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Role of the Alzheimer's predisposition factor CD2AP in brain endothelial cells

by

Colin William Gunn

A THESIS

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Abstract

Alzheimer disease (AD) is the most prevalent form of dementia in the elderly. Vascular degeneration plays a critical role in AD pathogenesis. For instance, APOE4 allele, the main genetic risk factor for AD, is linked to reduced cerebral blood flow and increased blood brain barrier (BBB) leakiness. CD2-associated protein (CD2AP) is an adaptor protein that regulates receptor endocytosis and recycling. Two single nucleotide polymorphisms in CD2AP are associated with higher risk for AD and levels of CD2AP are decreased in lymphocytes of sporadic AD patients. Recent human studies also show that CD2AP is linked to the burden of Aβ plaques, memory deficits and modulates AD symptoms in APOE4 patients. Interestingly, CD2AP is enriched in the brain microvascular endothelial cells (BMECs), a key component of the brain microvasculature. However, the basic functions of CD2AP in BMECs and its contribution to brain vasculature dysfunction in AD remain totally unknown.

Here we show that siRNA-mediated depletion of CD2AP in both human brain endothelial cell line and mouse primary BMECs leads to the deregulation of proteins involved in AD pathogenesis. Of particular interest, we found that CD2AP interacts with one of these proteins, ApoER2, the receptor for ApoE that is targeted to degradation upon ligation by AD allele ApoE4. Similar to ApoE4, CD2AP loss reduced the levels of ApoER2. Finally, we discovered that downregulation of ApoER2 in CD2AP-depleted cells is modulated by Rab5 GTPase activity.

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List of Symbols, Abbreviations, Nomenclature

Symbol	Definition
-/-	Homozygous Knockout
AD	Alzheimer's Disease
AJ	Adherens Junction
Alix	ALG-2-Interacting Protein X
ApoE	Apolipoprotein E
ApoER2	Apolipoprotein E Receptor 2
APP	Amyloid Precursor Protein
Arp2/3	Actin-Related Proteins 2/3
Aβ	Amyloid Beta
BBB	Blood-Brain Barrier
BCAR1	Breast Cancer Anti-Estrogen Resistance 1
BDCA2	Blood Dendritic Cell Antigen 2
BMEC	Brain Microvascular Endothelial Cell
Catl	Cathepsin L
cbl	Casitas B-lineage Lymphoma
CD2AP	CD2-Associated Protein
CDK-5	Cyclin-Dependent Kinase 5
CFBP	CIN85/CD2AP Family Binding Protein
CIN85	Cbl Interacting Protein of 85 kDa
Co-IP	Co-Immunoprecipitation
CP	Capping Protein
CSF	Cerebrospinal fluid
Dab1	Disabled-1
E-cadherin	Epithelial cadherin
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor

eNOS	Endothelial Nitric Oxide
F-actin	Filamentous Actin
Flt-1	Fms Related Tyrosine Kinase 1
FRS2b	Fibroblast growth factor Receptor Substrate 2b
G-actin	Globular Actin
GEEC	Glycosylphosphotidylinositol-anchored protein Enriched Compartments
GSK-3β	Glycogen synthase kinase-3β
hCMEC/D3	Human Cerebral Microvascular Endotheliacl Cell/D3
HGF	Hepatocyte Growth Factor
ICD	Intracellular Domain
LDL	Low-Density Lipoprotein
ILV	Intralumenal Vesicles
IP	Immunoprecipitation
LRP1	Low density lipoprotein Receptor-related Protein 1
LTP	Long Term Potentiation
MVB	Multi-Vesicular Bodies
NA-FITC	Sodium Fluorescein
NMDAR	N-methyl-D-aspartate receptor
NO	Nitric Oxide
pDC	Plasmacytoid Dendritic Cell
PI3K	Phosphoinositide 3-Kinase
PS1	Presenilin 1
Rac1	Ras-related C3 botulinum toxin substrate 1
RAGE	Receptor for Advanced Glycation Endproducts
RIN3	Ras and Rab interactor 3
SH3	SRC Homology 3
SHIP1	SH2 Domain-Containing Inositol 5-Phosphatase 1
siRNA	Small Interfering RNA
SNP	Single Nucleotide Polymorphism

SUMO	Small Ubiquitin-like Modifier
TGF	Transforming Growth Factor
TJ	Tight Junction
TrkA	Tropomyosin receptor kinase A
VE-cadherin	Vascular-Endothelial cadherin
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
VLDLR	Very Low Density Lipoprotein Receptor
WT	Wild Type

Chapter 1: Introduction

1.1 CD2AP

1.1.1 Identification and origin of CD2AP

CD2AP was first identified as interacting with the cytoplasmic domain of CD2, (a transmembrane protein of the immunoglobulin superfamily (Dustin et al., 1998). In T-cells, CD2 receptor engagement with its antigen induces CD2AP binding to CD2 resulting in clustering in the central region of the the immunological synapse. It was shown that expression of CD2AP dominant negative blocked CD2-induced cytoskeleton polarization and immunological synapse formation (Dustin et al., 1998).

1.1.2 Gene structure and regulation

Analysis of the primary structure of CD2AP reveals three N-terminal SH3 protein binding domains, a polyproline domain, C-terminal actin binding sites and a C-terminal coil-coil domain **(Figure 1).**

Figure 1. Diagram of CD2AP primary sequence and protein domains.

Schematic of CD2AP primary sequence and protein domains. Src homology 3 (SH3) domain; proline-rich region; putative actin binding regions; and a coiled-coil domain (CC) N and C represent the protein termini.

SH3 domains are protein binding domains that bind to their ligands at polyproline segments with the consensus sequence PXXP (where X is any amino acid). The canonical binding site on the SH3 domain for the recognition of polyproline sequences consists of a hydrophobic surface with three shallow pockets defined by the side chains of preserved aromatic residues (Roldan et al., 2011). SH3 domains were the first example of modular protein-binding domains that do not depend on post-translational modifications (i.e. phosphorylation) (Mayer, 2015). There are about 300 distinct SH3 domains identified thus far in humans, making it one of the most common protein domains known. SH3 domains act together with other binding domains to mediate the assembly of dynamic protein complexes (Mayer, 2015). Although the affinity of each individual SH3 binding domain to its target tends to be weak, multiple domains can work together to mediate tighter and more stable interactions. Indeed, many proteins contain multiple SH3 domains, up to five in some cases (Kärkkäinen et al., 2006), three in the case of CD2AP.

CD2AP expression has been shown to be altered in multiple ways. Firstly, high glucose conditions in concert with advanced glycation end products were found to decrease CD2AP protein levels and mRNA expression (Ha et al., 2015). Secondly, all-trans retinoic acid was shown to upregulate CD2AP gene expression by activating CD2AP promoter activity through the Sp1/3 response element (Xu et al., 2015). Lastly, it was discovered that the transcription factor E2F1 acts as a transcriptional activator, increasing expression of CD2AP (Zou et al., 2012).

1.1.3 Expression in cells

CD2AP was found to be expressed in the collecting ducts, the proximal and distal tubules of the kidney where it was concentrated apically (Li et al., 2000). CD2AP is also expressed by

enterocytes along villi in the small intestine. In the pancreas, CD2AP was present in acinar cells and ductal cells (Li et al., 2000). It is also expressed in the submandibular salivary gland, specifically in the secretory acini of the mucous component at epithelial cell borders (Li et al., 2000). In the brain CD2AP is most strongly expressed in endothelial cells but is expressed in other cell types including neurons to a much lesser extent (Zhang et al., 2014; Cochran et al., 2015). In the heart it is expressed in capillaries and platelet endothelial cell adhesion molecules (Li et al., 2000). Finally, the apical side of bronchial epithelial cells and capillaries were strongly positive for CD2AP as well as the mesothelial cells of the lung lobes (Li et al., 2000).

1.1.4 Protein structure

A recent study elucidated the structure of CD2AP using electron microscopy and single particle image analysis to determine the three dimensional structure of recombinant full-length CD2AP. This study found that the protein forms a tetramer with itself in solution. CD2AP was found to form a roughly spherical structure, consisting of a central coiled-coil domain forming the tetramer interface, surrounded by three globular domains corresponding to the three Src-homology-3 (SH3) domains. The spatial organization of CD2AP's tetrameric quaternary structure exposes the binding sites of all 12 SH3 domains in the tetramer, allowing simultaneous binding to multiple targets (Adair et al., 2014). This orientation allows for the spatial modulation of multiple target proteins demonstrating that CD2AP is functionally structured as a scaffold/adapter protein **(Figure 2)**.

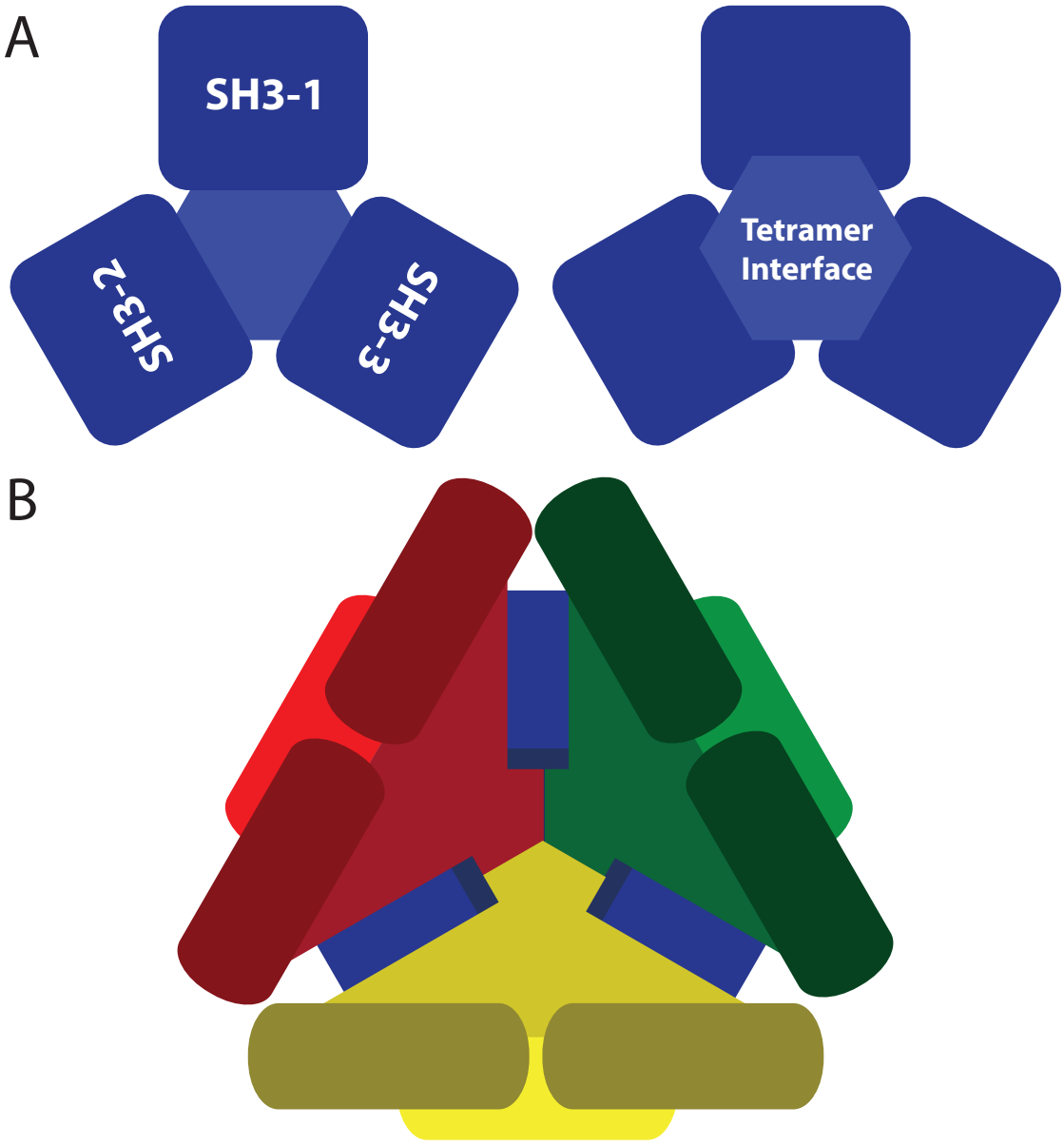

Figure 2. Schematic of three-dimensional structure of CD2AP

(A) Three-dimensional representation one CD2AP monomer (B) Three-dimensional representation of tetrameric CD2AP (each individual CD2AP protein is represented in a different colour where the blue monomer is hidden partially from view). Oriented externally are the three SH3 domains of each monomer extending outward symmetrically from the center of the tetramer.

A study by Rouka *et al.* (2015) showed that the different SH3 domains bind differentially (Rouka et al., 2015). This study demonstrated that significant differences exist in the recognition preferences of CD2AP's three SH3 domains for c-Cbl (Casitas B-lineage lymphoma), ALG-2-Interacting Protein X (Alix), and Ras and Rab interactor 3 (RIN3). This differential recognition is most likely preserved with other CD2AP binding partners explaining the multiple distinct functions characterized for CD2AP in the cell.

1.1.5 Homologue

CD2AP has a homologue, CIN85. Both CD2AP and CIN85 utilize three SH3 domains to bind various protein partners enacting their effects on the cell. Although CD2AP and CIN85 share a similar domain organization with a high degree of sequence conservation, they diverge in the binding modalities of their SH3 domains (Ceregido et al., 2013). For example, CD2AP binds to the E3 ubiquitin ligase Cbl-b at its first SH3 domain (SH3A) forming a dimer whereas two CIN85 proteins bind via their SH3A domains to one Cbl-b protein forming a trimer (Ceregido et al., 2013). Furthermore, CD2AP and CIN85 can bind proteins that the other cannot. For example, CD2AP bind Cbl-3/c whereas CIN85 does not (Calco et al., 2014). In addition, CD2AP but not Cin85 contains an actin binding domain (Welsch et al., 2005). The differential binding of these two orthologues as well as the lack of an actin binding domain on Cin85 may explain their divergent functions *in vivo.*

1.2 General roles for CD2AP

1.2.1 Actin dynamics

One crucial way that cells respond to stimuli is by rapidly and precisely remodeling their actin cytoskeleton. Actin exists in cells in one of two states: globular or G-actin (monomeric) and filamentous or F-actin (polymeric). Actin monomers are polar and bind end-to-end to form actin filaments. These filaments are consequently also polar and one end of the filament favours polymerization whereas the other end favours dissociation. The actin cytoskeleton is remodeled through tight spatial and temporal control over the balance of these two processes. This control is facilitated by the binding and dissociation of actin capping proteins (proteins that bind either end with high affinity) and the regulation of their action (Shekhar et al., 2016).

Two proteins that are known to participate in the regulation of actin remodeling are capping protein (CP) and cortactin. It was found that CP and cortactin form a complex with CD2AP at the cell edge and that by binding cortactin, CD2AP acts to recruit CP to the periphery of spreading cells. Cortactin acts to recruit Arp2/3 complex formation which facilitates actin branching, thus CD2AP plays a critical scaffolding role to enhance the ability of CP and cortactin to promote Arp2/3 complex-mediated actin assembly. Branched actin filaments are also important in vesicular trafficking and endocytosis, so these findings provide a potential link between CD2AP's role in receptor endocytosis and cytoskeleton remodeling (Zhao et al., 2013). Interestingly, CD2AP's binding site on CP is close to the location that confers CP's actin capping activity. As a result, binding of CD2AP with CP causes decreased capping activity of CP and also uncaps the actin filament leading to increased actin polymerization and reduced branching

adding another layer of complexity to the actin regulation network that CD2AP is involved in (Bruck et al., 2006).

In addition to interacting with actin capping proteins it has also been shown that CD2AP associates directly with actin via its C-terminus. In addition, CD2AP accumulations were found along the leading edge of the cell, colocalizing with F-actin, indicating that the distribution of CD2AP is related to the locomotory status of the cell (Lehtonen et al., 2002). In addition, a more recent study showed that CD2AP was found to be a capping protein itself that can effectively shut down fast actin dynamics at filament barbed ends. By stabilizing actin at the adherens junction (AJ), CD2AP plays an important role not only in cell–cell adhesion but also in suppression of cell migration. As stabilization of actin at the AJ suppresses cell migration, destabilization of actin is associated with increased actin dynamics, resulting in enhanced cell migration (Tang and Brieher, 2013). It can be postulated that through its ability to promote and reduce capping activity of other proteins as well as cap actin itself and associate with membrane bound proteins that CD2AP may act as a regulator of actin dynamics and either promote branching, polymerization or anchoring with the cell membrane based on upstream signalling.

1.2.2 Receptor Endocytosis

Based on CD2AP's specialized scaffolding structure and association with the actin cytoskeleton it is unsurprising that CD2AP is intricately involved in the regulation of receptor endocytosis. In one study epidermal growth factor (EGF) treatment of cells led to the formation of a complex containing CD2AP, the EGF receptor (EGFR), and Cbl (an E3 ubiquitin ligase), with cortactin being subsequently recruited. As previously mentioned cortactin is an actin cytoskeleton

regulator that interacts with CD2AP and also recruits the Arp2/3 complex, facilitating and stabilizing nucleation sites for actin polymerization. The study showed that through its ability to bind both CD2AP and the Arp2/3 complex, cortactin facilitated the activation-induced endocytosis of EGFR. This finding was the first indication that CD2AP may facilitate the trafficking of internalized growth factor receptors (Lynch et al., 2003). In addition, it was found that a novel protein dubbed CFBP (CIN85/CD2AP family binding protein) binds CD2AP and facilitates the recruitment of Cbl to the CD2AP/EGFR complex and may in fact be required for the downregulation of the receptor upon ligand stimulation (Konishi et al., 2006).

Another instance of CD2AP's role in receptor endosomal trafficking is the finding that vascular endothelial growth factor (VEGF)-activated Flt-1 binds to c-Cbl and Tyrosine 1333 in Flt-1 is the docking site for the c-Cbl/CD2AP complex. In this system the SH2 domain of c-Cbl is necessary for binding to Flt-1 and the second SH3 domain of CD2AP interacts with Flt-1 via c-Cbl. This c-Cbl/CD2AP complex binding to activated Flt-1 plays a crucial role in its endocytosis and subsequent degradation (Kobayashi et al., 2004, #4868).

