THE ROLE OF TBDN IN RETINAL ENDOTHELIAL CELL PERMEABILITY AND RETINAL HOMEOSTASIS

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The Role of Tbdn in Retinal Endothelial Cell Permeability and Retinal Homeostasis

By

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ABSTRACT

Tubedown (Tbdn) is a cortactin-binding acetyltransferase subunit which controls retinal endothelial permeability and promotes retinal vascular homeostasis. The Paradis-Gendron laboratory has generated a binary antisense *Tbdn (Astbdn)* transgenic mouse model (*TIE2/rtTA/Enh-TRE/ASTBDN-1)* that facilitates the conditional knockdown of Tbdn protein expression in retinal endothelial cells. The suppression of Tbdn expression in mouse retinal endothelium, facilitated by the introduction of doxycycline (Dox) to the binary system, results in several pathologic features that are also observed in human retinopathies. Tbdn protein expression is reestablished once doxycycline is removed from the system.

In study one, it was hypothesized that reexpression of Tbdn would occur in retinal blood vessels of *TIE2/rtTA/Enh-TRE/ASTBDN* mice upon Dox withdrawal and that Tbdn reexpression would correlate with decreased retinal pathology. In study one it was found through immunohistochemistry and morphological assessment that reexpression of Tbdn in retinal blood vessels resulted in a reduction of albumin leakage across the retinal blood vessel endothelium, along with a decrease in the extent of retinal vascularization and retinal pathologies compared to Tbdn knockdown mice. These results suggest that restoration of Tbdn protein expression after knockdown reduces retinal pathology at the morphological and functional level.

In this study it was shown that the proliferative retinal neovascularization and thickening resulting from induction of Tbdn knockdown in endothelium in transgenic

mice is associated with a significant increase in extravasation or leakage of albumin from abnormal retinal blood vessels *in vivo.* These results provide evidence that Tbdn is involved in the regulation of retinal endothelial cell permeability to albumin and implicates a functional role for Tbdn in blood vessel permeability dynamics. Study two explored the relationship between Tbdn and other known regulators of vascular endothelial permeability. Tbdn suppression in endothelial cells correlates with an increase in transcellular permeability pathway. Recent experimental evidence points to the importance of Src family protein tyrosine kinase (SFK) signaling in regulation of microvascular barrier function and vascular endothelial permeability.

In study two it is hypothesized that Tbdn expression regulates retinal endothelial cell permeability through regulation of the Src pathway. The Paradis-Gendron laboratory have previously generated RF/6A endothelial cell clones in which Tbdn expression had been suppressed by stable expression of the antisense *TBDN* cDNA construct ASTBDN. Src activation in retinal endothelial cell clones was measured by western blot and immunoprecipitation analysis. It was shown that when Tbdn is suppressed in endothelial cells, there is a higher level of activated Src compared to parental and negative control clones. This high level of activated Src suggests that Tbdn may regulate the transcellular permeability pathway for albumin transport through suppression of the Src pathway.

Based on the cumulative results of both studies I propose a mechanism whereby Tbdn plays an important role in regulating the permeability of retinal endothelial cells to albumin by interacting with other proteins in the transcellular albumin pathway.

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LIST OF ABBREVIATIONS

AMD	**age-related macular degeneration**
AP	**alkaline phosphatase**
Ard1	**arrest defective protein 1**
ASTBDN	**Antisense Tbdn**
BBB	**blood-brain barrier**
BM	**Bruch's membrane**
BRB	**blood-retinal barrier**
BSA	**bovine serum albumin**
CFH	**complement factor h**
CSK	**C-terminal Src kinase**
DMEM	**Dulbecco's modified eagle medium**
Dox	**Doxycycline**
EC	**endothelial cell**
ECL	**enhanced chemiluminescence**
FBS	**fetal bovine serum**
FITC	**fluorescein isothiocyanate**

GCL	**ganglion cell layer**
gp60	**60 kDa glycoprotein**
H & E	**hematoxylin and eosin**
HRP	**horseradish peroxidase**
INL	**inner nuclear layer**
IP	**immunoprecipitation**
IPL	**inner plexiform layer**
kDa	**kilodalton**
NAT-1	**n-terminal acetyltransferase**
NT	**non-treated**
ONL	**outer nuclear layer**
OPL	**outer plexiform layer**
PAR	**parental**
PBS	**phosphate buffered saline**
PDR	**proliferative diabetic retinopathy**
PHR	**photoreceptors**

PMSF	**phenylmethylsulfonyl fluoride**
PVDF	**polyvinylidene fluoride**
ROP	**retinopathy of prematurity**
RPE	**retinal pigment epithelium**
rtTA	**reverse tetracycline transactivation**
SD	**standard deviation**
SDS	**sodium dodecyl sulfate**
SDS-PAGE	**sodium dodecyl sulfate polyacrylamide gel electrophoresis**
SFK	**Src family kinase**
SH	**Src homology**
Tbdn	**Tbdn**
TBS	**tris buffered saline**
TBST	**tris buffered saline tween**
TGF-β	**transforming growth factor beta**
TRE	**tetracycline response element**
V	**vitreous body**

VEGF	**vascular endothelial growth factor**
WCL	**whole cell lysate**
ZO-1	**zonula occludens**

1.0 INTRODUCTION

1.1 The Eye

The eye is a highly specialized organ that contains tissues that vary greatly in structure and function (Figure 1a). The orbit and eyelids provide protection, while the cornea, lens, iris, and extra ocular muscles work together to focus light rays on photoreceptors of the retina, initiating the process of sight. A unique arrangement of vascular tissues within the eye provides the tissues with required nutrition and a means of gas exchange (Kaufman & Alm, 2003).

Light rays from an object enter the eye first through the cornea then progress through the pupil, which is the circular opening in the center of the colored iris. The light rays are bent or converged by the cornea, and then further by the lens (located behind the iris and the pupil). The light continues through the vitreous humor and then focuses on the retina behind the vitreous (Kaufman & Alm, 2003). It is suggested that as much as 80% of the visual sensory input in humans take place in the retina (Fatt & Weissman, 1992). The retina controls the initial steps of visual perception: a light stimulus is propagated through the eight layers of the retina (Figure 1b) to produce an image (Fatt & Weissman, 1992). Intricate neurons spanning the retinal layers allow for the propagation of signals through the retina until they reach the optic nerve, and then are sent along the visual pathway to the occipital cortex at the posterior of the brain. At the occipital cortex the electrical signals are interpreted by the brain as a visual image (Fatt & Weissman, 1992; Kaufman & Alm, 2003; Ferris & Tielsch, 2004).

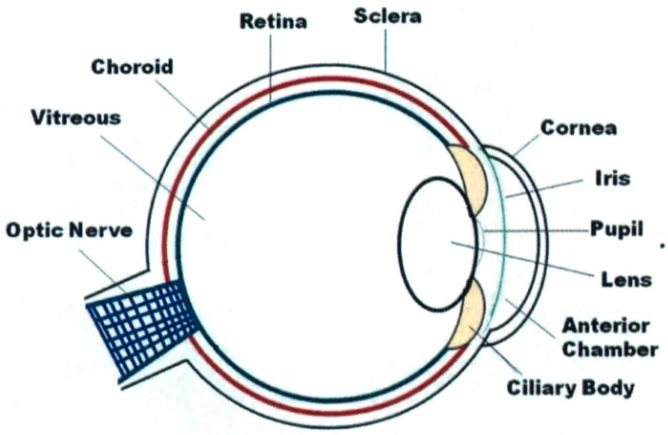

Figure 1a: Schematic diagram of the structural features of the eye

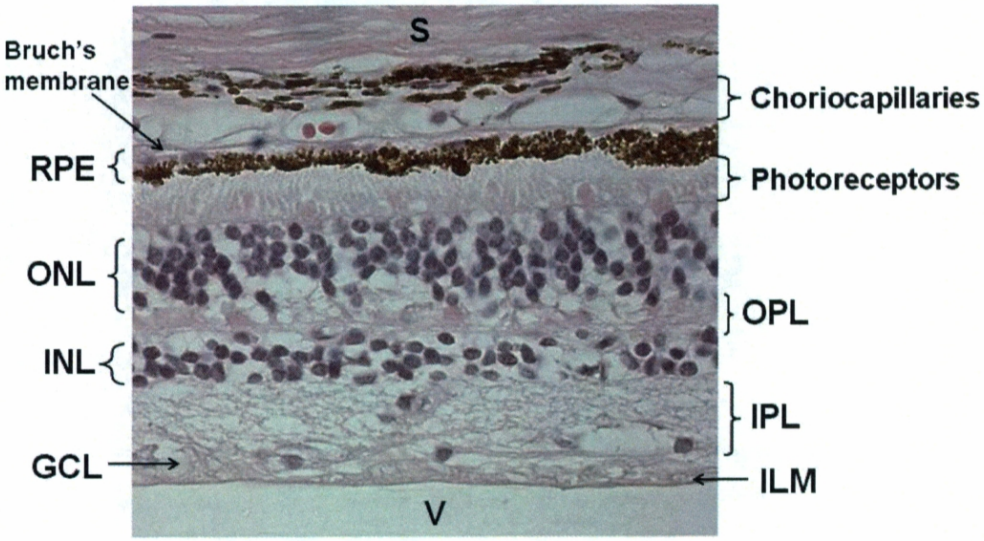

Figure 1b: Structural features of the human retina. Hematoxylin & Eosin staining showing different layers that comprise the retina. The inner limiting membrane (ILM) forms a diffusion barrier separating the retina from the vitreous (V). The other seven layers of the retina include: the ganglion cell layer (GCL), the inner plexiform layer (IPL), the inner nuclear layer (INL), the outer plexiform layer (OPL), the outer nuclear layer (ONL), the photoreceptors (PHR) and the retinal pigment epithelium (RPE). The IPL and OPL contain cell axons establishing synaptic connections while the INL and GCL contain cellular nuclei responsible for propagation and modification of the stimuli. The ONL contains cell bodies of the photoreceptor cells. The choriocapillaries nourish the avascular regions of the outer retina. The sclera (S) is the outermost structure. Magnification: 400X (Slide provided by Dr. Paradis and used with permission).

1.2 Vasculature of the eye

The vascular system makes up a continuous network through which blood transports oxygen and nutrients to all tissues of the body, while removing waste materials. The vascular system is comprised of blood vessels that are lined with endothelial cells, making up the endothelium. Endothelial cells derive from early precursors that proliferate and form blood vessels that continue to form vascular networks (Suburo & D'Amore, 2006). Two separate vascular systems are involved in the homeostasis of the retina: the retinal and the choroid vessels (D'Amore, 1994; Kaufman & Alm, 2003). The retinal vessels are arranged within the inner two thirds of the retina, and receive their blood supply from the central retinal artery. These blood vessels are lined with endothelial cells that form tight junctions, which make up the first component of the blood-retinal barrier (Vinores et al., 1999; Suburo & D'Amore, 2006). The outer layers of the retina, including the photoreceptors, are avascular and receive their metabolic requirements from the highly vascular choroid (Kaufman & Alm, 2003; Suburo & D'Amore, 2006).

The structure of the two vascular systems of the eye differs significantly, especially in regards to differences in permeability. The choroid vasculature is comprised of fenestrated capillaries that are permeable to allow the transport of nutrients from the blood to the outer layers of the retina. In contrast, the retinal vessels are impermeable to plasma proteins and even small water-soluble substances such as glucose, and nutrients have to be transported by means of a transport system (D'Amore, 1994; Kaufman & Alm, 2003). Retinal blood vessels have a supporting vascular network of cells called pericytes.

Pericytes are elongated, contractile cells found wrapped around pre-capilary arterioles outside the basement membrane of the blood vessel. Pericytes aid in the maintenance of endotheial cell structure. Pericytes co-localized with endothelial cells secrete Vascular Endothelial Growth Factor (VEGF), which under normal physiological conditions inhibits apoptosis, enhancing retinal endothelial cell survival (Wenbiao et al., 2002).

Retinal endothelial cells perform a number of functions that are necessary to maintain homeostasis of the eye, including regulation of barrier function by controlling the permeability to fluid, solutes and macromolecules into and out of the vessels (Pober & Min, 2006). Under normal physiological conditions the endothelium of a mature retinal blood vessel is stable and vascular leakage is limited. However, various pathological conditions can cause a disruption to the endothelium resulting in angiogenesis, a process in which new blood vessels develop from preexisting vascular beds, and endothelial cell hyper-permeability (Bergers & Benjamin, 2003; Suburo & D'Amore, 2006). Proliferative blood vessels can grow into other layers of the retina and disrupt the blood retina barrier. Leakage of blood, serum, and lipid from the pathological blood vessels can stimulate re-organization of cellular layers in the retina and promote photoreceptor damage, scarring and detachment of the RPE or retina. Disruption of retinal blood vessel endothelium can lead to various neovascular retinopathies causing visual loss and blindness.

1.3 Neovascular Retinopathies

Diseases involving ocular neovascularization can cause visual loss and blindness (Dorrell et al., 2007). Normal retinal vascularization is strictly regulated and proceeds in a highly ordered fashion: in response to signaling molecules, vascular endothelial cells

proliferate and migrate through the extracellular matrix, leading to the formation of new blood vessels. During pathologic neovascularization of the retina or choroid, vessels lose the balance between signaling molecules and this usually results in the formation of dysfunctional blood vessels. These newly formed vessels can leak fluid or are associated with fibrous proliferation leading to retinal edema, retinal hemorrhage, or retinal detachments, resulting in vision loss ((Bergers & Benjamin, 2003; Suburo & D'Amore, 2006; Dorrell et al., 2007).

Age-related macular degeneration (AMD) is the leading cause of blindness in people over 60 and its prevalence increases with age (Kohner, 1993; Vinores et al.,1999; Ambati et al., 2003; Ferris & Tielsch, 2004; Dorrell et al., 2007). The number of people suffering from vision loss is expected to increase dramatically in industrialized nations over the next decade as the population ages (Halpern et al., 2006; Dorrell et al., 2007; Klein, et al., 2007). AMD is a combination of age, genetic, and environmental factors that lead to degeneration of the outer layers of the central retina including the outer neural retina, RPE, Bruch's membrane (BM) and choriocapillaries (Bonnel et al., 2003; Gehrs et al., 2006; Edwards & Malek, 2007). Drusen formation in the macular region occurs in the majority of AMD patients. Drusens are pathological deposits that form between the basal lamina of RPE and BM (Ambati et al., 2003). In the early stages of AMD there is a characteristic thickening of the BM due to the accumulation of drusen deposits and RPE cell loss (dry form of AMD). This stage can progress into a neovascular form that involves neovascularization of the choroidal vasculature (wet form of AMD). These proliferative blood vessels may break through the BM and RPE thus disrupting the blood

retina barrier, and can invade and damage the photoreceptor layers. These pathological vessels are known to leak blood, serum, and lipid into the retinal tissues which can lead to disruption of retinal cells, scarring and detachment of the RPE or retina, and macular edema (D'Amore, 1994; Bonnel et al., 2003; Gehrs et al., 2006; Gendron et al., 2010).

Although the etiology of AMD remains largely unknown, numerous studies have shown that genetic predisposition significantly influences the risk of developing AMD (Ambati et al., 2003; Gehrs et al., 2006; Markiewsk & Lambris, 2007; Edwards & Malek, 2007; Katta et al., 2009). Growing evidence suggests that the complement system, an innate immune defense mechanism, plays a significant role in the development of AMD (Gehrs et al., 2006). The complement system is made up of over 40 proteins and cell surface receptors; many types of cells such as endothelial cells, epithelial cells, glial cells, monocytes, and neurons produce complements as part of a defense mechanism (Katta et al., 2009). Complement pathways are activated by different stimuli; once activated the pathway orchestrates a defense mechanism sending molecules to fight off unwanted microorganisms (Markiewski & Lambris, 2007).

Recently, several polymorphisms associated with AMD have been identified in the complement factor H gene *(CFH)* in a region called *LOC387715/HTRA1* (Klein, 2007; Katta et al., 2009; Gehrs et al., 2010; Donoso et al., 2010). This gene encodes the CFH protein, which is a major inhibitor of the complement system. The absence or low expression of CFH protein can lead to uncontrolled complement activation, leading to drusen formation (Gehrs et al., 2010; Donoso et al., 2010). Furthermore, environmental risk factors associated with AMD, such as smoking, are also known to decrease CFH

levels (Gehrs et al., 2010). When complement pathway regulatory proteins experience irregularities in their structures and/or functions the mechanisms of the complement system can be disrupted, which can result in damage to healthy cells and tissues, as seen in AMD (Klein, 2007; Gehrs et al., 2010; Donoso et al., 2010).

