THE ROLE OF THE ARD1 PROTEIN IN RETINAL ENDOTHELIAL CELL PERMEABILITY

By

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ABSTRACT

Tubedown (Tbdn) is a retinal homeostatic factor which is normally highly expressed in the retinal blood vessels which associates with the N-acetyltransferase Ard1. Tbdn also associates with Cortactin, a c-Src tyrosine kinase substrate. Downregulation of Tbdn correlates with neovascular retinal pathology in neovascular retinopathies. These diseases involve growth and hyperpermeability of retinal blood vessels leading to leakage of plasma proteins such as Albumin, inflammation and tissue damage. Knockdown of Tbdn in retinal endothelial cells results in co-suppression of Ard1, activation of c-Src/Fyn tyrosine kinase and increased permeability to Albumin. The focus of this study is to determine how Ard1 might regulate signaling pathways mediating the permeability of endothelial cells to Albumin as well as levels of phospho-Cortactin Tyr421 and activated c-Src/Fyn.

Ard1 was either knocked down using siRNA or overexpressed in RF/6A primate retinal endothelial cell line and the permeability to Albumin assayed. In parallel experiments, the activation status of components of the Albumin permeability pathway (phospho-Cortactin Tyr 421 and activated c-Src/Fyn) was monitored.

Ard1 knockdown reduced the permeability of the cells to FITC-Albumin but did not change the levels of phospho-Cortactin Tyr 421 or activated c-Src/Fyn when compared to controls. Cells transfected with the Ard1 expression vector overexpressed Ard1 and showed increased permeability of Albumin and increased levels of activated c-

Src/Fyn compared to controls. Our results suggest that Ard1-mediated increase of activated c-Src/Fyn does not require Tbdn.

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LIST OF ABBREVIATIONS

AMD	age-related macular degeneration
Acetyl CoA	Acetyl coenzyme A
ANOVA	analysis of variance
Ard1	arrest defective-1
Arp	actin related protein
ASTBDN	Antisense Tbdn
cdc42	cell division control protein 42
cDNA	complementary deoxyribonucleic acid
CO_2	carbon dioxide
CSK	C-terminal Src kinase
c-Src	cellular Src
CNV	choroidal neovascularization
CTR	control
DDW	Asp-Asp-Trp motif
DMEM	Dulbecco's modified eagle medium
DR	diabetic retinopathy
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
F-actin	filamentous actin
FBS	fetal bovine serum
FDA	Food and Drug Administration

FITC	fluorescein isothiocyanate
Flt-1	fms-like tyrosine kinase receptor
GFP	Green fluorescent protein
gp60	60-kDa glycoprotein
GTP	guanosine triphosphate
HCl	Hydrogen chloride
HIF-1α	hypoxia-inducible factor 1-alpha
HRP	horseradish peroxidase
IEM	embryonic endothelial cell line
JAM	junctional adhesion molecules
LMS	lenz micropthalmia syndrome
MLC	myosin light chain
MLCK	myosin light chain kinase
mTOR	mammalian target of rapamycin
M_r	molecular radius
NaCl	Sodium chloride
Nat1	N-terminal acetyltransferase
NLS	nuclear localization signal
nm	nanometer
NT	non-transfected
NTA	N-terminal acidic domain
N-WASP	Wiskott-Aldrich syndrome protein
PAK	p21-activated kinase
PDGF-B	platelet-derived growth factor B

PDR	proliferative diabetic retinopathy
PIX	PAK-interacting exchange factor
PTP1	protein tyrosine phosphatase 1
Rac1	Ras-related C3 botulinum toxin substrate
RF/6A	retinal endothelial cell line
ROP	retinopathy of prematurity
RPE	retinal pigment epithelium
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
s.e.m.	standard error of the mean
SH	Src homology
siRNA	small interfering ribonucleic acid
Tbdn	Tubedown
TGF-β	transforming growth factor β
TPR	tetratricopeptide repeat
VE	vascular endothelial
VEGF	vascular endothelial growth factor
v-Src	viral Src

Chapter 1 Introduction

1.1 The Visual System

1.1.1 The Eye

Vision is an important sense which allows us to perceive the world around us. Loss of vision may result in a loss of functional autonomy and depression in affected individuals. Light waves enter the eye through the cornea through an opening in the iris known as the pupil. The iris controls the diameter of the pupil and the amount of light that enters the eye (Davson and Perkins, 2014). These light rays then pass through the lens which is a structure that, along with the cornea, can refract light which will then focus on the retina. Before reaching the retina the light rays also pass through a gelatinous substance called the vitreous body (Davson and Perkins, 2014). The light rays then focus on the point of highest visual acuity in the retina, the fovea. The fovea is located within the macula and contains a high density of cones. The avascular fovea is responsible for clear central vision (Davson and Perkins, 2014).

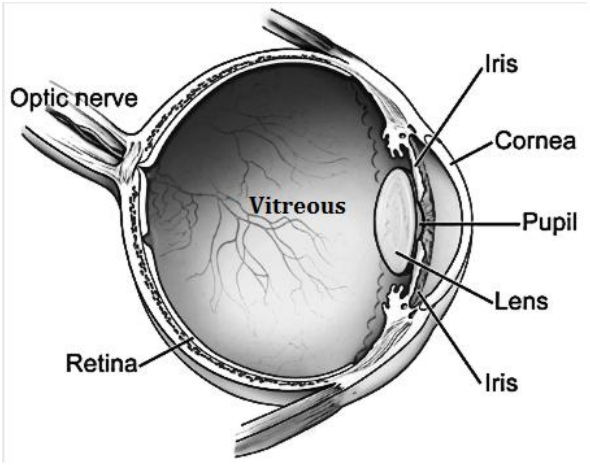
1.1.2 The Retina

The retina is a thin delicate tissue located in the inner layer of the globe of the eye. Located between the vitreous body and the retinal pigment epithelium (RPE), it consists of eight different layers. The nerve fiber layer (NFL) is located above the ganglion cell layer (GCL), followed by the inner plexiform layer (IPL) which contains the synapses between the bipolar cells and the ganglion cells (Bergman et al., 2014). The inner nuclear layer contains the nuclei of the bipolar cells. The outer plexiform layer (OPL) contains

the synapses between the rods and cones and the bipolar cells. Finally, the outer nuclear layer (ONL) consists of the cell bodies of the photoreceptors (Bergman et al., 2014). Beneath the photoreceptors lies the RPE (Bergman et al., 2014). Light rays pass through the ganglion cell layer and the bipolar cell layer to reach the photoreceptors. There are two types of photoreceptors; rods, which function in low light conditions and cones, which are responsible for color vision. The outer segments of the photoreceptors are composed of stacks of membranes which contain the visual pigment molecules required for vision. The photoreceptor outer segments experience photo-oxidative damage and they are maintained by the RPE (Strauss, 2014). The inner segments of the photoreceptors contain mitochondria and ribosomes and are responsible for the metabolism of the outer segments (Kolb, 2014). The photoreceptors transmit the signal to the bipolar cells, followed by transmission to the ganglion cells. There are both rod bipolar cells and cone bipolar cells. Cone bipolar cells can be divided into two types: OFF bipolar cells which are hyperpolarized by central illumination and ON bipolar cells which are depolarized by central illumination (Nelson and Connaughton, 2014). In humans, they have further differentiated cone bipolar cells into two types: midget bipolar cells, which contact one cone, and diffuse bipolar cells which interact with multiple cones (Wassle and Boycott, 1991). The differing connectivity with photoreceptors can result in variation of the functional outcomes of the visual pathway. Beneath the photoreceptors lies the RPE. The RPE contains pigment granules and has roles in light absorption and epithelial transport (Strauss, 2014). The axons of the ganglion cells converge to form the optic nerve where the visual signals leave the eye. These signals will travel to the visual cortex, where the information can be interpreted by the brain (Nelson, 2014). The blind spot of the retina is

due to the fact that there are no photoreceptors on the optic disc, which is where the optic nerve leaves the eye (Gamm, 2014).

A


Optic nerve
Iris
Cornea
Vitreous
Pupil
Lens
Retina
Iris

B

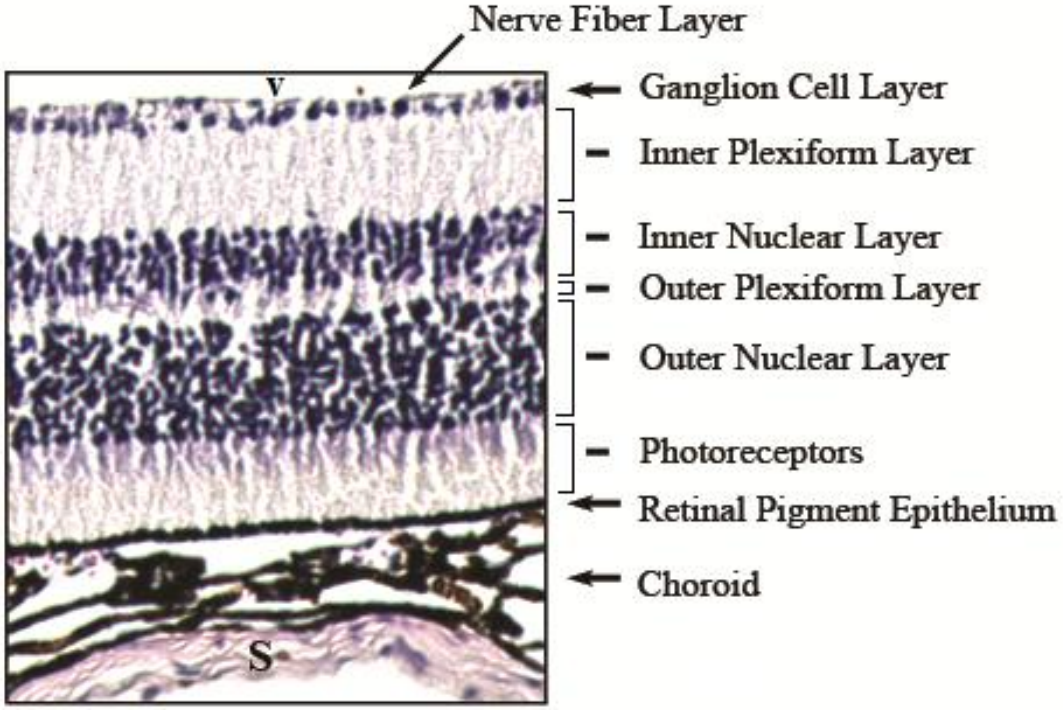

Nerve Fiber Layer
V
Ganglion Cell Layer
Inner Plexiform Layer
Inner Nuclear Layer
Outer Plexiform Layer
Outer Nuclear Layer
Photoreceptors
Retinal Pigment Epithelium
Choroid
S

Figure 1: (A) Anatomy of the human eye (a public domain image from (Anonymous). **(B) Hematoxylin & Eosin staining of the mouse retina.**

The retina consist of eight different layers. Beginning at the vitreous (V) the layers are as follows: nerve fiber layer (NFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), photoreceptors and the retinal pigment epithelium (RPE). The ONL contains the cell bodies of the photoreceptors. The choroid circulation supplies the avascular outer-retina. The outer layer of the eye is the sclera (S). Magnification: 10X.

http://www.wpclipart.com/medical/anatomy/eye/eye_diagram.png.html

1.2 Retinal Vasculature

There are two vascular supplies in the retina; intra-retinal blood vessels which supply the inner two-thirds of the retina while the choroidal circulation supplies the photoreceptors located in the avascular outer one-third of the retina (Kur et al., 2012). The central retinal artery enters the eye through the optic disc and branches into the retinal arteries. Endothelial cells with tight junctional complexes line the interior of the retinal blood vessels (Kur et al., 2012). The retinal blood vessels contain layers of smooth muscle which comprise the tunica media. Endothelial cells within the tunica intima line the interior of blood vessels and their tight junctions control the passage of solute from the lumen of the blood vessels to the interstitial space. Pericytes are contractile cells which surround the endothelial cells of capillaries. Their functions include regulation of

blood flow through capillaries as well as roles in angiogenesis and maintenance of normal blood vessels (Sims, 1986). Studies have shown that a variety of growth factors may be involved in recruitment of pericytes in blood vessels. These factors include growth factors such as vascular endothelial growth factor (VEGF), platelet-derived growth factor B (PDGF-B), transforming growth factor β (TGF-β) and their receptors as well as angiopoietin-1 and its receptor, the tyrosine kinase Tie-2 (Hammes, 2005). The retinal blood vessels have a much higher pericyte coverage (94.5%) than those of the choroid (11.5%; (Chan-Ling et al., 2011). The choroid vasculature is supplied by the long and short ciliary arteries. The choroid is composed of 5 layers, Bruch's membrane, three vascular layers (the choriocapillaries, Sattler's layer and Haller's layer) and the suprachoroidea (Kur et al., 2012). Haller's layer contains large arteries and veins while Sattler's layer contains medium and small arterioles that nourish the choriocapillaries. The blood vessels of the choroid are fenestrated, which means the endothelial cells contain small pores through which molecules can pass (Tornquist et al., 1990).