An interesting variation is seen with the RET receptor. In sympathetic neurons, CD2AP and Cbl-3/c both directly associate with the RET receptor and each other. CD2AP overexpression upregulated ubiquitination and degradation of the receptor while overexpressing both strongly upregulated degradation. It was concluded that CD2AP modulates Cbl-3/c ubiquitination of the RET receptor upon receptor activation (Tsui and Pierchala, 2008). Interestingly, the same lab in a more recent paper demonstrated that the C-terminal portion of CD2AP which contains the actin binding domain was not required for CD2AP's ability to promote ubiquitination of RET (Calco et al., 2014) indicating that CD2AP's role in receptor ubiquitination did not act through

cytoskeletal regulation. In addition, they showed that RET activation led to the subsequent downregulation of CD2AP indicating that the presence of a negative feedback loop.

Plasmacytoid dendritic cells (pDCs) are innate immune cells that circulate in the blood. In these cells it was shown that the first SH3 domain of CD2AP binds to the proline-rich domain of SHIP1. It was demonstrated that this CD2AP/SHIP1 complex negatively regulates the Cbl-mediated ubiquitination and degradation of the activated BDCA2 receptor and surprisingly Syk, which is a non-receptor tyrosine kinase (Bao et al., 2012). This finding provides another example of CD2AP's role in receptor tyrosine kinase degradation and indicates for that CD2AP's role in E3 ligase-dependent targeting of tyrosine kinases for degradation may not be exclusive to receptors.

Fibroblast growth factor receptor substrate 2b (FRS2b), another adapter protein, has also been shown to interact with CD2AP via PxPxxR motifs. PxPxxR motifs of FRS2b are essential for co-localization of FRS2b and CD2AP and for FRS2b inhibition of anchorage-independent cell growth. Knockdown of CD2AP or Cin85, or of Cbl, diminished the downregulating effect of FRS2b on ErbB2 (receptor tyrosine kinase involved in outgrowth and stabilization of peripheral microtubules) (Minegishi et al., 2013).

Finally, in a recent study CD2AP was found to complex with cytoplasmic p53 in vivo (Panni et al., 2015), which indicates another possible signaling role for CD2AP yet to be elucidated.

The studies described above indicate that CD2AP plays an important role in receptor endocytosis of multiple receptors in multiple cell types. So far it has been shown that CD2AP tends to associate with an ubiquitin ligase and either facilitates or inhibits the ubiquitination of tyrosine kinase receptors. Based on these studies the process is typically preceded by the

binding of a ligand to the receptor leading to a signaling cascade followed by the endocytosis of the receptor. This process is understood as a method of attenuating the initial signal (Haglund and Dikic, 2012). Although this conserved role of CD2AP has been shown in multiple studies with multiple receptors, the mechanism of CD2AP's action on receptor endocytosis has yet to be fully elucidated.

1.2.3 Cell adhesion/motility

One of the first studies involving CD2AP found that CD2AP and BCAR1 (a docking protein implicated in cell adhesion and induction of cell migration) interact *in vitro* and *in vivo*, and that CD2AP colocalizes with BCAR1 and F-actin at membrane ruffles (Kirsch et al., 1999). It has been demonstrated that phosphorylated BCAR1 localizes to the cell membrane and that BCAR1 has been shown to be a component of focal adhesions throughout their assembly and disassembly, a tightly regulated process involved in cell motility and adhesion (Barrett et al., 2013).

In addition, it was found that in epithelial cells CD2AP associates with Rac1 and that Rac1 activity induces CD2AP localization to epithelial cell-cell contacts. Rac1 is a small Rho GTPase whose family of proteins regulate many important cellular processes including cell proliferation, migration, and vesicle trafficking (van Duijn et al., 2010). In another study it was shown that phosphorylation of BCAR1 leads to activation of Rac1 and increased membrane ruffling and lamellipodium formation, events associated with cell migration (Sharma and Mayer, 2008). These results taken together indicate that CD2AP most likely acts in a complex with BCAR1 and Rac1 at focal adhesions and that this complex regulates actin dynamics involved in cell adhesion and motility.

CD2AP has also been shown to associate with F-actin at the leading edge of cells and interact with E-cadherin upon HGF stimulation. E-cadherin is an adhesion molecule whose homotypic interaction from cell to cell creates a cell-cell adhesion structure known as an adherens junction (AJ). It was found that CD2AP's function in this capacity is important for cell migration most likely by linking E-cadherin signaling and adhesion function with the regulation of the actin cytoskeleton (Mustonen et al., 2005). Conversely, CD2AP was found to be important for stabilizing of E-cadherin cell-cell adhesions at the AJ and conferring resistance to mechanical stress (Tang and Brieher, 2013).

These studies demonstrate that CD2AP is important for focal adhesions (BCAR1 and Rac1) and AJs (E-Cadherin) two structures involved in cell adhesion. It is known that the modulation of these adhesion structures is important for cell motility. These studies also demonstrate that CD2AP's involvement with these two adhesion structures is important for cell motility so it stands to reason that CD2AP may act in a regulatory complex that controls the modulation of cell adhesion based on the locomotry status of the cell. A summary of CD2AP's functions in receptor endocytosis and actin dynamics can be found below in **Table 1** and **Table 2** respectively.

Table 1. Receptors regulated by CD2AP

Receptor	Effector	Cell-type	Reference
Flt-1	c-Cbl	NIH3T3 Cells	(Kobayashi et al., 2004)
EGFR	Cbl, cortactin, CFBP	HEK 293	(Lynch et al., 2003), (Konishi et al., 2006)
RET	Cbl-3/c	Sympathetic Neurons	(Tsui and Pierchala, 2008)
BDCA2	SHIP1, Cbl	Plasmacytoid Dendritic Cells	(Bao et al., 2012)
ErB2	FRS2b, Cbl	HEK 293 & Primary Embryonic Neural Cells	(Minegishi et al., 2013)

Table 2. CD2AP's roles on the actin cytoskeleton

Effector	CD2AP Function	Cell Process	Cell-type	Reference
CD2	Clustering	Immunological synapse formation	T-cells	(Dustin et al., 1998)
BCAR1	Focal adhesion regulation	Cell motility/adhesion	COS-7	(Kirsch et al., 1999)
nephrin, podocin	Cell-cell adhesion stabilization	Slit Diaphragm integrity	Podocytes	(Schwarz et al., 2001)
F-actin	Actin binding	Cell locomotion	Kidney epithelial cells	(Lehtonen et al., 2002)
E-Cadherin, F-actin	Actin dynamics	Cell-cell adhesion/cell migration	Rat gastric mucosal cells	(Mustonen et al., 2005)
CP	CP inhibition	Actin capping	Cell-free assay	(Bruck et al., 2006)
Rac1, CAPZ, cortactin	Cell-cell adhesion stabilization	Cell-cell adhesion	Epithelial cells	(van Duijn et al., 2010)
CP, Cortactin, Arp2/3	Scaffold	Lamellipodia formation	Podocyte cell line	(Zhao et al., 2013)

1.3 Role of CD2AP in podocytes of the kidney

Several studies have found that CD2AP is extremely critical to the integrity of the renal glomerulus. CD2AP null mice exhibit renal failure at 6-7 weeks postnatally, characterized by podocyte effacement and proteinuria (Shih et al., 1999). Disease progression appears to begin with epithelial cell injury leading to a mesangial reaction consisting of hyperplasia and massive matrix deposition. Co-Immunoprecipitation studies suggest that CD2AP associates with nephrin, a protein critical for podocyte function (Shih et al., 1999). CD2AP is aberrantly expressed in podocytes exhibiting or undergoing foot process effacement, and loss of nephrin correlates with the loss of slit diaphragms that accompanies foot process effacement (Li et al., 2000).

One study showed that podocin, CD2AP, and nephrin associate with lipid rafts, the C-terminus of podocin interacts with CD2AP and that nephrin and podocin interact with CD2AP directly (Schwarz et al., 2001). The prevailing theory is that CD2AP, podocin and nephrin form a complex and that this complex is required for the integrity of the slit diaphragm **(Figure 3).**

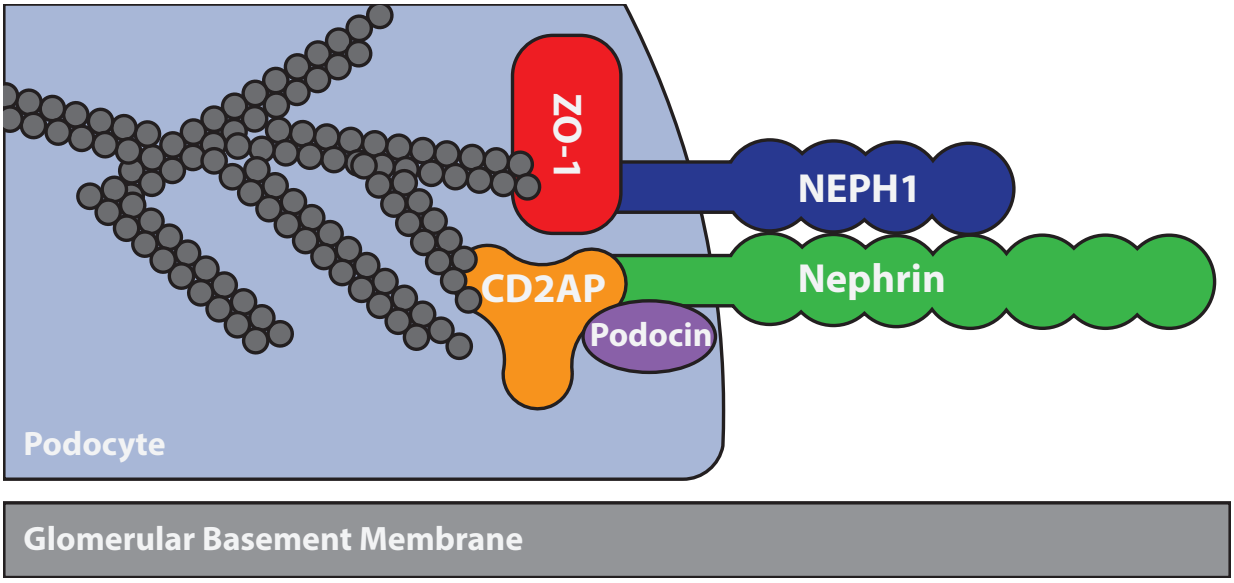

Figure 3. Schematic representation of the slit diaphragm

Diagram illustrating the interacting proteins that make up the slit diaphragm expressed in podocytes. See main text for details.

In terms of the signaling involved in this complex it was found that nephrin and CD2AP associate with the p85 regulatory subunit of phosphoinositide 3-kinase (PI3K) and induce phosphorylation and activation of the PI3K target protein kinase, AKT. In addition, both nephrin and CD2AP participate in PI3K/Akt signaling that safeguards podocyte viability (prevents against detachment induced apoptosis) (Huber et al., 2003). Interestingly, in mouse podocytes, bigenic heterozygosity of CD2AP and Fyn (a Src family kinase), as well as CD2AP and synaptopodin both lead to proteinuria. It was also determined that CD2AP interacts with Fyn and synaptopodin (Huber et al., 2006). In another study, CD2AP knockdown by siRNA inhibited podocyte's adhesion and cell body spreading, disturbed cell cycle leading to abnormal cell division, retarded podocyte proliferation, disrupted podocyte cytoskeleton and lowered nephrin expression and its phosphorylation (Zhang et al., 2009).

Experiments mimicking diabetic conditions, i.e. High glucose and high glucose plus advanced glycation end products (AGEs), have been shown to induce the relocalization of CD2AP away from the periphery in podocytes. High glucose plus AGEs also decreases CD2AP expression. In addition, LY294002, a phosphoinositide 3-kinase inhibitor, prevents the diabetic condition-induced CD2AP phenotype. These results suggest that diabetic conditions induce the phenotypical changes of podocyte CD2AP possibly via PI3K/Akt signalling (Ha et al., 2015).

A potential mechanism for podocyte effacement was elucidated by the finding that CD2AP downregulation results in Transforming Growth Factor (TGF)-β1 signalling to cause translocation of dendrin to the nucleus. Dendrin is a transcription factor for Catl and Catl was found to proteolytically degrade CD2AP, dynamin and synaptopodin. Lack of dynamin and

synaptopodin cause disorganization of actin cytoskeleton leading to hypersensitivity to apoptotic signals (Yaddanapudi et al., 2011).

Another potential mechanism which may act synergistically with the above mechanism involves dysregulation of CIN85, a homologue of CD2AP previously mentioned above. Full-length CIN85 was found to be strongly expressed in *CD2AP*$^{-/-}$ podocytes, leading to decreased anti-apoptotic signaling and enhanced endocytosis of nephrin. It was found that CIN85 is SUMOylated by SUMO-1, -2, and -3 and that SUMOylation is enhanced in the presence of CD2AP. Interestingly, mutating the lysine residue at amino acid 598 to arginine on CIN85 completely abolishes SUMOylation and leads to increased binding of CIN85 to nephrin (Tossidou et al., 2012). These results taken together indicate that CIN85 is post-translationally modified in CD2AP wild type (WT) cells and this modification is mediated by CD2AP which presumably protects podocytes against the detrimental effects of CIN85 binding to nephrin. This mechanism could be a contributing factor to the proteinuria and podocyte effacement phenotype found in CD2AP null mice.

1.4 CD2AP is important for blood-brain barrier integrity

Endothelial cells in the brain differ from endothelial cells in other tissues in that they are held together by highly electrically-resistive tight junctions and contain few transcytotic vesicles, limiting the flow of molecules from the blood into the brain both paracellularly and transcellularly (Daneman et al., 2010). The proper functioning of the blood-brain barrier requires the presence of specialized intercellular junctions (adherens and tight junctions) between these endothelial cells of the vasculature. Adherens junctions (AJs) are cell junctions

made up of the cadherin family of homotypic cell-cell adhesion proteins whose cytoplasmic domain is linked to the actin cytoskeleton (Rubin and Staddon, 1999). Tight junctions are a specialized cell-cell contact made up of claudins and occludins and restrain the passage of small polar molecule and large macromolecules (Abbott et al., 2010; Wolburg et al., 2009). Numerous studies, have demonstrated conclusively that tight junctions made up of proteins such as Claudin-5 depend very much on the existence of stable AJs (Rubin and Staddon, 1999). Not only is the cell-cell adhesion of the AJs required for the formation of tight junctions, their integrity depends on having intact AJs (Rubin and Staddon, 1999). Tight junctions and AJs are depicted in **Figure 4.**

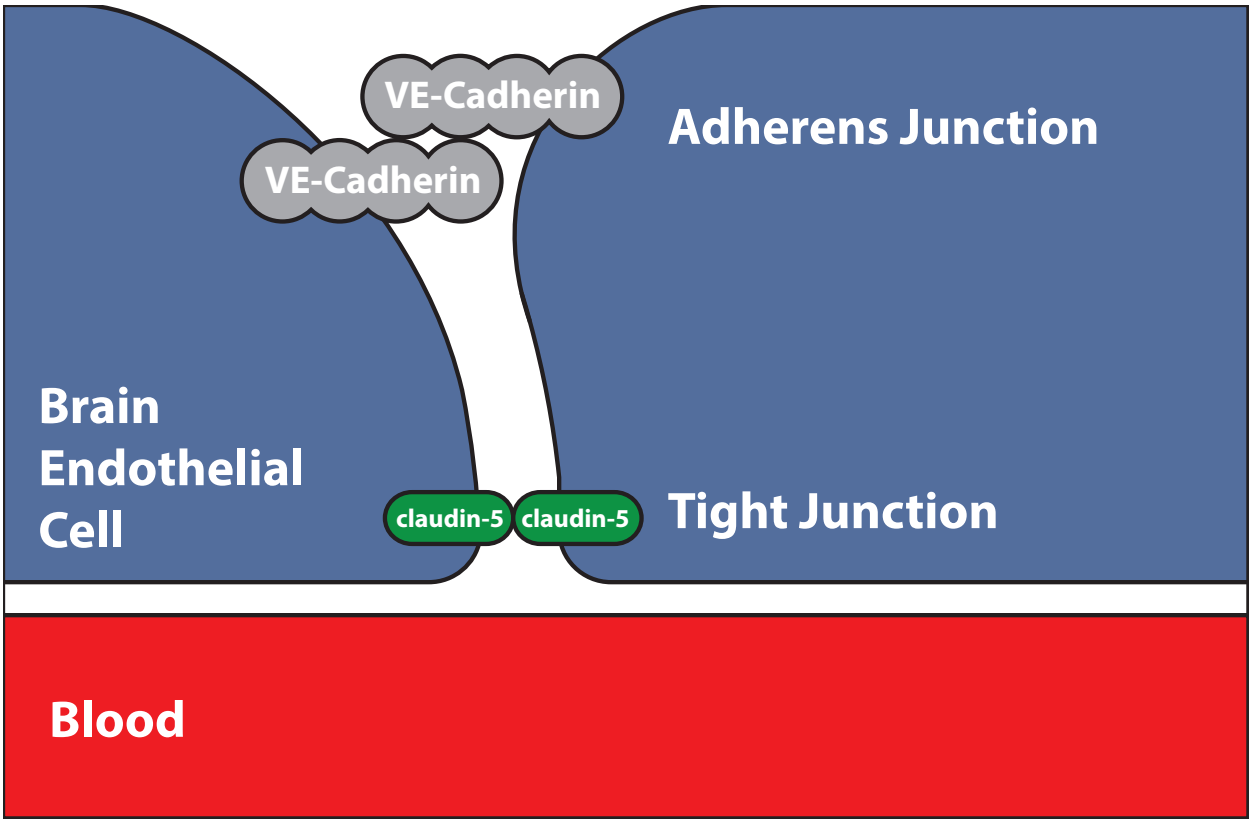

Figure 4. Schematic representation of the major cell-cell junctions expressed in brain microvascular endothelial cells.

Tight junctions in the brain are composed primarily of structured claudins and provide the solute restrictive barrier between endothelial cells. AJs composed of VE-Cadherin in brain endothelial cells are required for tight junction formation and provide structural stability to the cell-cell contact. See main text for details.