The leading cause of vision loss for people under the age of 65 is diabetes; 16 million people in the USA are diabetic and 40,000 new patients per year suffer from ocular complications of the disease, often as a result of retinal neovascularization (Dorrell et al., 2007, Qazi et al., 2009). Diabetic retinopathy is the result of microvascular retinal changes. The early stages of diabetic retinopathy involve capillary basement membrane thickening, loss of pericytes and loss of endothelial cells, which is thought to be induced by hyperglycemia. At advanced stages, neovascularization of the retina and optic disc occurs as part of proliferative diabetic retinopathy (PDR). As the disease progresses there can be breakdown of the blood retinal barrier resulting in vascular leakage and thickening of retina. Ultimately, PDR can cause blurred vision, macular edema, retinal detachment, and can eventually lead to complete vision loss (Paques et al., 1997; Vinores et al., 1999; Gendron et al., 2001; Gardner et al., 2002; Qazi et al., 2009).

Retinopathy of prematurity (ROP) is one of the leading causes of blindness in children (Lutty et al., 2006; Qazi et al., 2009). ROP results from the interruption of normal development of retinal blood vessels in late gestation followed by compensatory uncontrolled retinal neovascularization. The pathological vasculature includes abnormal, leaky new vessels and can cause scarring in the retina and eventually can cause retinal

detachment and blindness (Chen & Smith, 2007). New treatments have benefited patients; however, blindness, reduced visual acuity, myopia, late retinal detachments, and glaucoma remain threats to children who experienced severe ROP as infants (Good & Gendron, 2005; Lutty, et al., 2006; Good, 2006).

There are emerging therapies and treatments for neovascular retinopathies. Laser ablation has been used to treat neovascular lesions of ROP and PDR; however, laser surgery can destroy the normal, healthy retina as well (Lutty, et al.,2006; Chen & Smith, 2007). In recent years, the humanized VEGF antibody ranibizumab has been approved for treatment of neovascular AMD. Clinical trials are optimistic, indicating improvements in visual acuity in wet form AMD patients treated with the drugs (Boyer et al., 2007; Kaiser et al., 2007). However, ranibizumab is very expensive and must be repeatedly injected into the eye, which can lead to RPE tears (Apte, 2007; Kiss et al., 2007; Bakri & Kitzmann, 2007; Carvounis et al., 2007). Clinical trials using other types of humanized VEGF antibody, such as Pegatanib, VEGF Trap and Bevacizumab are currently underway (Dorrell et al., 2007).

New and more targeted treatments are needed to deal with the complexity of neovascular retinopathies. The molecular events leading to the neovascularization and vascular leakage in ROP, wet AMD, and PDR involve pro-angiogenic growth factors such as VEGF (Das & McGuire, 2003). Changes in extracellular matrix and glial cells have been suggested to contribute to neovascularization in various retinopathies (Paques et al.,1997; Robinson & Aiello, 1998; Hammes et al., 1998; Ambati et al., 2003; Campochiaro & Hackett, 2003). It is likely that there is a wide range of factors involved

in the development of neovascular retinopathies. Targeting one type of factor or element of the disease may not cover the whole scope of disease progression. By characterizing regulatory pathways acting on retinal endothelial cells we can have a better understanding of the mechanisms behind retinal neovascularization. This may lead to the development of new and alternative therapeutic targets.

1.4 Tbdn expression and function

Tbdn (Tbdn), also referred to as mNat1, NATH and Narg1, is a 100-kDa-acetyltransferase protein originally identified from an embryonic endothelial cell line (IEM) (Gendron et al., 2000). Tbdn displays homology to yeast Nat1, which forms a complex with the acetyltransferase Arrest Defective Protein (Ard1) to form an essential subunit of the yeast N-terminal acetyltransferase NatA (Park & Szostak, 1992; Gendron et al., 2000; Paradis et al., 2002; Willis et al., 2002; Sugiura et al., 2003; Gautschi et al., 2003; Kimura et al., 2003; Wang et al., 2004; Asaumi et al., 2005; Arnesen et al., 2005; Arnesen et al., 2006). In mammals, Tbdn exhibits 70% identity to mNat2 while Ard1 exhibits 81% identity with Ard2 (Sugiura et al., 2003; Arnesen et al., 2006). In both the yeast and mammalian cells, Tbdn acts in a complex with the acetyltransferase Ard1 and is involved in the regulation of a wide range of cellular processes including cell growth and differentiation (Park & Szostak, 1992; Paradis et al., 2002; Willis et al., 2002; Sugiura et al., 2003; Gautschi et al., 2003; Kimura et al., 2003; Wang et al., 2004; Asaumi et al., 2005; Arnesen et al., 2005; Arnesen et al., 2006).

Tbdn is widely expressed in blood vessels during embryogenesis, whereas in adults high levels are restricted to only a few tissues. These include the ocular endothelium, bone marrow capillaries, the choroid plexus endothelium, and blood vessels of regressing ovarian follicles (Gendron et al., 2000; Gendron et al., 2001; Paradis et al., 2002). The high levels of expression suggest that Tbdn may have a role in these particular areas. While the study of Tbdn is progressing, the role this protein plays in maintaining ocular homeostasis and the mechanism it uses is still not completely understood.

Several studies examined the level of Tbdn in retinal blood vessels of both healthy people and people suffering from ocular diseases. Expression of Tbdn is specifically suppressed in retinal blood vessels in neovascular retinal lesions in patients with PDR, ROP and AMD (Gendron et al., 2001; Paradis et al., 2002; Gendron et al., 2006; Gendron et al., 2010). For example, when Tbdn expression was examined in the eye specimens of diabetic adult patients, it was found that there is a significant suppression of Tbdn protein expression compared to eyes of healthy people (Gendron et al., 2001). Furthermore, samples from patients with ROP and AMD also showed a lower level of Tbdn compared to age-matched controls (Gendron et al., 2006, Gendron et al., 2010).

The effect of Tbdn suppression was investigated in both *in vitro* and *in vivo* models. The Paradis-Gendron laboratory has generated a binary antisense *Tbdn (Astbdn)* transgenic mouse model (*TIE2/rtTA/Enh-TRE/ASTBDN-1)* that facilitates the conditional knockdown of Tbdn protein expression in retinal endothelial cells (Wall et al., 2004). The suppression of Tbdn expression in mouse retinal endothelium results in several pathologic features that are also observed in human retinopathies, including retinal

neovascularization, fibrovascular proliferation, and retina–lens adhesions (Wall et al., 2004). The extent of the retinopathies correlated with the duration of Tbdn suppression (Wall et al., 2004). The Paradis-Gendron laboratory has also studied the targeted suppression of Tbdn in retinal endothelial cells *in vitro* by expression of an antisense cDNA construct specific for Tbdn (ASTBDN). This cell line displays a significant increase in the formation of capillary-like structures compared with controls, which could be an indication of an angiogenic-like process (Paradis et al., 2002). These findings suggest that Tbdn plays a role in the maintenance of retinal blood vessel homeostasis.

One important homeostatic function o Tbdn is the regulation of endothelial cell permeability. Previous work from the Paradis-Gendron laboratory suggests that Tbdn affects the permeability of RF/6A retinal endothelial cells *in vitro* (Paradis et al., 2008). Data collected from cellular permeability assays indicate a transcellular pathway for shuttling FITC-albumin across the RF/6A retinal endothelial cell monolayer. The knockdown of Tbdn in the cells leads to increased transcellular permeability to FITC-albumin (Paradis et al., 2008). A similar trend was seen *in vivo*: the induction of Tbdn knockdown in transgenic mice was associated with a significant increase in the leakage of albumin from abnormal retinal blood vessels (Paradis et al., 2008).

Paradis et al. (2008) showed that Tbdn co-localizes and interacts with cortactin. Cortactin is both a substrate of Src tyrosine kinase and an actin-binding protein, playing a role in regulating actin cytoskeleton assembly (Wu & Parsons, 1993; Huang et al., 2006). Cortactin is involved in processes requiring changes in the plasma membrane such as cell

migration and endocytosis and in intracellular movement of vesicles (Daly, 2004; Kessels & Qualmann, 2005; Cao et al., 2005; Huang et al., 2006). Through the regulation of actin cytoskeleton activity cortactin has been implicated in the regulation of endothelial cell permeability (Weed & Parsons, 2001; Daly, 2004; Malik & Mehta, 2006). Tbdn forms a complex with cortactin, thus providing a possible link between Tbdn and regulation of endothelial cell permeability (Paradis et al., 2008). Additionally, a recent study provides evidence that Tbdn may play a role in the regulation of endocytosis (Asaumi et al., 2005). Endocytosis is an essential step in transcellular permeability of macromolecules. Endocytosis of β–amyloid precursor transmembrane protein from cell surface can be inhibited by overexpression of the active NatA complex, which requires the overexpression of both Ard1 and Tbdn. Secretion of β -amyloid, which likely depends on the endocytosis of its precursor protein, was suppressed by overexpression of NatA (Asaumi et al., 2005). These studies suggest that when Tbdn/Ard1 is overexpressed there are changes in the permeability pathway since an essential step, endocytosis, is inhibited.

Since Tbdn was recently implicated in the regulation of the endocytosis (Asaumi et al., 2005), an essential step in transcellular permeability, along with the fact that Tbdn forms a complex with cortactin, a regulator of endothelial cell permeability, the role of Tbdn expression in the permeability of endothelial cells warrants further investigation.

1.5 Regulation of endothelial cell permeability

The regulation of endothelial cell permeability is known to be functionally important for maintaining vascular homeostasis (Minshall et al., 2002; Malik & Mehta, 2006). The transport of solutes and water across endothelial cell layer follows two distinct pathways, the paracellular and transcellular pathways. The paracellular pathway is mediated by tight junctions, which are an important structure in membrane barrier formation. The blood–retinal barrier (BRB), composed of tight junctions, is tightly regulated and allows minimal transport across the retinal barrier (Kaufman & Alm, 2003). The paracellular pathway may be regulated directly through modification of tight junction proteins, such as claudins, occludins and zonular occludins (ZO-1 and ZO-2) or indirectly through effects on the cytoskeleton (Harhaj & Antonetti, 2004; Kumar et al., 2009). Inflammatory stimuli such as VEGF can cause separation of tight junctions between endothelial cells as well as cytoskeleton contraction, leading to an intercellular gap that facilitates increased leakage of plasma fluid and protein (Kumar et al., 2009).

The transcellular pathway allows for the transportation of macromolecules, including albumin, from the luminal to abluminal cell surfaces (Rippe et al., 2002). The transport of macromolecules maintains osmotic pressure across capillaries while delivering important vascular solutes to the interstitial space (Lum & Malik, 1996).

Transcellular permeability of albumin is of particular interest since transcytosis of albumin has been shown to occur in the eye (Vinores et al., 1998). Albumin is the most abundant protein in human plasma and acts as a carrier protein for a variety of molecules, enzymes and hormones across the endothelium (Malik & Mehta, 2006). The

mechanism(s) by which endothelial cells internalize and transport albumin from the luminal to abluminal side are not completely understood. However, it is widely accepted that albumin binds to the 60 kDa glycoprotein (gp60) on the endothelial cell surface, which induces clustering of gp60 and facilitates an interaction between gp60 and Caveolin-1. Src tyrosine kinase, which is bound to Caveolin-1, is activated when albumin binds to gp60. Activated Src, in turn, phosphorylates Caveolin-1, gp60, and Dynamin-2 to initiate vesicle formation at the cell membrane and pinching off of the albumin containing-vesicles. These vesicles are subsequently transported to the basal membrane and release their contents via exocytosis into the interstitial space (Minshall et al., 2002; Malik & Mehta, 2006; Kumar et al., 2009; Hu & Minshall, 2009) (Figure 2).

Vascular transport of protein between the blood and interstitial compartments is essential for the maintenance of tissue homeostasis and normal organ functions. However, when the blood retinal barrier breaks down as seen in ocular disorders such as ROP, PDR, and AMD, staining for extravascular albumin reveals leakage through the tight junctions and an upregulation in transcellular vesicles carrying albumin (Vinores et al., 1998). Increased vascular permeability disrupts homeostasis, leading to an imbalance in nutrients and oxygen supply that can lead to angiogenesis and further proliferation of the disease (Erickson et al., 2007).

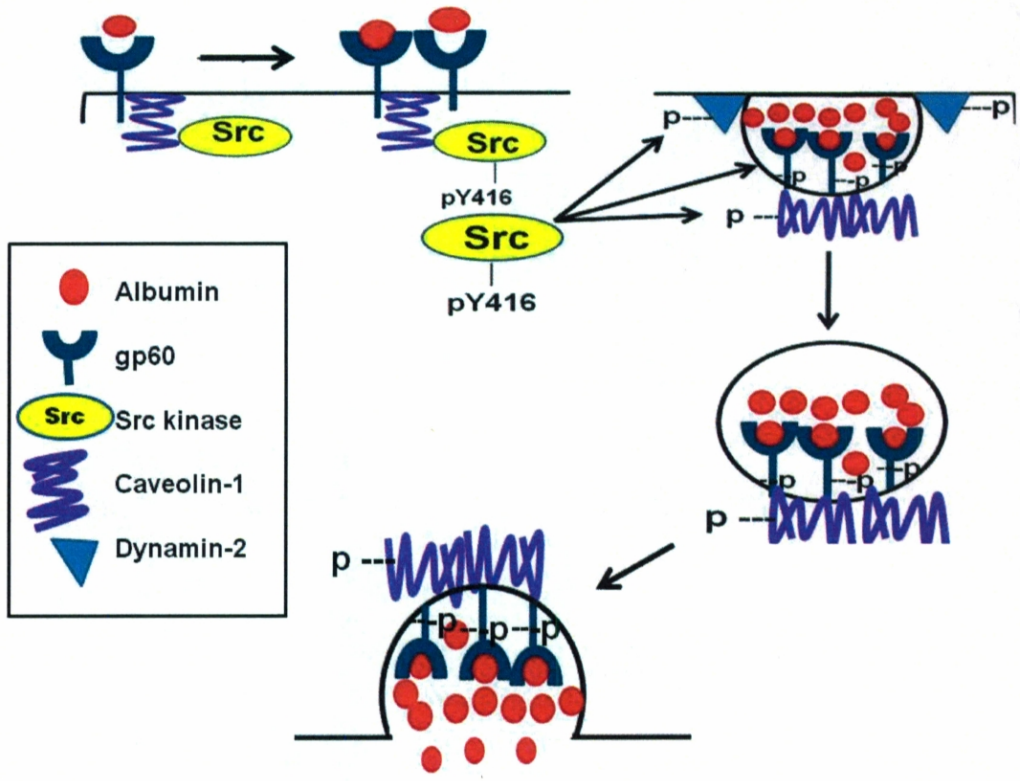

Figure 2: Schematic diagram depicting the transcellular transport of albumin.
Albumin binds to the 60 kDa glycoprotein (gp60) on the endothelial cell surface, which induces clustering of gp60 and facilitates an interaction between gp60 and caveolin-1. Src tyrosine kinase, which is bound to caveolin-1, is activated (phosphorylated, -p) when albumin binds to gp60. Activated Src, in turn, phosphorylates caveolin-1, gp60, and dynamin-2 to initiate plasmalemmal vesicle formation. The albumin containing-vesicles are subsequently transported to the basal membrane and release their contents via exocytosis into the interstitial space. (Adapted from Malik & Mehta, 2006).

Studies in the Paradis-Gendron laboratory showed maintenance of Tbdn expression is important for retinal blood vessel homeostasis and for controlling retinal vascularization (Wall et al., 2004; Paradis et al., 2008; Gendron et al., 2010). They also suggested that Tbdn affects albumin permeability of retinal endothelial cells *in vitro* and *in vivo* (Paradis et al., 2008). In order to understand the role Tbdn may have in regulating albumin permeability, it would be of importance to investigate the relationship between Tbdn and known components of the transcellular permeability pathway for albumin.

