complexes and the ability of immune cells to infiltrate the CNS by moving across the BBB in mice with EAE and potentially in MS patients. This novel complex provides an intriguing avenue for exploration of unconventional ways to prevent/inhibit/reduce neuroinflammation. However, future studies are needed to address the mechanistic and functional nature of the complex, which would allow for developing a viable roadmap towards disease prevention and development of new and effective therapies for MS patients.

Considering that the calnexin C-terminal domain provides an interaction site for at least seven proteins, the discovery of the calnexin C-terminal domain and Fabp5 complex alone does not exclude the fact that there could be additional interacting partner(s) mediating/contributing to the overall protein complex. Protein-protein interactions play an essential role in many cellular mechanisms, therefore it is important to understand the functional interplay between interacting proteins, which can be part of multiple dynamic and overlapping complexes that have distinct functions. Thus, mapping out potential additional interacting partners of the calnexin C-terminal domain and Fabp5 should provide additional mechanistic insight into how the BBB immune cell permeability is regulated. One of the strong candidates is a phosphatase PTP1B which is known

to bind calnexin C-terminal domain (26). It has also been shown that PTP1B dephosphorylates activated STAT3 (27) therefore potentially linking calnexin C-terminal domain and STAT3 signaling.

Furthermore, physiological roles and downstream functional consequences of the calnexin/Fabp5 complex formation should be explored under healthy conditions, far beyond just the biology of the BBB. This will expand the scope and understanding of the overall nature of the calnexin C-terminal domain/Fabp5 complex. Additionally, since calnexin is involved in ER protein quality pathways, it is critical to explore the crosstalk between calnexin and Fabp5 under pathological conditions and its implications to protein folding. Moreover, any information on human calnexin and Fabp5 gene mutation and clinical phenotype relationships might provide additional levels of insight into a more global role of the complex, as well as the individual proteins.

Immune cell trafficking across inflamed BBB is controlled by dynamic interplay between the integrity of junctional complexes, integrin-mediated adhesion and pro- and anti-inflammatory signaling events. Therefore, all these avenues should be explored as a potential cause and/or contributing factor for defective T-cell movement in the absence of the calnexin C-terminal domain/Fabp5 complex. To address the integrity of the junctional complexes between adjacent endothelial cells it is important to evaluate the expression and localization of the specific proteins that form junctional complexes i.e. tight junction proteins that include transmembrane proteins claudin and occludin, and the cytoplasmic scaffolding proteins zonula occludens. Furthermore, integrin-mediated adhesion to ICAM-1 and VCAM-1 should be evaluated on the endothelial cells that lack calnexin and Fabp5 interaction. Also, a more global and broadly-targeted approach could be taken to evaluate the changes of the endothelial cells by using both total RNA sequencing and

protein mass spectrometry. These approaches could provide some clues of the alterations of the transcriptional and translational profile in endothelial cells that result from calnexin and/or Fabp5 deletion.

Moreover, metabolic and signaling pathways should be investigated with a primary focus on the STAT3 signaling pathway. Previously it has been shown that under certain conditions, the calnexin C-terminal domain is being cleaved and released, with the fragment migrating to the nucleus where it acts as an enhancer of STAT3-mediated transcriptional response (28). Therefore, the ability of Fabp5 to bind to the calnexin C-terminal domain might modulate the proteolytic cleavage of calnexin and in turn affect STAT3 signaling.

The mechanistic nature of the calnexin C-terminal domain and Fabp5 interaction is not known, therefore, the complex interface and the exact amino acids involved on both the calnexin C-terminal domain and Fabp5 should be identified in future studies. Additionally, Fabp5, being a member of the family of fatty acid binding proteins, binds various lipophilic ligands and can exist in both the apo- and holo-form. Therefore, it should be addressed if the interaction of Fabp5 with the calnexin C-terminal domain is fatty acid dependent. The calnexin C-terminal posttranslational modifications should be taken into account to evaluate if the status of specific post-translational modifications has any effects on the complex stability. Specifically, two calnexin C-terminal domain juxtamembrane cysteines that undergo palmitoylation could be of primary interest since Fabp5 is able to bind lipophilic ligands and might position itself in a way to accommodate its fatty acid binding pocket to be oriented towards the palmitoylated cysteines in the calnexin C-terminal domain. This adds multiple factors to possibly consider when evaluating the stability and dynamics of the complex. Also, other members of the Fabp family should be screened and evaluated for calnexin C-terminal domain binding.

The unique structural and functional topology of calnexin and the observation that the C-terminal domain is spatially separated by the ER membrane from the ER lumenal domain provides an ideal scenario for drug design targeting the C-terminal domain without affecting the chaperone activity of the ER luminal domain of the protein. Combined knowledge acquired in the future studies could be used to reach the ultimate goal of this study, i.e. to find a pharmaceutical way to modulate the calnexin C-terminal domain/Fabp5 complex and reduce and/or prevent immune cell infiltration into the CNS in MS patients. Brain drug delivery is often challenging and has the poorest success rates compared to other therapeutic areas. In most of the cases, the drug needs to be delivered into the parenchyma of the brain. In contrast, the potential drugs modulating the calnexin C-terminal domain/Fabp5 complex do not need to cross the BBB, but rather need to be taken up by the endothelial cells of the BBB to be effective. This might still be challenging due to extremely low pinocytotic activity of BBB endothelial cells, but worthy of further study.

3.3. References

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