Recently, increases in blood-brain barrier permeability were observed in CD2AP knockout mice (Cochran et al., 2015). Therefore, a possible impact of CD2AP on AD pathogenesis could be its impact on the BBB integrity.

1.5 CD2AP in Alzheimer's Disease (AD)

1.5.1 CD2AP levels and polymorphisms in AD

AD is the most prevalent form of dementia in the elderly (2015). More then 38% of people over 85 years old suffer from AD (2015). Despite recent advances in research, the only treatments available for patients suffering from the disease remain symptomatic (Hogan, 2014). Therefore, every effort to understand the basis of the disease is considerable. The exact cause for the disease remains undetermined for most cases, however several modifiable and non-modifiable risk factors for the disease have been identified, giving clues about the disease progression. Genetic factors play a pivotal role in the development of AD. Twin studies revealed that heritability accounts for 60 to 80% of AD cases (Bergem et al., 1997; Gatz et al., 2006; Raiha et al., 1996). The genotype for apolipoprotein E (*APOE*) protein is the principal genetic risk factor for late-onset AD. Carrier of the epsilon 4 allele have up to a 15 times greater risk for developing AD (Farrer et al., 1997). However, *APOE4* carriers account for 50% of AD cases (Tanzi, 2012), indicating that other genetic factors could influence AD risk. Recent GWAS studies were able to identify several common single polymorphism variants that affect AD risk. Interestingly, An association between CD2AP polymorphism rs9349407 and AD was found in two GWAS study (Hollingworth et al., 2011; Naj et al., 2011) and that association was later confirmed by a meta-analysis (Chen et al., 2015). A very recent study confirmed that the

previously described SNP: rs9349407 and a SNP not previously described: rs9296559 were associated with increased AD risk (Tao et al., 2017). Importantly this study also showed for the first time that in human CD2AP gene expression is reduced in peripheral blood lymphocytes of AD patients compared to healthy controls (Tao et al., 2017).

1.5.2 CD2AP in amyloid beta (Aβ) production and clearance

Aβ is a peptide produced from the successive cleavage of amyloid precursor protein (APP) by β and γ-secretase (Goate et al., 1991; Zhang and Song, 2013). Soluble Aβ can assemble into insoluble fibrils and then aggregate in amyloid plaques (Burdick et al., 1992; Gravina et al., 1995; Kim et al., 2007). Amyloid plaques are a major hallmark for AD. The only mutations leading to autosomal dominant AD are linked to an increase accumulation of Aβ plaques (Harvey et al., 2003; Karch et al., 2014; Ryman et al., 2014). Plaques in the brain of AD patients are well correlated with the clinical manifestation of the disease (Blessed et al., 1968; Tremblay et al., 2007). Using 725 patients from the Religious Orders Study (ROS) and Rush Memory and Aging Project (MAP), Shulman showed that CD2AP polymorphism is associated to neuritic plaque pathological burden (Shulman et al., 2014).

Two mechanisms might explain the effect of CD2AP polymorphism on Aβ concentration. First, as discussed later in the section entitled cerebrovascular function, CD2AP knockout mice exhibit changes in the blood-brain barrier (Cochran et al., 2015). CD2AP polymorphism could therefore affect Aβ clearance from the brain. Another potential mechanism explaining the effect of CD2AP polymorphism on Aβ pathology is through endocytosis. It was previously shown that CD2AP is associated with clathrin-mediated endocytosis (Havrylov et al., 2008; Huber et al., 2003; Kobayashi et al., 2004; Schiffer et al., 2004; Wasik et al., 2012). Interestingly, several

studies showed that APP processing occurred in endocytic vesicles (Cole and Vassar, 2007; Vassar et al., 1999; Lah and Levey, 2000; Koo et al., 1996; Koo and Squazzo, 1994). Therefore, a change in CD2AP function or reduced expression could affect the production of Aβ in neuronal cell. Accordingly, a recent study, suppressing CD2AP expression using shRNA in N2a-APP695 cells results in decreased membrane-bound APP, increased intracellular Aβ40 and 42 and reduced Aβ40, Aβ42 and Aβ42/Aβ40 ratio in the extracellular media, suggesting that CD2AP might play a role in Aβ production (Liao et al., 2015). In the same study, using 1-month old APP/PS1 mice, the authors showed that complete loss of CD2AP in brain resulted in a decreased Aβ42/Aβ40 ratio in brain tissue lysates

1.5.3 CD2AP in tau pathology

Less is known concerning the link between CD2AP and tau pathology. A recent study showed that genetic manipulation of cindr, the fly ortholog of human CD2AP, robustly enhanced tau toxicity. Thus, the results suggest that cindr protects against tau retinal toxicity in drosophila, identifying a potential mechanism for the association of human CD2AP with AD susceptibility (Shulman et al., 2014). Therefore, further investigation in different models would be necessary to unveil the possible role of CD2AP in tau pathology.

1.5.4 Interactions between CD2AP and AD risk factors

The most important genetic risk factor for late onset AD is the genotype of *APOE* gene. Being a carrier of the *APOE4* allele increases the risk of AD by 15 times (Farrer et al., 1997), in addition to a reduction in the age of first symptom presentation (Corder et al., 1993). *APOE* genotype can affect almost all aspects of AD pathogenesis. *APOE4* carriage is associated with impaired Aβ clearance, reduced brain glucose uptake and impaired cerebrovascular function (Bell et al.,

2009; Castellano et al., 2011; Kim et al., 2009a; Kim et al., 2009b). Studies showed that the concentration of ApoE is reduced in the plasma and CSF of *APOE4* carrier (Cruchaga et al., 2012; Gupta et al., 2011). ApoE concentration in the plasma is associated with cognitive performance and plaque accumulation in the brain of patients (Shinohara et al., 2013). Recently a study revealed that the relation between Pittsburgh compound B (PiB) Positron Emission Tomography (PET) and plasma ApoE is lost in patients carrying the rs9349407 CD2AP polymorphism (Lazaris et al., 2015). One possible explanation for this loss of relation is that loss of CD2AP in carriers of the polymorphism might reduce the endosomal recycling of ApoE protein and Aβ clearance and consequently change the association between plasma ApoE concentration and PIB binding.

1.6 Endosomal Trafficking

1.6.1 Overview of the endocytic machinery

Endocytosis is a cellular process which is utilized to internalize fluids, solutes, macromolecules and plasma membrane components (Huotari and Helenius, 2011). This cellular process induces the invagination of the cell membrane and the formation of vesicles and vacuoles resulting from cell fission (Huotari and Helenius, 2011). Endosomes can sort, process, recycle, activate, silence and degrade substances and receptors and therefore are important regulators of several cellular pathways (Huotari and Helenius, 2011). The basic endosome pathway has three major components: a recycling system for the membrane component, a degradative system for the digestion of macromolecules and a connective feeder pathway. The majority of cargo internalized in mammalian cells are recycled back to the plasma membrane in early endosomes. Every hour, about 50 to 180% of the area of the plasma membrane is recycled in a typical

mammalian cell (Steinman et al., 1983). Therefore, transport to lysosome for degradation through late endosome accounts for a minority of the internalised fluid and membrane component. Early endosomes are the main sorting station of the endocytic pathway. Early endosomes initially receive the incoming cargo through several pathways including caveolar, GEEC, and ARF6-dependent pathways (Mayor and Pagano, 2007). Vesicles are normally included in the early endosome for 10 min and are then recycled. A few vesicles stay for the life time of the early endosome to be include into the late endosomes (Maxfield and McGraw, 2004). Late endosomes form from early endosomes. After their formation late endosomes undergo a series of transformational processes including exchange of membrane components, movement to the perinuclear area, a shift in composition of fusion partners, formation of additional intralumenal vesicles (ILVs), a drop in lumenal pH, acquisition of lysosomal components, and a change in morphology. Most mature late endosomes undergo a fusion with lysosomes. However, a few of them contribute to the formation of lysosomes (Luzio et al., 2007, #12192)

Endosomes are very mobile in the cell. Their distance from the nucleus is related to their maturation stage and function (Collinet et al., 2010). Endosomes in the periphery are generally small and numerous and endosome closer to the nucleus are generally larger and in a smaller number (Collinet et al., 2010). The endosomes move from the periphery to the nucleus along the microtubules using dynein and kinesin motor proteins (Murray et al., 2000); (Soppina et al., 2009).

1.6.2 The role of the Rab GTPases

The endocytic pathway is a tightly regulated and conserved cellular process in which the plasma membrane and its associated components are internalized and recycled to achieve proper cell membrane composition and homeostasis. In this pathway, early endosomes are a considered a major hub where sorting of internalized cargo is performed (D'Souza et al., 2014). Cargo can be recycled back to the plasma membrane, to the Trans-Golgi Network (TGN), or be routed to lysosomes for degradation depending and the regulation of where cargo ends up is regulated by various components of the pathway (D'Souza et al., 2014). Here the focus will be on the small rab GTPases.

In an elegant study it was demonstrated that cargo moves through distinct domains on endosomes and that these domains are occupied by different Rab GTPases (Sönnichsen et al., 2000). This revealed compartmentalization of endosomes within the same continuous membrane and that differential compartmentalization conferred distinct identity and function to the endosome (Sönnichsen et al., 2000).

Indeed, the prevailing functional model for the Rab GTPases is that they are required for conferring the structural and functional identity of organelles in the endosomal pathway (Wandinger-Ness and Zerial, 2014). Their role in this capacity depends on a number of functions.

Firstly, it relies on the cycling of bound GTP/GDP by interacting proteins, which activates and inactivates them respectively. This GTP/GDP mediated regulation is required for the properly localized assembly of multiprotein machineries on the cytoplasmic surface of the various endosomal compartments (Wandinger-Ness and Zerial, 2014).

Secondly, Rab GTPase can exist as either a soluble or integral-membrane protein depending on its state. Rab GTPases are kept soluble in the cytosol and in the inactive conformation through association with Rab GDP-dissociation inhibitors (Rab-GDI) (Gavriljuk et al., 2013); (Wandinger-Ness and Zerial, 2014). Conversly, prenylation of specific carboxy-terminal cysteine residues together with hypervariable regions on the protein serve to promote specific and stable membrane association (Wu et al., 2010); (Wandinger-Ness and Zerial, 2014).

Third, in the active, GTP-bound conformation, Rab GTPases bind effector proteins that carry out their downstream effects. The main function of Rab effectors is facilitating efficient membrane trafficking between compartments (Wandinger-Ness and Zerial, 2014).

Fourth, Rab GTPases regulate each other, and are regulated by a complex signaling network. This signaling network consists of downstream Rab GTPases, as well as upstream GTPases of separate subfamilies, namely the Arf, Rho, and Arl family GTPases (Wandinger-Ness and Zerial, 2014).

Through their diverse functionality Rab GTPase protein assemblies are tightly regulated in space and time, can vary over time, and are reversible, thus allowing for the modulation of the membrane composition and intracellular fate of endosomal cargo (Wandinger-Ness and Zerial, 2014).

1.6.3 Introducing the Rabs

In this section a few of the key Rab GTPases will be introduced and an overview of how they function together in the endocytic pathway will be given. Four of the most ubiquitously expressed Rab GTPases are Rab4, Rab5, Rab7 and Rab11 which will be the focus of this section. Rab5, Rab4 and Rab11 all function on the early endocytic pathway whereas Rab7 functions on

the late endocytic pathway (Wandinger-Ness and Zerial, 2014). Rab5 can also be detected on the cell membrane, where it also acts in the formation of clathrin-coated vesicles (CCVs), CCV fusion with early endosomes, and fusion between early endosomes (McLauchlan et al., 1998). Indeed, evidence suggests that Rab5 may in fact be required for clathrin-mediated endocytosis (McLauchlan et al. 1998). Rab7 however, acts downstream from Rab5 regulating transport from early to late endosomes and lysosomes (Jäger et al., 2004); (Wandinger-Ness and Zerial, 2014). The endocytic pathway in general consists of repetitive fusion and fission events between vesicles and endosomes, where endocytosed cargo is transported into early endosomes, becomes concentrated in fewer and larger endosomes, which move along microtubules towards the cell centre (Wandinger-Ness and Zerial, 2014). During this process the levels of Rab5 on the endosomes increase until they reach a peak, followed by the complete loss of Rab5 on the membrane and its replacement with Rab7. This conversion of Rab5 to Rab7 in the endocytic pathway marks the transition of cargo and endosome identity from early to late endosomes (Wandinger-Ness and Zerial, 2014).

Rab4 and Rab11 on the other hand regulate the transport of cargo along the recycling pathway, from early and recycling endosomes to the cell surface (Ullrich et al., 1996). Rab4 is involved in the recycling of integrins, receptors, ubiquitin ligases, and other transport machinery (Cui and Zhang, 2013), (Wandinger-Ness and Zerial, 2014). Rab11 and its downstream effectors have critical roles in cell polarity (Jing and Prekeris, 2009), (Wandinger-Ness and Zerial, 2014).

1.6.4 CD2AP as a Rab Effector

CD2AP was found to be an effector of the small GTPase Rab4, binding to GTP-bound Rab4 and be localizing to vesicular structures present throughout the cytoplasm as well as partially

aligned along actin fibers or ruffles. CD2AP was also found to induce enlargement of early endosomes when coexpressed with Rab4 or (Casitas B-lineage lymphoma) c-Cbl. The expression of CD2AP with Rab4 alters the morphology of late endosomes and interferes with the degradation of Platelet-derived growth factor (PDGF) receptors most likely by affecting the sorting of the receptors into the late endosomes (Cormont et al., 2003). This was the first indication of CD2AP's role in endosomal formation and regulation and suggests that CD2AP is involved in a sorting or transport step distal to early endosomes.

In another study it was found that in podocytes CD2AP associates with vesicles and dynamic actin and that CD2AP was required for the formation of these vesicles. It was also demonstrated that CD2AP was dynamically recruited to these vesicles indicating a tightly regulated process. They also showed that these vesicles were rab4 positive and had a "membranous sub-organization in the vesicle interior" indicating they were most likely Multi-Vesicular Bodies (MVB) of the late endosomal degradation pathway (Welsch et al., 2005). Interestingly, MVB formation is required for downregulation of activated signaling receptors (Katzmann et al., 2002) in which CD2AP plays an important role.

In addition, it was found that in neurons, CD2AP is associated with Rab5-bound endosomes and positively regulates TrkA (Tropomyosin receptor kinase A) localization to endosomes (Harrison et al., 2016). Finally, it was shown that CD2AP binds a Rab5-activating guanine nucleotide exchange factor RIN3. RIN3 was shown to recruit CD2AP to Rab5 positive early endosomes (Rouka et al., 2015).

Based on CD2AP's implication in the endosomal trafficking of receptors, the specialized trafficking functions of brain endothelial cells in the blood brain barrier (Abbott et al., 2010), as

well as CD2AP's enrichment in endothelial cells compared to other cells in the brain (Cochran et al., 2015), the as of yet uncharacterized role of CD2AP in the regulation of intracellular trafficking in brain endothelial cells remains an unanswered and intriguing question.

1.7 Vascular function and AD

1.7.1 Vascular impairments in aging and AD

Increasing evidence suggests that vascular degeneration is an integral part of AD pathogenesis (Girouard and Iadecola, 2006). It is estimated that between 60 and 90% of AD patients show vascular abnormalities (Bell and Zlokovic, 2009; Jellinger and Mitter-Ferstl, 2003). Vascular changes in AD patients include amyloid angiopathy, microinfarcts, ischemic lesions, changes in the blood-brain barrier and microvascular degeneration (Bell and Zlokovic, 2009; Jellinger and Mitter-Ferstl, 2003). Defects in the neurovascular unit can have a great impact on the modulation of Aβ uptake and clearance from the brain.

1.7.2 Neurovascular coupling and AD

The energy requirements of the brain vary with respect to changes in neuronal activity. In order to meet these demands, tightly regulated and rapid cerebral blood flow (CBF) adjustments are required to maintain cellular homeostasis and function (Enager et al., 2009); (Tarantini et al., 2016). This is achieved through a process called neurovascular coupling, which is carried out by an intercellular signaling network made up of neurons, astrocytes, smooth muscle cells/pericytes and microvascular endothelial cells (Chen et al., 2014); (Tarantini et al., 2016). It has been demonstrated in both human patients and rodent models that aging substantially impairs neurovascular coupling responses (Fabiani et al., 2014); (Balbi et al., 2015); (Tarantini et

al., 2016). Furthermore, AD patients and mouse models of AD have been shown to exhibit significant impairment of neurovascular coupling responses (Hock et al., 1997); (Rombouts et al., 2000); (Tarantini et al., 2016). There is also strong evidence that endothelial NO production contributes to neurovascular coupling (Toth et al., 2015). This is interesting as CD2AP is enriched in brain endothelial cells and is important for blood-brain barrier integrity (Zhang et al., 2014; Cochran et al., 2015).

1.7.3 Vascular clearance of Aβ

Low density lipoprotein receptor-related protein 1 (LRP1) modulates the transport of Aβ in neurons (Kanekiyo et al., 2011), astrocytes (Koistinaho et al., 2004), microglia (Laporte et al., 2004) and endothelial cells (Deane et al., 2004; Yamada et al., 2008). LRP1 knockout mice have a reduction of Aβ clearance from the brain (Kanekiyo et al., 2013). A reduction of LRP1 might also contribute to the accumulation of Aβ peptide in the brain of AD patients (Donahue et al., 2006; Miller et al., 2008). Interestingly, LRP1 expression levels are significantly reduced in brain endothelial cells in normally aging and AD in humans and animal models (Ramanathan et al., 2015). Aβ binds to LRP1 receptors and is then internalized in early followed by late endosome before being degraded in the cell (Hu et al., 2009; Li et al., 2012). Since CD2AP plays a role in endocytosis (Havrylov et al., 2008; Huber et al., 2003; Kobayashi et al., 2004; Schiffer et al., 2004; Wasik et al., 2012), altered CD2AP expression could impair transport and clearance of Aβ peptide out of the brain through this mechanism.