1.6 Src Family Kinases and the albumin permeability pathway

The internalization and transportation of albumin by endothelial cells involves several steps, controlled by Src protein activation (Figure 2). It is becoming increasingly evident that the Src family kinases (SFKs) play key roles in the regulation of microvascular barrier function and various endothelial responses including permeability of albumin (Minshall et al., 2000; Shajahan et al., 2004; Kim et al., 2009; Hu & Minshall, 2009). SFKs are non-receptor cytoplasmic and membrane-associated protein tyrosine kinases.

All SFKs share a similar domain arrangement, which have been classified into eight distinct functional regions (Figure 3). From the N-terminus to C-terminus, these regions include a myristylation site, *Src* homology (SH4) domain, unique region, SH3 domain, SH2 domain, linker, the catalytic (SH1) domain, and a regulatory domain (Hubbard & Till, 2000).

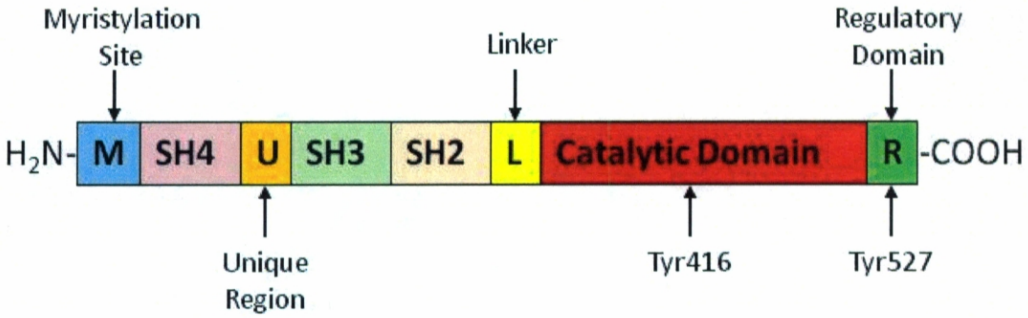

Figure 3: Src family kinase domain structure

All SFKs share a similar domain arrangement, which have been classified into eight distinct functional regions. From the N-terminus to C-terminus (left to right) these regions include a myristylation site (M), *Src* homology (SH4) domain, unique region (U), SH3 domain, SH2 domain, linker (L), the catalytic (SH1) domain, and a regulatory domain (R). (Adapted from Hubbard & Till, 2000).

Src myristylation leads to its association with the cell membrane. The unique region is specific for different Src family members and may determine the interaction between individual Src family members and other cellular proteins. SH3 and SH2 domains are protein–protein interaction domains shared with other Src family kinases and many other signaling proteins. The SH1 domain is the region of tyrosine kinase activity. There are two major phosphorylation sites on Src: Tyr416 located in the SH1 domain and Tyr527 in the regulatory domain near the carboxyl terminus. Both phosphorylation sites play a key role in regulating the activity of SFKs (Martin, 2001; Hu et al., 2008).

Src activity is regulated by intramolecular interactions of the SH2 and SH3 domains and through control of the balance in the phosphorylation states of Tyr416 and Tyr527 (Boggon & Eck, 2004). The inactive state of the Src kinases is maintained by a short sequence at the C-terminus containing the regulatory Tyr527. Src is activated upon phosphorylation at Tyr416 and/or dephosphorylation at Tyr527 (Schlessinger, 2000). Phosphorylation of Tyr527 by the C-terminal Src-kinase (Csk) creates a binding site for the SH2 domain, resulting in an intramolecular association that negatively regulates Src activity. The autophosphorylation of Tyr416 causes change in the conformation of the activation loop which upregulates kinase activity (Gonfloni et al., 2000).

Transcellular permeability to albumin has been shown to be tightly regulated by Src. Phosphorylation of Src at Tyr416 activates the protein which in turn phosphorylates the other components of the permeability pathway, including caveolin-1, gp60, and dynamin-2 (Shajahan et al., 2004a, 2004b), leading to the internalization of albumin within caveolae from the endothelial plasma membrane (Figure 2). Hence, measuring the

level of Src phosphorylated at Tyr416 is equivalent to measuring levels of activated Src and reflects Src activity and possibly the activation of the albumin permeability pathway, since Src activation is an essential step in the albumin transcellular permeability pathway.

Exploring the relationship between Tbdn and known components of the transcellular permeability pathway for albumin will help understand the role Tbdn plays in regulating albumin permeability. Activation of Src kinase results in the activation of all other components of the albumin permeability pathway, which eventually leads to albumin transport (Shajahan et al., 2004a, 2004b). Examining the effect of Tbdn suppression on Src activity levels would provide more insight into the role of Tbdn in the pathway and therefore the mechanism by which Tbdn functions in maintaining vascular homeostasis.

1.7 Rationale for Current Study

Several lines of evidence suggest that Tbdn is a necessary contributor to the maintenance of normal retinal vascular homeostasis and may play role in endothelial cell permeability (Gendron et al., 2000; Gendron et al., 2001; Asaumi et al.,2005; Gendron et al., 2006; Paradis et al., 2008; Gendron et al., 2010). Tbdn knockdown in the antisense *Tbdn* (*Astbdn*) transgenic mouse model *TIE2/rtTA/Enh-TRE/ASTBDN* has been associated with retinal thickening, vascularization and increased permeability of albumin (Wall et al., 2004; Paradis et al., 2008). However, the effects of reexpressing Tbdn in this model have not been yet examined. In this study Tbdn will be reexpressed in mice that have undergone Tbdn suppression for a period of six weeks. It is hypothesized that

reexpression of Tbdn in retinal blood vessels of *TIE2/rtTA/Enh-TRE/ASTBDN* mice will lead to a decrease in albumin permeability and a reversal of retinal pathology. An examination of the consequences of Tbdn reexpression is valuable not only in furthering our understanding of the protein, but also in exploring therapeutic options for neovascular retinopathies.

In vitro work suggests that Tbdn affects the permeability of RF/6A retinal endothelial cells to albumin, and the knockdown of Tbdn in these cells leads to increased albumin permeability across cell monolayer (Paradis et al., 2008). We speculate that increased permeability of proteins such as albumin across Tbdn-suppressed endothelial cells of retinal blood vessels is a contributing factor to the retinal pathology present in the knockdown mouse model. Studying Tbdn and its role on the albumin permeability pathway of retinal endothelial cells could shed more light on the mechanism by which Tbdn functions in maintaining vascular homeostasis.

In the present study the relationship between Tbdn and known regulators of the albumin permeability pathways are also investigated. The second hypothesis to be tested is that Tbdn plays a regulatory role in this permability pathway, and aids in the maintainence of a basal level of albumin permeability. One possible mechanism of action by which suppression of Tbdn may lead to up-regulation of the albumin permeability pathways includes affecting the stability and/or activity of proteins involved in the pathway.

In this study the effect of Tbdn suppression on a key component of the permeability pathway, Src kinase, is investigated. Activation of Src kinase results in the activation of all other components of the albumin permeability pathway, which leads to albumin transport. Low levels of Src activity are required to maintain microvascular homeostasis and increased levels of Src activity indicate that the albumin permeability pathway is active and transporting albumin from the luminal to abluminal side of blood vessels (Minshall et al., 2002; Malik & Mehta, 2006; Hu & Minshall, 2009; Kumar et al., 2009). Examining the effects of Tbdn suppression on Src activity levels would provide more insight into the mechanism by which Tbdn functions in maintaining vascular homeostasis.

1.8 Overview of Methodology for Current Study

Characterization of the role of Tbdn in retinal homeostasis and retinal endothelial cell permeability is analyzed in this study using both *in vivo* and *in vitro* techniques. The Paradis-Gendron laboratory has generated a binary antisense *Tbdn (Astbdn)* transgenic mouse model (*TIE2/rtTA/Enh-TRE/ASTBDN-1)* enabling the conditional knockdown of endothelial Tbdn (Figure 4) (Wall et al., 2004). The mouse model utilizes two separate gene constructs driven by two distinct promoters: the Tie-2 promoter and the tetracycline response element (TRE) promoter. Tie-2 is an endothelial cell specific promoter and controls the expression of the reverse tetracycline transactivator protein (rtTA) (Wall et al, 2004). The TRE promoter drives the expression of a specific *ASTBDN* cDNA fragment. The introduction of doxycycline (Dox) to the binary system prompts a decrease in Tbdn protein expression. Dox binds to the rtTA protein resulting in a conformational

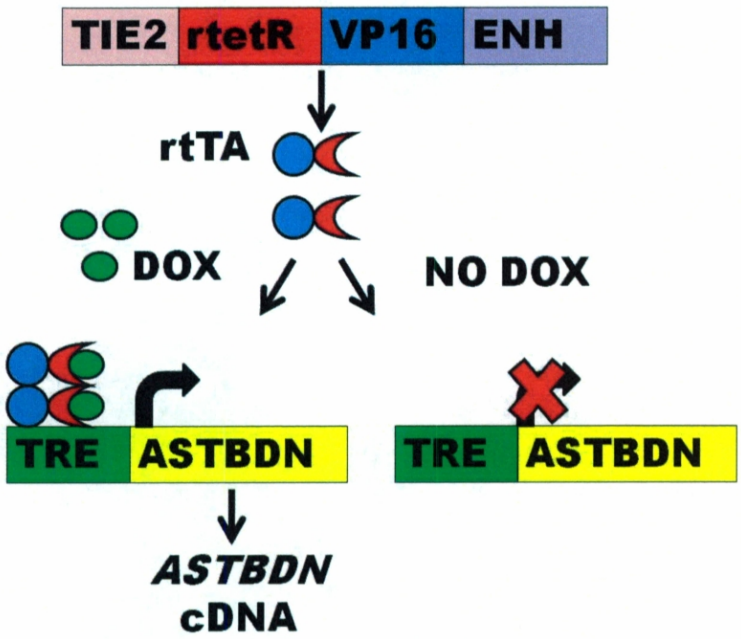

Figure 4: Schematic of transgenic system allowing conditional endothelial knockdown of Tbdn protein expression. The Tie2 promoter directs the endothelial specific expression of the rtTA protein. Administration of Dox allows for the rtTA protein to activate the TRE driving the expression of ASTBDN cDNA and decreasing Tbdn expression. In the absence of Dox, and during Dox withdrawal, the rtTA protein does not activate the TRE, and therefore, the expression of ASTBDN cDNA does not occur.

change, which enables rtTA to bind specifically at a site within the TRE promoter allowing the expression of *ASTBDN* cDNA, decreasing Tbdn protein expression (Wall et al., 2004). In the absence of Dox, as in the case of the Dox-withdrawn specimens, rtTA does not bind to the TRE promoter and consequently Tbdn protein expression is not suppressed. Analysis of the specimens of this mice model is accomplished using histological and immunohistochemical techniques. The effect of Tbdn suppression on retinal blood vessel homeostasis was examined by assessing vessel integrity and permeability *in vivo*. There are a variety of methods that can be used to assess blood vessel integrity and permeability; the technique utilized depends on the type of tissue that is being examined (Vinores et al., 1990). Tracer molecules, like Evans Blue Dye or radiolabelled molecules can provide insight to various pathologies. However, there are limitations associated with the use of tracer substances to assess blood vessel permeability. The use of tracers in retinal blood vessels is impractical for studies using animal models. The introduction of foreign material may disrupt the structure and integrity of retinal blood vessel, and may cause a complete eruption of the vessels (Vinores et al. 1990).

The immunolocalization of endogenous extravascular albumin offers many advantages for assessing blood vessel permeability. The technique can be used with many types of specimens (fixed, autopsy, or archival), no exogenous substance is introduced, and it can be used at microscopic levels. Since albumin is confined within the vessels in the retina, the immunohistochemical demonstration of extravascular albumin is a useful tool for determining the site and extent of blood vessel permeability. The location and

intensity of albumin staining generally correlates with the location and severity of pathologies (Vinores et al., 1990). Immunohistochemical analysis of extravascular albumin has been used to assess vessel integrity and permeability in a variety of ocular disorders and is used in this study to analyze the effect of Tbdn suppression on retinal blood vessel permeability to albumin.

The effects of downregulating the level of Tbdn expression on endothelial cell permeability *in vitro* were studied in RF/6A retinal endothelial cells. RF/6A cells (American Type Culture Collection, Manassas, VA) are an immortalized endothelial cell line derived from rhesus macaque fetal choroid–retinal tissue (Lou & Hu, 1987). The cells were transfected using lipofection with the vector pcDNA3.1/ Zeo (Invitrogen) alone, or with a construct of the pcDNA3.1/Zeo vector harboring *TBDN* cDNA nucleotide sequences 1–1413 in an antisense orientation (*ASTBDN*). The ASTBDN construct was described previously and shown to suppress Tbdn protein expression in RF/6A clones (Gendron et al., 2000; Paradis et al., 2002; Paradis et al. 2008).

To assess the relationship between Tbdn and other known regulators of vascular endothelial permeability, components of the albumin permeability pathway were investigated in RF/6A clones. Pathway components were chosen by reviewing the literature to determine the overall significance in pathway mechanics as well as antibody availability. Recent experimental evidence points to the importance of Src family protein tyrosine kinase (SFK) signaling in regulation of microvascular barrier function and vascular endothelial permeability of albumin (Hu & Minshall, 2009; Kim et al.,2009). Phospho tyrosine 416-Src levels in parental cells, ASTBDN knockdown clones and

negative controls were measured under normal growth conditions and after stimulation with albumin by Western blot analysis.

2.0 MATERIALS AND METHODS

2.1 Study One

2.1.1 Animals

The care and use of animals in this study followed the guidelines set by the Canadian Council on Animal Care and were approved by the Institutional Animal Care Committee of Memorial University of Newfoundland.

The Paradis-Gendron laboratory has generated a binary antisense *Tbdn* (*Astbdn*) transgenic mouse model driven by the *Tie-2* pan-endothelial promoter, which enables conditional knockdown of Tbdn protein expression in endothelial cells (Wall et al., 2004). Retinal endothelial Tbdn expression was suppressed in young adult TIE2/*rtTA/Enh-TRE/ASTBDN* bitransgenic mice for a period of six weeks. Conditional suppression of Tbdn was facilitated by feeding the mice with commercially prepared mouse chow containing Doxycycline (Dox) at 600 mg/kg (Bio-Serv, New Jersey). Control aged mice were the same age but not treated with Dox. Alternatively, single transgenic mice were fed Dox for similar lengths of time to serve as additional controls. Dox fed bitransgenic and control mice were sacrificed at appropriate time points and examined grossly and histologically (See Figure 5 for experimental summary). Morphometric and immunohistochemical analysis of ocular tissues and blood vessels was preformed as previously described (Wall et al., 2004; Section 2.1.2 and 2.1.3) to determine the extent and progression of the ocular pathology.

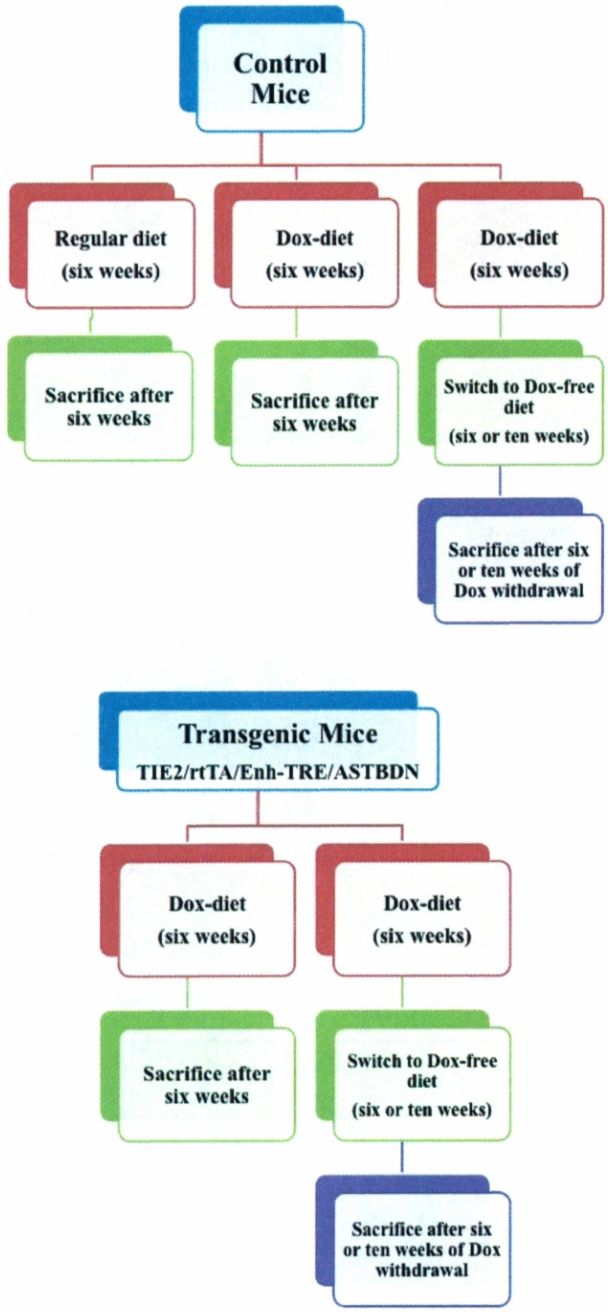

Figure 5: Sequence of experimental events for setting up control and transgenic mice systems.