1.3 Vascular Permeability

A monolayer of endothelial cells known as the vascular endothelium lines blood vessels. The tunica intima layer consists of vascular endothelium, basement membrane and sometimes an internal elastic lamina which also play a role in the barrier function of blood vessels. The vascular endothelium, together with vascular smooth muscle cells, can regulate angiogenesis, smooth muscle tone and tissue fluid homeostasis (Mehta and Malik, 2006). Generally, the endothelial barrier is controlled by inflammatory cells and

mural cells, transcellular transport and by intercellular junctions via paracellular permeability (Goddard and Iruela-Arispe, 2013). Mural cells include pericytes and smooth muscle cells. The vascular endothelium is semi-permeable and can transport large molecules and fluid from the lumen of the blood vessels to the interstitial space. Plasma proteins in the blood are at a higher concentration within the vessels than in the interstitial space (Mehta and Malik, 2006). In general, molecules smaller than 3nm molecular radius (M_r) cross the endothelium through the paracellular pathway while molecules greater than 3nm M_r as well as water cross the endothelium by the transcellular pathway. Albumin, which is the most abundant of the plasma proteins, crosses the vascular endothelium under normal conditions via the transcellular pathway (Mehta and Malik, 2006). Extracellular matrix (ECM) proteins which lie beneath the vascular endothelium play a role in its barrier function. The endothelial cell layer regulates transport of albumin, thus it also regulates the transendothelial oncotic pressure gradient. Endothelial cells are connected by junctional proteins which make up adherens junctions, tight junctions and gap junctions. Adherens junctions are composed of vascular endothelial (VE)-Cadherin, while tight junctions are formed from claudins, occludin as well as junctional adhesion molecules (JAM) (Mehta and Malik, 2006). Gap junctions are composed of transmembrane hydrophilic proteins called connexins (Stout et al., 2004). Vascular permeability can be regulated by thrombin, histamine, bradykinin and VEGF. VEGF causes an increase in both paracellular and transcellular permeability. Paracellular permeability is the transit of solutes between the cells via interendothelial junctions while transcellular permeability is transit of solutes through the cells via caveolae-mediated transport (Mehta and Malik, 2006). Initially, VEGF binds the fms-like tyrosine kinase

(Flt-1) receptor which results in an increase in vascular permeability (Mehta and Malik, 2006). Specifically, VEGF treated bovine retinal microvascular cells caused a significant increase in permeability of albumin across a cell monolayer (Chang et al., 2000).

Endothelial cell permeability can also be regulated by Src family of kinases. Src phosphorylates dynamin and caveolin which are required for caveolar endocytosis (Mehta and Malik, 2006). Transcellular transport of albumin is dependent on endocytosis of albumin bound to the 60-kDa glycoprotein receptor (gp60). Albumin binds gp60 which is a required step in albumin transcytosis. gp60 then activates Src through activation of the βγ subunit of Gi (Mehta and Malik, 2006). This is followed by Src-dependent tyrosine phosphorylation of dynamin-2, which interacts with caveolin-1 to initiate caveolae mediated endocytosis and transport of albumin (Shajahan et al., 2004).

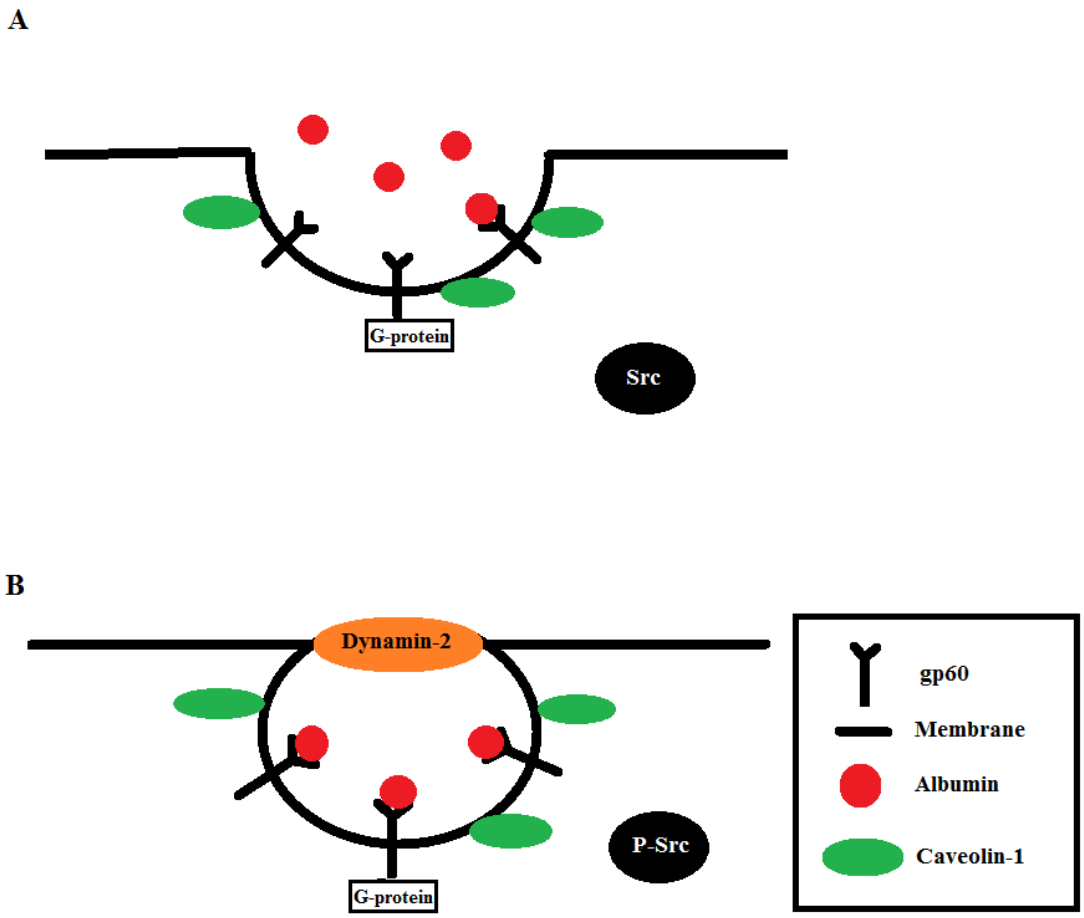


Figure 2: Schematic of caveolar endocytosis and transport of albumin.

(A) Albumin binds gp60 which is a required step in albumin transcytosis. gp60 then activates Src through activation of the βγ subunit of Gi. (B) This is followed by phosphorylated Src (P-Src)-dependent tyrosine phosphorylation of dynamin-2, which interacts with caveolin-1 to initiate caveolae mediated endocytosis and transport of albumin.

1.4 Neovascular Retinopathies

Neovascular retinopathies are characterized by growth of abnormal blood vessels in the retina which can lead to vision loss. Visually impaired people have a higher incidence of depression than people with normal vision (Evans et al., 2007). A possible reason for this higher incidence of depression could be the loss of independence that often occurs when one loses their vision as well as adverse side effects from treatment. One example of a neovascular retinopathy, Age-related macular degeneration (AMD), has been reported to be the leading cause of blindness in the aged population in developed countries and also the third leading cause of blindness worldwide (Resnikoff et al., 2004). AMD can occur in both dry and wet forms. Consequences of the dry form of AMD include the appearance of drusen deposits between Bruch's membrane and the retinal pigment epithelium located in the macula (Ambati et al., 2003). Drusen are yellow or white deposits of lipids and are a common early sign of dry AMD. Accumulation of drusen can lead to geographic atrophy of RPE cells and death of the photoreceptors (Green and Key, 2005). AMD also consists of thickening of Bruch's membrane as well as hyper and hypopigmentation of RPE and cell death, which leads to vision loss (Green, 1999). The dry form of AMD can be asymptomatic with the possibility of developing into wet AMD. The wet or exudative form of AMD involves neovascularisation of the choroidal blood vessels which can either remain beneath the RPE or pass through Bruch's membrane and the RPE to enter the neural retina (Ambati et al., 2003). The more aggressive classic type of choroidal neovascularization (CNV) is often associated with early and substantial vision loss due to loss of photoreceptors while the less severe occult

type of CNV are present with long term maintenance of vision until deterioration of the RPE occurs (Bressler et al., 1990;Schmidt-Erfurth et al., 2007). Topographic angiography showed that classic CNV appeared as a well-demarcated lesions with steep, craterlike borders, frequently surrounded by a halo, which suggests leakage of choroidal blood vessels and perfusion changes (Schmidt-Erfurth et al., 2007). Occult CNV was documented by topographic angiography as a convex lesion with flat, ill-defined borders and intraretinal leakage which is most likely less pronounced that classic CNV (Schmidt-Erfurth et al., 2007). These vessels are abnormal because they are hyperpermeable and may leak blood and serum proteins such as albumin from the retinal blood vessels to the interstitial space. This leakage of albumin can result in inflammation, scarring and possible retinal detachment (Ambati et al., 2003;Bonnel et al., 2003).

Another common cause of vision loss is diabetic retinopathy (DR). There are approximately 93 million people globally suffering from DR, specifically 17 million with proliferative diabetic retinopathy (PDR; (Yau et al., 2012). DR can be either nonproliferative or proliferative in which there is neovascularisation of retinal blood vessels. DR involves capillary basement membrane thickening as well as loss of endothelial cells and pericytes. This can lead to capillary occlusion as well as microaneurysms. In the more advanced PDR, there can be neovascularisation, retinal detachment and vision loss (Gardner et al., 2002;Gendron et al., 2001). Hyperglycemia in patients with diabetes is thought to be correlated with DR. Several different pathways have been proposed to link hyperglycemia to the microvascular complications seen in DR patients: Increased polyol pathway flux, increased advanced glycation end-product

formation, activation of protein kinase C and increased flux though the hexosamine pathway (Hammes, 2005). The polyol pathway metabolizes excess glucose and can result in an accumulation of sorbitol. Sorbitol can have damaging effects to retinal cells (Fong et al., 2004;Gabbay, 1973).

Retinopathy of prematurity (ROP) is one of the leading causes of blindness in children (Qazi et al., 2009). Retinas of premature infants are incompletely vascularised followed by delayed vascular growth after birth (Smith, 2004). Hypoxia in the peripheral areas of the retina causes an increase in VEGF expression and retinal blood vessels grow toward the VEGF stimulus (Smith, 2004). Angiogenesis occurs by initial vasodilation of existing vessels which is followed by increased permeability as well as degradation of the surrounding matrix. Endothelial cells can then migrate and proliferate in order to form new blood vessels (Conway et al., 2001).

Several current treatments for neovascular retinopathies include laser photocoagulation and anti-VEGF drugs (Bandello et al., 2013). VEGF is involved in increased permeability in animal cells as well as retinal endothelial cells in vitro (Aiello et al., 1994). Anti-VEGF drugs are anti-angiogenesis agents which inhibit the function of VEGF (Chan et al., 2007). Anti-VEGF drugs such as ranibizumab and bevacizumab have been shown to be effective in some patients with AMD (Kanoff and Miller, 2013). Bevacizumab is a humanized monoclonal antibody with strong anti-angiogenic activity approved by the Food and Drug Administration (FDA) for treatment of colorectal cancer in 2005 (McCormack and Keam, 2008). Ranibizumab is a monoclonal antibody fragment derived from bevacizumab (Ferrara et al., 2006). While ranibizumab is approved for

treatment of neovascular AMD, it is much more expensive than bevacizumab which is used "off label" to treat neovascular AMD (Raftery et al., 2007). Recent studies showed bevacizumab and ranibizumab had equal effects on visual acuity (CATT Research Group et al., 2011;IVAN Study Investigators et al., 2012). Anti-VEGF drugs are also used to treat patients with PDR (Bandello et al., 2013). ROP is normally treated with laser ablation of the peripheral retina (Fleck, 2013). However, some patients with AMD do not respond to anti-VEGF treatments (Kanoff and Miller, 2013). There have also been reports of patients developing RPE tears after being treated with anti-VEGF drugs (Chang et al., 2007). Several other side effects including inflammation (Georgopoulos et al., 2009), retinal ischemia and ocular irritation have also been reported (Wong et al., 2008). New, more effective treatments with less risk to the patient are required to help patients suffering from AMD, PDR and ROP. In order to develop new treatments, researchers must be able to better understand the molecular pathways involved in neovascularisation in neovascular retinopathies.