1.8 Reelin

1.8.1 Reelin in neuronal migration during development

Reelin is a large 400 kDa extracellular matrix glycoprotein that is produced and secreted by Cajal-Retzius cells (D'Arcangelo et al., 1995b; Ogawa et al., 1995) and is important for neuronal migration during development. Cajal-Retzius cells reside in the marginal zone of the cortex during development meaning full-length Reelin and its fragments are highly concentrated at the top of the developing cortex. In this capacity Reelin secretion controls the migration of cortical neurons from the ventricle to the cortical surface where they create an inside-out layering of the cortex (Nadarajah et al., 2001). Inside-out meaning that later migrating neurons migrate past the layer formed by earlier cells creating a new layer above (Nadarajah et al., 2001). Indeed, human mutations in the Reelin (*RELN*) gene cause neuronal migration defects, producing deficits in the structure and layering of neurons of the cortex and cerebellum (Hong et al., 2000); (Reddy et al., 2011). Lissencephaly (smooth cortex with lack of gyri and sulci), hippocampal abnormalities, and underdevelopment of the cerebellum are structural phenotypes associated with these mutations and are associated with clinical phenotypes of hypotonia (low muscle tone), ataxia (lack of voluntary control of muscle movement), and severe mental retardation (Hong et al., 2000); (Reddy et al., 2011). The lack of Reelin (D'Arcangelo et al., 1995a), its receptors Very Low Density Lipoprotein Receptor (VLDLR) and Apolipoprotein E Receptor 2 (ApoER2) (Trommsdorff et al., 1999), or the cytoplasmic adaptor protein Dab1 (Sheldon et al., 1997); (Howell et al., 1997) all result in the same phenotype, namely cerebellar dysplasia and scrambling of the neuronal layers in the neocortex (Weeber et al., 2002).

1.8.2 Reelin Signaling Pathways

The canonical Reelin receptors are ApoER2 and VLDLR (Hiesberger et al., 1999) which are both members of the Low-Density Lipoprotein (LDL) receptor family. Upon Reelin binding, the signaling cascade is initiated by the phosphorylation of Disabled-1 (Dab1) by Src and Fyn leading to the Dab1 activation of a number of downstream effectors depending on the site of Dab1 tyrosine phosphorylation (Feng and Cooper, 2009). The canonical Reelin signaling pathways in neurons are summarized and described in **Figure 5.**

1.8.3 Reelin is involved in long term potentiation

So far only Reelin signaling during development and neuronal migration has been discussed, however both Reelin receptors: ApoER2 and VLDLR, remain expressed in the brain during adulthood (Weeber et al., 2002). Upon investigation of Reelin signaling in the adult brain it was determined that Reelin signaling plays an important role in memory formation and long term potentiation. Knockout mice lacking ApoE/Reelin receptors VLDLR and ApoER2 have marked deficits in memory formation and LTP (Weeber et al., 2002). Also, it was demonstrated that Reelin greatly enhances LTP in hippocampal slice cultures (Weeber et al., 2002). These data imply a role for ApoE receptors and for Reelin in synaptic function as well as learning and memory, which most likely acts to enhance stability and maintenance of synapses in the brain (Weeber et al., 2002).

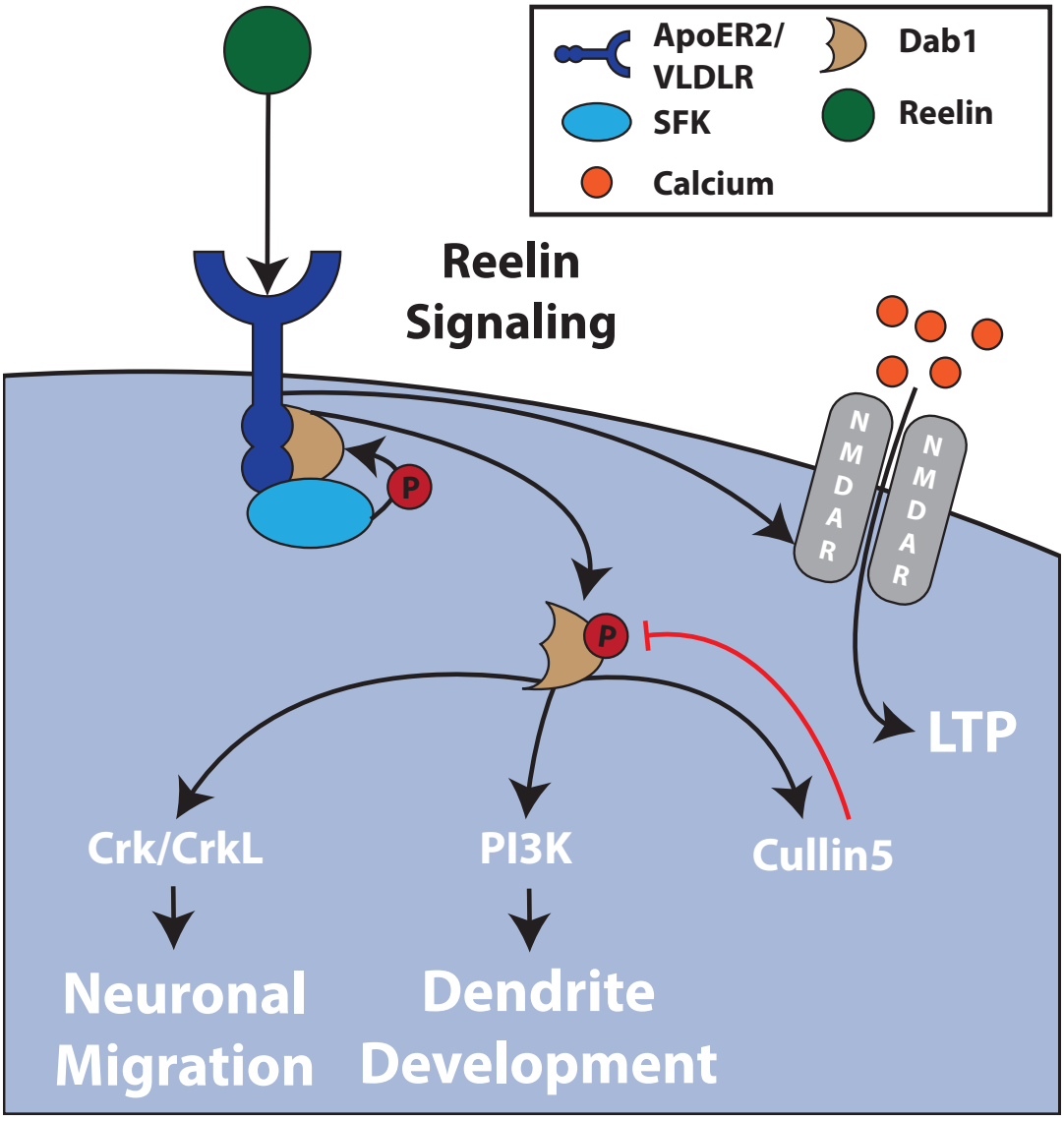

Figure 5. **Schematic representation of a model for the actions of Reelin signaling in neurons.**

Reelin binding to ApoER2 and the VLDL receptor stimulating Reelin signaling. Non-receptor tyrosine kinases of the Src family (SFKs) are thus activated which activate Dab1 which signals through multiple pathways including a positive feedback loop further activating SFKs. Dab1 activates the Crk/CrkL pathway which modulates adhesion molecules related to cell migration. It also activates PI3K-mTOR pathway implicated in dendrite and spine development. There exists a negative feedback loop via Cullin5 where Dab1 is ubiquitinated and degraded. Reelin signaling has also been shown to stimulate NMDA receptor activity, thereby increasing Ca^{2+} influx and LTP (Lee and D'Arcangelo, 2016).

It has been demonstrated that Reelin acts on NMDA receptor (NMDAR) signaling through ApoER2 (Herz and Chen, 2006), (Ishii et al., 2016). It has also been shown that an alternatively spliced variant of ApoER2 modulates NMDAR activity via Reelin signaling. In this mechanism, exon 19, which encodes a section of the intracellular domain of ApoER2 (ApoER2-ICD), is required for tyrosine phosphorylation of the NMDA receptor subunit NR2 in response to Reelin. Further, mice expressing a mutant ApoER2 lacking exon 19 displayed pronounced cognitive disturbances which were related to NMDAR function (Beffert et al., 2005); (Ishii et al., 2016).

Interestingly, the Reelin pathway also controls learning and memory via activation of transcriptional factors. Proteolytic cleavage of ApoER2 is a key component of the nuclear signaling activated by Reelin binding. In this cascade Reelin binds to ApoER2 causing the nuclear translocation of the ApoER2-Intracellular Domain (ICD). ApoER2-ICD then binds to transcription factors MEF2 and CREB in the nucleus, resulting in the expression of key genes involved in synaptic plasticity (Telese et al., 2015); (Ishii et al., 2016).

1.8.3 Disrupted ApoER2-Reelin signaling in AD

Evidence suggests that NMDA dysfunction is related to cognitive impairment in AD (Ishii et al., 2016). A study conducted by Chen *et al.* (2010) demonstrated that the Reelin-ApoER2-NMDAR pathway contributes to the pathophysiology of AD. They showed that ApoE4, reduces ApoER2 expression on the neuronal synapse after ligand-induced receptor endocytosis via Reelin binding, and that this impaired Reelin signaling mediated synaptic plasticity and NMDAR phosphorylation (Chen et al., 2010); (Ishii et al., 2016). In addition, it was uncovered that Reelin signaling protects against Aβ toxicity-induced synaptic dysfunction in the early stages of AD and

the associated impairment in learning and memory (Lane-Donovan et al., 2015); (Ishii et al., 2016).

1.8.4 ApoER2 interactions with *APOE* alleles

Studies performed using *APOE* transgenic mice found that long term potentiation (LTP) was significantly greater in animals expressing mouse ApoE or human ApoE3 than in ApoE knockout (Trommer et al., 2004); (Hoe et al., 2006). In another study it was shown that Oligomeric Aβ1–42 to a much greater extent than soluble Aβ1–42 inhibited long-term potentiation (LTP), and that the susceptibility reflected clinical ApoE isoform dependent AD risk where ApoE4 > ApoE3 = ApoE-KO > ApoE2 in terms of susceptibility (Trommer et al., 2005); (Hoe et al., 2006). These results taken together suggest a potential mechanism in which the promotion of memory dysfunction by ApoE4 might involve an impairment of ApoER2 dependent signaling pathways, thereby accelerating synaptic loss and the onset of AD (Weeber et al., 2002).

Furthermore, evidence suggests that ApoE stimulates Aβ production via the coordinated endocytosis of ApoE, ApoER2, APP, and β-secretase. This mechanism also involves an adaptor protein which facilitates complex formation between ApoER2 and APP. In addition, it was found that, ApoE4 produces more Aβ than does ApoE2 or ApoE3 in this system (He et al., 2007) providing an additional link between ApoER2 and AD.

Many effects of ApoE interactions with the cells of the vasculature have been elucidated. Despite ApoER2 and VLDLR being both ApoE receptors as well as Reelin receptors there little is known about Reelin signaling in the brain vasculature. In addition, CD2AP is highly enriched in brain endothelial cells which form blood vessels a major component of the neurovascular unit

and blood-brain barrier (Cochran et al., 2015). This presents a unique opportunity to probe the effect of Reelin signaling on brain endothelial cells and how CD2AP may contribute.

1.9 Hypothesis and Aims

Single nucleotide polymorphisms in the *CD2AP* gene confer an increased risk for AD (Hollingworth et al., 2011; Naj et al., 2011). Further, decreased levels of CD2AP are found in lymphocytes of AD patients. CD2AP is enriched in brain endothelial cells by ~20 fold (vs brain) and CD2AP knockout mice have increased blood-brain barrier permeability (Cochran et al., 2015). Brain endothelial cells represent a major component of the blood-brain barrier and neurovascular unit (Chen et al., 2014) that are both altered in AD (Di Marco et al., 2015; Girouard and Iadecola, 2006). Until now, the few studies related to CD2AP functions in the brain have been performed in neurons (Harrison et al., 2016; Liao et al., 2015; Ubelmann et al., 2017). Based on these rationales I present the following hypothesis:

> **Hypothesis:** CD2AP functionally interacts with proteins related to the pathogenesis of AD; loss of CD2AP function affects the biology of these proteins in brain endothelial cells.

We will particularly focus on:

1) LDL receptors (ApoER2, LRP1), PICALM for their known implication

2) Cell adhesion / tight junction molecules for their implication in basic CD2AP-mediated functions of cell-celli interactions

In order to test this hypothesis, I propose the following 3 aims:

1) To Investigate the roles of CD2AP on LDL receptors (ApoER2, LRP1), PICALM and cell adhesion molecules through loss of function studies in human (cell line) and mouse (primary cells) brain endothelial cells.
2) To investigate the effect of CD2AP-depletion on brain endothelial cell monolayer permeability.
3) To determine the mechanisms by which CD2AP regulates the expression of receptors and cell-cell adhesion molecules.

Chapter 2: Materials and Methods

2.1 Antibodies

Antibodies used in this study were: anti-CD2AP (Protein Tech, catalogue 51046-1-AP), anti-ApoER2 full-length (Abcam catalogue ab108208; rabbit), anti-ApoER2 fragment (Abcam catalogue ab86548; rabbit), anti-actin (mouse); anti-claudin5 (Life Tech catalogue 352500; mouse), anti-VE-cadherin (Santa Cruz catalogue sc6458; goat), anti-LRP1 (Abcam catalogue ab92544; rabbit), anti-rabaptin5 (BD Biosciences catalogue 610676; mouse), anti-GFP (Santa Cruz catalogue sc9996; mouse), anti-picalm (Santa Cruz catalogue sc6433; rabbit), anti-dynamin II (Abcam catalogue ab3457; rabbit), anti-PI3K (Transduction Laboratories catalogue P13020; mouse), anti-AKT (Santa Cruz catalogue sc-8312; rabbit).

2.2 Constructs and siRNA

Rab5 mutant constructs express full length GFP-tagged Rab5 cloned into a EGFP vector. The two mutants are single nucleotide substitution mutations (Q79L & N133I). Four siRNA's were used in total: one human CD2AP siRNA (Santa Cruz, sc-29984) two mouse CD2AP siRNA's. The first mouse CD2AP siRNA (Santa Cruz, sc-29985) was used for all experiments and all data presented is from this siRNA and the second siRNA ON-TARGETplus Cd2ap siRNA (Dharmacon, L-041647-01-0005).

2.3 Western Blotting

Cells were lysed directly on plate in RIPA Buffer (50mM Tris-Cl ph 8.0, 150mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1mM EDTA, 0.2mM PMSF, cOmplete™ tablet (Roche)) using a cell scraper, brains were lysed in RIPA Buffer using a Dounce tissue grinder.

Protein concentration was determined by DC assay (BioRad Laboratories) according to manufacturer's instructions. Proteins were separated using SDS-PAGE and transferred to PVDF membranes (Millipore) for western blot analysis. Membranes were blocked using 5% skim-milk powder and incubated overnight in primary antibodies. Secondary anti-HRP conjugate antibodies were used and immunoreactivity was visualized using Western LightingTM (PerkinElmer). Immunoreactivity was developed on film or using a ChemiDoc™ imaging system, band intensity was quantified using ImageJ or Image Lab and normalized to actin as a loading control. Values were then adjusted by normalizing control intensity to 100 and knockdown therefore expressed as a percent reduction from the control. Statistical analysis was performed as described in section 2.12.

2.4 Cell culture

2.4.1 hCMEC/D3

Cells were cultured as per supplier's protocol (EMD Millipore, SCC066) in EndoGRO™-MV Complete Media Kit (EMD Millipore, SCME004). Cells were cultured on Collagen I (EMD Millipore, 08-115) coated T75 75cm^2 culture flasks and passaged at 90% confluency. Cells were passaged via 1min incubation in trypsin-EDTA (0.25%) to detach cells, quenched with media, pelleted and resuspended in growth media and either passaged onto another T75 flask or seeded onto plates for experiments as described in section 2.6.1.

2.4.2 Mouse Primary Brain Endothelial Cell (BMEC)

Cells were purified and cultured as described in section 2.5. Cells were plated directly for experiments and not passaged as phenotype was lost with passaging.

2.5 Primary mouse brain microvascular endothelial cell (BMEC) purification

Mice were sacrificed and brains removed and placed in petri dish of ice cold PBS where cerebellum was removed and discarded. Meninges was removed by rolling brains on UV sterilized 12x12" kimwipes until meninges no longer visible. Brains were transferred to dry petri dish and diced thoroughly with a scalpel forming pieces about $1mm^3$ in size. Brain material was then added to enzymatic digestion buffer (250 U/ml collagenase type II (Worthington, LS004174), 10 U/ml dispase (Worthington, LS02100), 10 U/ml DNAase (Worthington, LS006343) in DMEM) and incubated for 90 mins at 37°C. After digestion brain material was pelleted by centrifugation at 200g for 3 mins, supernatant aspirated and pellet resuspend in 4°C DMEM and pelleted again at 200g for 3 mins. Supernatant was aspirated and brain material resuspended in DMEM + 20% BSA and homogenized by passing through a 5ml pipette followed by a flame-rounded Pasteur pipette. Suspension was then centrifuged at 1500g for 20mins at 4°C pelleting the brain capillaries. Supernatant and floating white matter was aspirated and capillaries resuspend in Medium 131 (Gibco, M-131-500) + MVGS supplement kit (Gibco, S00525) and passed through a 70um cell strainer to dissociate the capillary cells. Capiillary cell suspension was then supplemented with 4ug/ml of puromycin (SigmaAldrich, P8833-10MG) plated on collagen IV (Santa Cruz, sc-29010) coated culture plates and placed in a CO_2 incubator at 37°C. After 2 days the media is changed to puromycin-free growth media and cells are cultured without additional media changes until reaching appropriate confluency.