For Dox withdrawal experiments, bitransgenic and control mice underwent six weeks of Dox induction and were then switched to a Dox-free diet for six to ten weeks. This time period is referred to as Dox-withdrawal and facilitates the reexpression of retinal endothelial Tbdn. The specimens will be herein referred to as six weeks Dox-withdrawal and ten week Dox-withdrawal or collectively as Dox-withdrawn mice (Figure 5). The animals were sacrificed at appropriate time points and analyzed grossly and histologically. Additionally, paraffin sections of eyes from Dox-withdrawn mice were assessed for the presence of fibrovascular retinal lesions, Tbdn expression levels and albumin extravasation compared to control and Dox-treated mice (Sections 2.1.2 and 2.1.3).

2.1.2 Immunohistochemistry

Immunohistochemistry was performed on paraformaldehyde-fixed, paraffin-embedded sections of mouse eye specimens. The sections used for staining were always taken at the eye equator in order to maintain consistency in section locations between eye specimens. The eye sectioning procedure can be quite tedious and much damage can occur to the specimens. Specimens were only chosen for immunohistochemistry if they had intact retinal tissue and little to no damage to the rest of the section. A common artifact that is exhibited in most sections seen in this study is detachment of the choroid from the retina. This has no effect on the outcome of any investigations performed.

Tbdn expression was analyzed by immunohistochemistry using OE5 mouse monoclonal anti-Tbdn antibody (Martin et al., 2007). Sections from paraffin-embedded tissues were deparaffinized, post-fixed in 4% paraformaldehyde for ten minutes, followed

by washing in TBS (10mM Tris-HCl pH 7.6, 150 mM NaCl) three times for five minutes in duration. Slides were then incubated for one hour in 2% ECL Advance blocking agent (Amersham) in TBST (10mM Tris-HCl pH 7.6, 150 mM NaCl with 0.05% Tween 20). Sections were then incubated overnight with OE5 primary antibody at the concentration of 15 ug/mL with control isotype match IgG2a antibody (X0943, Dako) at the same concentration in 2% ECL Advance blocking agent (Amersham) in TBST. Sections were washed in TBST three times for five minutes in duration, and then incubated for one hour with anti-mouse IgG2a alkaline phosphotase (AP)-conjugated antibody (115-055-206; Jackson ImmunoResearch) used at 1:250 dilution in 2% ECL advance blocking agent in TBST. Sections were washed in TBST three times for five minutes in duration and then developed using Vector Red substrate kit (Vector Laboratories) according to the manufacturer's instructions. Sections were then air-dried and mounted in Permount (Fisher Scientific, Pittsburg, PA). Adjacent sections were stained with hematoxylin and eosin (H & E) to assess tissue integrity and pathology. Sections were photographed using a Leica DMIRE2 microscope system with a QImaging RETIGA Ex*i* camera and Openlab software (Version 5, Improvision; Lexington, MA, USA).

Retinal albumin expression/localization was analyzed by immunohistochemistry using goat anti-albumin horseradish peroxidase (HRP)-conjugated antibody (GeneTex; GTX 19195; Lot #15293). Sections from paraffin-embedded tissues were deparaffinized, post-fixed in 4% paraformaldehyde for ten minutes, followed by washing in TBS three times for five minutes in duration. For melanin bleaching, sections were incubated with 0.25% $KMnO_4$ for five minutes, rinsed with TBS followed by five minute incubation in

1% oxalic acid and another cycle of washes in TBS. Endogenous peroxidases were blocked in 0.3% H_2O_2 for ten minutes followed by post-fixation with 4% paraformaldehyde for ten minutes and three washes in TBS. Sections were incubated for 1 hour with 6% fat-free skim powdered milk in TBST for blocking nonspecific binding sites followed by incubation with goat anti-albumin HRP-conjugated antibody at the concentration of 1 ug/mL in 3% powdered milk/TBST overnight at room temperature. Goat anti-rabbit HRP-conjugated antibody (Promega Lot # 237671) at the same concentration was used on control and Tbdn knockdown specimens. The peroxidase activity was detected using NovaRed substrate kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. Sections were then air-dried and mounted in Permount (Fisher Scientific, Pittsburg, PA). Adjacent sections were stained with H &E to assess of tissue integrity and pathology. Sections were photographed using a Leica DMIRE2 microscope system with a QImaging RETIGA Ex*i* camera and Openlab software (Version 5, Improvision; Lexington, MA, USA).

2.1.3 Morphometric Analysis and Blood Vessel Counting

For retinal tissue analysis, digital photographs were taken in lesional areas of the central retina where the most pathology was observed, as this is the area of interest for this study. Morphometric analysis of the retinal pathological lesions was performed using the Improvision Openlab software area outline tool. The retinal area measurements in all specimens were acquired within an invariable histological reference width

encompassing the central retina of micrographs of equivalent magnification (50X). Relative retinal areas were expressed in micrometers ± standard error of means (s.e.m).

Retinal blood vessel counts were performed by counting albumin-stained blood vessels with the assistance of the Improvision Openlab software counting tool on digital micrographs of equivalent magnification (50X) (QImaging RETIGA Ex*i* camera and Openlab software; Improvision). Blood vessel counts for all specimens were acquired within the same histological reference width used for measuring retinal area.

2.1.4 Data and statistical analyses

All tissue sections were viewed and photographed using a Leica DMIRE2 microscope system (Leica; Bannockburn, IL, USA) equipped with a QImaging RETIGA Exi camera (QImaging; Surrey BC, Canada) and Improvision Openlab (version 5) software (Improvision; Coventry, UK) for the quantification of the Tbdn and albumin staining. Between three to six representative digital images of eye sections from Tbdn-knockdown mice, Dox-withdrawn mice and control age-matched mice were processed for each antibody. All staining quantification was performed on micrographs of equivalent dimensions taken at equivalent magnification and microscope and camera parameters.

Tbdn expression was measured by the presence and intensity of anti-Tbdn antibody staining in retinal blood vessels using color spy tool of Openlab software to detect red staining intensity. For control purposes, background staining intensity levels were taking from areas outside the blood vessels in the neural retina to normalize

intensity values in all specimens. Tbdn staining intensity was expressed in means of arbitrary intensity units. Micrographs shown in Figure 5 are representative of the experiments quantified and presented in Figure 6 (section 3).

Intensity of albumin staining in the inner neural retina tissue was measured by determining brown color intensity using color spy tool of Openlab software. Background measurements, measured from outer neural retina layers, were subtracted from inner neural retina tissue staining measurements. Albumin staining intensity was expressed in means of arbitrary intensity units. Micrographs shown in Figure 7 are representative of the experiments quantified and presented in Figure 8 (section 3).

Differences between experimental groups were analyzed for statistical significance using one-way ANOVA by SPSS 17 Software (SPSS Inc, Chicago, IL). Differences were considered significant at $P < 0.05$

2.2 Study Two

2.2.1 Cell Culture

RF/6A cells (American Type Culture Collection, Manassas, VA) are an immortalized endothelial cell line derived from rhesus macaque fetal choroid–retinal tissue (Lou & Hu, 1987a,b). RF/6A cells were previously transfected (Paradis et al., 2002) by using lipofection with the vector pcDNA3.1/ Zeo (Invitrogen) alone, or with a construct of the pcDNA3.1/Zeo vector harboring *TBDN* cDNA nucleotide sequences 1–1413 in an antisense orientation (*ASTBDN*). The *ASTBDN* construct in RF/6A cell clones was described previously and shown to block Tbdn protein expression in RF/6A cells (Gendron et al., 2000; Paradis et al., 2002; Paradis et al., 2008).

RF/6A cells (parental cells, stable AS*TBDN* clones and control clones) were cultured in Dulbecco's Modified Eagle Media (DMEM) (Invitrogen) supplemented with 2 mM glutamine plus 10% fetal bovine serum (FBS) and 50 μM of nonessential amino acids and Zeocin. Cells were cultured on 100 mm tissue culture dish at 1.0×10^6/plate and were maintained at 37°C in 10% CO_2 atmosphere.

2.2.2 Level of Activated Src Family Kinases (SFK)

Src activity levels in parental cells, ASTBDN knockdown clones and negative controls were measured under normal growth conditions and after stimulation with albumin (BSA). After 24 hours, the integrity of the cellular monolayer was evaluated for confluence by phase contrast light microscopy. Confluent cell monolayers were further cultured for 48 hr in growth media with reduced FBS concentration (0.5%) and again in

serum free media for two hours to deplete albumin. The monolayers were then treated for 5 and 10 minutes with either 20% FBS or 5 μg/ml of BSA to activate the albumin permeability pathway. To stop the stimulation cells were washed twice in cold TBS.

2.2.3 Western Blot and Immunoprecipitation Analysis

Protein extraction was performed as described previously (Gendron et al., 2000). Cell extracts were prepared using 0.5% lysis buffer (50 mM Tris, pH 7.8, 150 mM NaCl, 0.5% Brij 96) supplemented with 1 mM DTT, protease inhibitors (1 mM PMSF, 0.3 U/ml aprotinin, and 10 μg/ml leupeptin) and phosphatase inhibitors (1 mM sodium orthovanadate, 25 mM sodium fluoride, and 10 mM beta-glycerophosphate). Lysates were clarified by centrifugation at 4°C, supernatants were removed and stored at -80°C for further analysis. Protein concentration was measured using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA) with a BSA standard curve (0 to 60 ug/ml).

RF/6A protein samples (100 ug) were separated on 7% SDS-polyacrylamide gels and transferred to PVDF membrane (Bio-Rad). The membranes were blocked with 2% ECL Advanced Blocking Agent in TBST for 1 hour at 55 °C and then incubated with appropriate antibodies overnight at room temperature. For detection of total Src and activated Src (phospho tyrosine 416-Src) in RF/6A cells mouse monoclonal anti-Src antibody (Clone 327 ab16885; Abcam) and rabbit polyclonal anti-phospho-Src antibody (phospho-Src Family (Tyr416); Cell Signaling) were used, respectively. For detection of Tbdn an affinity-purified rabbit polyclonal anti-Tbdn (MI- 755-766) antibody was used (Paradis et al., 2008). Development of blots was performed by using HRP-conjugated secondary antibodies (Promega) and chemiluminescence detection kits (ECL Advanced

Reagent and ECL Plus Reagent; Amersham Pharmacia Biotech, Piscataway, NJ). To ensure equal loading of protein samples, western blots were stripped and reprobed with rabbit polyclonal anti-STAT3 antibody (Stat3 C-20; Santa Cruz Biotechnology). Densitometry analyses were performed using the Kodak Gel Logic 200 Imaging System (Eastman Kodak Company, Rochester, NY) and band intensities were analyzed using Kodak Molecular Imaging Software (Version 4.0, Eastman Kodak Company, Rochester, NY).

To determine which Src kinase family members are detected in the phospho tyrosine 416-Src western blot, immunoprecipitations were performed using antibodies raised against three SFK members: Src (Clone 327 ab16885; Abcam), Fyn (sc-434; Santa Cruz) and Lyn (sc-7274; Santa Cruz). RF/6A protein extracts were prepared in cold immunoprecipitation buffer supplemented with protease inhibitors and phosphatase inhibitors as described above. Protein concentration was determined using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). Appropriate antibodies (2.5 µg-10µg) were added to protein samples (100 ug). Samples were than adjusted to equal volumes (250µl-1ml) with immunoprecipitation buffer and incubated 4°C for 4-16 hours on a nutator.

Antibody-Antigen complexes were recovered using sepharose beads coupled to protein G (Amersham). Beads were first centrifuged at 4°C for one minute at 1000X*g* and then washed twice with cold TBS, followed by two washes with cold immunoprecipitation buffer. One volume of immunoprecipitation buffer was added to the

beads to prepare a 50% suspension. Samples are incubated with washed beads (50% slurry) for 90 minutes at 4°C on nutator. After the wash the supernatant was aspirated completely and protein-loading buffer (5X) was added to the samples. Protein samples were boiled for five minutes, and then separated on SDS-polyacrylamide gels, transferred to PVDF membranes and probed with anti-Src and anti-phospho-Src antibodies as described above.

2.2.4 Data and statistical analyses

Densitometric analyses were conducted of bands on various western blot using the Kodak Gel Logic 200 Imaging System (Eastman Kodak Company, Rochester, NY). Intensities of the expressed bands were analyzed using Kodak Molecular Imaging Software (Version 4.0, Eastman Kodak Company, Rochester, NY). The western blot shown in Figure 12 is representative of the experiments quantified and presented in Figure 13 and 14 (section 3).

Differences between experimental groups were analyzed for statistical significance using one-way ANOVA by SPSS 17 Software (SPSS Inc, Chicago, IL). Differences were considered significant at $P < 0.05$

3.0 RESULTS

3.1 Study One

Tbdn expression, retinal pathology and retinal endothelial cell permeability to albumin

Analyses have shown that the level of Tbdn expression in retinal blood vessels can be re-established by restoring a Dox-free diet for Dox–treated Tbdn knockdown (bitransgenic *TIE2/rtTA/Enh-TRE/ASTBDN*) mice (Figures 6 and 7). Tbdn expression was analyzed by immunohistochemistry using OE5 mouse monoclonal anti-Tbdn antibody (Figure 6). In Dox-treated Tbdn knockdown specimens, the intensity of Tbdn staining was 60% of the intensity seen in controls (Figures 6 and 7). In both the six-week and ten week Dox-withdrawn specimens the intensity of Tbdn staining was 90-95% of the control group. Tbdn levels of expression in controls were not different than the levels in either the six week and ten week Dox-withdrawn specimens ($p > 0.05$, ANOVA; control (n=10) 0.4±0.02; six week Dox-withdrawn (n=6) 0.4±0.02; ten week Dox-withdrawn specimens (n=6) 0.4±0.03), indicating that Tbdn expression could be re-established to the of levels found in control mice (Figure 7). Statistical analysis also confirmed that Tbdn levels in the Dox-treated mouse were significantly different compared to all other treatment groups ($p < 0.05$, ANOVA; (n=7) 0.2±0.03) (Figure 7).