1.5 Arrest Defective-1 and Tubedown

Arrest Defective-1 (Ard1, also referred to as Naa10) is a 30 kDa N-terminal acetyltransferase originally discovered in yeast (Park and Szostak, 1992). In yeast, it interacts with a protein known as Tubedown (Tbdn, also referred to as Narg1, NATH, Naa15) to form the N-terminal acetyltransferase NatA complex (Park and Szostak, 1992). Tbdn has been found to be downregulated during capillary-like formation of IEM

embryonic endothelial cells (Gendron et al., 2000). Tbdn is homologous to the yeast Nat1 N-terminal acetyltransferases (Gendron et al., 2000). The Tbdn protein sequence contains tetratricopeptide (TPR) repeats which are known to mediate protein-protein interactions (Fig. 2). This suggests that Tbdn may interact with other proteins (Main et al., 2005;Paradis et al., 2002;Paradis et al., 2008;Willis et al., 2002). There are mammalian homologues for Ard1 (Ard2, 81% identity) and Tbdn (mNat2, 70% identity) which have been previously described (Arnesen et al., 2006;Sugiura et al., 2003). The NatA complex in yeast likely mediates co-translational acetylation of nascent polypeptides from ribosomes after initial cleavage of methionine by methionine aminopeptidases (Gautschi et al., 2003). The NatA complex also plays roles in cell growth and differentiation (Arnesen et al., 2006;Asaumi et al., 2005;Lim et al., 2006;Park and Szostak, 1992). It has also been shown that the putative acetyltransferase Nat5 can interact with NatA and has also been reported to interact with Nat1 in the ribosome of yeast (Arnesen et al., 2006;Gautschi et al., 2003;Hou et al., 2007). A study has shown that the binding of Tubedown to Ard1 to form the NatA complex causes a structural change in the active site of Ard1 which allows it to acetylate an α-amino group on nascent polypeptide chains with N-terminal alanine, cysteine, glycine, serine, threonine or valine (Liszczak et al., 2013). This suggests that Tubedown plays a role in regulating activity of Ard1. Ard1 has the ability to acetylate lysine residues and potential substrates for this acetylation have been reported (Geissenhoner et al., 2004;Kimura et al., 2003;Lim et al., 2006;Polevoda and Sherman, 2003;Wang et al., 2004).

Ard1 has been reported to acetylate and interact with many different proteins in the literature. Ard1 activates the cyclin D1 promoter through activation of Activator Protein-1 proteins c-Jun and c-Fos to ultimately increase cellular proliferation (Seo et al., 2010). Hypoxia-inducible factor 1-alpha (HIF-1α) binds a specific mouse isoform of Ard1 and prevents the acetylation of β-catenin (Lim et al., 2008). Ard1 acetylates and activates β-catenin promoting lung cancer cell proliferation (Lim et al., 2006). Ard1 can also bind the p21-activated kinase (PAK)-interacting exchange factor (PIX) and disrupt binding between PIX and G protein-coupled receptor kinase interacting proteins (Hua et al., 2011). This prevents the localization of PIX to the plasma membrane, as well as inhibits Ras-related C3 botulinum toxin substrate 1 (Rac1) and Cell division control protein 42 homolog (cdc42) proteins which are involved in regulation of cell growth ultimately inhibiting cellular migration (Hua et al., 2011). Furthermore, Ard1 binds and acetylates myosin light chain kinase (MLCK), inactivating it and resulting in reduced levels of phosphorylated myosin light chain (MLC) and reduced cell motility (Shin et al., 2009). Ard1 can also acetylate Tuberous sclerosis protein 2 and reduce mammalian target of rapamycin (mTOR) activity leading to autophagy (Kuo et al., 2010). Ard1 interacts with DNA methyltransferase which silences the E-cadherin tumour suppressor promoter by methylation (Lee et al., 2010). Ard1 is overexpressed in several types of cancer including breast, lung and colorectal cancer (Yu et al., 2009). Currently, the NatA complex is a possible attractive target for cancer treatment.

Tbdn is expressed during embryogenesis and is regulated during the differentiation of vascular and neuronal tissues (Gendron et al., 2001;Sugiura et al.,

2003). In mice, Tbdn and Ard1 are highly expressed during brain development in areas of cell division and migration and are downregulated as neurons differentiate (Sugiura et al., 2003). However, high levels of Tbdn protein in adults are restricted to few vascular beds including blood vessels of regressing ovarian follicles and the ocular endothelium (Gendron et al., 2000;Gendron et al., 2001;Paradis et al., 2002).

A mutation in the *Ard1* gene which causes Ogden syndrome has been found to be lethal in human infant males. The disorder is comprised of a combination of symptoms including an aged appearance as well as prominent eyes (Rope et al., 2011). Another mutation in the *Ard1* gene resulting in Lenz Micropthalmia Syndrome (LMS) which is characterized by anothalmia or micropthalmia also involves dysregulation of the *STRA6* gene (Esmailpour et al., 2014). STRA6 is a retinol binding protein receptor which mediates cellular uptake of retinol and is important in regulating the retinoic acid signalling pathway (Kawaguchi et al., 2007). In LMS, expression array studies showed decreased expression of genes involved in the retinoic acid signalling pathway including *STRA6* (Esmailpour et al.,2014). These studies indicate that different mutations in the *Ard1* gene can have different effects.

There is evidence that Tbdn plays a role in regulation of retinal homeostasis as well as in growth and differentiation. The Gendron/Paradis lab has characterized Tbdn as a novel homeostatic factor which has been shown to be involved in blood vessel growth and homeostasis (Gendron et al., 2000;Gendron et al., 2001;Paradis et al., 2002). An earlier study reports that Tbdn is found to be downregulated during capillary-like formation of IEM embryonic endothelial cells suggesting that it plays a role in dampening

blood vessel formation (Paradis et al., 2002). The knockdown of Tbdn expression in RF/6A rhesus macaque choroid-retina endothelial cells resulted in an increase in formation of capillary-like structures, supporting the idea that Tbdn negatively regulates angiogenesis (Paradis et al., 2002). Drs. Paradis and Gendron have created cell clones in which Tbdn levels are suppressed by expression of an antisense *TDBN* cDNA construct (Paradis et al., 2002). Knockdown of Tbdn using these clones resulted in co-suppression of Ard1 protein levels and increased permeability of FITC-Albumin across a cellular monolayer (Paradis et al., 2008). Drs. Paradis and Gendron have also created a bitransgenic mouse model that enables conditional knockdown of Tbdn in endothelial cells (Wall et al., 2004). These mice exhibited retinal and choroidal neovascularisation and intra- and preretinal fibrovascular lesions resembling those seen in human proliferative retinopathies (Wall et al., 2004). Conditional knockdown of Tbdn in endothelial cells of transgenic mice also resulted in retinal neovascularization and thickening and extravasation of albumin from retinal blood vessels in vivo (Paradis et al., 2008). In addition, a study has found that endocytosis of β-amyloid precursor transmembrane protein can be inhibited by overexpression of the NatA complex (Tbdn and Ard1; (Asaumi et al., 2005). Tbdn has been found specifically suppressed in retinal blood vessels of neovascular retinal lesions of patients with AMD, PDR and ROP (Gendron et al., 2001;Gendron et al., 2006;Gendron et al., 2010;Paradis et al., 2002). Furthermore, Tbdn has been found to co-localize with Cortactin and Filamentous (F)-actin in the cytoplasm and in the cortex of endothelial cells (Paradis et al., 2008). All of these studies together suggest that the NatA complex plays a role in regulation of endothelial cell permeability.

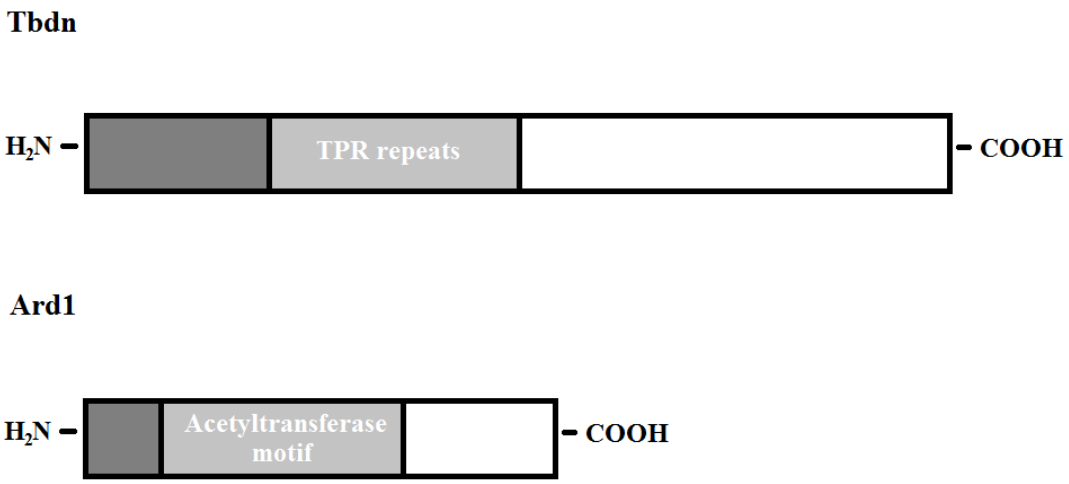


Figure 3: The molecular organisation of Tbdn and Ard1.

Tbdn and Ard1 interact to form the NatA complex. Tbdn contains a series of TPR repeats while Ard1is the catalytic subunit of the NatA complex containing an acetyltransferase motif.

1.6 Cortactin

Tbdn has been found to colocalize with Cortactin and F-actin in cytoplasmic regions and at the cortex of endothelial cells (Paradis et al., 2008). Cortactin is an 80-85 kDa cortical actin binding protein which is a substrate of viral Src (v-Src) transformed cells (Wu et al., 1991). Cortactin structure consists of an N-terminal acidic domain (NTA), six and a half tandem repeats, an α-helical region, a proline-rich region and a Src

homology (SH3) domain (Fig. 2). Cortactin is also known to regulate cellular permeability through its interaction with the actin cytoskeleton (Daly, 2004;Mehta and Malik, 2006). Within the NTA domain, Cortactin has been shown to bind and activate actin-related protein (Arp) 2/3 which increases N-WASp (Wiskott-Aldrich syndrome protein) – mediated actin nucleation and polymerization (Ammer and Weed, 2008).

Cortactin can be phosphorylated by cellular Src (c-Src), v-Src and Fyn on three separate residues (Tyr 421, Tyr 466 and Tyr 482). Phosphorylation of Cortactin at Tyr 421 creates a binding site for c-Src which will result in phoshphorylation of Tyr 466 and Tyr 482 (Weed and Parsons, 2001). Increased phosphorylation of Cortactin by c-Src led to increased cellular migration and cancer metastasis (Ammer and Weed, 2008;Daly, 2004;Huang et al., 1998).

c-Src phosphorylation of Cortactin has been shown to regulate endocytosis through actin polymerization (Ammer and Weed, 2008). Tbdn has also been reported to colocalize with Cortactin at the cortex of endothelial cells (Paradis et al., 2008). Therefore, it would be beneficial to look into the interaction between the NatA complex and Cortactin phosphorylation in the Tbdn-mediated regulation of retinal endothelial permeability of albumin.

Figure 4: The molecular organisation of Cortactin.

Arp2/3 binds the DDW-motif within the NTA domain. The actin-binding domain contains six-and-a-half tandem repeats. c-Src phosphorylation of Y421, Y466 and Y482 occurs in the proline-rich region.