2.6 Transfection

2.6.1 hCMEC/D3 Cells

hCMEC/D3 Cells were seeded at 42,000 cells/cm^2 and transfected 24 hours later, yielding a confluency of about 50%. Transfection was performed with lipofectamine 2000 (Invitrogen, 11668027) reagent. For one well of a 24-well plate (1.9cm^2 growth area) 2uL of lipofectamine 2000 was added to 50uL of Opti-MEM (Gibco, 31985062) and incubated at RT for 30mins. This solution was then added to a solution of 50uL of Opti-MEM + 5uL of 10uM siRNA and/or 500ng DNA and incubated for 30mins at RT. Cell media was aspirated and replaced with 500uL of Opti-MEM followed by dropwise addition of 100uL of transfection solution and placed back in 37^oC CO_2 incubator. Transfection media was then aspirated and replaced with growth media after 3 hours. Cells were either harvested, fixed or used for assays 48 hours post-transfection.

2.6.2 Mouse Primary BMECs

Mouse primary BMECs were transfected 5 days after plating, yielding a confluency of about 50%. Transfection was performed with HiPerFect (QIAGEN, CA) reagent. For one well of a 24-well plate (1.9cm^2 growth area) 3uL of HiPerFect and 1.692uL of 10uM siRNA were added to 100uL of Opti-MEM (Gibco, CA) and incubated at RT for 10mins. Cell media was aspirated and replaced with 100uL of Opti-MEM followed by dropwise addition of 100uL of transfection solution and placed back in 37^oC CO_2 incubator. Transfection media was then aspirated and replaced with growth media after 3 hours. Cells were either harvested, fixed or used for assays 7 days post-transfection.

2.7 Immunoprecipitation

2.7.1 Cell Lysate

Working with plate on ice, ice-cold homogenization buffer (experiment-dependent formulation, see results section) was added to cells after rinsing with PBS. Cells were scraped thoroughly with a cell scraper to detach and lyse cells. Cell lysate was then sonicated, centrifuged at 13,000 rpm, decanted off pellet to fresh tube and protein concentration was measured by DC assay (BioRad Laboratories). 1mg of protein completed to 500uL with homogenization buffer was added to 10uL of packed beads: Protein G Sepharose 4 Fast Flow (GE Healthcare Life Sciences) for mouse antibodies or Protein A Sepharose 4 Fast Flow (GE Healthcare Life Sciences) for rabbit antibodies and inverted at 4^{o}C for 1hr as a preclear step. At the same time 1ug of antibody was added to 500uL of IP Wash Buffer (20mM Tris-Cl ph 7.5, 150mM NaCl, 0.1% Triton X-100, 0.1% NP40, 0.5mM EDTA, 0.2mM PMSF, cOmplete™ tablet (Roche)) and added to 10uL of packed beads and inverted at 4^{o}C for 1hr to bind antibodies to the beads. Tubes were centrifuged to pellet beads and antibody solutions were aspirated and replaced with precleared cell lysate and inverted at 4^{o}C for 2hr to immunoprecipitates protein of interest. Beads were rinsed 3 times with IP Wash Buffer and 40uL of 2x Laemlli Buffer was added to packed beads and boiled for 5 minutes at 95^{o}C pelleted and supernatant used for western blot.

2.7.2 Mouse Brain Homogenate

All steps identical to cell lysate IP except that whole mouse brain is removed and homogenized in a Dounce tissue grinder on ice. Homogenate was fractionated into two fractions. First fraction was generated by homogenizing in BF1 buffer (150mM NaCl, 1% NP-40, 50mM Tris-Cl (pH 8.0), cOmplete™ tablet (Roche)) and centrifuged for 15 mins at 12,000g. The supernatant

was designated as brain fraction 1 and the pellet was resuspend in BF2 buffer (150mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50mM Tris-Cl (pH 8.0), cOmplete™ tablet (Roche)) and centrifuged at 10,000g and the supernatant designated as brain fraction 2.

2.8 Cell monolayer permeability assay

Cells were seeded and transfected as described in section 2.6 on either 0.4um or 1um pore-sized PET trans-well inserts (EMD Millipore, MCHT24H48 or MCRP24H48). Media in both chambers of the insert was then aspirated and 500uL of media + 50ug/mL sodium fluorescein (Sigma-Aldrich, F6377) was added apically and 1700uL media without sodium fluorescein was added basally (for hCMEC/D3 cells growth media is used and for mouse primary BMECs phenol red-free Opti-MEM (Gibco, 11058021) is used). The cells were returned to the incubator and every 30 mins 400uL of media was removed from the basal side and replaced with fresh media. The concentration of sodium fluorescein from this 400uL sample was determined by measuring the absorbance at 480nm using a spectrophotometer. The permeability of the monolayer to sodium fluorescein was then calculated based on these concentrations (Gaillard et al., 2001) and permeability between conditions was compared using the unpaired Student's t-test.

2.9 Immunostaining

Cells were rinsed in HBSS and fixed in 4% PFA for 15mins at room temperature. Cells were then permeabilized in 0.5% Trition X-100 in PBS for 10 mins at room temperature with gentle agitation. Cells were then incubated with primary antibody diluted in 0.1% Triton X-100 in PBS overnight. Cells were then washed 3 times for 5mins with PBS, then incubated in secondary antibody diluted in 0.1% Trinton X-100 in PBS for 2hrs. Cells were washed 2 times for 5mins then incubated in 1ug/mL DAPI in PBS, rinsed once with PBS for 5mins and mounting media was

added to coat the bottom of the well and allowed to dry overnight at room temperature protected from the light.

2.10 Fractionation

Mouse brain or cultured endothelial cells were homogenized in (10mM HEPES, 0.32M sucrose, 0.2 mM PMSF, 1 mM EGTA, and a cOmplete™ tablet (Roche)). The homogenate was then centrifuged at 1000g at 4 °C for 10 min. Separated from the pellet (P1), the supernatant (S1) was then centrifuged again for 15 min at 12,000g. The resulting pellets (P1 & P2) corresponds to nuclear, mitochondrial and membrane bound protein whereas the supernatants (S1 & S2) corresponds to the cleared soluble cytosolic fraction.

2.11 Reelin Production and Purification

Reelin was produced by purifying conditioned media from stably transfected HEK 293 cells which excrete Reelin. These cells were cultured in low glucose DMEM (Gibco, 11885084) which was switched to phenol red-free Opti-MEM once the cells were confluent. After 3 days the conditioned Opti-MEM was harvested and centrifuged at 5000g for 15mins to pellet any cell debris. The clarified media was then sterile filtered and concentrated by 30 times using Amicon Ultra-15 100kDa Centrifugal Filter Units (EMD Millipore). The concentrate was diluted in PBS and concentrated again using an Amicon Ultra-4 50kDa Centrifugal Filter Unit (EMD Millipore) to replace media with PBS while retaining the dissolved Reelin. The concentration of Reelin was measured by DC Assay (BioRad Laboratories) and the presence and purity of Reelin confirmed by western blot.

2.12 Statistical Analysis

All data are presented as Mean ± SEM. Results of statistical analysis (Mean, t-value, degree of freedom and p-value) are presented in the text. For western blot analysis each sample was compared to a theoretical mean of 100 using a one-sample t-test (as values for control and knockdown in each experiment were normalized to yield a value of 100 for the control). For the permeability assay an unpaired two-sample t-test was used. Statistical significance was set at $p<0.05$. All statistical analyses were performed with Prism 7 (GraphPad software, San Diego, CA, USA) software.

Chapter 3: Results

3.1 Characterization of *in-vitro* altered protein expression in CD2AP-depleted human brain endothelial cell line

3.1.1 CD2AP siRNA-induced knockdown

In order to determine potential roles for CD2AP in brain microvascular endothelial cells a loss of function approach was utilized where CD2AP expression was knocked down and the effect that this has on the cell's biology/function was observed. This is an approach widely used to uncover unknown roles for a protein of interest but is especially relevant for the purposes of this study. This is because this study aims to elucidate CD2AP's impact on AD pathology and to that effect, it has been shown that: CD2AP gene expression is reduced in human AD patients (Tao et al., 2017), CD2AP protein levels in the brain are reduced in AD patients (Frédéric Calon Lab) lower CD2AP levels correlate with increased Tau pathological burden (Frédéric Calon Lab).

In order to utilize this powerful approach, it was first determined whether CD2AP expression could be knocked down via siRNA transfection in a human brain endothelial cell line. The human brain endothelial cell line hCMEC/D3 is a well characterized *in vitro* model of human brain microvascular endothelial cells. hCMEC/D3 cells were cultured on collagen I coated plates and transfected with a pool of three siRNA oligomers selectively targeting different sections of human CD2AP mRNA. Western blot analysis of the cell lysate two days post-transfection revealed that CD2AP protein expression was decreased on average by 71.86%; (Mean = 28.14, $t(8) = 21.58$, $p < .0001$), in cells transfected with CD2AP siRNA versus cells transfected with control siRNA **(Figure 6)**.

This result indicates that CD2AP protein levels can be robustly reduced in this system allowing for effective loss of function studies.

3.1.2 Altered expression of VE-Cadherin and claudin-5

Based on a study performed by Cochran *et al.* (2015) showing that CD2AP knockout mice exhibit increased permeability across the BBB (Cochran et al., 2015) protein expression of critical barrier forming cell-cell adhesion molecules was investigated. Interestingly it was found that cells with decreased CD2AP expression had lower levels of the adherens junction (AJ) forming protein, VE-cadherin compared to the control (Mean = 62.04, $t(8) = 6.543$, $p = .0002$). Conversely CD2AP-depleted cells had increased levels of the tight junction forming protein, claudin-5 (Mean = 141.5, $t(5) = 3.469$, $p = .0179$) **(Figure 6)**.

3.1.3 Altered expression of LRP1

Mutations in CD2AP have been shown to confer increased risk for AD by genome wide association studies (Hollingworth et al., 2011; Naj et al., 2011). LRP1 is a receptor expressed by the brain vasculature and is responsible for clearance of Aβ from the brain to the blood. LRP1 modulates the transport of Aβ in endothelial cells (Deane et al., 2004; Yamada et al., 2008) and LRP1 knockout mice have a reduction of Aβ clearance from the brain (Kanekiyo et al., 2013). Based on LRP1's clear implication in Aβ clearance through endothelial cells the effect of CD2AP-depletion on LRP1 expression was evaluated. It was determined that in CD2AP-depleted cells, protein levels of full length LRP1 was reduced (Mean = 44.92, $t(8) = 13.36$, $p < .0001$) while a lower molecular weight LRP1 band was increased (Mean = 272.6, $t(8) = 8.008$, $p < .0001$) **(Figure 6)**. One possible explanation is that CD2AP reduction leads to the proteolytic cleavage of LRP1. Interestingly, this lower molecular weight LRP1 fractionates in the soluble fraction exclusively

while the full length exists in both soluble and membrane associated fractions and is enriched in P1 **(Figure 7)**.

3.1.4 Reduction of ApoER2

Based on CD2AP's implication in AD and its established role in receptor endocytosis and degradation, the possibility that CD2AP alters expression of other AD implicated receptors was entertained. One such receptor ApoER2 is an ApoE and Reelin receptor. ApoE is a lipoprotein with well established roles in AD pathophysiology and Reelin is an excreted protein whose signaling through Reelin receptors VLDLR and ApoER2 has been shown to protect against Aβ toxicity-induced synaptic dysfunction in AD (Lane-Donovan et al., 2015; Ishii et al., 2016). Through siRNA knockdown of CD2AP it was shown that CD2AP-depleted hCMEC/D3 cells exhibit decreased expression of full length ApoER2 (Mean = 53.56, $t(8) = 7.006$, $p = .0001$) **(Figure 7).**

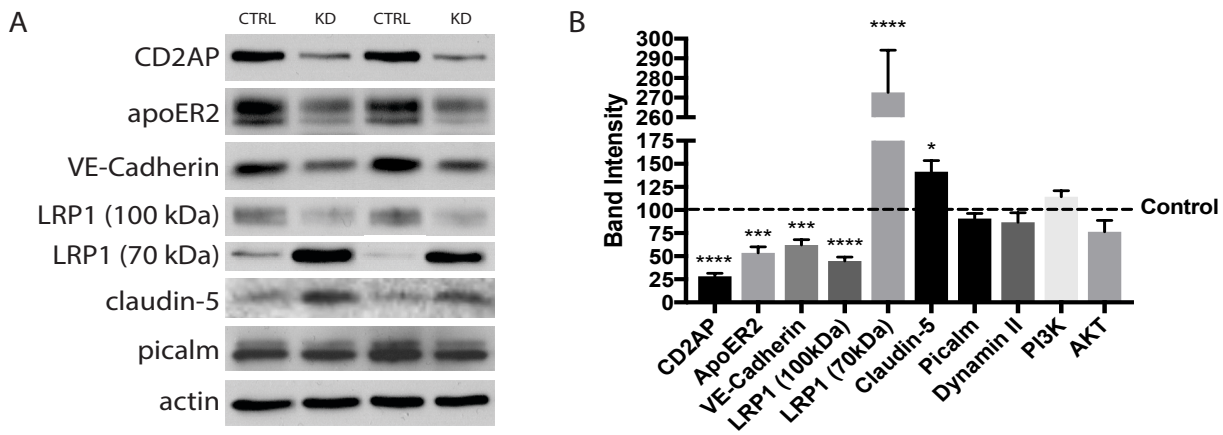

Figure 6. **Western blot analysis of CD2AP-depleted hCMEC/D3 cells.**

(A) representative western blot images showing protein expression for two experiments each of control siRNA transfected cells (CTRL) and CD2AP siRNA transfected cells (KD). Only blots of significantly altered protein expression is shown except for PICALM and actin. (B) quantification of the western blot data of CD2AP siRNA transfected cell lysate. Intensity was normalized to actin expression and adjusted to yield control intensity of 100 for each protein. Mean values are shown an error bars represent standard error of the mean. P-value is represented by number of stars (**** = p < .0001, *** = p < .001, ** = p < .01, * = p < .05).

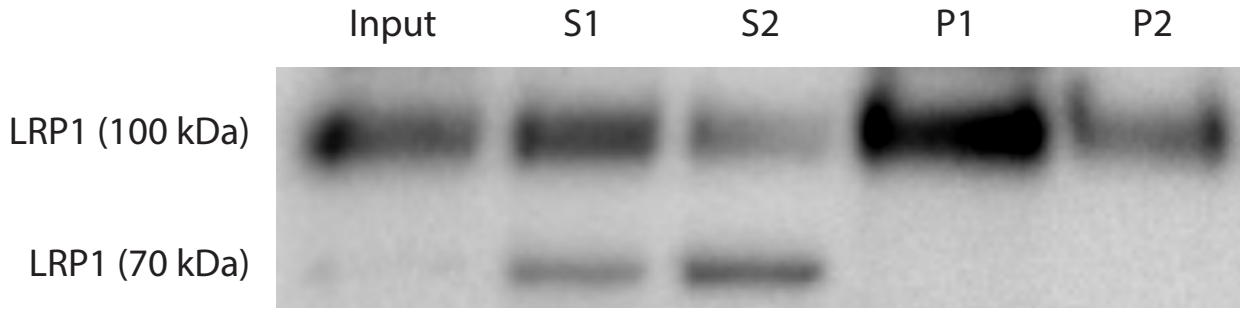

Figure 7. **Western blot image of fractionation experiment.**

Input represents the non-fractionated cell lysate, S1 & S2 represent the two supernatant fractions and P1 & P2 represent the two pellet fractions.

3.2 Characterization of *in-vitro* altered protein expression in CD2AP-depleted primary mouse brain endothelial cells

3.1.1 Primary brain microvascular endothelial cell culture validation

Next, validation of the results obtained from hCMEC/D3 were carried out in primary mouse brain microvascular endothelial cells (BMEC). In order to obtain a culture of these primary cells they had to be purified from mouse brain which has not been published as a protocol that does not require use of an ultracentrifuge. A working protocol was adapted from a capillary extraction protocol provided to us by a collaborator's lab (Dr. Frédéric Calon's Lab) and a published rat brain endothelial cell purification protocol (Perrière et al., 2005) and optimized through multiple iterations. The cultures were then assessed for purity through western blot and immunostaining for presence of various endothelial specific markers (VE-cadherin and claudin-5) and absence of contaminating cell markers (GFAP, CaMKII, GAP43) **(Figure 8)**. Interestingly CD2AP protein levels are markedly enriched in this brain endothelial culture relative to whole brain.

3.1.2 CD2AP siRNA-induced knockdown

Primary cells are notoriously difficult to transfect which held true for these primary mouse BMECs. It was found that lipofectamine 2000 and lipofectamine 3000 reagents as well as electroporation were highly cytotoxic and that HiPerFect was the only transfection reagent that was viable (data not shown). Once a protocol was optimized for transfection, cells transfected with mouse CD2AP siRNA were depleted of CD2AP protein by an average of 79.91% (Mean = 20.09, $t(8) = 18.45$, $p < .0001$), compared to control siRNA **(Figure 9)**.

3.1.2 Reduction of VE-Cadherin

Next, the decrease in VE-Cadherin levels were replicated in the primary mouse BMEC system. It was found through western blot analysis that CD2AP-depleted mouse primary BMECs had a significant reduction in VE-Cadherin compared to control (Mean = 83.69, $t(7) = 4.289$, $p = .0036$). These data are consistent with the results observed in hCMEC/D3 cells.

3.1.3 Altered expression of LRP1

In addition, the effect on LRP1 expression was investigated. Surprisingly, and opposite to the effect seen in hCMEC/D3 cells, CD2AP-depleted mouse primary BMECs showed a significant increase of the full length LRP1 (Mean = 143.5, $t(6) = 3.967$, $p = .0074$) and a decrease of the lower molecular weight LRP1 band compared to the control (Mean = 53.33, $t(7) = 5.496$, $p = .0009$). **(Figure 9)**. This indicates that the CD2AP-depletion mediated effect for LRP1 processing is species or at least cell-type dependent

3.1.4 Reduction of ApoER2

Finally, the effect of CD2AP depletion on ApoER2 was analyzed. The results from western blot analysis show that full length ApoER2 was significantly reduced in CD2AP-depleted cells compared to the control (Mean = 61.92, $t(7) = 4.289$, $p = .0036$) **(Figure 9)**. This result is in agreement with the results obtained from hCMEC/D3 cells and was the most robust effect observed leading to the investigation into its potential mechanism.