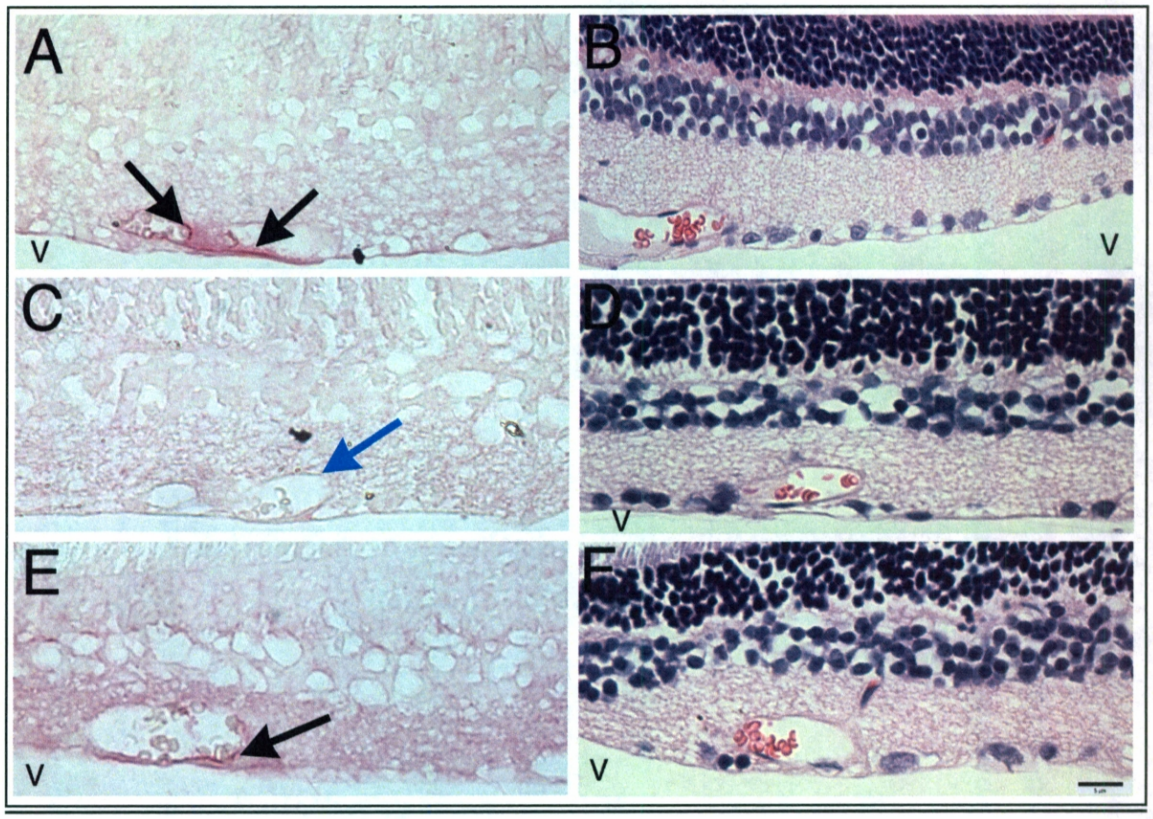

Figure 6: Immunohistochemical analysis of Tbdn expression in retinal blood vessels of Tbdn-knockdown mice, Dox-withdrawn mice and control age-matched mice. Specimens were stained with OE5 monoclonal anti-Tbdn antibody (A, C, E), which yields a bright red reaction product in retinal blood vessels when developed using AP-conjugated secondary antibody and Vector Red AP substrate. All images show the inner and some of the outer layers of the neural retina and are oriented with the vitreous cavity (v) of the eye at the bottom of the panel. Compared with control (single transgenic is shown) age-matched mice (A), 6 weeks endothelial-specific-Tbdn-knockdown eyes (C) showed significantly less staining for Tbdn. The 6 weeks Dox-withdrawn mice (E) showed retinal endothelial Tbdn staining levels resembling those of control mice, indicating that Tbdn has been re-expressed to normal levels in these specimens. Black arrows indicate positively stained retinal blood vessels of controls and Dox-withdrawn mice while the blue arrow exemplifies the low levels of staining of Tbdn-knockdown retinal blood vessels. Adjacent sections (B, D, F) were stained with H&E in order to enable assessment of tissue integrity and pathology. Red blood cells are clearly visible within the blood vessels (stained dark pink in H&E). Representative images are shown. Magnification: 400X.

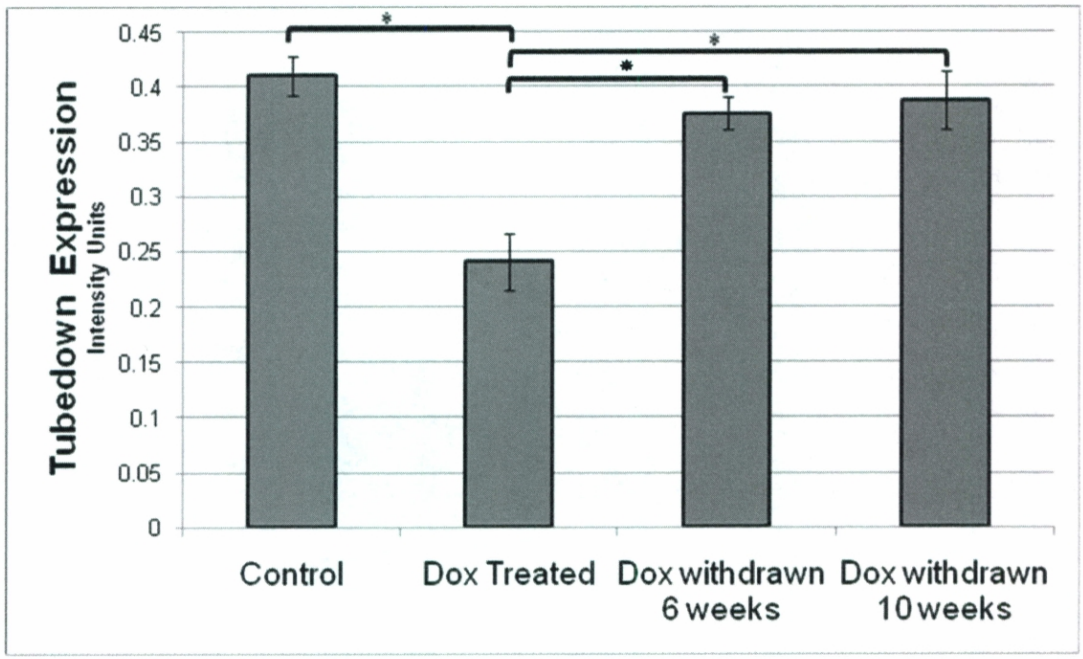

Figure 7: Quantitative analysis of retinal endothelial Tbdn expression in Dox-treated (Tbdn knockdown) mice, Dox-withdrawn mice and control mice. Six weeks of Dox induction in bitransgenic mice leads to knockdown of retinal endothelial Tbdn expression (Dox-treated) in comparison to control mice. However, eyes from mice which were Dox-induced for six weeks and allowed to recover after Dox-withdrawal for additional six weeks (Dox-withdrawn 6 weeks) or ten weeks (Dox-withdrawn 10 weeks) showed significant rescue of retinal endothelial Tbdn expression. Average Tbdn levels in endothelial cells of retinal blood vessels were quantified by measuring staining intensities using Improvision OpenLab software after immunohistochemical analyses of Tbdn protein expression using monoclonal OE5 anti-Tbdn mouse antibody. A representative experiment is shown in Figure 6 A, C, E; Average intensity for control specimens equals 0.4105 arbitrary intensity units. Bars represent standard deviations (SD); significantly different groups marked with *

Wall et al. (2004) previously showed, by morphologic assessment of retinas in Dox-induced *TIE2/rtTA/ Enh-TRE/ASTBDN* mice, the presence retinal lesions characterized by thickening of the retina. In study one I have shown shown that Dox-induced/Tbdn suppressed mice had retinal lesions, thickened retinal layers and subsequent increase in retinal area (Figures 8 and 9), as well as an increase in numbers of retinal blood vessels (Figure 10). In contrast, there were no ocular pathologies exhibited in the control mice specimens (Figure 8 and 9). Re-establishment of Tbdn expression in the retinal tissues by restoring a Dox-free diet for Dox-treated mice showed a significant decrease in retinal area (Figures 8 and 9), as well as lower numbers of retinal blood vessels within a histological reference width compared to Tbdn suppressed mice (Figure 10).

Statistical analysis confirmed that the retinal area measurements were significantly different between the Dox-treated and Dox-withdrawn mice and control-age matched mice ($p < 0.05$, ANOVA; Dox-treated specimens (n=7) 1580.1 μm^2 ± 152.2; Dox withdrawn six weeks (n=6) 991 ± 196.5; Dox-withdrawn ten weeks (n=6) 592.7 μm^2 ± 24.1; control age-matched mice (n=10) 621.8 μm^2 ± 55.1). There was no significant difference between retinal area in the control and ten week Dox-withdrawn

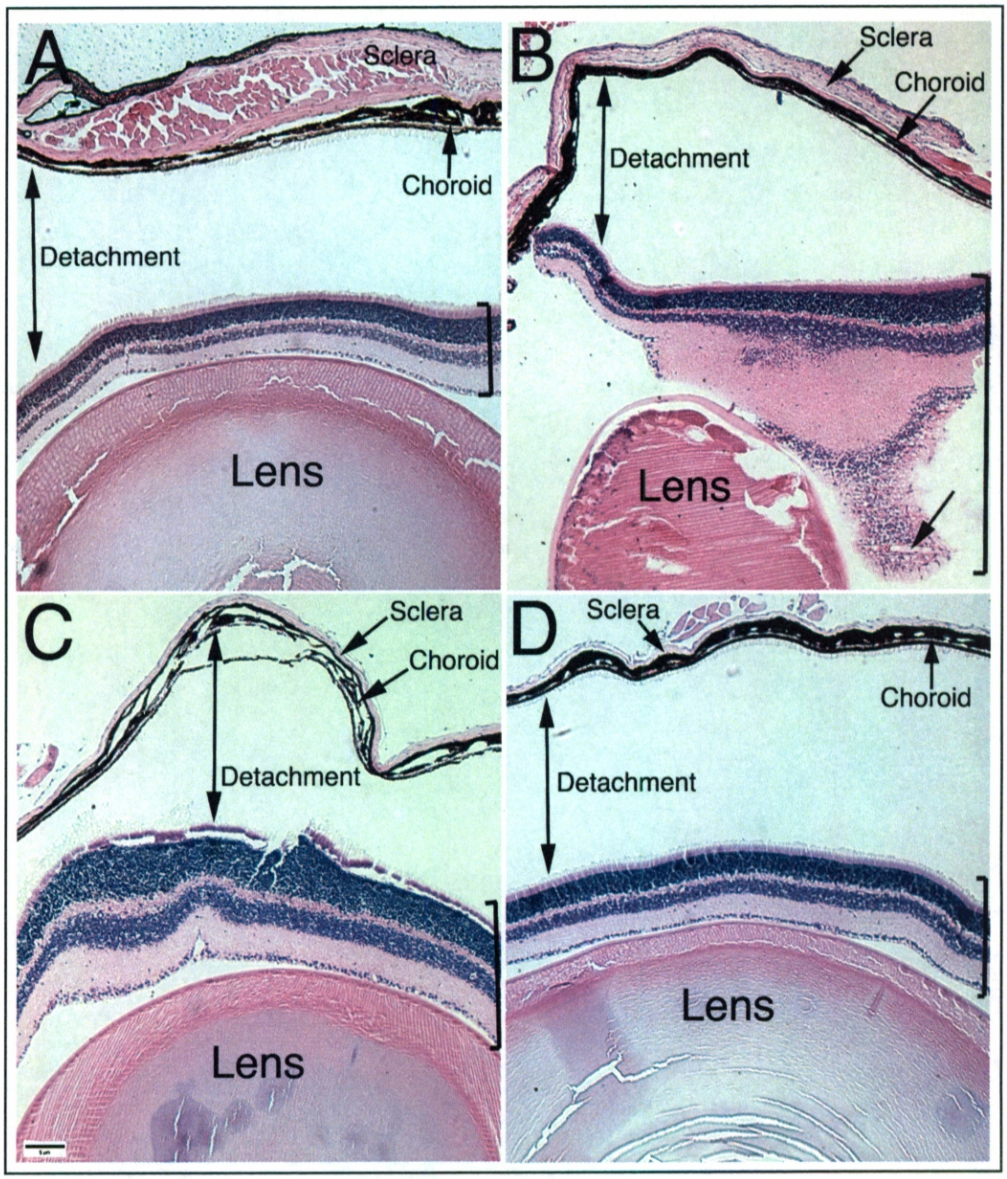

Figure 8: Morphologic analysis of retinas in Dox-treated (Tbdn-knockdown mice), Dox-withdrawn mice and control age-matched mice. The micrographs are taken of paraffin-embedded H & E stained eye specimens encompassing the central retina. The retina spans the width of the image and is designated by a black bracket. The choroid (black in color) and sclera (pink in color) are labeled. The choroid and retinal layers are usually attached but can separate during tissue processing, as indicated on the micrographs (detachement). Compared with control (single transgenic is shown) age-matched mice (A), endothelial-specific-Tbdn-knockdown mice eyes (B) showed thickening of the retinal layers, fibrovascular growth, and thus an overall increase in retinal area within a histological reference width. (***Continued on next page)***

The black arrow on panel B indicates a retinal blood vessel in a region of fibrovascular growth. Six week Dox-withdrawn specimens (C) typically showed thickening of the retina however not to the extent seen in Tbdn-knockdown mice. The ten-week Dox-withdrawn specimens (D) typically resembled the control mice, with little thickening of the retina. Representative images are shown. Magnification: 50X

Note: Detachment of the retina from the choroid is a common result of the sectioning and fixation process. This type of artifact is seen across all types of eye specimens.

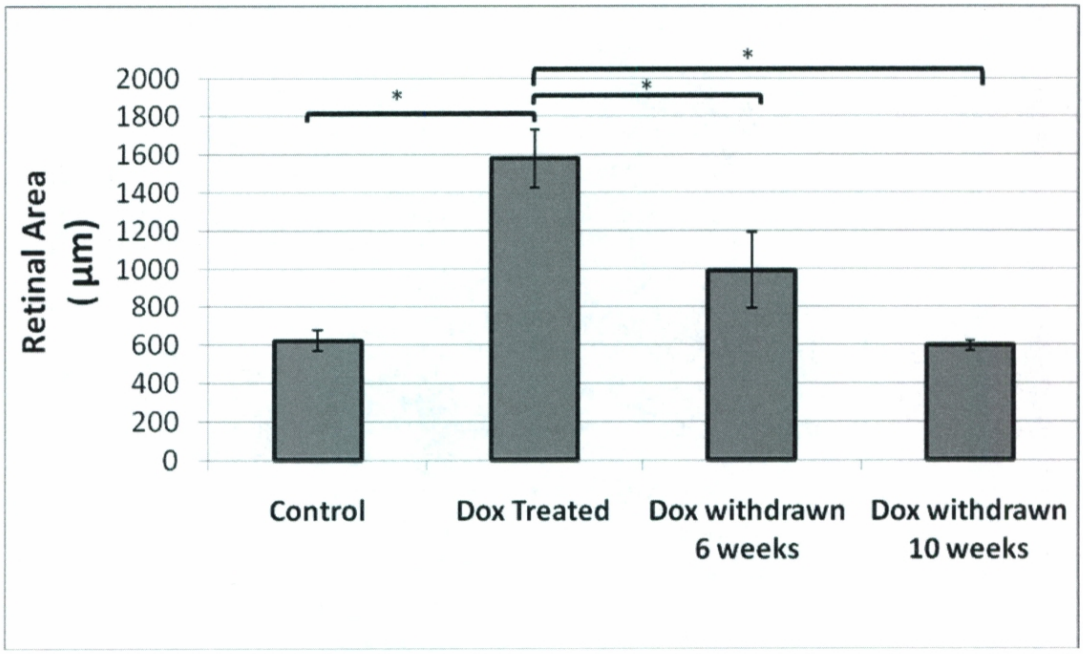

Figure 9: Morphometric analysis of retinal area in Dox-treated (Tbdn-knockdown mice), Dox-withdrawn mice and control age-matched mice. Morphometric analysis of the retinal area in albumin stained paraffin sections of mouse eyes (representative shown in Figure 10) were measured using the Improvision Openlab software area outline tool. The retinal area measurements in all specimens were acquired within an invariable histological reference width encompassing the central retina of micrographs of equivalent magnification of 50X; significantly different groups marked with *