1.7 Src Family Kinases

Src family kinases are a family of non-receptor tyrosine kinases that includes nine kinases: c-Src, Fyn, Lyn, Blk, Yes, Lck, Hck, Fgr and Yrk. The Src family kinases play roles in regulation of cellular proliferation and cellular permeability and endocytosis (Dehm and Bonham, 2004;Mehta and Malik, 2006;Sverdlov et al., 2007). Specifically, the protein tyrosine phosphatase DEP-1 (density-enhanced phosphatase) is phosphorylated in a Src- and Fyn- dependent manner. This allows DEP-1 to dephosphorylate Src on its inhibitory residue Y529 and allows VEGF induced Src phosphorylation of VE-cadherin and Cortactin. Furthermore, knockdown of DEP-1

impairs Src-dependent VEGF-mediated responses including regulation of permeability and capillary formation (Spring et al., 2012). Another Src family kinase known as Lyn is expressed in B cells and has both positive and negative roles in B cell receptor induced signal transduction (Xu et al., 2005).

v-Src is a gene found in Rous Sarcoma virus that produces a tyrosine kinase that causes cancer in chickens (Smart et al., 1981). c-Src, a 60kDa Src Family Kinase, has been reported to show increased activation in colon, liver, lung, breast and pancreatic cancers (Dehm and Bonham, 2004).

c-Src contains a myristoylated N-terminus, a SH4 domain, a unique region, a SH3 domain, a SH2 domain, a linker region, a catalytic domain (SH1) as well as a regulatory domain (Fig. 3). In the inactive confirmation the SH2 domain of c-Src interacts with the Tyr 527 residue in the regulatory domain. Phosphorylation at the Tyr 416 residue in the catalytic domain changes c-Src to its active conformation in which it can phosphorylate its targets. Carboxy-terminal Src kinase (Csk) and protein tyrosine phosphatase 1 (PTP1) can phosphorylate and dephosphorylate Tyr 527 respectively while Tyr 416 undergoes autophosphorylation (Roskoski, 2005). v-Src lacks the Tyr 527 inhibitory residue and is therefore constitutively active as opposed to normal c-Src (Hunter and Cooper, 1985).

c-Src also plays a role in regulation of transendothelial permeability of albumin. When albumin binds to the glycoprotein 60 receptor (gp60) it induces clustering of the receptor and activation of c-Src through βγ subunit of Gi. Activated c-Src then phosphorylates caveolin-1, the Guanosine triphosphate (GTP)ase dynamin-2 and the gp60

receptor in order to induce transendothelial vesicular transport of albumin through the cell (Mehta and Malik, 2006). The Caveolin-1 Tyr 14 residue is phosphorylated by Src to initiate caveolar invagination from the plasma membrane (Kim et al., 2009;Shajahan et al., 2004). Dynamin Tyr 597 was identified as an important residue phosphorylated by c-Src responsible for caveolae-mediated endocytosis (Shajahan et al., 2004). Another known substrate of c-Src is Cortactin. c-Src has been reported to phosphorylate Cortactin and regulate endocytosis through actin polymerization (Ammer and Weed, 2008). Therefore, it would be beneficial to explore the possible relationship between the NatA complex, c-Src and phosphorylated Cortactin and their roles in Tbdn-mediated regulation of retinal endothelial permeability of albumin.

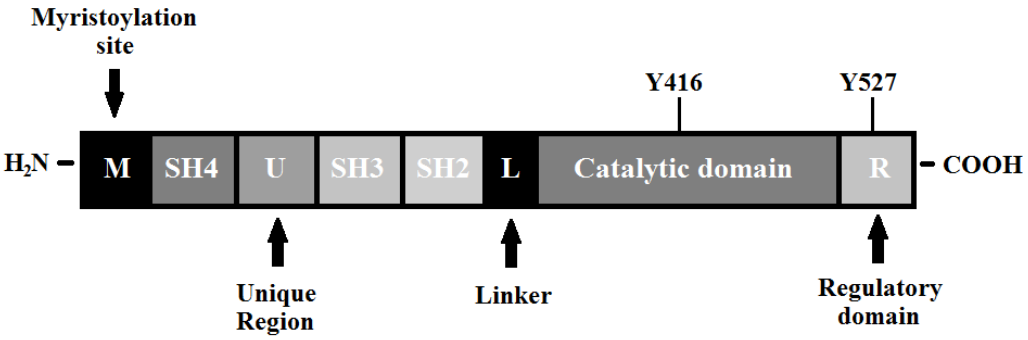


Figure 5: The molecular organisation of c-Src.

Src contains a myristoylation site, SH4 domain, unique region, SH3 domain, SH2 domain, linker, catalytic SH1 domain and a regulatory domain. The two main phosphoryation sites are Y416 in the catalytic domain and Y527 in the regulatory domain.

1.8 Rationale Behind Current Study

Many people around the world suffer from vision loss and other complications associated with AMD, PDR and ROP. These diseases involve neovascularisation and hyperpermeability of retinal blood vessels. Changes in endothelial permeability can result in leakage of plasma proteins such as albumin from the retinal blood vessels into the interstitial space, resulting in inflammation and vision loss. The Tbdn protein has been shown to regulate endothelial permeability in the retinal blood vessels (Gendron et al., 2000; Gendron et al., 2001; Paradis et al., 2008; Gendron et al., 2010). Knockdown of Tbdn has been reported to lead to co-suppression of Ard1 protein levels. (Paradis et al., 2008). Also, Tbdn is known to form the NatA complex with Ard1. c-Src phosphorylation of Cortactin regulates endocytosis through actin polymerization (Ammer and Weed, 2008). This study will examine the effects of knockdown and overexpression of Ard1 on Tbdn, phosphorylated Cortactin Tyr 421 and phosphorylated c-Src Tyr 416 levels as well as permeability of retinal endothelial cells to albumin.

Learning more about the role Tbdn/Ard1 plays in regulation of retinal endothelial cell permeability could possibly lead to development of drugs to treat those suffering from AMD, PDR and ROP. My hypothesis is that Tbdn-mediated regulation of retinal endothelial permeability requires the acetyltransferase Ard1.

Aim 1: Examine the effect of Ard1 knockdown in RF/6A cells.

Aim 2: Examine the effect of Ard1 overexpression in RF/6A cells.

Aim 3: Examine the effect of knockdown of Ard1 and overexpression of Ard1 on permeability of RF/6A cells to albumin.

Chapter 2 Materials and Methods

2.1 Cell Culture

RF/6A retinal endothelial cell line (CRL-1780; American Type Culture Collection) from *Macaca mulatta* were grown in Dulbecco's modified eagle medium (DMEM; Life Technologies Inc., Burlington, ON) supplemented with 10% fetal bovine serum (FBS) plus 2nM glutamine and nonessential amino acids. RF/6A cell clones in which Tbdn expression is suppressed by stable expression of the antisense *Tbdn* cDNA construct *AS-TBDN* were grown in DMEM supplemented with 10% FBS, 2nM glutamate, nonessential amino acids and in the presence of zeocin (Life Technologies Inc., Burlington, ON). RF/6A TE2 #9 cell clones which overexpress Myc-tagged human ARD1 construct provided by Dr. Paradis (Faculty of Medicine, Memorial University of Newfoundland) were also grown in culture in DMEM supplemented with 10% FBS, 2nM glutamate, nonessential amino acids and in the presence of 750ug/100ml G418 (Life Technologies Inc., Burlington, ON) and analysed using permeability assays and western blot. All cells were cultured on 100mm tissue culture dishes and were maintained at 37°C in 10% CO_2 atmosphere. The cells were counted by trypsinizing the cells followed by counting using a hemocytometer.

2.2 Antibodies

Mouse monoclonal anti-Ard1 (sc-373920) and anti-NARG1 (sc-81643) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-STAT3 (sc-482) was also obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-Phospho-Cortactin (Tyr421) antibody (#4569) and rabbit monoclonal anti-Phospho-Src Family (Tyr416) antibody (#6943) were obtained from Cell Signalling Technology (Danvers, MA). Other antibodies used included mouse monoclonal anti-Cortactin 4F11 (Millipore, Billerica, MA), anti-c-Src clone 327 (Abcam, Toronto, ON) and anti-Fyn (sc-434; Santa Cruz, CA). Additional antibodies used included mouse monoclonal anti-α-tubulin DM1A (Sigma, St. Louis, MO). We used horseradish peroxidase (HRP) conjugated anti-mouse IgG and anti-rabbit IgG secondary antibodies for analysis by western blot (Promega, Madison, WI).

2.3 Transfection

Ard1 was knocked down using duplex siRNA corresponding to nucleotides 193-211 of *Macaca mulatta* Ard1 cDNA within the coding region (5'-CCAGAUGAAAUACUACUUCUU-3') and scrambled control siRNA (5'-ACUAACGUUACGUACAUCAUU-3') which were purchased from Dharmacon (Fisher Scientific, Ottawa, ON). *Macaca mulatta* RF/6A cells were electroporated with 10nM concentrations of either control or Ard1 siRNA in addition to pmaxGFP vector obtained from Lonza (Basel, Switzerland). RF/6A cells were also transfected with 7.5ug/1.33 x 10^6

cells of an Ard1-Myc/His Tagged expression vector pcDNA3.1 Neo –*ARD1*/MYC-HIS provided by Dr. Paradis (Faculty of Medicine, Memorial University of Newfoundland). The transfection was done using the Neon Transfection System (Life Technologies Inc., Burlington, ON) following the protocols specified by the manufacturer. The electroporated cells were then cultured at a density of 1.33×10^6 cells/100 mm plate. After 48 hours, the cells were both plated for the permeability assay and plated for harvesting after an additional 24 hrs. Transfection efficiencies were determined by co-transfecting the cells with a green fluorescent protein (GFP) plasmid followed by counting the number of fluorescent and total cells and expressing the transfection efficiencies as a percentage of fluorescent cells over total cells.

2.4 Western Blot

Protein extraction was performed as on cells allowed to grow for 24 hrs after being replated 48 hours post-transfection. Cells were washed twice with 25mM Tris pH 7.6, 150nM NaCl, then harvested and suspended in cell lysis buffer (20mM Tris-HCl pH 7.4, 10% glycerol, 137mM NaCl, 0.1% SDS, 1% Triton X100, 2mM EDTA, 0.5% Na Deoxycholate) which contains protease inhibitors (1mM phenylmethylsulfonyl fluoride, 0.3 U/ml aprotinin and 10ug/ml leupeptin), phosphatase inhibitors (1mM sodium orthovanadate, 50mM sodium fluoride and 25mM β-glycerophosphate) and 1mM dithiothreitol. Lysates were clarified by centrifugation then a Bio-Rad protein assay was performed to determine protein concentration (Bio-Rad Laboratories, Hercules, CA).

Western blots were then performed by loading equal quantities of protein from the samples onto a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to nitrocellulose membrane (GE Water & Process Technologies, Trevose, PA). Proteins were detected using specific primary and horseradish peroxidase conjugated secondary antibody. Western blotting analysis was completed using chemiluminescent detection by use of Lumiglo and ECL Advance chemiluminescent substrates (KPL, Inc., Gaithersburg, MD). Densitometry analyses were conducted using the Kodak Gel Logic 2200 imaging system (Eastman Kodak Company, Rochester, NY) and intensities of the bands were analyzed using Kodak Molecular imaging software (Version 4.0, Eastman Kodak Company, Rochester, NY). The bands were compared to loading controls and expressed as a percentage of non-transfected cells. At least 3 western blots were averaged per experiment.

2.5 Transcellular Permeability Assay

For fluorescein isothiocyanate (FITC)-Albumin permeability assays, RF/6A cells in which ARD1 has been knocked down by transfection with ARD1 siRNA were seeded onto transwell inserts (Costar Transwell, no. 3470, 6.5-mm diameter, 0.4-µm pore size; Corning, Tewksbury, MA) and grown in the same conditions as described in the cell culture section depending on the specific cell type. The assay was completed using cells transfected with 10nM ARD1 siRNA and 10nM Control siRNA and cells transfected with an Ard1-MYC-HIS overexpression vector. Permeability assays were also completed

using RF/6A parental cells and ASTBDN #20 cells. 40000 cells per insert (0.4×10^6 cells/ml) were plated for transfected cells to account for cell death due to transfection while 30000 cells per insert (0.3×10^6 cells/ml) were plated for non-transfected cells. The cells were grown to confluence on the inserts for 24 hours before being washed 3 times with DMEM. The integrity of the cellular monolayers were evaluated for confluence before use. The inserts were then transferred to new plates containing DMEM and incubated for 2 hours. FITC-albumin (Sigma A9771, Sigma, St. Louis, MO) was added at a final FITC-albumin concentration of 50uM. The rate of transit of FITC-albumin across the cell monolayer was determined by measuring the increase in the amount of FITC-albumin in the lower well after 40 minutes of incubation. Using the FLUOstar optima spectrofluorometer (BMG LABTECH), fluorescence intensities were measured at an excitation wavelength of 485 nm and an emission wavelength of 520 nm and FITC-albumin was quantified against a standard curve of FITC-albumin. Transcellular permeability assays and harvesting of cells for analysis by western blot were completed in parallel at 72 hours post transfection.

2.6 Data and Statistical Analysis

Statistical analysis of western blot protein quantitations were compared using the one-way analysis of variance (ANOVA), followed by the Fisher's exact statistical test. The *P* values were calculated using PRISM program and the data was determined to be statistically significant if the *P* value was less than 0.05.