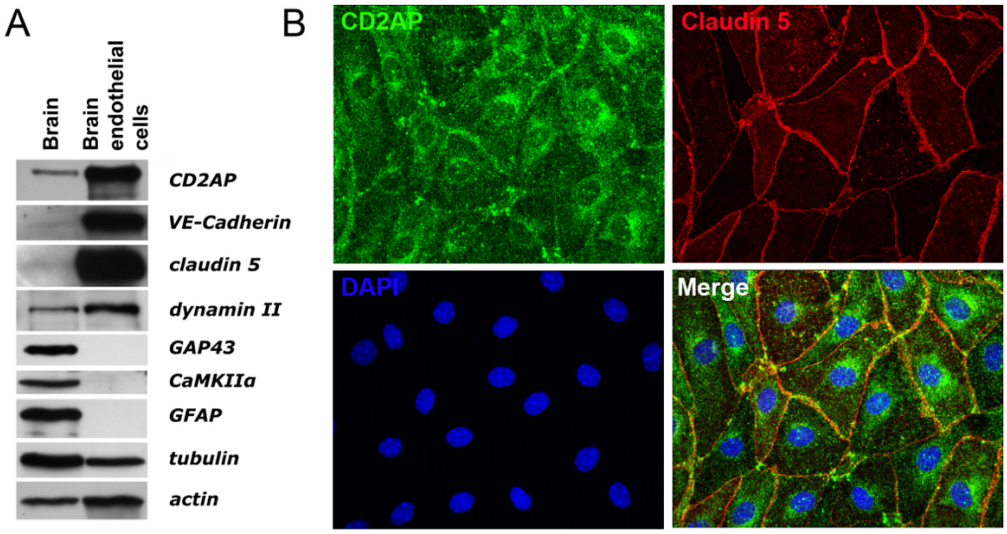

Figure 8. **Validation of culture purity and endothelial cell specific markers.**

(A) Western blot showing that culture is enriched in endothelial cell markers: VE-Cadherin and claudin-5. Absence of neuronal markers GAP43 and CaMKII and astrocytic GFAP confirm absence of contaminating cells. (B) immunostaining of confluent monolayer of purified BMEC culture showing the expression of functional tight junctions expressed at cell-cell contacts.

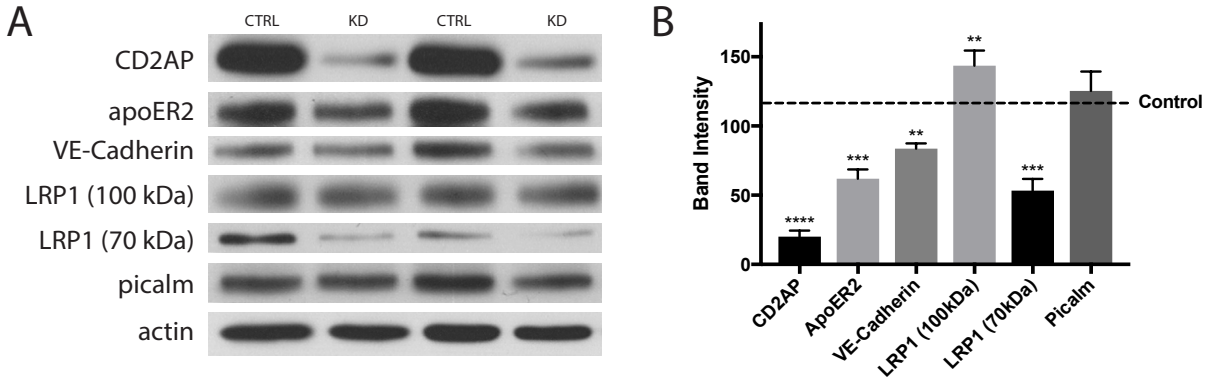

Figure 9. **Western blot analysis of CD2AP-depleted mouse primary BMECs.**

(A) representative western blot images showing protein expression for two experiments each of control siRNA transfected cells (CTRL) and CD2AP siRNA transfected cells (KD). (B) quantification of the western blot data of CD2AP siRNA transfected cell lysate. Intensity was normalized to actin expression and adjusted to yield control intensity of 100 for each protein. Mean values are shown and error bars represent standard error of the mean. P-value is represented by number of stars (**** = $p < .0001$, *** = $p < .001$, ** = $p < .01$, * = $p < .05$).

3.3 Investigation into mechanism driving CD2AP-depletion induced reduction of ApoER2

3.3.1 CD2AP interacts with ApoER2 in human endothelial cells and whole mouse brain

Since CD2AP can promote endocytosis of receptors, and acts as an adapter protein, it was hypothesized that it may affect levels of ApoER2 through a protein-protein interaction. In order to investigate whether ApoER2 and CD2AP interact, an immunoprecipitation was performed on hCMEC/D3 cell lysate harvested in four different buffers of varying detergent compositions. CD2AP was precipitated using a CD2AP antibody bound to protein A sepharose beads and the eluted protein was analyzed through western blot analysis. In all four conditions ApoER2 was detected in the precipitate indicating for the first time to our knowledge that ApoER2 co-precipitated with CD2AP **(Figure 10B)**. In addition, this result revealed a novel interaction between CD2AP and dynamin II which although unexpected, was unsurprising considering CD2AP's role in receptor endocytosis. In order to confirm the ApoER2 interaction, the experiment was repeated with additional controls. ApoER2 protein immunoprecipitated with CD2AP antibody bound beads but not not antibody-free protein A or protein G beads eliminating the chance that ApoER2 simply bound non-specifically to the beads **(Figure 10A)**. Finally, the co-immunoprecipitation was replicated in whole mouse brain homogenate that was separated into two fractions by differential centrifugation. In both fractions CD2AP antibodies immunoprecipitated both CD2AP and ApoER2. In addition, in the first fraction (low speed centrifuge clarified supernatant representing the soluble cytosolic fraction) the reverse co-immunoprecipitation was observed with ApoER2 antibodies pulling down both ApoER2 and CD2AP **(Figure 10C)**. These data indicate that CD2AP may act in complex with ApoER2 in cells.

Taken together with the results showing a decrease of ApoER2 upon CD2AP depletion these data may indicate a functional link between ApoER2 and CD2AP.

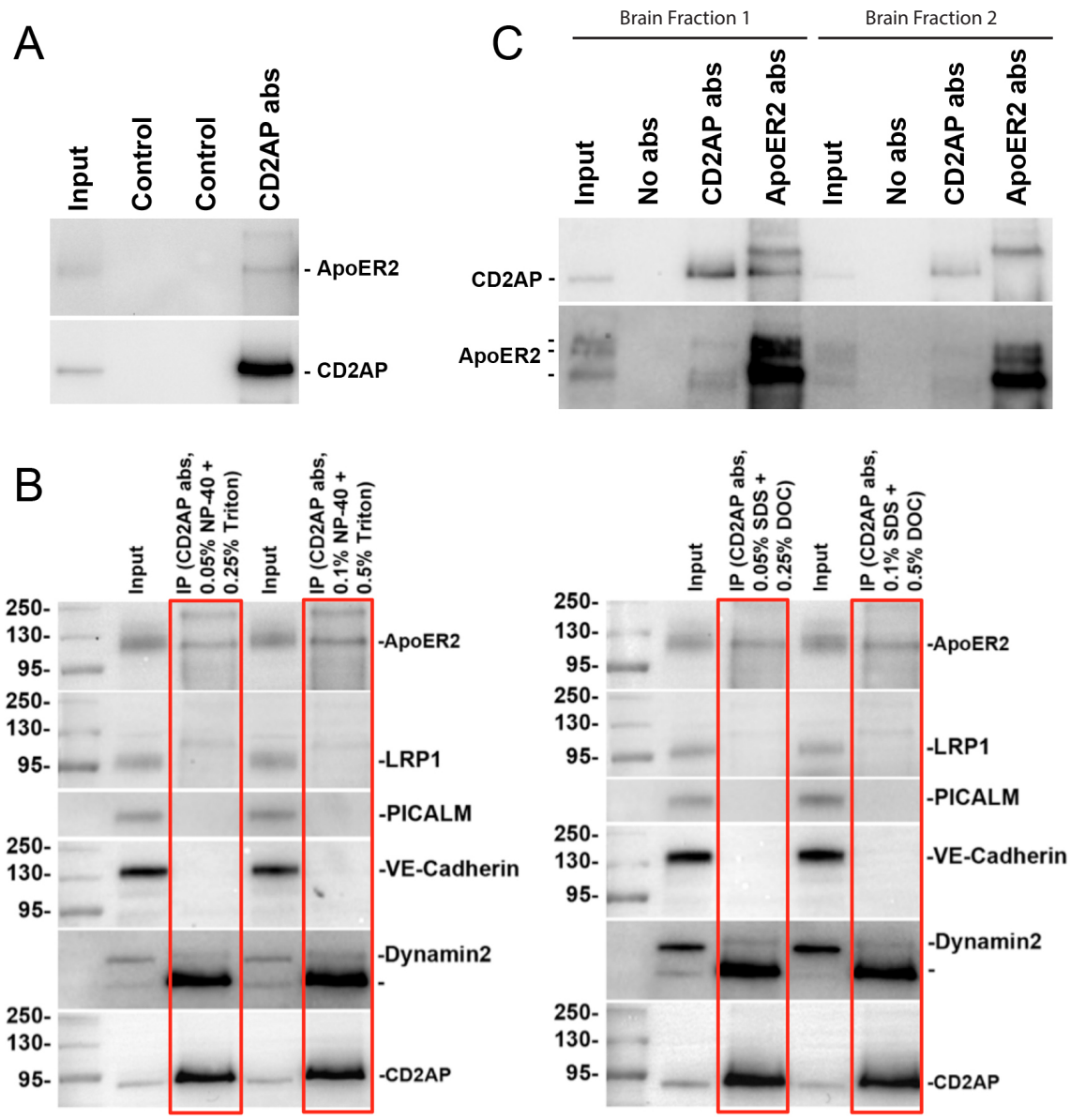

Figure 10. **Co-immunoprecipitation results showing interaction between CD2AP and ApoER2 in hCMEC/D3 cell lysate and mouse brain homogenate.**

(A) Input represents hCMEC/D3 lysate, first control is IP with antibody-free Protein A, second control is IP with antibody-free Protein G and last lane shows that ApoER2 is pulled out by CD2AP antibodies. (B) Same experiment done with different detergent compositions for the homogenization buffer. CD2AP antibodies pull out ApoER2 and dynamin II but not LRP1, PICALM or VE-Cadherin. (C) Same experiment performed on mouse brain homogenate. Brain fractions are the two different centrifugal separated fractions (as described in section 2.7.2).

3.3.2 Rab5 activity modulates CD2AP depletion-induced reduction of ApoER2

Based on the finding that CD2AP and ApoER2 exist in a complex and considering that CD2AP has been implicated in receptor endocytosis and subsequent degradation it was hypothesized that CD2AP depletion altered endosomal trafficking within the cell and that this was the cause of the ApoER2 reduction. In order to study this, wild-type, overactive (Q79L) and inactive (N133I) mutants of a key endosomal trafficking regulator, Rab5 was cotransfected with CD2AP siRNA to determine whether the CD2AP knockdown-mediated reduction processing of ApoER2 could be blocked. Overexpression of WT Rab5 (producing an overall modest increase in activity) or overexpression of the inactive enzyme have no effect on the levels of ApoER2 cleaved fragment in brain endothelial cells. In contrast, ectopic expression of an overactive Rab5 mutant reduces the levels of cleaved ApoER2 fragment **(Figure 11)**. It noteworthy that CD2AP knockdown led to decreased expression of all three cotransfected Rab5 constructs. Taken together, these results indicate that CD2AP loss reduces exogenous Rab5 levels, and most importantly, increasing Rab5 activity is sufficient to rescue ApoER2 cleavage.

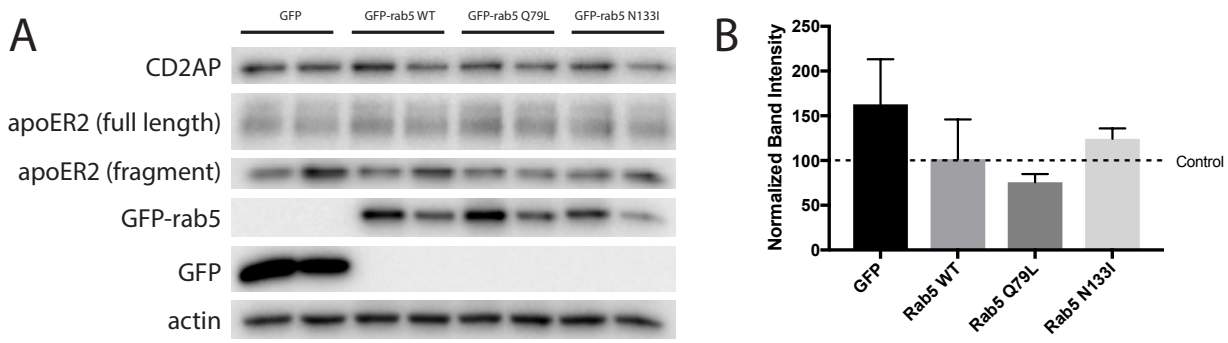

Figure 11. Rab5 WT and mutant co-transfection experiment.

(A) Western blot of Rab5 cotransfection experiment. For each condition of transfected construct, the two lanes represent control siRNA (first lane) and CD2AP siRNA (second lane). The cells transfected with either control siRNA or CD2AP siRNA and either GFP, WT Rab5, overactive Rab5 (Q79L) or inactive Rab5 (N133I). (B) Quantification of the western blot. Means are plotted with error bars representing the standard deviation (N=2).

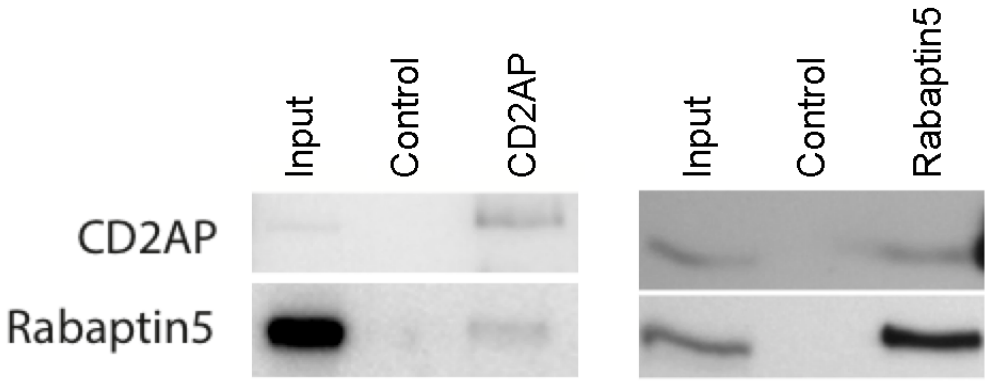

Figure 12. **CD2AP interacts with rabaptin5**

Co-ip performed on whole mouse brain homogenate showing homogenate (input) and precipitate from: antibody free beads (Control) rabaptin5 and CD2AP antibody bound beads. CD2AP signal is present in the rabaptin5 precipitate and rabaptin5 signal is present in the CD2AP precipitate of indicating an interaction between the two proteins.

3.3.3 CD2AP interacts with Rab5 effector rabaptin5

In order to better understand how CD2AP could possibly modulate the activity of Rab5 the idea that CD2AP could be interacting with effectors of Rab5 was visited. Effectors of Rab-GTPases bind to the Rabs and modulate their localization and activity. One well known Rab5 effector is rabaptin5. To uncover whether CD2AP is acting through an interaction with rabaptin5 an immunoprecipitation was performed. In whole mouse brain homogenate, it was found that rabaptin5 antibodies coimmunoprecipitated CD2AP and and rabaptin5 **(Figure 12)**. And in a membrane fraction of whole mouse brain CD2AP antibodies but not ApoER2 or antibody-free antibodies pulled out Rabaptin5 **(Figure 12)**. These results taken together indicate that CD2AP and rabaptin5 most likely interact in mouse brain.

3.4 Investigation into CD2AP's role in brain endothelial cell monolayer integrity

In the brain, endothelial cells are the barrier forming cell-type of the blood-brain barrier and the extremely low permeability between the cells is dependent on the proper expression and function of cell-cell adhesion molecules (eg. VE-cadherin) and tight junction proteins (eg. Claudin-5). Based on the above data showing that CD2AP regulates the expression of cell-cell adhesion and tight junction proteins it was hypothesized that CD2AP depletion may alter permeability across the endothelial cell monolayer. In order to test this mouse primary BMECs were cultured and transfected on trans-well inserts allowing for an assay measuring trans-endothelial permeability to be conducted. Trans-well inserts consist of an upper chamber and lower chamber separated by a porous membrane on which endothelial cells are cultured until confluent. Then the extravasation of sodium fluorescein across the cell monolayer was measured and used to calculate the trans-endothelial permeability. It was found that mouse

primary BMECs transfected with CD2AP siRNA formed a monolayer with lower mean permeability (Mean $_{CD2AP\ siRNA}$ = 1.149 x 10^{-5}) than cells transfected with control siRNA (Mean $_{control\ siRNA}$ = 1.746 x 10^{-5}, t(6) = 4.291, p = .0051) **(Figure 13).**

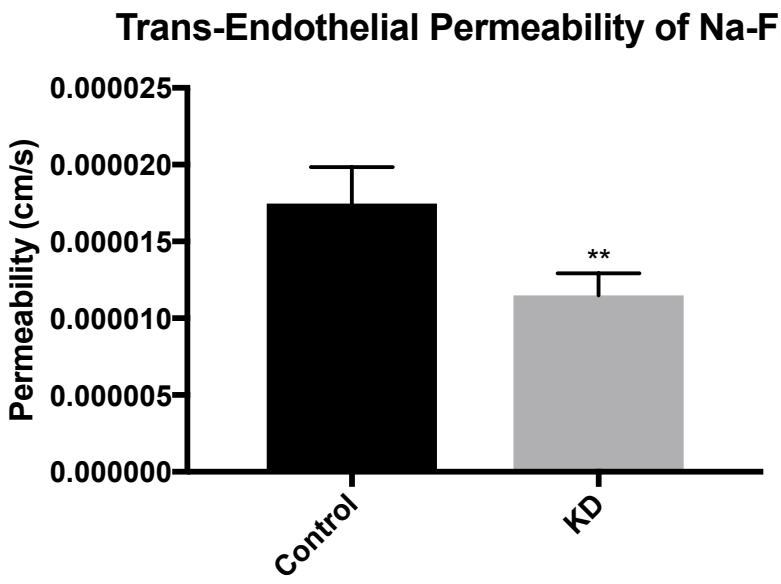

Figure 13. **Quantification of permeability assay**.