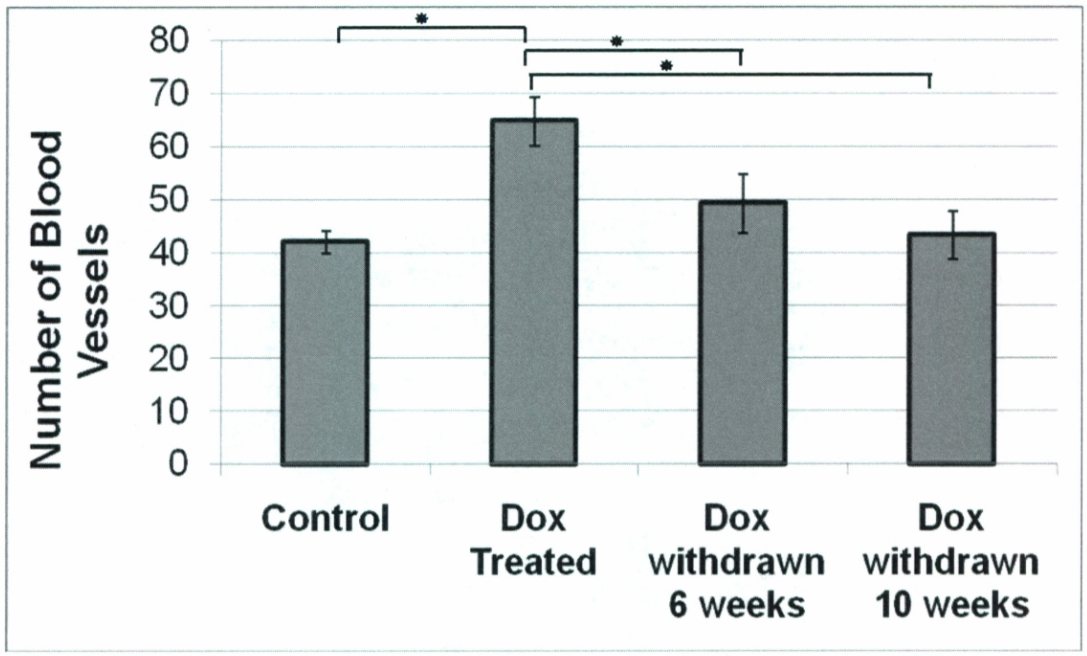

Figure 10: Quantification of retinal blood vessels in Dox-treated (Tbdn-knockdown) mice, Dox-withdrawn mice and control age-matched mice. Morphometric analysis of the retinal blood vessels in albumin-stained paraffin sections of mouse eyes (for which a representative is shown in Figure 10) was performed using the Improvision Openlab software counting tool. The blood vessel counts in all specimens were acquired within an invariable histological reference width encompassing the central retina of micrographs of equivalent magnification of 50X; significantly different groups marked with *

specimens ($p > 0.05$, ANOVA). However, there was a significant difference between retinal areas in the six week Dox-withdrawn specimens and all other treatment groups ($p < 0.05$, ANOVA; Dox withdrawn six weeks (n=6) 991 ± 196.5; Dox-treated specimens (n=7) 1580.1 μm^2 ± 152.2; Dox-withdrawn ten weeks (n=6) 592.7 μm^2 ± 24.1; control age-matched mice (n=10) 621.8 μm^2 ± 55.1). These results indicate that when Tbdn is reexpressed for a period of at least ten weeks the pathology size is reduced, and retinal area returns to a size similar to that seen in the control specimens.

In Dox-treated specimens the blood vessel counts were significantly different compared to the Dox-withdrawn mice and control age-matched mice ($p < 0.05$, ANOVA; Dox-treated (n=7) 64.7± 4.7; control (n=10) 42 ±2.1; six week Dox-withdrawn (n=6) 49.3 ±5.5; ten week Dox-withdrawn (n=6) 43.2 ±4.50) (Figure 10). Furthermore, it was also determined that there was no significant difference between retinal area in the control, six week Dox-withdrawn specimens and ten week Dox-withdrawn specimens ($p > 0.05$, ANOVA) (Figure 10). These results indicate the presence of retinal vascularization during suppressed Tbdn suppression.

The retinal lesions of Tbdn-knockdown mice were analyzed by immunostaining of serum albumin as a measure of retinal blood vessel permeability and integrity. In control mouse, the plasma albumin is limited to the intravascular area (blood vessel lumen) (Figure 11A). In contrast, the retinal lesions of endothelial Tbdn knockdown mice

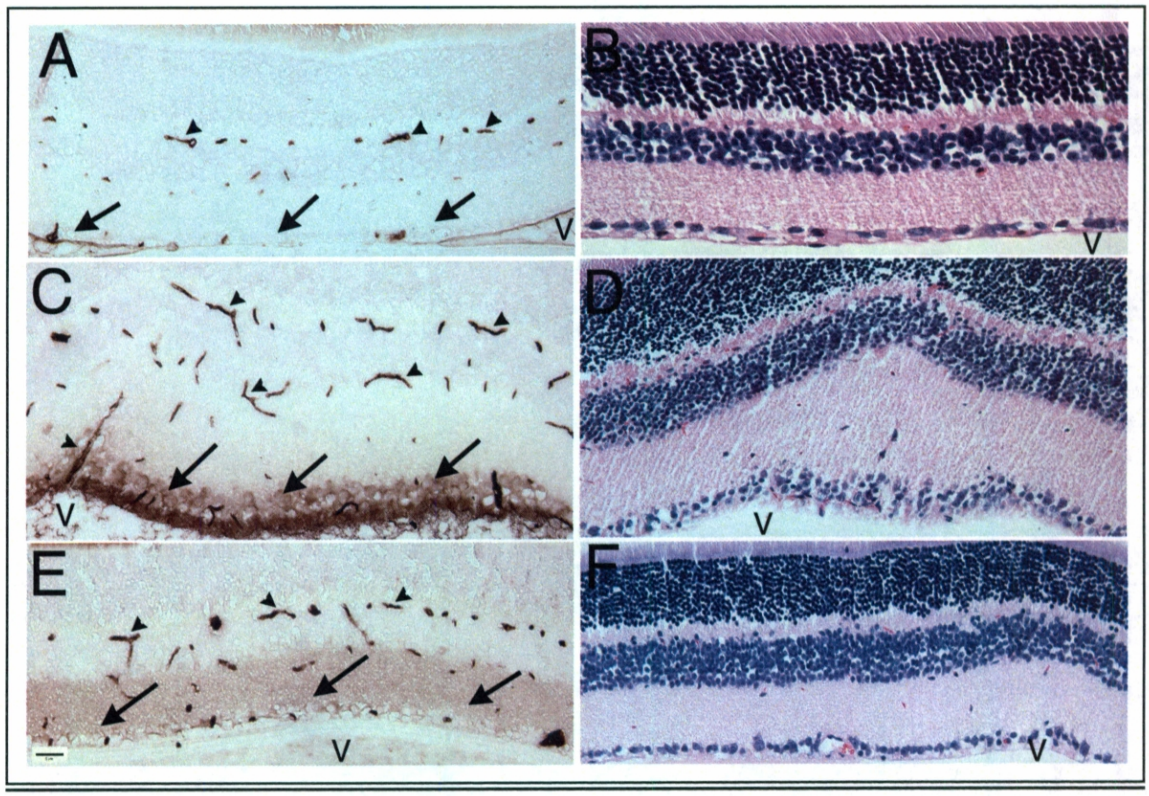

Figure 11: Immunohistochemical analysis of retinal albumin extravasation in endothelial-specific-Tbdn-knockdown mice, Dox-withdrawn mice and control age-matched mice. Staining of retinal tissue for albumin was performed using a HRP-conjugated goat anti-albumin antibody, which yields a brown reaction product (panels A, C, E). Compared with control (single transgenic is shown) age-matched mice (A), endothelial-specific-Tbdn-knockdown eyes (C) showed significant leakage or extravasation of albumin from retinal blood vessels. Brown albumin staining is confined mainly to blood vessel lumens in control retinas (A), whereas in Tbdn-knockdown eyes (C) it is observed in extravascular locations both in and around blood vessels and in neural retinal tissues. Mice that underwent Dox withdrawal for six weeks allowing for Tbdn reexpression (E) showed less extravasation of albumin from retinal blood vessels compared to endothelial-specific-Tbdn-knockdown eyes (C). Control sections stained with negative-control HRP-conjugated goat anti-rabbit IgG at the same concentration as the anti-albumin antibody showed no staining (not shown). All images show the inner and some of the outer layers of the neural retina and are oriented with the vitreous cavity (v) of the eye at the bottom of the panel. Representative images are shown. Small black arrowheads are used to show blood vessels, large black arrows point to neural retina. Adjacent sections (B, D, F) were stained with hematoxylin and eosin in order to enable assessment of tissue integrity and pathology. Magnification: 200X.

specimens exhibited albumin staining in extravascular locations both in and around blood vessels and in neural retinal tissues (Figure 11C). Albumin staining intensity in the extravascular neural retina tissues of the Dox-treated mouse specimens was significantly different compared to the control specimens ($p < 0.05$, ANOVA; Dox-treated (n=7) 55.1± 4.1; control (n=10) 13.04 ±2.3) (Figure 12).

Next I tested if Dox-withdrawal and reexpression of Tbdn in the bitransgenic mouse model affected the levels of albumin permeability through retinal blood vessels caused by induced suppression of Tbdn expression. There was a significant decrease in extravascular albumin staining intensity in both the six and ten week Dox-withdrawn specimens compared to the Dox-treated specimens ($p < 0.05$, ANOVA; Dox-treated (n=7) 55.1± 4.1; Dox-withdrawn six weeks (n=6) 30.3 ±3.6; Dox-withdrawn ten weeks (n=6) 36.5± 4.48) (Figures 11E and 12). It was also determined that extravascular albumin staining intensity in both Dox-withdrawn treatment groups was significantly different from that of the control group ($p < 0.05$, ANOVA; Dox-withdrawn six weeks (n=6) 30.3 ±3.6; Dox-withdrawn ten weeks (n=6) 36.5± 4.48; control (n=10) 13.04 ±2.29) (Figures 11E and 12). There was no significant difference in albumin staining intensity between two Dox-withdrawn groups ($p >0.05$, ANOVA) (Figure 12). These results indicate that the hyperpermeability of the retinal blood vessels during Tbdn suppression is reversible.

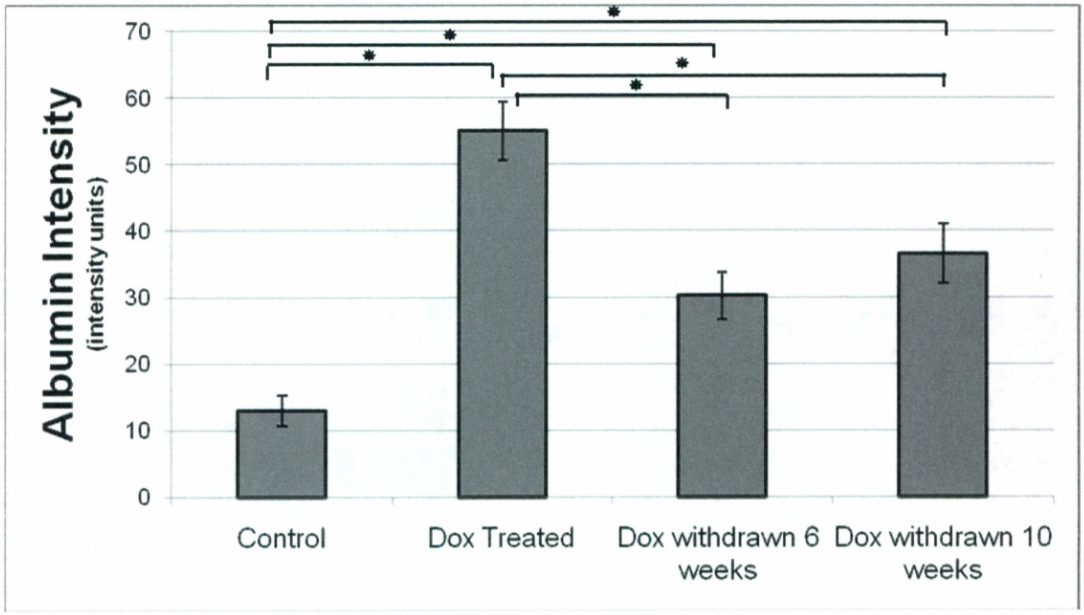

Figure 12: Quantitative analysis of albumin extravasation in Control, Dox-treated and Dox-withdrawn mice. Compared with control age-matched mice, endothelial-specific-Tbdn-knockdown eyes (Dox-treated) showed significant leakage of albumin, represented by an increase in brown staining intensity in neural retinal tissues. However, eyes from mice which were Dox-induced for six weeks and allowed to recover Tbdn levels for an additional six weeks (Dox-withdrawn 6 weeks) or ten weeks (Dox-withdrawn 10 weeks) showed significantly less extravasation of albumin (less brown staining intensity) in neural retinal tissues compared to endothelial-specific-Tbdn-knockdown eyes. Average albumin staining intensity levels were quantified using Improvision OpenLab software after immunohistochemical analyses of albumin localization/expression using goat anti-albumin antibody for which a representative experiment is shown in Figure 5. Average albumin staining intensity for Dox-treated specimens equals 55.14 arbitrary intensity units. Error bars represent standard deviations (SD). The formula used to generate the histogram: average staining intensity of inner neural retina (along inner limiting membrane) minus the intensity in outer neural retina (devoid of staining). Significantly different groups marked with *

3.2 Study Two

Transcellular permeability of RF/6A endothelial cells

To determine if retinal endothelial RF/6A cell clones (harboring the antisense *TBDN* construct) knockdown for Tbdn have a different levels of activated (Tyr416-phosphorylated) src Western blots were performed. Western blot using Tyr416-phospho-src family antibody detected three bands in Tbdn knockdown RF/6A cell clone (Figure 13, WCL lane).

To determine which Src kinase family members are detected by the Tyr416-phospho-Src Family antibody immunoprecipitations (IP) were performed using either Src, Lyn or Fyn antibodies followed by Western Blot analyses with both Src (Figure 13, right panel) and Tyr416-Phohspho-Src (Figure 13, Left panel) antibodies. The Src IP samples revealed the presence of 60-kDa band by Tyr416-phosopho-Src and Src Western blot. The Fyn immunoprecipitated samples revealed the presence of 60 kDa band by Tyr416-phospho-Src western blot suggesting that Src and Fyn are co-migrating on SDS-PAGE. The Lyn immunoprecipitated samples revealed the presence of two bands, 53 and 56 kDa by Tyr416-phospho-Src western blot. Thorne et al. (2006) also demonstrated that Lyn migrates at 53 and 56 kDa. These analyses confirm that the phospho-Src antibody detects Src, Fyn, and Lyn in Tbdn knockdown RF/6A clone.

Figure 14 is representative example of a Western blot analysis of control and Tbdn knockdown RF/6A cell clones for Tbdn, Tyr416-phosphorylated (activated) Src

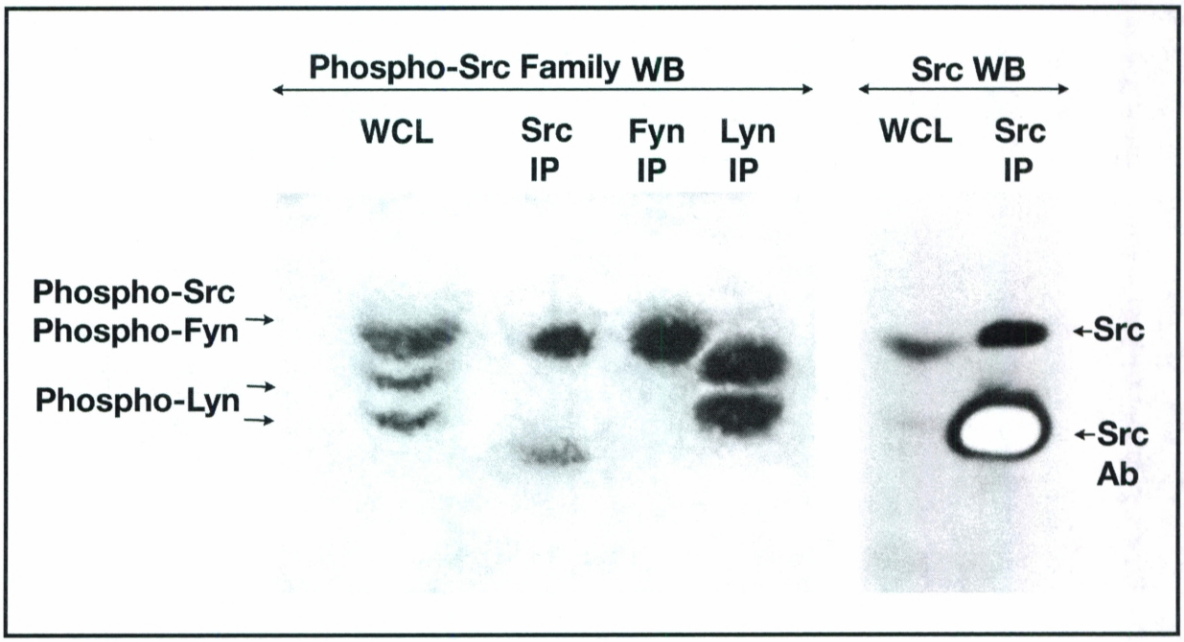

Figure 13: Detection of activated Src, Lyn and Fyn in whole cell lysate of Tbdn knockdown RF/6A clone cells. Western blot of whole cell lysate of RF/6A cells knockdown for Tbdn using Tyr416-phospho-Src family antibody revealed three bands (WCL). IP were performed using antibodies against three Src kinase family members: Src, Fyn and Lyn. Subsequently, the IPs were analyzed by Western blot with both Src (right panel) and phospho-Src (left panel) antibodies.