Chapter 3 Results

3.1 Ard1 is significantly knocked down and overexpressed in RF/6A retinal endothelial cells.

Tbdn has been shown to play a role in regulation of retinal endothelial cell permeability to albumin (Paradis et al., 2008). In addition, the effect of Tbdn in regulation of retinal endothelial cell permeability is supported by the role Tbdn plays in regulation of retinal homeostasis and neovascularisation (Wall et al., 2004). Furthermore, the role of Tbdn in the regulation of endocytosis (Asaumi et al., 2005), which is a necessary step in the transcellular permeability of albumin, indicates that Tbdn is involved in transcellular permeability regulation (Paradis et al., 2008). Therefore, it was only logical that we investigate the role of the other half of the NatA complex, Ard1, in regulation of the transcellular permeability of albumin. In order to examine the role of Ard1 in albumin permeability, we knocked down Ard1 in RF/6A cells. Furthermore, we transfected RF/6A cells with an Ard1 overexpression plasmid to analyse the effect of Ard1 overexpression on albumin permeability. For each experiment, transfection efficiencies were calculated for each plate of GFP transfected cells. The transfection efficiencies varied from a mean of 42% ± 9% for cells only transfected with GFP to 62% ± 2% for cells transfected with both the Ard1 vector and GFP (Fig. 6). There was no significant difference in the transfection efficiencies among the different groups of transfected cells. Ard1 was significantly knocked down by transient siRNA transfection of 10nM of Ard1 siRNA

when compared to non transfected, control siRNA transfected and GFP transfected cells ($P < 0.01$; Fig. 7B). Ard1 was also significantly overexpressed by transfection of RF/6A cells with Ard1 expression vector when compared to non transfected, control siRNA transfected and GFP transfected cells ($P < 0.01$; Fig. 7). Ard1 was overexpressed by 3.7 ± 0.48 fold in RF/6A retinal endothelial cells when compared to GFP transfected cells ($P < 0.01$; Fig. 7B) and by 1.4 fold in 10nM CTR siRNA transfected cells compared to GFP transfected cells and non transfected cells ($P < 0.01$; Fig. 7B).

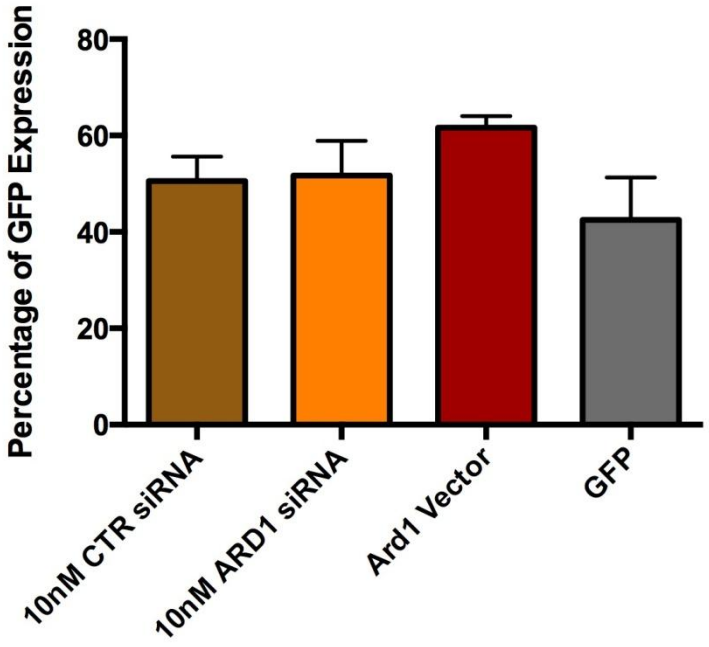
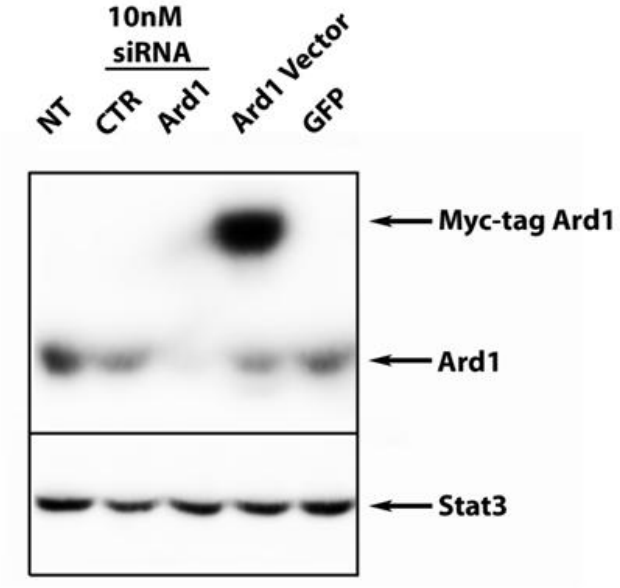


Figure 6: Relative transfection efficiencies.

Transfection efficiencies for control siRNA transfected cells (CTR), Ard1 siRNA transfected cells (Ard1), GFP transfected cells (GFP) and cells transfected with Ard overexpression plasmid (Ard1 vector). Transfection efficiencies were determined as a percentage of fluorescent cells over total cells. There was no significant difference in the transfection efficiencies between groups. Data shown is expressed as the mean ± s.e.m in each group (n=3).

A


10nM
siRNA
NT
CTR
Ard1
Ard1 Vector
GFP
Myc-tag Ard1
Ard1
Stat3

B

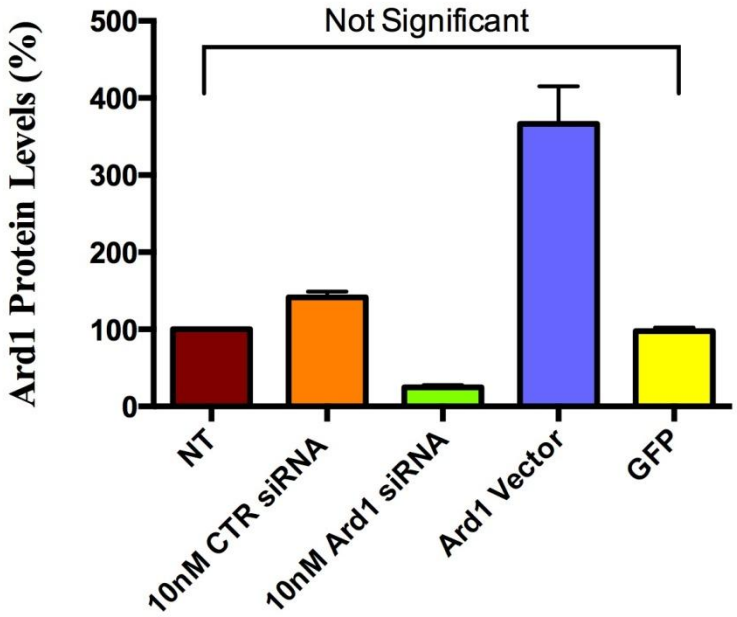

Ard1 Protein Levels (%)
500
400
300
200
100
0
Not Significant
NT
10nM CTR siRNA
10nM Ard1 siRNA
Ard1 Vector
GFP

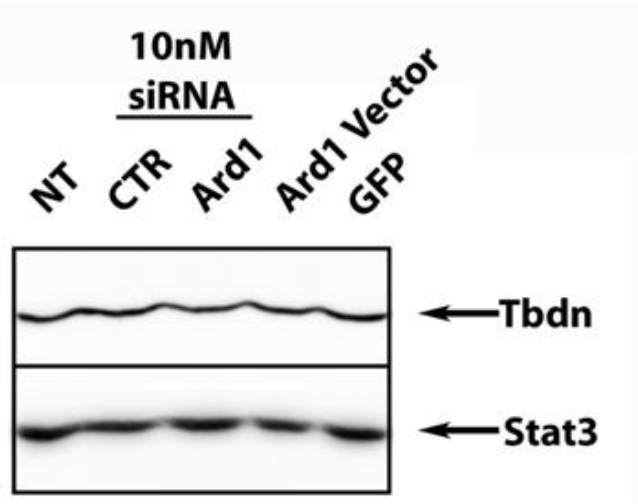
Figure 7: siRNA-mediated knockdown of Ard1 and Ard1 overexpression in RF/6A retinal endothelial cells.

Non transfected cells (NT), control siRNA transfected cells (CTR), Ard1 siRNA transfected cells (Ard1), cells transfected with Ard1 overexpression plasmid (Ard1 vector) and GFP transfected cells (GFP) protein levels were analyzed by western blot for Ard1 expression. The blot was re-probed and analyzed for Stat3 as a loading control. The only groups that were not significantly different were NT and GFP. Ard1 was significantly knocked down by 10nM Ard1 siRNA ($P < 0.01$) when compared to NT, CTR and GFP. Ard1 was significantly overexpressed by transfection with Ard1 expression vector ($P < 0.01$) when compared to NT, CTR and GFP. (A) Representative experiment; (B) the average of Ard1 levels ± s.e.m. of experiments are shown (n=5). Ard1 protein levels in B are relative to loading controls and expressed as a percentage of non-transfected cells.

3.2 Ard1 knockdown and Ard1 overexpression have no effect on Tbdn levels.

Knockdown of Tbdn in endothelial cells resulted in co-suppression of Ard1 protein levels (Paradis et al., 2008). To investigate if Ard1 levels regulate Tbdn levels, we examined the effect of Ard1 knockdown and Ard1 overexpression on Tbdn levels. The results showed that the knockdown of Ard1 or the overexpression of Ard1 resulted in no significant effect on Tbdn levels in RF/6A retinal endothelial cells when compared to non transfected cells (Fig. 8). However, Tbdn levels of 10nM CTR siRNA transfected cells were found to be increased compared to 10nM Ard1 siRNA and GFP transfected cells by approximately 1.5 fold ($P < 0.05$; Fig. 8B).

A


10nM
siRNA
NT
CTR
Ard1
Ard1 Vector
GFP
Tbdn
Stat3

B

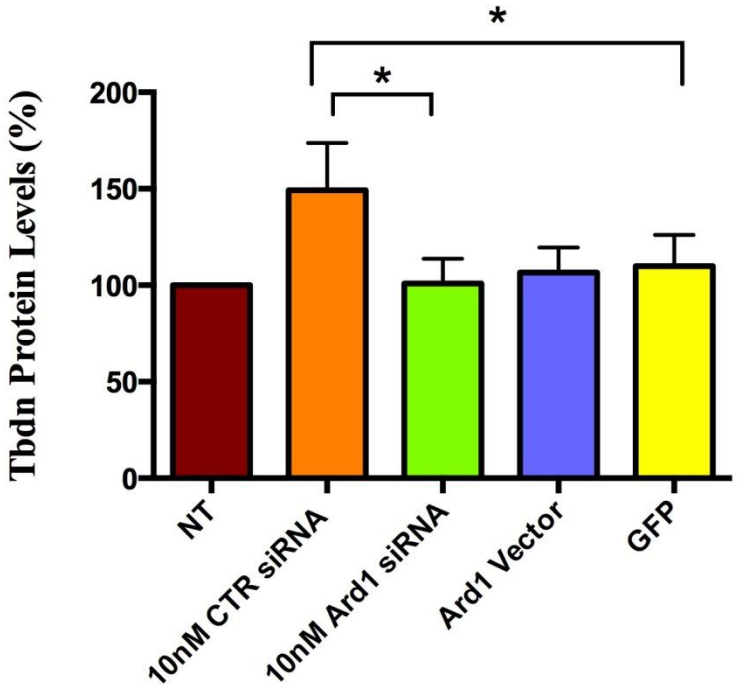

Tbdn Protein Levels (%)
200
150
100
50
0
*
*
NT
10nM CTR siRNA
10nM Ard1 siRNA
Ard1 Vector
GFP

Figure 8: Ard1 knockdown and Ard1 overexpression in RF/6A retinal endothelial cells has no effect on Tbdn levels.

Non-transfected cells (NT), control siRNA transfected cells (CTR), Ard1 siRNA transfected cells (Ard1), cells transfected with Ard overexpression plasmid (Ard1 vector) and GFP transfected cells (GFP) protein levels were analyzed by western blot for Tbdn expression. The blot was re-probed and analyzed for Stat3 as a loading control. Ard1 knockdown or overexpression resulted in no significant effect on Tbdn levels when compared to NT. (A) Representative experiment; (B) the average of Tbdn levels ± s.e.m. of experiments are shown (n=5). Tbdn protein levels in B are relative to loading controls and expressed as a percentage of non-transfected cell protein levels. (* is $P < 0.05$).