The mean permeability of sodium fluorescein (NA-FITC) across a mouse BMEC monolayer is represented in the units cm/s. Control represents mouse BMECs transfected with control siRNA and KD represents cells transfected with CD2AP siRNA. Error bars represent standard error of the mean.

3.5 Summary of Results

Through the data presented above the following key findings have been made:

1) siRNA induced knockdown of CD2AP lead to a reduction of VE-cadherin in both a human BMEC cell line (hCMEC/D3 cells) and mouse primary BMECs as well as an increase of claudin-5 in hCMEC/D3 cells
2) siRNA induced knockdown of CD2AP lead to a reduction of full-length LRP1 (100kDa) and an increase in a 70kDa fragment in hCMEC/D3 cells while this knockdown lead to the opposite effect in primary mouse BMECs (increase of 100kDa band and decrease of 70kDa band)
3) siRNA induced knockdown of CD2AP lead to a decrease of ApoER2 in both hCMEC/D3 cells and primary mouse BMECs and that CD2AP interacts with ApoER2 in both hCMEC/D3 cells and whole mouse brain
4) Rescuing activity but not levels of Rab5 lead to a rescue of ApoER2 expression
5) CD2AP interacts with an effector of Rab5 known to modulate its activation and function.

These results taken together indicate that CD2AP plays a non-trivial role in the regulation of a wide array of integral membrane proteins in brain microvascular endothelial cells. The data show that one of these proteins, ApoER2, interacts with CD2AP and that the CD2AP-depletion-induced reduction in its levels may be mediated by the endosomal regulator Rab5, whose activity may be modulated by CD2AP through a novel CD2AP/Rabaptin5 interaction.

Based on the emerging roles these proteins in Alzheimer's Disease progression and recent findings tying CD2AP to AD, these data present important clues to CD2AP's currently unclear contribution to Alzheimer's Disease pathogenesis.

Chapter 4: Discussion

4.1 CD2AP regulates receptor expression through endocytosis and endosomal trafficking

In both hCMEC/D3 cells and mouse primary BMECs CD2AP depletion led to the altered expression of a number of membrane bound proteins and an interaction was found between CD2AP and ApoER2 but not any other receptors. Based on the wide array of receptors affected by CD2AP depletion in these cells it is likely that CD2AP acts as a generalized regulator of receptor sorting in the endosomal pathway in brain endothelial cells.

Further support for this contention is that CD2AP has been found to regulate receptor endocytosis and subsequent endosomal sorting in a wide array of cell types with a large number of receptors (Lynch et al., 2003; Konishi et al., 2006; Kobayashi et al., 2004; Tsui and Pierchala, 2008; Bao et al., 2012; Minegishi et al., 2013). It is unlikely, however that CD2AP's only method of action is through receptor ubiquitination as staining of CD2AP in brain endothelial microvascular endothelial cells gives a strong endosomal localization as opposed to being restricted to the cell surface (data not shown). In addition, CD2AP has been found to colocalize with the endosomal regulators Rab4 and Rab5 as well as their effectors and also modulates their activity (Cormont et al., 2003; Welsch et al., 2005; Rouka et al., 2015).

Based on the above data it is likely that CD2AP acts at both the cell surface and on endosomes through its interactions with E3 ubiquitin ligases, the actin cytoskeleton, rab-GTPases and their effectors to regulate the destination of internalized receptors. However, the way that CD2AP coordinates this complex network in order to target receptors to the right pathway is currently unknown.

I have shown that CD2AP depletion-mediated cleavage of ApoER2, a correlate of ApoER2 degradation can be rescued by the vector expression of an overactive mutant of Rab5 (Rab5 Q79L), but not overexpression of the WT or inactive Rab5 mutant.

This leads to the hypothesis that CD2AP depletion likely induces the inactivation of Rab5 or inhibits its activation. Thus, by rescuing Rab5 activation the effect of CD2AP-depletion on ApoER2 levels can be reversed. This leads to the question, how does CD2AP regulate Rab5 activity? Typically, Rab5 is activated and localized to endosomes through its association with its effectors. One well known effector of Rab5 is Rabaptin5 and this study has shown through immunoprecipitation experiments that CD2AP and Rabaptin5 form a complex in whole mouse brain and in hCMEC/D3 cells. In this respect CD2AP could potentially recruit Rabaptin5 to early endosomes inducing activation of Rab5 and the in absence of CD2AP this recruitment-induced activation of Rab5 cannot take place, or more simply, CD2AP acts as an adaptor protein bringing Rapbaptin5 and Rab5 together on early endosomes, allowing Rab5 to mediate its trafficking functions.

Based on a recent study, it is possible that the complex between Rabaptin5 and CD2AP is regulated by the poly-ubiquitination of the receptor as well as activated Rab4 (Rab4-GTP). A recent study found that Rabaptin5 can be recruited to early endosomes by the presence of ubiquitinated cargo and activated Rab4 (Rab4-GTP) (Kälin et al., 2016). Considering CD2AP is involved in E3 ubiquitin ligase-mediated receptor ubiquitination and subsequent internalization it is very possible that CD2AP remains bound to the receptor and acts to recruit Rabaptin5, subsequently activating and recruiting Rab5 and/or Rab4 and through its function in this complex target the receptor for degradation or recycling.

This proposed mechanism would functionally integrate two of CD2AP's known roles in cell biology and explain the wide array of receptors affected by CD2AP depletion. A depiction of this potential mechanism can be found in **Figure 14.**

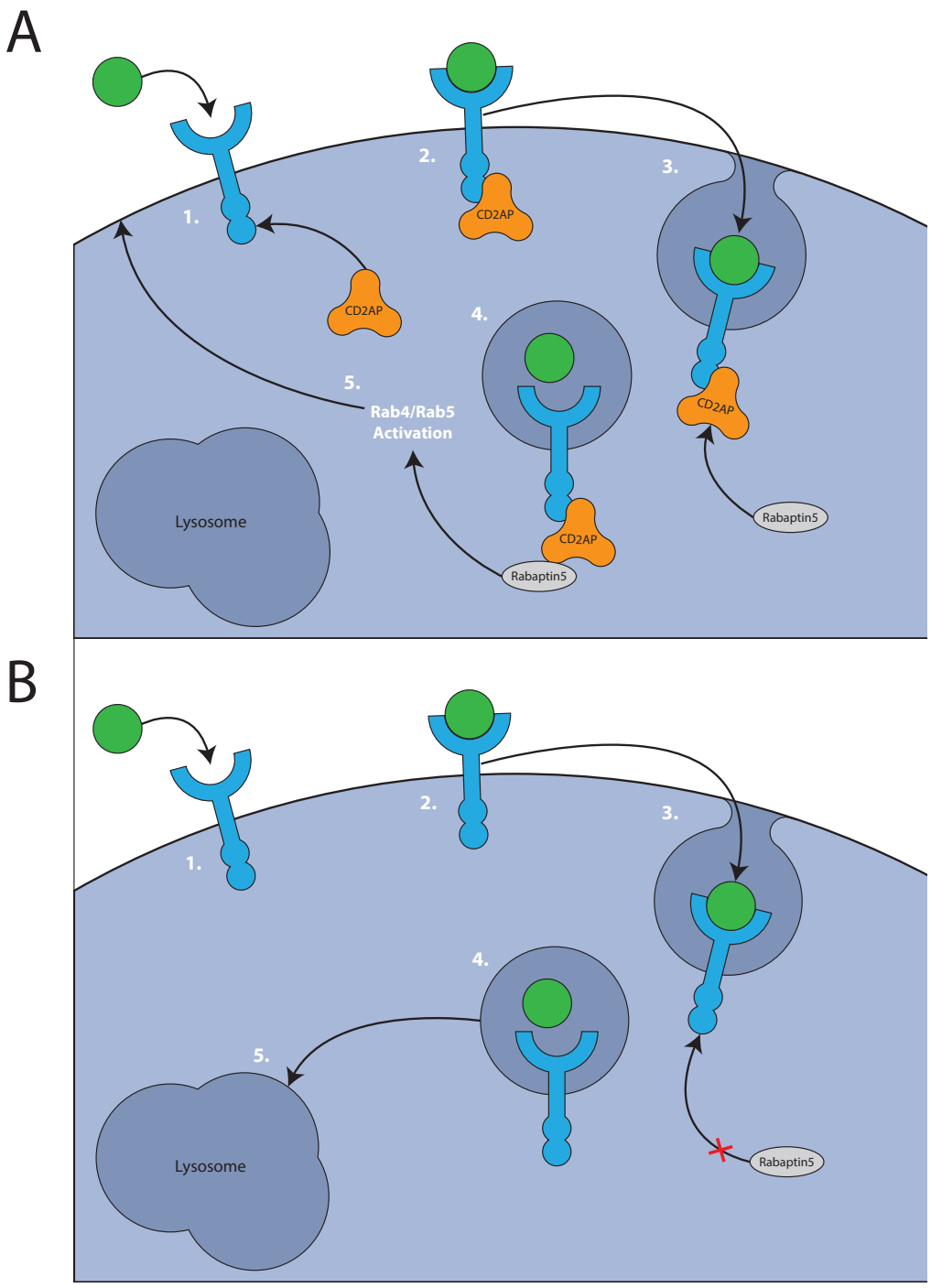

Figure 14. **Schematic representation of proposed hypothetical mechanism**.

(A) Wild type condition where CD2AP interacts with rabaptin5 causing activation of Rab5 and subsequent recycling of ApoER2.

(B) CD2AP knockdown condition where there is no interaction with rabaptin5, thus no activation of Rab5 and endosome containing ApoER2 is targeted for degradation.

Aside from the proposed mechanism above, it is also possible that CD2AP modulates the function of other proteins responsible for the recycling of certain receptors back to the membrane thus directing for recycling. SNX17 is an adaptor protein that has been shown to rescue ApoER2 and LRP1 from the degradation pathway by transferring them to recycling endosomes (Sotelo et al., 2014). It is conceivable that CD2AP is required for this process and the absence of CD2AP leads to the breakdown of this recycling mechanism.

Additionally, it is possible that CD2AP depletion is inducing the proteolytic processing of these receptors. It is known that ApoER2 is proteolytically cleaved by α-secretase and γ-secretase (Balmaceda et al., 2014) and LRP1 is cleaved by γ-secretase (Zurhove et al., 2008). This possibility is made more likely by the finding that in CD2AP knockdown cell lysate, there is an increase in the expression of lower molecular weight bands corresponding with proteolytic cleavage of the full length receptor. It may be that CD2AP activity is somehow sequestering these secretases away from the corresponding receptors and upon depletion of CD2AP the secretase can access and cleave the receptors leading to the reduction of the full length receptor and the accumulation of the proteolytic fragments observed in my results. In support of this it has been shown recently that CD2AP depletion leads to the trapping of APP at the early endosomal membrane rather than trafficking it through to the lysosomal pathway for degradation which occurs when CD2AP is present (Ubelmann et al., 2017). Trapping APP at the endosomal membrane led to increased processing by γ-secretase which is a protease that cleaves both ApoER2 and LRP1 (Ubelmann et al., 2017). It was found that CD2AP was required for the trafficking of APP into the lumen of the endosome, a process required for lysosomal degradation. Thus in brain microvascular endothelial cells, CD2AP depletion may lead to the

retention of ApoER2 and LRP1 at the endosomal membrane, leading to an increase in their proteolytic processing. The processing of ApoER2 and LRP1 may be performed by γ-secretase so it would be very interesting to inhibit γ-secretase activity with DAPT to determine if it can block the formation of their proteolytic fragments.

Based on my Rab5 mutant data it is quite possible that the disrupted lysosomal trafficking mechanism is due to a CD2AP-depletion induced reduction in Rab5 activity or localization. Alternatively, CD2AP could be modulating trafficking of endocytic vesicles itself through its regulation of the actin cytoskeleton. In support of this, it was shown that CD2AP forms F-actin structures on early endosome and is required for the trafficking of vesicles to the early endosome (Gauthier et al., 2007). Rab5 also modulates the trafficking of vesicles to, and fusion with the early endosome so it is possible that the expression of overactive Rab5 in cells was enough to compensate for the lack of reduction CD2AP levels, reversing the CD2AP-depletion effect.

4.2 Vascular dysfunction induced by CD2AP depletion

This study has also shown that CD2AP depletion leads to a decrease in the cell-cell adhesion protein VE-cadherin, an increase in the tight junction protein claudin-5 and a decrease in brain endothelial cell permeability. This differential expression of important vascular proteins and properties may represent CD2AP depletion-mediated vascular dysfunction. VE-cadherin is required for the structural stability of the cell-cell contacts between endothelial cells of the brain vasculature. In this system, the reduction of CD2AP leads to an increase in claudin-5 which may explain the decrease in permeability across the BMEC monolayer. Although *in-vitro* these

results seem to indicate that reduction of CD2AP leads to a tighter BBB, *in vivo* where the endothelial barrier is subjected to forces that are just not present *in vitro* (stretching of the vessel wall due to vasodilation and fluctuating blood pressure, as well as shear stress from blood flow) the higher expression of claudin-5 coupled to the lower expression of VE-cadherin (critical for cell-cell adhesion) may create an unstable and rigid vessel wall, unable to withstand these forces leading to breakdown of the vasculature mimicking conditions seen in aging and AD (Girouard and Iadecola, 2006).

It has been well known for some time now that the vasculature is not simply a static conduit for blood. There exists a strong relationship between the cells of the vasculature and neurons/astrocytes that is known as the neurovascular unit. Neuronal activity is coupled to vasodilation and astrocyte calcium signaling through a complex signaling network that is not yet fully understood. This coupling is known as neurovascular coupling and it has been demonstrated in both human patients and rodent models that aging substantially impairs neurovascular coupling responses (Fabiani et al., 2014); (Balbi et al., 2015); (Tarantini et al., 2016). Furthermore, AD patients and mouse models of AD have been shown to exhibit significant impairment of neurovascular coupling responses (Hock et al., 1997); (Rombouts et al., 2000); (Tarantini et al., 2016).

Importantly, neurovascular uncoupling in aging was found to be reversible through interventions that improve endothelial function and reactivity (Park et al., 2007; Toth et al., 2014). This indicates that endothelial dysfunction contributes strongly to neurovascular uncoupling. Logically then, it can be hypothesized that CD2AP-depletion mediated dysregulation of VE-cadherin which make up the adherens junctions and claudin-5 which

contribute to tight junctions may contribute to neurovascular uncoupling (Komarova et al., 2017).

In addition to the regulation of AJs and TJs there is strong evidence that endothelial NO production contributes to neurovascular coupling (Toth et al., 2015) so it stands to reason that dysregulation of NO production may lead to neurovascular uncoupling. Nitric oxide is produced in the endothelium by endothelial Nitric Oxide Synthase (eNOS). eNOS can be phosphorylated following activation of various signaling pathways, such as the VEGF and Insulin pathways, increasing its activity (Fulton et al., 1999). Reelin signaling has recently been shown to blunt VEGF induced activation of eNOS via the Reelin receptor ApoER2 (Ding et al., 2016) and ApoER2 knockdown was shown to decrease basal eNOS activity (Ulrich et al., 2014). Conversely, Reelin signaling was shown to increase NF-kB activity (Ding et al., 2016). NF-kB is a transcriptional factor for eNOS expression (Zhu et al., 2016) suggesting that Reelin signaling likely both dampens VEGF induced activation of eNOS activity while modulating eNOS levels. The CD2AP data presented here show that knockdown of CD2AP leads to a reduction of one of the two canonical Reelin receptors, ApoER2. A reduction of this receptor would conceivably lead to a dysregulation of Reelin signaling and thus regulation of eNOS activity via Reelin signaling.

Also, it was shown in bovine aortic endothelial cells (BAOEC) that ApoE3 signaling through ApoER2 increases eNOS activity (Ulrich et al., 2014) uncovering a distinct ApoE/ApoER2-regulated eNOS pathway separate from the VEGF pathway. Conversely ApoE4 does not stimulate eNOS, and instead blocks the ApoE3/ApoER2 increase in eNOS activity in a concentration dependent manner (Ulrich et al., 2014). Based on the CD2AP-depletion mediated

reduction of ApoER2 it is likely that this ApoE/ApoER2 singaling and the resultant increase in eNOS activity would be disrupted, mimicking the effect of the *APOE4* allele.