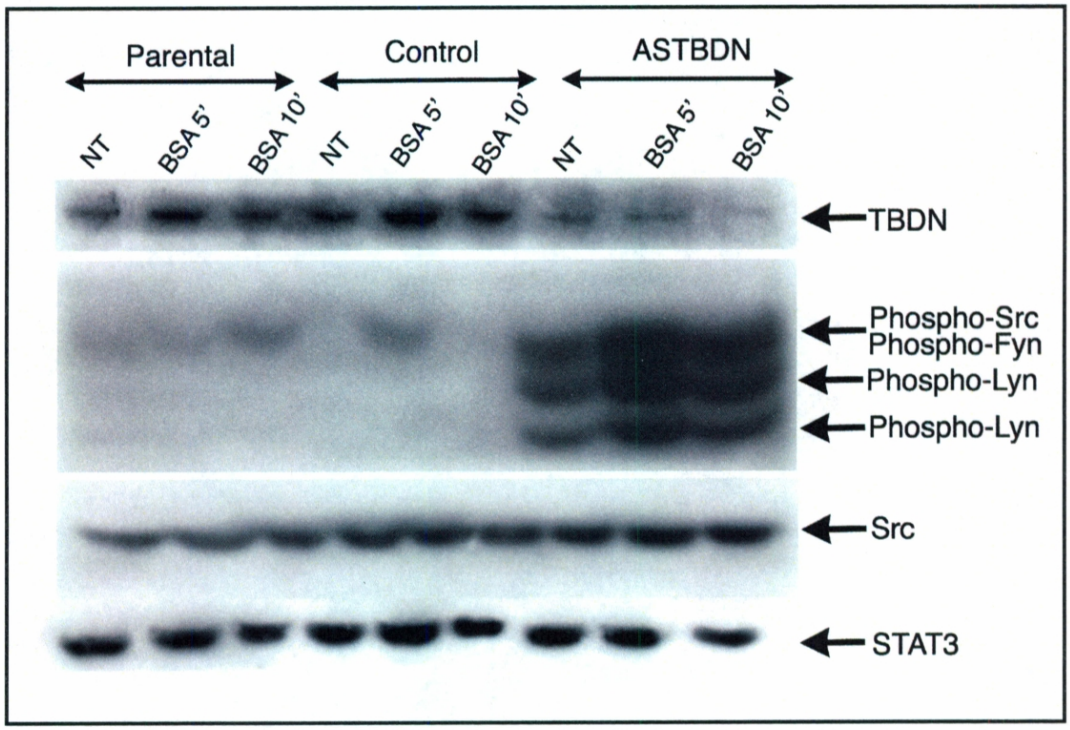

Figure 14 :Western blot analysis of Tbdn, activated Src family kinases and Src kinase levels in Tbdn knockdown clones, ASTBDN control clones and RF/6A parental cells. RF/6A parental endothelial cells (Parental), Tbdn knockdown and control cell clones were analyzed by Western blot for Tbdn, activated Src family kinase (Tyr416-phospho-Src family), and Src kinase expression (Top and middle panels, respectively). Blots were reprobed and analyzed for STAT3 as loading control (Bottom panel). Upper panels show Tbdn levels are suppressed in Tbdn knockdown clones, and that Tbdn knockdown clone have a higher constitutive activation of Src/Fyn pathway than the parental and control cell lines. Analyses were performed on non-treated rested cells (NT) and after stimulation with albumin (BSA) for 5 or 10-minute time intervals.

family kinase, and total Src kinase expression (Figure 14, Top and middle panels respectively). Blots were reprobed and analyzed for STAT3 as loading control (Figure 14, bottom panel). Tbdn levels were suppressed in Tbdn knockdown RF/6A cell clones, as indicated by the reduced signal of protein bands in Figure 14. Western blot using Tyr416-phospho-Src Family antibody detected three bands in Tbdn knockdown RF/6A cell clone ASTBDN (Figure 14). These bands were determined to be Src (60 kDa), Fyn (60 kDa), and Lyn (53 kDa and 56 kDa) (Figure 13). Levels of activated Src/Fyn detected by anti-Tyr416-phospho-Src family antibody relatively to the loading control Stat3 were up-regulated in the Tbdn knockdown RF/6A cell clones compared to the parental and control clones (Figures 14 and 15) ($p < 0.05$, ANOVA). Levels of activated Src/Fyn detected by anti-Tyr416-phospho-Src family antibody relatively to the total Src levels were up-regulated in the Tbdn knockdown RF/6A cell clones compared to the parental and control clones ($p < 0.05$, ANOVA) (Figure 16). The levels of total Src relative to the loading control Stat3 did not vary across the different cell clones, and were not significantly different ($p > 0.05$, ANOVA) (Figures 14 and 17). Western blot using Fyn antibody determined that the levels of Fyn relatively to the loading control Stat3 were also consistent across the Tbdn knockdown cell clone, parental cell and control cell clone (H. Paradis, Memorial University of Newfoundland, personal communication, 2010).

Stimulation of RF/6A parental endothelial cells, Tbdn knockdown clones and control clones using BSA resulted in a seemingly transient increase of active Src at 5 or 10 minutes (Figure 14). However, this increase is not significant, as seen in Figure 15 ($p > 0.05$, ANOVA).

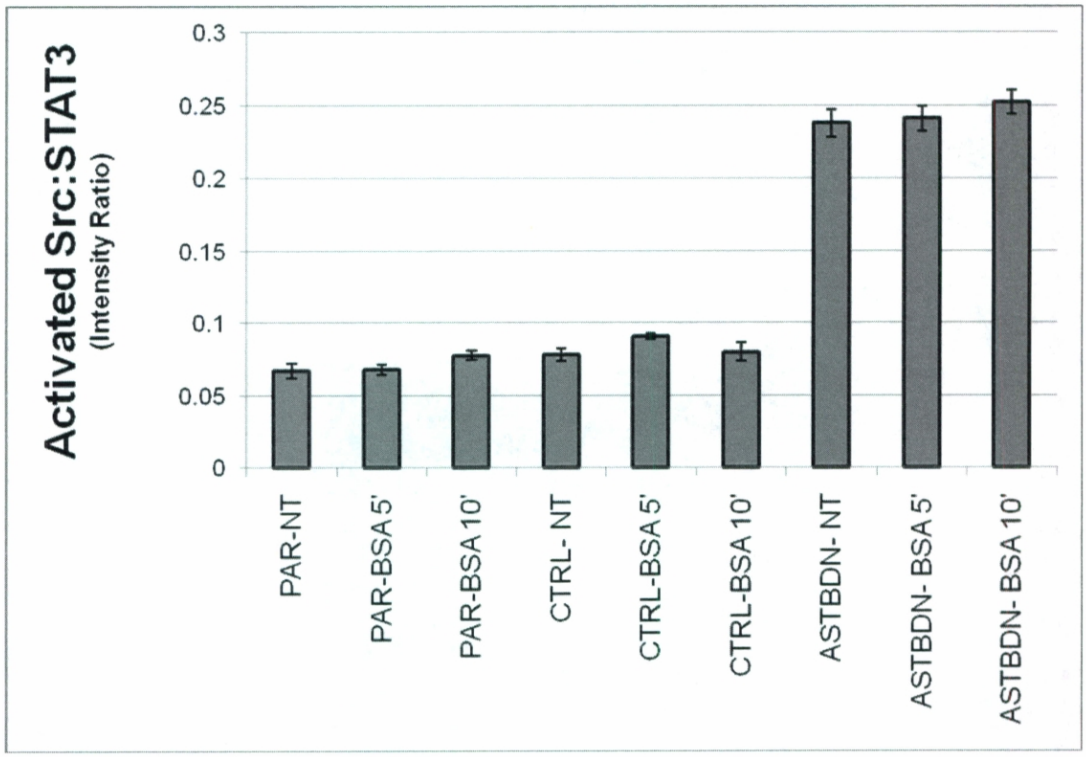

Figure 15: Quantitative analysis of activated Src/Fyn levels in RF/6A parental endothelial cells (Par), Tbdn knockdown clones (ASTBDN) and control clones (CTRL). Densitometric analyses of activated Src levels and the loading control (STAT3) in RF/6A parental endothelial cells (Par), Tbdn knockdown clones (ASTBDN) and control cell clones (CTRL) were done using the Kodak Gel Logic 200 Imaging System and intensities of the expressed bands were analyzed using Kodak Molecular Imaging Software (Version 4.0). Phospho-Src and STAT3 levels were measured under normal growth conditions (NT) and after stimulation with bovine serum albumin (BSA) for ten minute time intervals. Tbdn knockdown clones had a significantly higher constituent activation of Src/Fyn pathway than the parental and control cell lines. Activated Src levels were normalized to STAT3 levels. Bars represent the mean of triplicate samples; error bars represent standard deviations (SD).

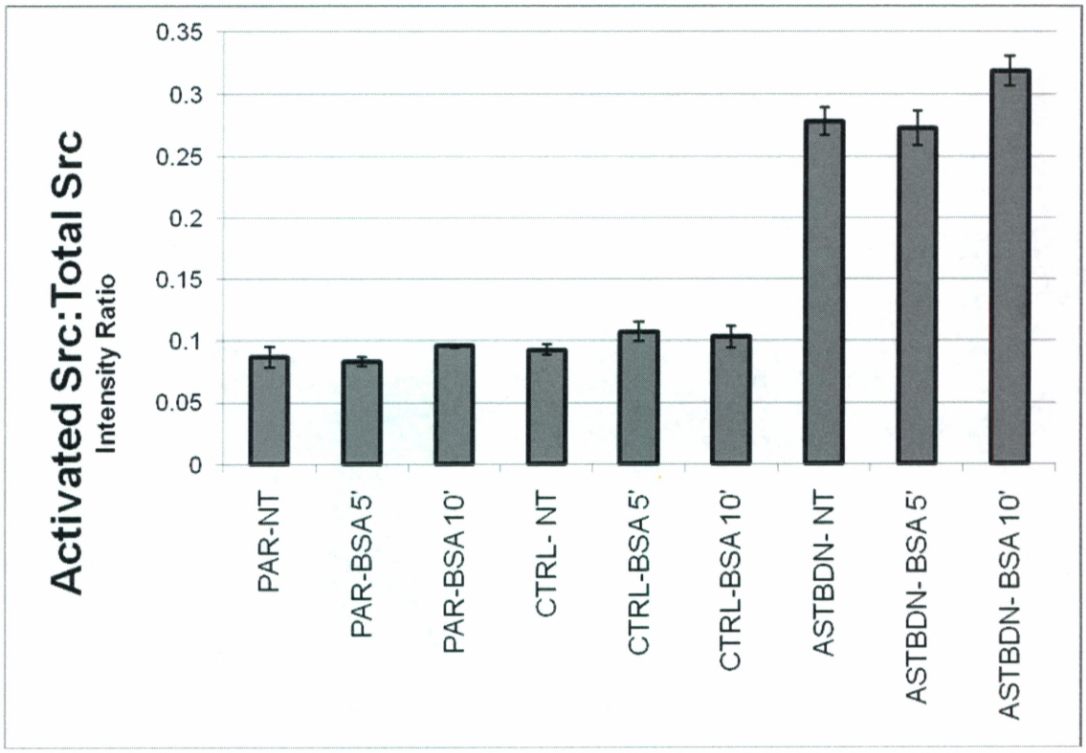

Figure 16: Quantitative analysis of Activated Src over total Src levels in RF/6A parental endothelial cells (Par), Tbdn knockdown clones (ASTBDN) and control clones. Densitometric analyses of activated Src and total Src levels in RF/6A parental endothelial cells (Par), Tbdn knockdown clones (ASTBDN) and control cell clones (CTRL) were done using the Kodak Gel Logic 200 Imaging System and intensities of the expressed bands were analyzed using Kodak Molecular Imaging Software (Version 4.0). Activated and total Src levels were measured under normal growth conditions (NT) and after stimulation with bovine serum albumin (BSA) for ten-minute time intervals. Activated Src levels were normalized to total Src levels. Bars represent the mean of triplicate samples; error bars represent standard deviations (SD).

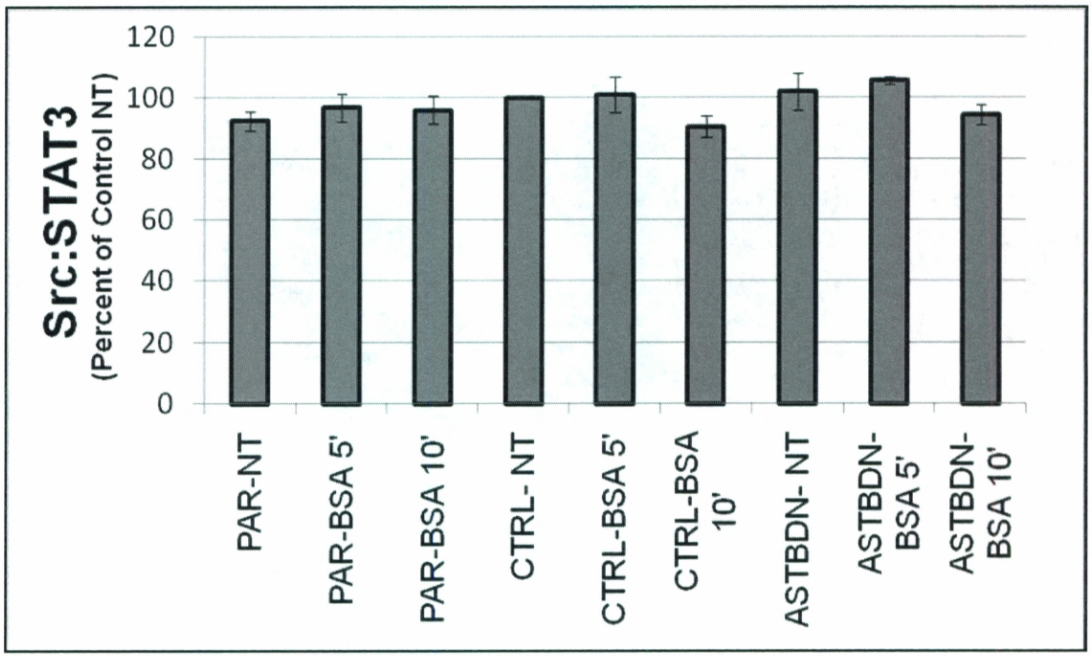

Figure 17: Quantitative analysis of total Src levels in RF/6A parental endothelial cells (Par), Tbdn knockdown clones (ASTBDN) and control clones. Densitometric analyses of total Src levels and the loading control (STAT3) in RF/6A parental endothelial cells (Par), Tbdn knockdown clones (ASTBDN) and control cell clones (CTRL) were done using the Kodak Gel Logic 200 Imaging System and intensities of the expressed bands were analyzed using Kodak Molecular Imaging Software (Version 4.0). Total Src levels were measured under normal growth conditions (NT) and after stimulation with bovine serum albumin (BSA) for ten-minute time intervals. Src levels were normalized to STAT3 level and expressed as a percentage of the control non-treated samples (CTRL-NT). Bars represent the mean of triplicate samples; error bars represent standard deviations (SD).

This study shows when Tbdn is knockdown in endothelial cells activated Src/Fyn levels are upregulated. Since Src is activated in the albumin permeability pathway, these results suggest that the transcellular permeability pathway to albumin may be hyperactive when Tbdn is suppressed.