3.3 Transfection of RF/6A cells with Ard1 overexpression plasmid results in a significant decrease in the amount of cells at 48 hrs post-transfection.

Transfection often produces increased levels of cell death. Knockdown and overexpression of genes and proteins by transfection can affect the number of cells present after transfection and both transfection as well as the aberrant expression of genes that can affect the post-transfection cell count. During this experiment, the cells of each cell type were counted at 48 hours post-transfection. The cell count of cells transfected with the Ard1 overexpression plasmid were significantly decreased when compared to non transfected, GFP transfected, 10nM Ard1 siRNA transfected and 10nM CTR siRNA transfected cells despite the fact that the same amount of cells were transfected at time 0 ($P < 0.05$; Fig. 9). The cell count of cells transfected with the Ard1 overexpression plasmid was 51% lower than that of cells transfected with GFP ($P < 0.05$; Fig. 9).

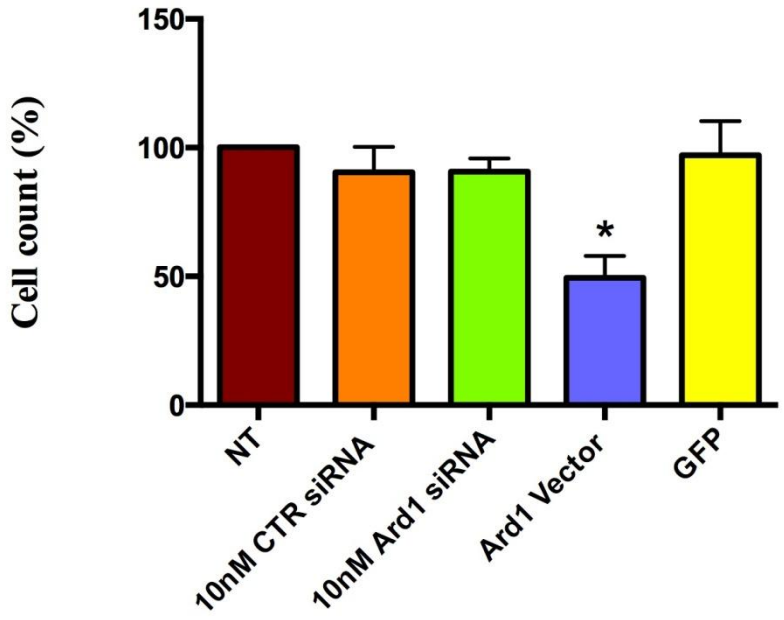


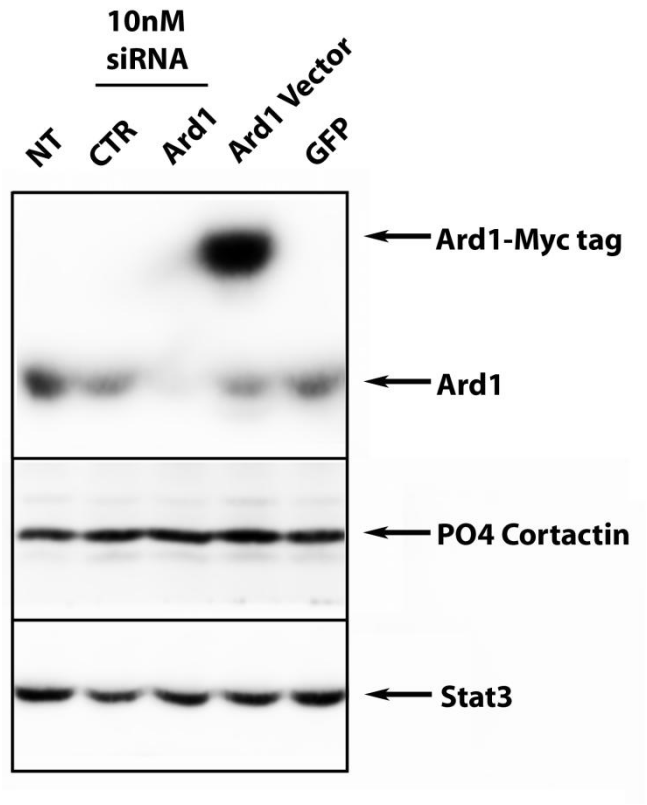
Figure 9: Transfection of RF/6A cells with Ard1 overexpression plasmid results in a significant decrease in the amount of cells at 48 hrs post-transfection.

Cells transfected with Ard1 vector have significantly lower cell counts than all other experimental groups ($P < 0.05$). Cell count 48 hrs after transfection of RF/6A cells with 1.33 x 10^6 cells per 100mm plate. Control siRNA transfected cells (CTR), Ard1 siRNA transfected cells (Ard1), GFP transfected cells (GFP) and cells transfected with Ard1 overexpression plasmid (Ard1 vector) were all transfected with GFP. The average cell count ± s.e.m. of experiments are shown (n=3). Non transfected (NT) cells were plated at 0.8 x 10^6 cells per 100mm plate at the time of transfection to prevent the cells from being over confluent at 48 hrs. (* is $P < 0.05$).

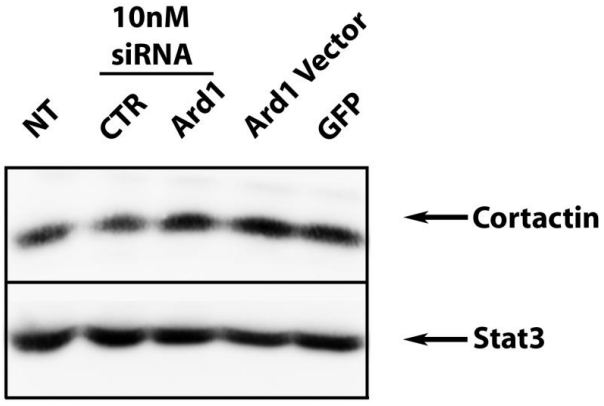
3.4 Ard1 knockdown and Ard1 overexpression have no effect on phospho-Tyr421 Cortactin levels.

Cortactin has been shown to co-localize with Tbdn along the actin cytoskeleton and at the cortex of endothelial cells (Paradis et al., 2008). Cortactin is also known to regulate cellular permeability through its interaction with the actin cytoskeleton (Daly, 2004; Mehta and Malik, 2006). We examined the effects of Ard1 knockdown on phospho-Tyr421 Cortactin and total Cortactin levels. The levels of phospho-Tyr421 Cortactin and total Cortactin were measured by western blotting techniques using specific antibodies. Ard1 knockdown by transient siRNA transfection or Ard1 overexpression resulted in no significant change in phospho-Tyr421 Cortactin over total Cortactin levels relative to loading controls (Fig. 10C).

A


10nM
siRNA
NT
CTR
Ard1
Ard1 Vector
GFP
Ard1-Myc tag
Ard1
PO4 Cortactin
Stat3

B


10nM
siRNA
NT
CTR
Ard1
Ard1 Vector
GFP
Cortactin
Stat3

C

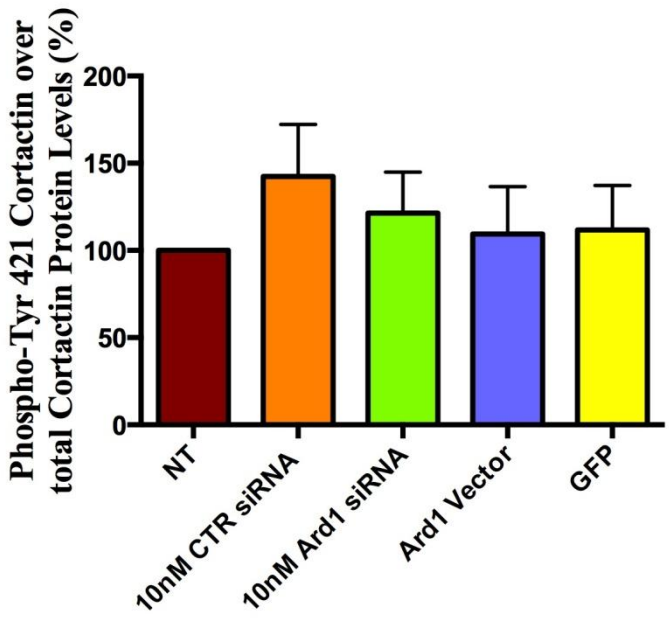


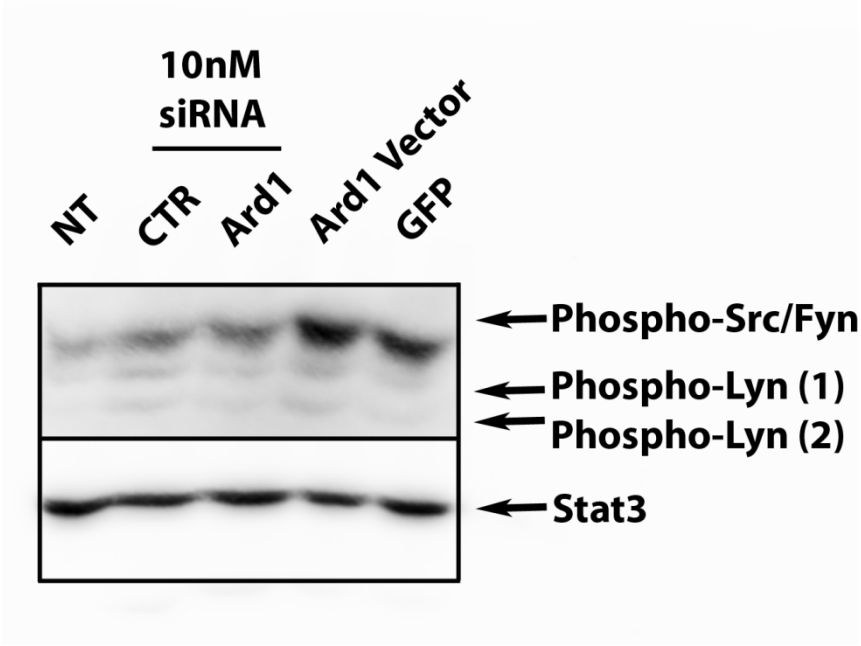
Figure 10: Ard1 knockdown and Ard1 overexpression in RF/6A retinal endothelial cells has no significant effect on phospho-Tyr421 Cortactin levels.

Non-transfected cells (NT), control siRNA transfected cells (CTR), Ard1 siRNA transfected cells (Ard1), cells transfected with Ard overexpression plasmid (Ard1 vector) and GFP transfected cells (GFP) protein levels were analyzed by western blot for phospho-Tyr421 Cortactin and total Cortactin levels. The blot was re-probed for Stat3 as a loading control. Cortactin protein levels in C are relative to loading controls and expressed as a percentage of non-transfected cell protein levels. There was no significant difference between the groups. (A) Representative experiment for phospho-Tyr421 Cortactin; (B) Representative experiment for total Cortactin (C) average of phospho-Tyr421 Cortactin levels over total Cortactin levels ± s.e.m. of experiments are shown (n=3).

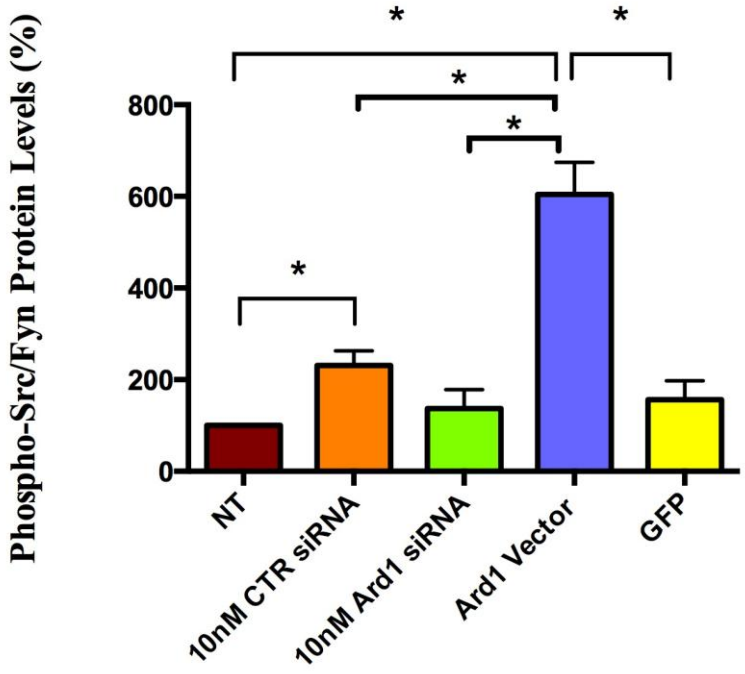
3.5 Ard1 knockdown has no effect on phospho-c-Src/Fyn levels while Ard1 overexpression results in increased levels of phospho-c-Src/Fyn.