The effect of CD2AP levels on these two distinct pathways taken together indicate that CD2AP depletion may create a situation where both the activation, inhibition and transcription of eNOS are deregulated. It stands to reason that the disruption of two layers of eNOS regulation could significantly alter the homeostasis of the eNOS system. Considering that eNOS is critical for neurovascular coupling it is apparent that aberrant CD2AP expression could potentially contribute to neurovascular uncoupling. A summary of ApoER2 signaling in endothelial cells is presented in **Table 3.**

Table 3. Summary of ApoER2 signaling in endothelial cells

Ligand	Function	Cells involved	Tissue Origin	Reference
Reelin	Increases monocyte adhesion and ICAM1, VCAM1, and E-selectin expression. Suppresses endothelial nitric oxide synthase (eNOS) activity. Increases nuclear factor κB (NF-κB) activity that favours eNOS expression	Endothelial cells, Monocytes	Aorta	(Ding et al., 2016)
ApoE	ApoE3 increases eNOS activity whereas ApoE4 does not affect eNOS and blocks ApoER2 signaling	Bovine Aorta Endothelial Cells	Aorta	(Ulrich et al., 2014)
Thrombin	ApoER2 was required for Activated Protein C (APC) antiapoptotic function upon thrombin stimulation. APC can bind to ApoER2 and APC activation lead to Dab1 phosphorylation	Immortalized HUVECs	Umbilical Vein	(Sinha et al., 2016)
Reelin	Reelin was found in trancytotic vesicles and on the luminal plasma membrane in brain endothelial cells indicating that Reelin can cross the blood-brain barrier via active transport.	Rat Brain Endothelial Cells (brain slice)	Brain	(Perez-Costas et al., 2015)

Endothelial derived nitric oxide however, is just one signaling pathway that regulates neurovascular coupling. Upstream of endothelial cells, both neurons and astrocytes respond to increased glutamate at the synapse to transmit signals to regulate vessel dilation. One such way that neurons contribute to neurovascular coupling is through glutamate-induced activation of NMDARs on neurons which can stimulate the activation of neuronal NO synthase that provides NO to act directly on the vasculature as a dilator (Phillips et al., 2016). Astrocytes on the other hand respond to glutamate through metabotropic glutamate receptors (mGluR), which can induce the production of arachidonic acid which then produces epoxyeicosatrienoic acid (EET) and prostaglandins which both serve to dilate the vessel, while arachidonic acid itself can act on the smooth muscle to produce vasoconstriction upon its conversion to 20-HETE (Phillips et al., 2016). Recent work has shown that the astrocytic component may not be necessary for the initial alteration in blood flow upon neuronal activity (functional hyperemia) and that astrocytic calcium signaling occurs slightly after vasodilation indicating that it could not be responsible for the initiation of functional hyperemia (Rosenegger and Gordon, 2015). Instead it is beginning to be thought that astrocytes play an important modulatory role to influence the size of the neuron induced response and that they may be critical for basal blood flow control (Rosenegger and Gordon, 2015). It is also important to emphasize that the component of the neurovascular unit that carries out the the physical change in the vasculature is the smooth muscle cell/pericyte and that intact signaling machinery in this cell-type is critical for effective for transducing the input from neurons, astrocytes and endothelial cells. In this context it would be of great interest to investigate CD2AP's role in these critical signaling pathways and cell-types to obtain a larger picture of CD2AP's impact on neurovascular coupling.

4.3 CD2AP's effect on LRP1 function in Aβ clearance

Through another mechanism CD2AP may contribute to AD through the modulation of Aβ vascular clearance via Aβ transporter LRP1. LRP1 knockout mice have a reduction of Aβ clearance from the brain (Kanekiyo et al., 2013) and a reduction of LRP1 may contribute to the accumulation of Aβ peptide in the brain of AD patients (Donahue et al., 2006; Miller et al., 2008). Interestingly, LRP1 expression levels are significantly reduced in brain endothelial cells in normally aging and AD in humans and animal models (Ramanathan et al., 2015). Reducing CD2AP protein levels in human and mouse BMECs led to a decrease in LRP1. This decrease may contribute strongly to AD pathogenesis by decreasing Aβ clearance from the brain leading to accumulation and subsequent aggregation. Conversely the RAGE transporter is involved in transport of Aβ from the periphery into the brain (Deane et al., 2004). It would be interesting to analyze the effect of CD2AP-depletion on RAGE levels as it would be important to know the which way the balance of Aβ transport would be shifted when CD2AP levels are altered. LRP1's method of action and CD2AP's possible involvement are summarized in **Figure 15.**

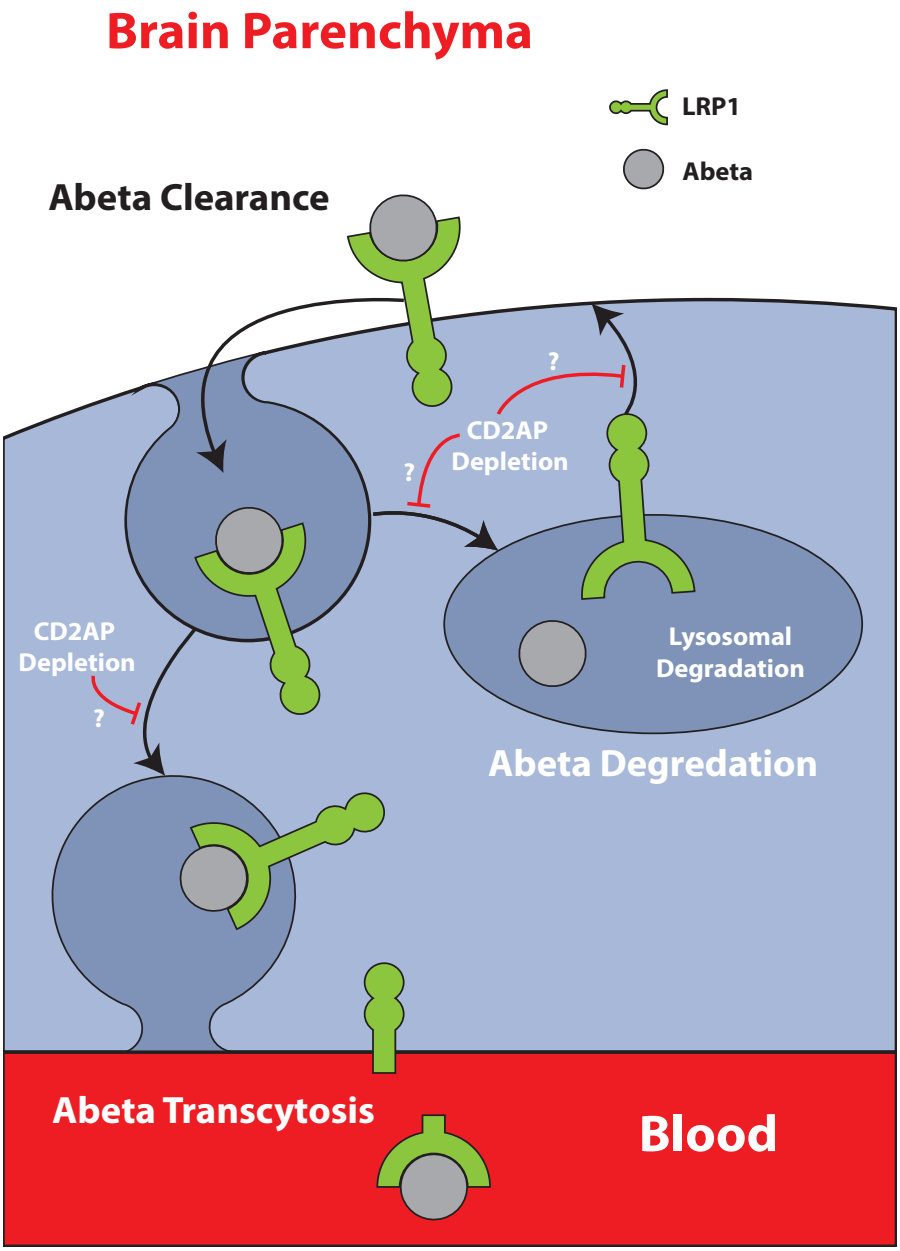

Figure 15. Schematic representation of the function of LRP1 in the brain vasculature.

LRP1 binds free Aβ in the brain and is internalized into the cell. From there the receptor is either targeted for degradation, recycling or transcytosis. Transcytosed Aβ-LRP1 complex is expressed on the luminal side of the BBB where the extracellular domain of LRP1 is cleaved releasing sLRP1 bound to Aβ into the blood where it is degraded later by the liver effectively clearing it from the body. sLRP1 acts as an Aβ "sink" creating a gradient that drives Aβ out of the brain opposed to into the brain. sLRP1 binds 70-90% of plasma Aβ.

Furthermore, Aβ transport from the brain to the blood via LRP1 was dramatically decreased in ApoE4 expressing mice compared to mice expressing the ApoE3 variant (Deane et al., 2008). This suggests that carriers for AD implicated CD2AP SNPs and/or *APOE4* alleles have aberrant Aβ clearance mechanisms and that CD2AP SNPs may further increase AD risk in *APOE4* allele carriers.

4.4 CD2AP depletion may decrease Reelin signaling via ApoER2 downregulation

A study conducted by Chen *et al.* (2010) demonstrated that the Reelin-ApoER2-NMDAR pathway contributes to the pathophysiology of AD. They showed that ApoE4, reduces ApoER2 expression on the neuronal synapse after ligand-induced receptor endocytosis via Reelin binding, and that this impaired Reelin signaling mediated synaptic plasticity and NMDAR phosphorylation (Chen et al., 2010), (Ishii et al., 2016). This ApoE4-mediated pathway greatly resembles the effect observed in CD2AP-depleted brain microvascular endothelial cells. These results show that CD2AP depletion decreases levels of ApoER2 and that this depletion is modulated by the endosomal pathway.

If the same role for CD2AP uncovered here in BMECs is conserved in neurons than CD2AP depletion-mediated downregulation of ApoER2 would mimic the effect of the *APOE4* allele on surface expression of ApoER2. SNPs in the CD2AP gene that confer AD risk may then possibly act in concert with the *APOE4* allele having a multiplicative decrease in ApoER2 leading to more profound cognitive deficits via the ApoER2/NMDAR-dependent pathway.

In addition to cognitive deficits there is strong evidence suggesting that Reelin signaling through ApoER2 is critical for neuronal survival during aging (Beffert et al., 2006). This could not only

contribute to ApoE4's impact on AD pathology by way of reducing ApoER2 at the cell surface, but could also explain the increased risk for AD in carriers of genetic variants of CD2AP. This study has shown that reduced CD2AP expression leads to a reduction in ApoER2. Also, CD2AP expression has been shown to be lower in patients with AD (Tao et al., 2017). Taken together these data create a link between lower CD2AP expression and a susceptibility to AD due to a decrease in the protective signaling of Reelin through ApoER2. It is possible then, that genetic variants of CD2AP may increase AD risk by reducing CD2AP protein expression or function leading to a reduction of ApoER2 dependent Reelin signaling.

In addition, it was uncovered that Reelin signaling protects against Aβ toxicity-induced synaptic dysfunction in the early stages of AD and the associated impairment in learning and memory (Lane-Donovan et al., 2015), (Ishii et al., 2016). The CD2AP depletion-mediated ApoER2 downregulation in endothelial cells could potentially sensitize them to Aβ toxicity. Considering vascular breakdown is a contributing factor to AD this may contribute to AD pathogensis.

Finally, it was found that Reelin signaling inhibits two kinases responsible for tau phosphorylation, GSK-3β and CDK-5 (Ohkubo et al., 2003, #51264). As hyperphosphorylated tau aggregate to form neurofibrillary tangles, a hallmark of AD, this inhibition is protective against AD. The CD2AP-depletion mediated reduction in ApoER2 could contribute to AD pathology by decreasing this inhibition causing the increase in tau phosphorylation. In fact, data shared with us by a collaborator indicates that decreasing CD2AP levels in human AD patients correlate with increased tau pathological burden (Dr. Frédéric Calon's Lab) providing a strong footing for the above proposed mechanism.

The methods of action for CD2AP in brain endothelial cells proposed in this discussion are summarized schematically in **Figure 17.**

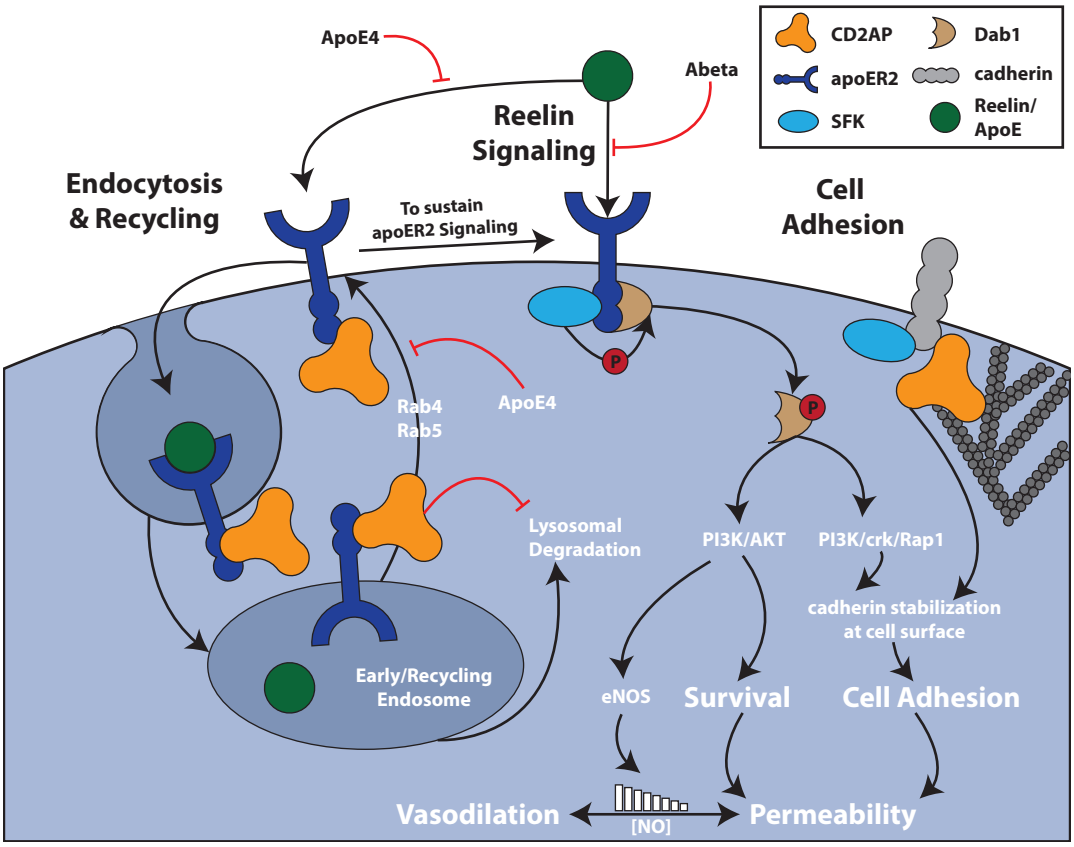

Figure 16. Schematic representation of a hypothetical model of CD2AP's role in brain microvascular endothelial cells.

This study has uncovered that CD2AP is involved in the endosomal processing of ApoER2 and possible proteolytic cleavage of LRP1 and that CD2AP-depletion leads to: a reduction of ApoER2 and VE-Cadherin in both hCMEC/D3 cells and mouse primary BMECs, a dysregulation of LRP1 levels in both cells types and an increase of claudin-5 in hCMEC/D3 cells. In addition, a decrease in the permeability of sodium fluorescein across a mouse primary BMEC monolayer was observed when CD2AP was knocked down. It was also discovered that CD2AP interacts with ApoER2 in both hCMEC/D3 cells and whole mouse brain and that the CD2AP-depletion mediated cleavage of ApoER2 was modulated by Rab5 activity. These results imply a generalized role for CD2AP in the regulation of certain membrane-bound proteins through its role in receptor endocytosis and trafficking. Through these roles in BMECs CD2AP is proposed to modulate the effect of a number of signaling pathways leading to dysregulation of important cellular processes. The most notable implications of this are:

1) Potential impairment of Aβ clearance via LRP1.
2) Potential loss of Reelin signaling which protects cells from Aβ toxicity, regulates cell adhesion via Crk/Rap1 and neurovascular coupling via eNOS.
3) Potential blood-brain barrier breakdown due to dysregulation of key cell-cell junction proteins.

4.5 Future Directions

Loss of CD2AP affects brain endothelial cell biology by 1) altering proteolytic processing of LRP1, 2) promoting degradation of ApoER2 via disturbance of endosomal trafficking/recycling pathway and 3) deregulating the expression of key cell–cell interaction proteins. The potential implications of these misegulations have been explored in this discussion but the question remains: does CD2AP depletion modulate AD pathology through its loss of function in brain endothelial cells? The data presented here points to a serious consequential breakdown of critical vascular machinery at the molecular level. Additional in vivo studies are required to address. the following critical outstanding questions:

1) Does CD2AP depletion impair Reelin signaling?
2) Does CD2AP depletion impair endothelial cell-cell adhesion integrity?
3) Does CD2AP and the Reellin/ApoE/ApoER2 signaling regulate brain vascular functions and how these functions are altered with ApoE4, in the presence of Abeta or upon loss of CD2AP or expression of CD2AP polymorphic genes
4) Does CD2AP depletion affect Aβ clearance?

First, to determine whether CD2AP depletion impairs Reelin signaling *in vitro,* cultured brain endothlelial cells can be incubated with exogenous Reelin and the phosphorylation status of Dab1 (the first downstream effector to become phosphorylated upon Reelin signalling) analyzed.

Secondly, to determine if CD2AP-depletion can disrupt blood-brain barrier integrity, brain endothelial cell-specific CD2AP knockout model can be generated and subjected to extravasation experiments using various known tracers (sodium fluorescein, FITC-dextran)

across the blood-brain barrier. The fluorescent tracer would be injected into the blood stream of control and knockout mice and its levels in the brain would be analyzed to determine if blood-brain-barrier permeability is altered.

Third, to determine if this loss of Reelin signaling has a functional consequence *in vivo* the same CD2AP mutant mouse model could be utilized to investigate eNOS regulation, neurovascular coupling, cell survival or adhesion. If these defects are found and can be partially rescued by exogenous addition of Reelin to increase signaling, then a strong case would be made that these brain endothelial cell-specific CD2AP knockout-induced deficits are a result of aberrant Reelin signaling. In addition, a system for live *in vivo* imaging of the neurovascular unit in awake mice has been developed (Tran and Gordon, 2015). This would allow for the application of exogenous Reelin by injection into the brain or the blood an observing the vascular response (dilation or constriction).

Finally, in order to address whether or not CD2AP is important for LRP1-mediated Aβ clearance, an Aβ transport assay could be done using BMEC coated trans-well inserts and incubating the cells with fluorescent Aβ in the upper chamber and calculating flux across the monolayer. In addition, crossing the brain endothelial cell-specific CD2AP null mice with an AD mouse model with Aβ would allow us to study accumulation and clearance of Aβ *in vivo*.

4.6 Conclusion

The data presented here indicate that CD2AP is critical for a number of cellular processes in brain endothelial cells and that CD2AP depletion affects a number of pathways important for vascular function and implicated in AD. Going forward it will be important to determine the

ultimate impact of CD2AP on vascular functions *in vivo* as well as on AD pathogenesis. This work represents an important step toward our understanding of CD2AP's functions in the brain and its implication in the vascular defects in AD.

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