4.0 DISCUSSION

Study One

The transgenic mouse model (*TIE2/rtTA/Enh-TRE/ASTBDN)* used in the first study enables the conditional knockdown of endothelial- Tbdn facilitated by the introduction of Dox to the system (Wall et al., 2004). In the absence of Dox, rtTA does not bind to the TRE promotor and Tbdn expression in not suppressed. Upon Dox withdrawal for 6 to 10 weeks, Tbdn levels were reexpressed to normal levels after Tbdn suppression in this mouse model (Figure 6 and 7).

In the first study, examination of endothelial specific Tbdn-knockdown mice revealed retinal areas that were significantly larger and had a higher blood vessel count than that of the control age matched mice (Figure 8, 9, and 10). These results support previous findings (Wall et al., 2004; Gendron et al., 2010) that the endothelial Tbdn–suppressed mice exhibit retinal neovascularization with fibrovascular lesions and changes in the thickness of retinal layers, all which account for the increase in retinal area (Wall et al., 2004).

Several studies have reported successful reversal of pathological effects after Dox- withdrawal in transgenic mouse models utilizing the Tet-ON system (Gardner et al., 2002; Huang et al., 2006; Sander et al., 2007). In study one it was found that upon Tbdn reexpression (demonstrated in Dox-withdrawn mice) there was a significant decrease in both the number of blood vessels and retinal area compared to the Tbdn-suppressed mice (Figures 8, 9 and 10). These results support previous findings (Wall et al., 2004; Gendron et al., 2010) that Tbdn plays an important role in controlling retinal neovascularization

(Paradis et al., 2008). These findings suggest that reexpression of Tbdn may reverse retinal pathologies caused by loss of Tbdn expression and thus the restoration of Tbdn protein expression may be a potential therapeutic approach for treating proliferative retinopathies.

In study one, Tbdn-suppressed specimens exhibited significant albumin staining in the extravascular neural tissues compared to control specimens in which albumin was confined to blood vessel lumens (Figure 11). When Tbdn is suppressed, there may be a change in the regulation of microvascular integrity and the upregulation of albumin permeability pathway may occur, as suggested by the increase in albumin extravasation (Figure 11). This increased albumin extravasation into tissue could have detrimental effects and may disrupt the tissues and cells of the retina and could cause breakdown of vascular basement membranes. In other words, the increased transfer of serum albumin across Tbdn-suppressed endothelial cells may be a contributing factor to the above-mentioned retinal pathologies (retinal thickening and vascularization) present in the Tbdn knockdown mouse model of neovascular retinopathy.

It could be argued that the increased extravascular albumin staining demonstrated in Tbdn-knockdown mice is attributed to damaged blood vessels, spilling blood and serum outside the vasculature. However, if the blood vessels did break and spill their contents into the extravascular space, the retinal tissues would be filled with blood and scattered with red blood cells. By examining the H&E stained sections of the retina it was evident that the blood vessels were indeed structurally intact and no spillage of blood and

serum was present in the tissues. In fact the red blood cells were seen confined within the blood vessels of Tbdn-knockdown mice (Figure 6F).

In this first study, it was found that upon Tbdn reexpression (demonstrated in Dox-withdrawn mice) there was a significant decrease in extravascular albumin staining intensity compared to the Tbdn-suppressed mice (Figures 11 and 12). This is evidence that Tbdn plays an important role in regulating the transcellular albumin permeability pathway.

Transcellular transport is the primary mechanism by which albumin crosses the restrictive vessel barrier in retinal blood vessels (Minshall et al. 2000; Minshall et al. 2002). The paracellular pathway is normally impermeable to albumin, and electron micrographic studies have shown that this pathway is closed to macromolecule tracers like albumin (Predescu & Palade, 1993; Predescu et al., 2004). Many studies have established that endothelial albumin transport is mediated primarily by caveolae via transcytosis (Figure 2) (Schnitzer et al., 1994; Minshall et al., 2000; Vogel et al., 2001; Predescu et al., 2004; Tiruppathi et al., 2003; Malik & Metha, 2006).

I hypothesize that Tbdn plays an essential role in regulating the transcellular albumin permeability pathway. When Tbdn protein expression is suppressed, the permeability pathway is disrupted, leading to an upregulation of albumin permeability, as suggested by the increased extravascular albumin staining in the Dox-treated specimens. When Tbdn is reexpressed, demonstrated here in the Dox-withdrawn specimens, the

transcellular albumin permeability pathway regains proper function, and albumin permeability is limited.

Study Two

In order to understand the roles Tbdn may have in regulating albumin permeability we investigated the relationship between Tbdn and known components of the transcellular permeability pathway for albumin. The internalization and transportation of albumin by endothelial cells involves several steps (Minshall et al., 2000). Albumin binding to the 60 kDa glycoprotein (gp60) on the endothelial cell surface induces clustering of gp60 and facilitates an interaction between gp60 and caveolin-1. Src tyrosine kinase, which is bound to caveolin-1, is activated when albumin binds to gp60. Activated Src, in turn, phosphorylates caveolin-1, gp60, and dynamin-2 to initiate caveolae formation, fission and transendothelial vesicular transport of albumin (Tiruppathi et al., 1997; Minshall et al., 2000; Shajahan et al., 2004a, 2004b).

It is becoming increasingly evident that the Src family kinases (SFKs) play key roles in the regulation of microvascular barrier function and various endothelial responses including hyperpermeability of albumin (Tiruppathi et al., 1997; Niles & Malik 1999; Minshall et al., 2000; Shajahan et al., 2004; Kim et al., 2009; Hu & Minshall, 2009). Since Src plays such an important part in mediating albumin permeability, and Tbdn has been implicated in endocytosis (Asaumi et al., 2005), we examined the effect of Tbdn knockdown on Src activity in endothelial cells. It was found that activated Src/Fyn (phosphorylated at Tyr416) was significantly upregulated in Tbdn suppressed endothelial

cells compared to parental and control clones (Figures 14 and 15). Since Src activation is one of the initial steps of transcellular albumin transport, it is implied that this upregulation in activated Src is indicative of an increase in albumin permeability. Previous studies have shown that the knockdown of Tbdn in the same retinal endothelial cell clones used here results in increased permeability to FITC -albumin (Paradis et al., 2008). This new finding provides further evidence that the suppression of Tbdn in retinal endothelial cells leads to an increase in transcellular albumin permeability, possibly by upregulating Src/Fyn activity.

Interestingly, the significant upregulation of activated Src in Tbdn knockdown cell clones sheds some light on the possible mechanism Tbdn may have in regulating the transcellular albumin pathway. Recent evidence suggests that Tbdn binds to the actin binding protein cortactin (Paradis et al., 2008). Cortactin is known to be important for regulating the actin cytoskeleton dynamics (Weed & Parsons, 2001; Daly 2004) and by doing so it is involved in processes such as cell migration, endocytosis and movement of vesicles (Weed & Parsons, 2001; Daly, 2004; Kessels & Qualmann, 2005; Kowalski et al., 2005). Since cortactin is implicated in endothelial permeability and migration (Daly, 2004; Kowalski et al., 2005; Mehta & Malik, 2006) the idea of a role for Tbdn in the vesicle transport mechanism seems plausible, especially when considering these recent Src findings.

With this accumulating evidence that Tbdn plays a role in retinal homeostasis and is a participant in the transcellular permeability pathway of albumin, the next step would be to determine the mechanism(s) by which Tbdn functions. While the exact mechanism

by which Tbdn functions is beyond the scope of this project, I would like to now speculate on the topic with regards to the findings of both of my studies. Tbdn, in a complex with Ard1, functions as an acetyltransferase, and knockdown of Tbdn expression in endothelial cells leads to the co-suppression of Ard1 protein expression (Paradis et al., 2008). We speculate that the substrates of this acetylatranferase activity are involved in the regulation of retinal endothelial cell permeability to albumin.

Protein acetylation has many consequences, including effects on DNA binding, protein stability and protein–protein interaction (Deribe, Pawson & Dikic, 2010). Acetylation can also affect other posttranslational modifications of proteins, like phosphorylation, by having an effect on the proteins overall physical properties and activity (Polevoda & Sherman, 2002; Matsuzaki et al., 2005). One possible mechanism by which suppression of Tbdn may lead to upregulation of the albumin permeability pathways includes affecting the stability and/or activity of proteins involved in the pathway through acetylation. When Tbdn expression is supressed it may bring upon a subsquent change in the regulation of the pathway, which could disrupt the stability and/or activity of pathway components, and could cause the pathway to be upregulated.

The search for a possible mechanism of action by which suppression of Tbdn pathway may lead to increase albumin permeability is a complicated one. The significant upregulation of activated Src in Tbdn knockdown clones prompts speculations that Tbdn could be regulating Src activation via acetylation. However, current literature does not support or refute an acetylation site on Src kinase. Therefore, it remains possible that the Ard1/Tbdn complex directly regulates Src kinase activity via acetylation. Src kinase

interacts with various other proteins that could be potentially acetylated by Ard1/Tbdn complex, and thereby indirectly influencing Src kinase activity by the Ard1/Tbdn complex. With this in mind, I would like to further elaborate on other possible substrates for the Ard1/Tbdn complex.

The first possible substrate for the Ard1/Tbdn complex is the actin-cytoskeleton-binding protein cortactin. Paradis et al. (2008) preformed immunoprecipitation of Tbdn from endothelial cells followed by gel electrophoresis and mass spectrometry and identified cortactin as a co-immunopurifying species. It was also shown by laser scanning confocal microscopy that Tbdn colocalizes with cortactin and F-actin in cytoplasmic regions and at the cortex of endothelial cells (Paradis et al., 2008).

This work by Paradis et al. (2008), together with previous studies suggesting that Tbdn plays a role in the regulation of endocytosis (Asaumi et al.,2005), which is a necessary process for transcellular transport of albumin, support the idea that Tbdn is involved in the regulation of transcellular permeability of albumin. Cortactin is involved in cell migration, endocytosis and movement of vesicles (Weed & Parsons, 2001; Daly, 2004; Kessels & Qualmann, 2005; Kowalski et al., 2005). Therefore, there is evidence showing a relationship between Tbdn and cortactin, and that both play a role in transcellular permeability. The possibility that cortactin is a substrate for Ard1/Tbdn acetylation is supported by the fact that cortactin does have acetylation sites (Zhang et al., 2007). Acetylation of lysine on cortactin affects actin dynamics and cell motility. More specifically, acetylation reduces the interaction between cortactin and F-actin, resulting in decreased cell migration (Zhang et al., 2009). Knowing that cortactin has been

demonstrated to interact with proteins participating in the vesicular flux of molecules across endothelial layers (Daly, 2004; Kowalski et al., 2005), I speculate that Tbdn may be exerting a regulatory role on cortactin by acetylating the protein and decreasing its action in vesicular transport. When Tbdn is suppressed, there may be changes in its acetylation activity, which could allow for changes in the interaction between cortactin and F-actin, facilitating changes in endocytosis or vesicle movement. This could lead to an increase in albumin transcellular transport via vesicles and thus upregulation of the permeability pathway, as indicated by the increased levels of activated Src.

Alternatively, perhaps acetylation of cortactin modifies its ability to be phosphorylated. It has been established that Src phosphorylates cortactin and phosphorylated cortactin promotes cytoskeleton remodeling (Huang et al., 1997; Zahradka et al., 2009). The acetylation of cortactin by Tbdn/Ard1 could possibly lead to a reduced ability to be phosphorylated by Src, which may aid in maintaining basal levels of albumin permeability. When Tbdn is suppressed there may be changes in its acetylation activity which could allow for increased phosphorylation of cortactin by Src kinase, leading to changes in cortactin protein interactions, and consequently to changes in endocytosis or vesicle movement. Again, this could promote an increase in albumin transcellular transport via vesicles and upregulation of the permeability pathway activity, as indicated by the increased levels of activated Src.

Another possible substrate for the Ard1/Tbdn complex is caveolin-1. Caveolin-1 is the primary structural component of endothelial caveolae, which mediate the transport of albumin across the vascular endothelium (Minshall et al., 2003; Figure 2). While the

structure and functions of Caveolin are well characterized (Minshall et al., 2000; Vogel et al., 2001; Minshall et al., 2003; John et al., 2003; Tiruppathi et al., 2003; Malik & Metha, 2006) the number and localization of posttranslational modifications in caveolin are not completely defined. A recent study by Vainonen et al. (2004), characterized the N-terminal acetylation of Caveolin-1. It was found that both isoforms of Caveolin-1, Caveolin-1α and Caveolin-1β, are processed *in vivo* by removal of starting methionines before acetylation of their N-termini (Vainonen et al., 2004). Caveolin-1β was found in both the acetylated and non-acetylated forms, which has lead to the suggestion that this modification plays a regulatory role (Vainonen et al., 2004). While this exact role of Caveolin-1 acetylation remains unknown, these findings lead me to suggest that N-terminal acetylation of Caveolin-1 could be facilitated by the Ard1/Tbdn complex.

Studies have shown that phosphorylation of caveolin-1 by Src family kinases initiates caveolae formation and facilitate the uptake and transport of albumin through endothelial cells (Tiruppathi et al., 1997; Minshall et al., 2000; Tiruppathi et al., 2003; Shajahan et al., 2004; Kim et al., 2009; Hu & Minshall, 2009). It has been shown that protein acetylation can affect protein phosphorylation (Polevoda & Sherman, 2002; Matsuzaki et al., 2005), and so perhaps acetylation of caveolin-1 by Ard1/Tbdn regulates its ability to become phosphorylated by Src kinase. When Tbdn is expressed at normal levels, as seen in RF/6A parental cells and control cell clones, it may be exerting a regulatory role on caveolin-1 via acetylation, mediating its ability to become phosphorylated by Src kinase and maintaining basal levels of albumin permeability. When Tbdn expression is suppressed, there could be a change in Ard1/Tbdn complex

acetylation activity, which could lead to changes in the activity of caveolin-1. Not only could it alter the way caveolin-1 interacts with other components of the albumin permeability pathway, but changes in acetylation activity could also alter its ability to be phosphorylated by Src kinase. Changes in acetylation activity could increase Src phosphorylation of caveolin-1, which would lead to an increase in caveolae formation and subsequent increase in albumin transcellular permeability as indicated by the increased levels of activated Src.

5.0 CONCLUSIONS

In study one, I found that reexpression of Tbdn in retinal blood vessels upon Dox withdrawal results in reduced leakage of retinal endothelial albumin along with a decrease in the extent of retinal vascularization and retinal pathologies compared to Tbdn knockdown mice. These results suggest that restoration of Tbdn protein expression reduces retinal pathology at the morphological and functional level. Tbdn protein expression regulates the albumin transcellular permeability pathway, and when Tbdn protein expression is suppressed, the pathway is upregulated. When Tbdn is reexpressed the transcellular albumin permeability pathway regains proper function, and albumin permeability is limited. These results provide evidence that Tbdn is involved in the regulation of retinal endothelial cell permeability to albumin and implicates a functional role for Tbdn in blood vessel permeability dynamics.

In the second study, I investigated expression of active Src kinase in RF/6A endothelial cells, since recent experimental evidence points to the importance of Src signaling in regulation of vascular endothelial permeability (Minshall et al., 2000; Shajahan et al., 2004; Kim et al., 2009; Hu & Minshall, 2009; Adam et al., 2010). It was shown that when Tbdn expression is suppressed in RF/6A endothelial cells, Src/Fyn activity is up regulated, suggesting the transcellular permeability pathway is active.

Based on the results of both study one and two, I proposed a mechanism where Tbdn may play an important role in regulating the permeability of retinal endothelial cells to albumin by interacting with other proteins in the transcellular albumin permeability pathway.

The findings of both studies expand upon and strongly support cumulative evidence generated by the Paradis-Gendron laboratory that Tbdn plays a role in regulating the transcellular permeability pathway for albumin transport, possibly by regulating Src/Fyn activity. Together, this work implicates a functional role for Tbdn in blood-vessel permeability dynamics that are important for vascular homeostasis. Loss of Tbdn could have clinically relevant significance and Tbdn may be used as a future target for treatment of ocular neovascularization diseases.

6.0 REFERENCES

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