We examined the effects of Ard1 knockdown and overexpression on c-Src protein levels. The tyrosine kinase c-Src has been reported to play a role in regulation of cellular permeability and caveolae-mediated endocytosis (Mehta and Malik, 2006;Shajahan et al., 2004). Ard1 was knocked down as well as overexpressed in RF/6A cells and levels of phospho-c-Src/Fyn, total c-Src and total Fyn were analysed using western blotting techniques (Fig. 11A,C,E). Three bands were detected by western blot using the anti-phospho-Src Family (Tyr416) antibody. The highest 60kDa band corresponds to activated c-Src and activated Fyn which co-migrate on the SDS-PAGE (Ho et al., 2012). The two lower bands represent the two isoforms of activated Lyn (Ho et al., 2012). Only the top band representing phosphorylated c-Src and phosphorylated Fyn was quantified. This study focuses on c-Src rather than Lyn levels due to the involvement of activated c-Src in the regulation of endothelial cell permeability (Mehta & Malik, 2006). Knockdown of Ard1 was found to have no significant effect on phospho-c-Src/Fyn levels relative to loading controls (Stat3 or Tubulin). However, Ard1 overexpression increased activated c-Src/Fyn levels by 6 fold ± 0.7 fold compared to non transfected cells ($P < 0.0001$; Fig. 10B). Knockdown and overexpression of Ard1 had no significant effect on total c-Src (Fig. 11D) or total Fyn levels (Fig. 11F). Phospho-c-Src/Fyn levels of 10nM CTR siRNA transfected cells were increased by 2.3 fold ± 0.32 fold compared to non-transfected cells ($P < 0.05$; Fig. 11B). Total c-Src levels of 10nM CTR siRNA transfected cells were increased by 1.3 fold compared to GFP transfected cells ($P < 0.05$; Fig. 11D).

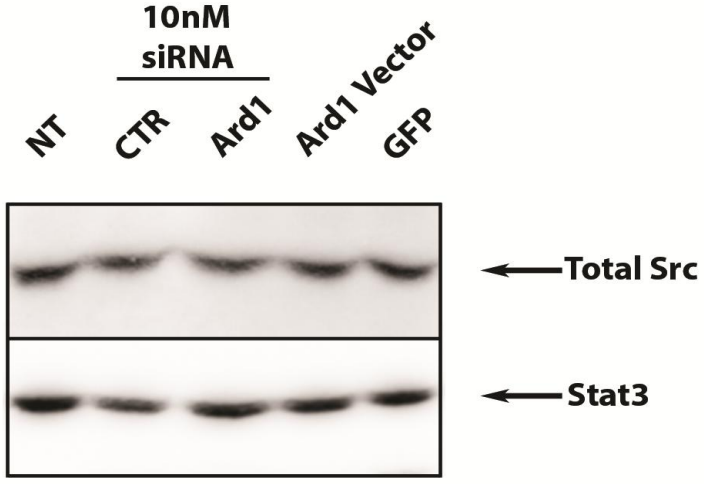
A


10nM
siRNA
NT
CTR
Ard1
Ard1 Vector
GFP
Phospho-Src/Fyn
Phospho-Lyn (1)
Phospho-Lyn (2)
Stat3

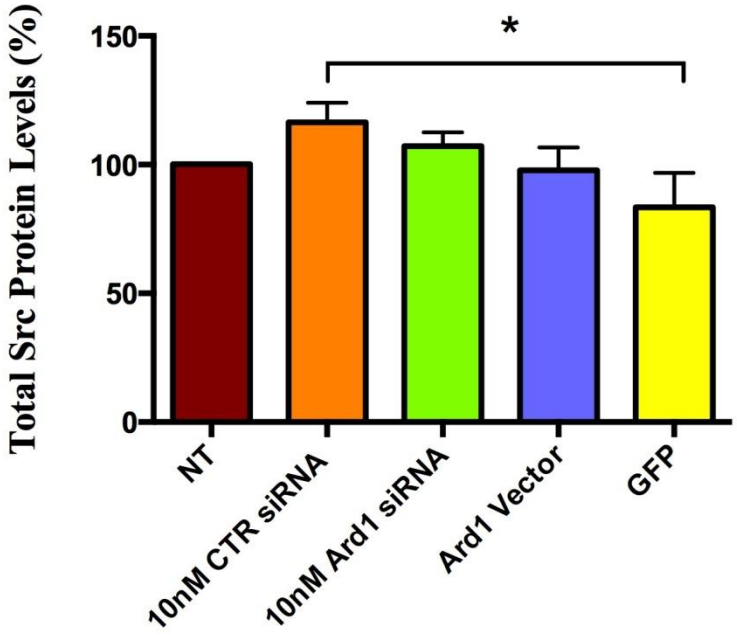
B


Phospho-Src/Fyn Protein Levels (%)
800
600
400
200
0
*
*
*
*
*
NT
10nM CTR siRNA
10nM Ard1 siRNA
Ard1 Vector
GFP

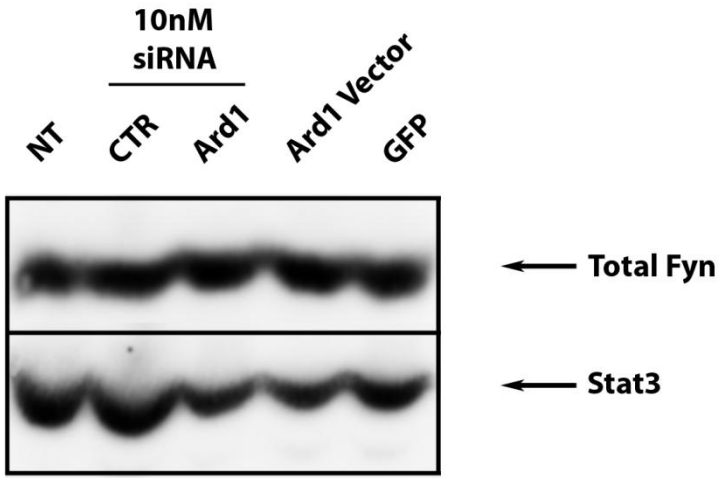
C


10nM
siRNA
NT
CTR
Ard1
Ard1 Vector
GFP
Total Src
Stat3

D


Total Src Protein Levels (%)
150
100
50
0
*
NT
10nM CTR siRNA
10nM Ard1 siRNA
Ard1 Vector
GFP

E


10nM
siRNA
NT
CTR
Ard1
Ard1 Vector
GFP
Total Fyn
Stat3

F

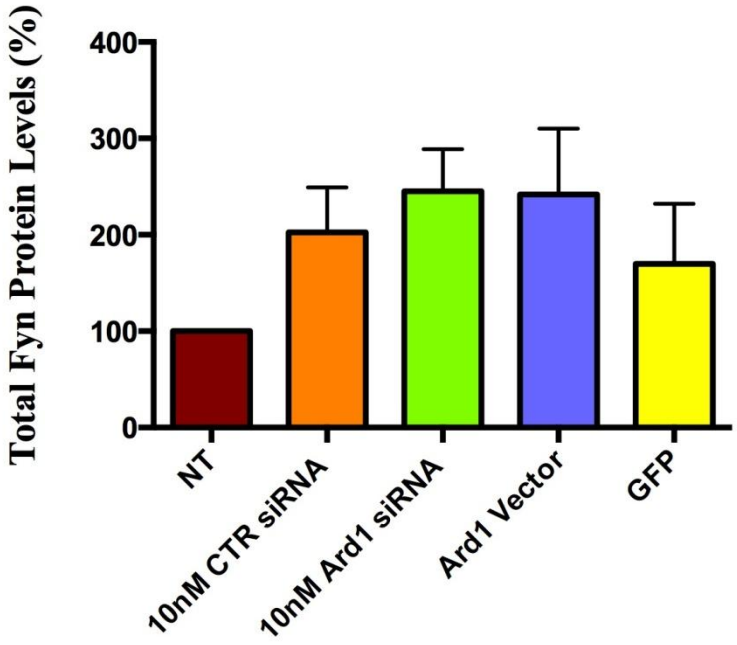

Total Fyn Protein Levels (%)
400
300
200
100
0
NT
10nM CTR siRNA
10nM Ard1 siRNA
Ard1 Vector
GFP

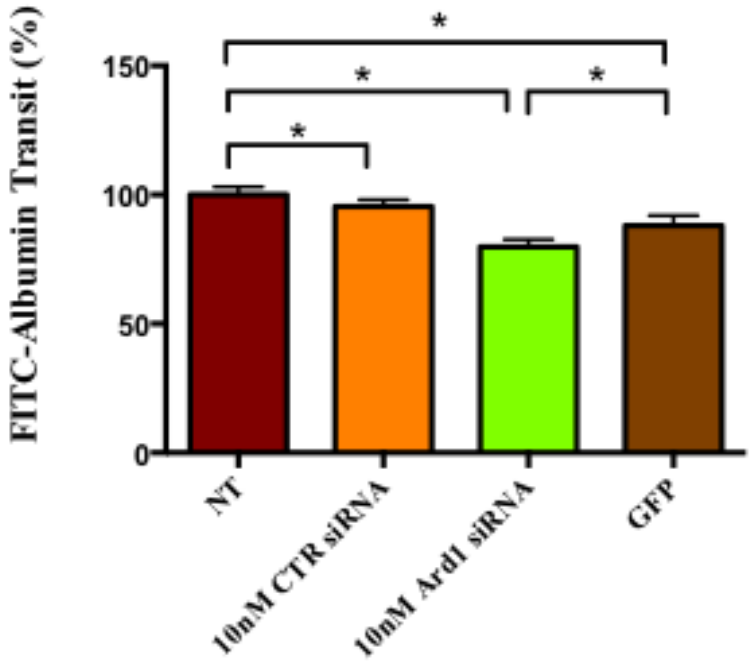
Figure 11: Ard1 knockdown has no effect on phospho-c-Src/Fyn levels while Ard1 overexpression results in increased levels of phospho-c-Src/Fyn.

Non-transfected cells (NT), control siRNA transfected cells (CTR), Ard1 siRNA transfected cells (Ard1), cells transfected with Ard1 overexpression plasmid (Ard1 vector) and GFP transfected cells (GFP) protein levels were analyzed by western blot for phospho-c-Src and phospho-Fyn (phospho-c-Src/Fyn) levels, total Src levels and total Fyn levels. The blots were re-probed for Stat3 as a loading control. Ard1 overexpression resulted in a significant increase in phospho-c-Src/Fyn levels ($P < 0.0001$) when compared to NT, CTR and GFP. Protein levels in B,D and F are relative to loading controls and expressed as a percentage of non-transfected cell protein levels. (A) Representative experiment for phospho-c-Src/Fyn. (B) average of Phospho-c-Src/Fyn levels ± s.e.m. of experiments are shown (n=3). (C) Representative experiment for total Src. (D) average of total c-Src levels ± s.e.m. experiments are shown (n=4). (E) Representative experiment for total Fyn. (F) average of total Fyn levels ± s.e.m. of experiments are shown (n=3). (* is $P < 0.05$).

3.6 Ard1 knockdown in RF/6A cells results in a decrease in albumin permeability while Ard1 overexpression results in an increase in albumin permeability.

Transcellular permeability assays were performed to determine the effect of Ard1 knockdown and Ard1 overexpression on permeability (Fig. 12). As shown in Fig. 12A, when Ard1 was knocked down, a significant decrease in permeability of retinal endothelial cells to albumin was observed when compared to controls ($P < 0.05$). Specifically, Ard1 knockdown reduced permeability of retinal endothelial cells to albumin by 20% ± 3% when compared to non transfected cells ($P < 0.05$), by 17% ± 3% when compared to control siRNA transfected cells ($P < 0.05$), and by 9% ± 4% when compared to GFP transfected cells ($P < 0.05$). However, when Ard1 is overexpressed, there was a significant increase in permeability of retinal endothelial cells to albumin when compared to controls ($P < 0.01$; Fig. 12B). Specifically, Ard1 overexpression increases the permeability of retinal endothelial cells to albumin by 21% ± 5% when compared to non transfected cells ($P < 0.01$) or by 33% ± 6% when compared to GFP transfected cells ($P < 0.01$). Knockdown of Tbdn levels using Tbdn knockdown clones resulted in an increase in permeability of 30% ± 4% of retinal endothelial cells to albumin when compared to non-transfected cells ($P < 0.0001$; Fig. 12B) or by 38% ± 4% when compared to GFP transfected cells ($P < 0.0001$). This is in agreement with data that has been described previously (Paradis et al., 2008). Permeability of GFP transfected cells was slightly decreased by 12% ± 4% compared to non-transfected cells ($P < 0.05$; Fig. 12B).

A


FITC-Albumin Transit (%)
150
100
50
0
*
*
*
*
NT
10nM CTR siRNA
10nM Ard1 siRNA
GFP

B

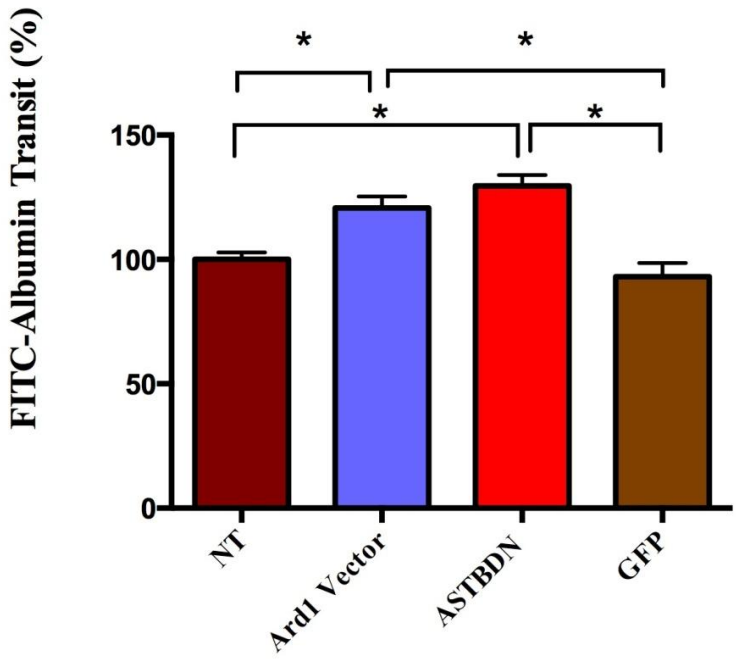

FITC-Albumin Transit (%)
150
100
50
0
*
*
*
*
NT
Ard1 Vector
ASTBDN
GFP

Figure 12: Ard1 knockdown in RF/6A cells results in a significant decrease in albumin permeability while Ard1 overexpression results in a significant increase in albumin permeability.

FITC-albumin transit across monolayers of non-transfected cells (NT), control siRNA transfected cells (CTR), Ard1 siRNA transfected cells (Ard1), GFP transfected cells (GFP), cells transfected with Ard overexpression plasmid (Ard1 vector) and Tbdn-knockdown clone (ASTBDN) were expressed as a percentage of NT cells after 40 minutes. (A) Significantly lower percentages of FITC-albumin transit were observed in Ard1 knockdown cells than NT, CTR and GFP ($P < 0.05$). (B) Significantly higher percentages of FITC-albumin transit were observed in Ard1 vector cells and ASTBDN cells when compared to NT and GFP ($P < 0.01$). FITC-Albumin transit across RF/6A monolayers is expressed as a percentage of non-transfected cells at 40 minutes. Data shown is expressed as the mean ± s.e.m. of experiments in each group (n=4). (* is $P < 0.05$).

Chapter 4 Discussion

In order to understand the possible role of Ard1 in regulation of retinal endothelial permeability, we examined the link between Ard1 and known proteins involved in regulation of transcellular permeability of albumin. These proteins are Cortactin and c-Src. Ard1 is an N-terminal acetyltransferase known to be involved in regulation of cell growth and differentiation of neuronal tissues (Arnesen et al., 2006;Lim et al., 2006;Lim et al., 2008;Sugiura et al., 2003). Acetylation is defined as the transfer of acetyl groups from acetyl coenzyme A (acetyl CoA) onto the ε amino acid groups of lysine residues which alters the biological properties of proteins (Deribe et al., 2010). This study focused on the relationship between Ard1 and phosphor-Tyr421 Cortacin and activated c-Src. Interestingly, results by the Gendron/Paradis lab reported that knockdown of Tbdn which is associated with a 50% reduction in Ard1 expression, results in increased levels of phospho-Tyr421 Cortactin and phospho-Tyr416 activated c-Src/Fyn levels and increases permeability to albumin. Therefore, we hypothesized that Tbdn regulation of retinal endothelial permeability requires Ard1.

In order to test this hypothesis, I knocked down and overexpressed Ard1 in RF/6A primate retinal endothelial cells using transient transfection techniques. There was no significant change in the transfection efficiencies of the cells transfected with either Ard1 siRNA or Ard1 overexpression vector when compared to controls (Fig. 6). However, there was a significant decrease in the cell count of cells transfected with the Ard1 overexpression vector when compared to controls (Fig. 9). A possible explanation for the reduced cell count is that overexpression of Ard1 in RF/6A cells results in an increase in

cell death following transfection. There are several different processes involved in death of cells which include apoptosis, senescence and necrosis. The most likely cause of cell death is an increase in apoptosis due to gene changes caused by overexpression of Ard1. Increased levels of Ard1 may alter the normal apoptotic pathways in the cells. Knockdown of Ard1 triggers apoptosis in human cell lines (Arnesen, Gromyko et al., 2006). Furthermore, in lung cancer cells, knockdown of Ard1 results in reduced cyclin D1 expression and suppressed cellular proliferation (Lim, Park, & Chun, 2006). However, breast cancer cell lines which overexpress Ard1 showed reduced cell growth (Kuo et al., 2010). In addition, knockdown of Ard1 was shown to have no effect on cell count following transfection of RF/6A cells with Ard1 siRNA. It seems that overexpression of Ard1 in this experiment resulted in an increase in cell death following transfection. However, knockdown of Ard1 did not reduce the cell number and does not appear to lead to cell death in retinal endothelial cells. Ard1 appears to have different roles in regulation of cell death depending on the cellular context studied. The next logical step for future experiments would be to explore the role of Ard1 in regulation of apoptosis and cell death in retinal endothelial cells which is beyond the scope of this MSc thesis.

Past research has shown that knockdown of Tbdn resulted in an increase in activated c-Src/Fyn levels and phospho-Tyr421 Cortactin levels (Ho, 2012). Research by Drs. Gendron and Paradis has also shown that knockdown of Tbdn results in co-suppression of Ard1 levels to approximately 50% and increased permeability of retinal endothelial cells to albumin (Paradis et al., 2008). However, knockdown of Ard1 by approximately 75% resulted in a small decrease in permeability of retinal endothelial cells

to albumin (Fig.12). The results suggest that Tbdn knockdown-mediated increase in permeability is either independent of Ard1 levels or is overcoming the effect of Ard1 suppression to 50%. It is possible that the 50% Ard1 knockdown resulting from Tbdn knockdown might be a feedback response of the cells to Tbdn knockdown in order to re-establish the normal permeability. On the other hand, overexpression of Ard1 resulted in a significant increase in activated c-Src/Fyn levels (Fig. 11) as well as an increase in permeability of retinal endothelial cells to albumin (Fig. 12). All together these studies indicate that Tbdn decreases permeability while Ard1 increases it. However, the role of Ard1 on c-Src/Fyn and Cortactin regulation is less clear. Past results have shown that knockdown of Tbdn resulted in an increase in activated c-Src/Fyn levels and phospho-Tyr421 Cortactin levels (Ho, 2012). My results revealed no significant change in the levels of phosphorylated Cortactin (Y421) or activated c-Src/Fyn associated with the knockdown of Ard1 (Fig. 10, Fig. 11). It is possible that the effects of Ard1 knockdown on these two components of the albumin permeability pathway are too minimal to be observed under the tested conditions. In addition, the results suggest that Ard1-mediated increase of activated Src/Fyn does not require Tbdn. Overexpression of Ard1 was not associated with an increase in Y421-Phospho-Cortactin levels. This could suggest that Tbdn is required to regulate Y421-phospho-Cortactin levels or alternatively that the effects of Ard1 overexpression on Y421-Phospho-Cortactin are too minimal to be observed under the tested conditions. Overexpression of Ard1 might alter endothelial albumin permeability via a mechanism that differs from Tbdn knockdown in that it does not involve phosphorylation of Cortaction but may involve c-Src activation. It is also

possible that the co-suppression of Ard1 during Tbdn knockdown might oppose the decrease in permeability observed under such circumstances.

The mechanisms by which Ard1 regulates permeability are not clear. Ard1 knockdown has been reported to result in increased apoptosis (Arnesen, Gromyko et al., 2006). Moreover, both Ard1 and Tbdn are known to play roles in cell growth (Arnesen et al., 2006;Asaumi et al., 2005;Lim et al., 2006;Park and Szostak, 1992). Knockdown and overexpression of Ard1 could cause abnormal regulation of cell growth or apoptosis depending on the cellular context. Src family kinase members Fyn and Lyn are cleaved in their unique region in hematopoietic cells undergoing apoptosis (Luciano et al., 2001). This could result in changes in permeability of retinal endothelial cells to albumin in response to changes in Ard1 levels since the Src family kinases c-Src and Fyn have been shown to be involved in regulation of permeability (Gong et al., 2008). One possible explanation for the increase in phosphorylated c-Src/Fyn and increase in permeability could be in relation to the active site of Ard1. A study has shown that the binding of Tbdn to Ard1 to form the NatA complex causes a structural change in the active site of Ard1 which allows it to acetylate different targets than when Ard1 is not in complex with Tbdn (Liszczak et al., 2013). When Ard1 is overexpressed, more Ard1 may exist on its own rather than in complex with Tbdn creating a structurally different active site which can acetylate different targets than when Tbdn is bound to Ard1. It is possible that Ard1 can acetylate different targets when not bound to Tbdn, which could provide an alternate mechanism of regulation of permeability of retinal endothelial cells to albumin.

Tbdn levels of 10nM CTR siRNA transfected cells were found to be significantly higher than 10nM Ard1 siRNA transfected cells (Fig. 7). Furthermore, Ard1 levels of 10nM CTR siRNA transfected cells were found to be significantly higher than GFP transfected cells and non-transfected cells (Fig. 6). Phosphorylated c-Src/Fyn levels of 10nM CTR siRNA transfected cells were also found to be significantly higher than non-transfected cells (Fig. 10). Transfection with this specific CTR siRNA is somehow increasing Tbdn and Ard1 levels. When the CTR siRNA was ordered, the siRNA sequence was entered into a nucleotide blast to determine if there was any homology to any proteins other than Ard1. When designing the scrambled CTR siRNA, sequences with more than 14 contiguous base pairs of homology to other coding sequences were excluded. Although the program BLAST was used to compare the CTR siRNA to both human and *macaca mulatta* genomes, it is possible that the CTR siRNA is knocking down another protein that could result in the increased levels of Tbdn and Ard1. To overcome this problem, the experiment could be completed with a different scrambled CTR siRNA to see if the effects on Tbdn and Ard1 levels are repeated. Permeability analysis also showed that permeability of GFP transfected cells was significantly decreased compared to non-transfected cells. It is also possible that the transfection process itself might affect the permeability to albumin.

Further research is required to determine the role of Ard1 in regulation of retinal endothelial cell permeability. Possible future directions could include overexpression of Tbdn to then observe the effects on Ard1 levels, activated c-Src levels and permeability.

Chapter 5 Conclusions

This study examined the effects of knockdown and overexpression of Ard1 on Tbdn, phosphorylated Cortactin Tyr 421 and phosphorylated c-Src Tyr 416 levels as well as permeability of retinal endothelial cells to albumin. Previous work in the laboratory of Drs. Gendron and Paradis have shown Tbdn to be involved in regulation of retinal endothelial permeability (Paradis et al., 2008).

The results revealed that knockdown of Ard1 resulted in a decrease in permeability of retinal endothelial cells to albumin, however had no effect on phospho-c-Src/Fyn or phosphorylated Cortactin Tyr 421 levels. Overexpression of Ard1 resulted in increased levels of phospho-c-Src/Fyn and increased permeability of retinal endothelial cells to albumin. This suggests that Ard1 overexpression may alter endothelial albumin permeability through a pathway which does not involve phosphorylation of Cortactin but may involve c-Src activation.

It appears that the relationship between Ard1 and permeability is more complicated than expected and will require further study. Understanding more about the role of Ard1 and Tbdn in the regulation of retinal endothelial permeability could lead to development of drugs to help treat those suffering from neovascular retinopathies.

Chapter 6 References

Anonymous Eye diagram: A public domain PNG image.

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