SIGNALING PATHWAYS INVOLVED IN TUBEDOWN REGULATION OF RETINAL ENDOTHELIAL PERMEABILITY

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by

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ABSTRACT

Increased vascular permeability is the early physical manifestation of diseases such as diabetic retinopathy (PDR) and age-related macular degeneration (AMD), which are the leading causes of blindness. Previous studies in Drs. Gendron-Paradis lab have suggested that Tubedown (Tbdn) is an important regulator of endothelial permeability in the retina. Tbdn is a member of the Nat1 family of proteins that associate with the acetyltransferase Ard1. Tbdn protein expression is suppressed in eye specimens from patients with PDR and AMD. Moreover, the conditional endothelial specific Tbdn knockdown mouse model (*TIE2/rtTA/Enh-TRE/ASTBDN-1)* was found to mimic the pathological features of PDR and AMD and to have increased extravasation of Albumin. Previously, we found that Tbdn co-immunoprecipitated with the actin binding protein Cortactin and co-localized with Cortactin and F-actin in endothelial cytoplasmic and cortex regions. Cortactin plays an essential role in the Albumin permeability pathway and is a well-known substrate of c-Src. Tyrosine kinase c-Src not only phosphorylates Cortactin but also takes part in signaling to increase the permeability of endothelial cells.

Based on this knowledge, we hypothesized that Tbdn knockdown would lead to an increase in phospho-Cortactin and phospho-Src. Tbdn knockdown *in vitro* by siRNA and stable transfection of an antisense *Tbdn* cDNA construct significantly increased phospho-Cortactin and phospho-Src levels. *In vivo,* using the Tbdn knockdown mouse model, we confirmed the increase in phospho-Src. Furthermore, human PDR specimens, known for downregulation of Tbdn, also revealed robust levels of phospho-Src. These results support the hypothesis that Tbdn regulates signaling pathways mediating retinal endothelial cell permeability to Albumin by influencing Cortactin and c-Src. Future

therapies for neovascular retinopathies could target Tbdn with the hope of preventing, and not simply treating, these devastating causes of blindness.

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ABBREVIATIONS

Arp2/3	**Actin related protein 2/3**
AMD	**Aged related macular degeneration**
AP	**Alkaline phosphatase**
ASTbdn	**Antisense-Tubedown**
Ard1	**Arrest defect 1**
BSA	**Bovine serum Albumin**
BM	**Bruch's membrane**
Cav-1	**Caveolin-1**
Cbp	**Csk binding protein**
CTR	**Control**
Csk	**C-terminal src kinase**
Dox	**Doxycycline**
Dyn-2	**Dynamin-2**
FAK	**Focal adhesion kinase**
FBS	**Fetal bovine serum**
FITC	**Fluorescein isothiocyanate**
gp60	**60 kDa glycoprotein**
GCL	**Ganglion cell layer**
AGEs	**Glycation end products**
H&E	**Haematoxylin and eosin**
HRP	**Horse radish peroxidase**

IEM	**Mouse embryonic Endothelial Cell line**
ILM	**Inner limiting membrane**
INL	**Inner nuclear layer**
IPL	**Inner plexiform layer**
kDa	**Kilo-Dalton**
KD	**Knockdown**
NT	**Non-treated**
NATA	**N-terminal acetyltransferase**
NTA	**N-terminal acidic domain**
ONL	**Outer nuclear layer**
OPL	**Outer plexiform layer**
PAR	**Parental**
PCAF	**P300/CBP-associated factor**
PDGF-B	**Platelet-derived growth factor**
PDR	**Proliferative diabetic retinopathy**
PRR	**Proline rich region**
PTP-1	**Protein tyrosine phosphatase-1**
RPE	**Retinal pigment epithelium**
ROP	**Retinopathy of prematurity**
rtTA	**Reverse tetracycline transactivation**
S	**Sclera**
SDS	**Sodium dodecyl sulfate**
SFK	**Src Family Kinases**
SH2	**Src homology domain 2**

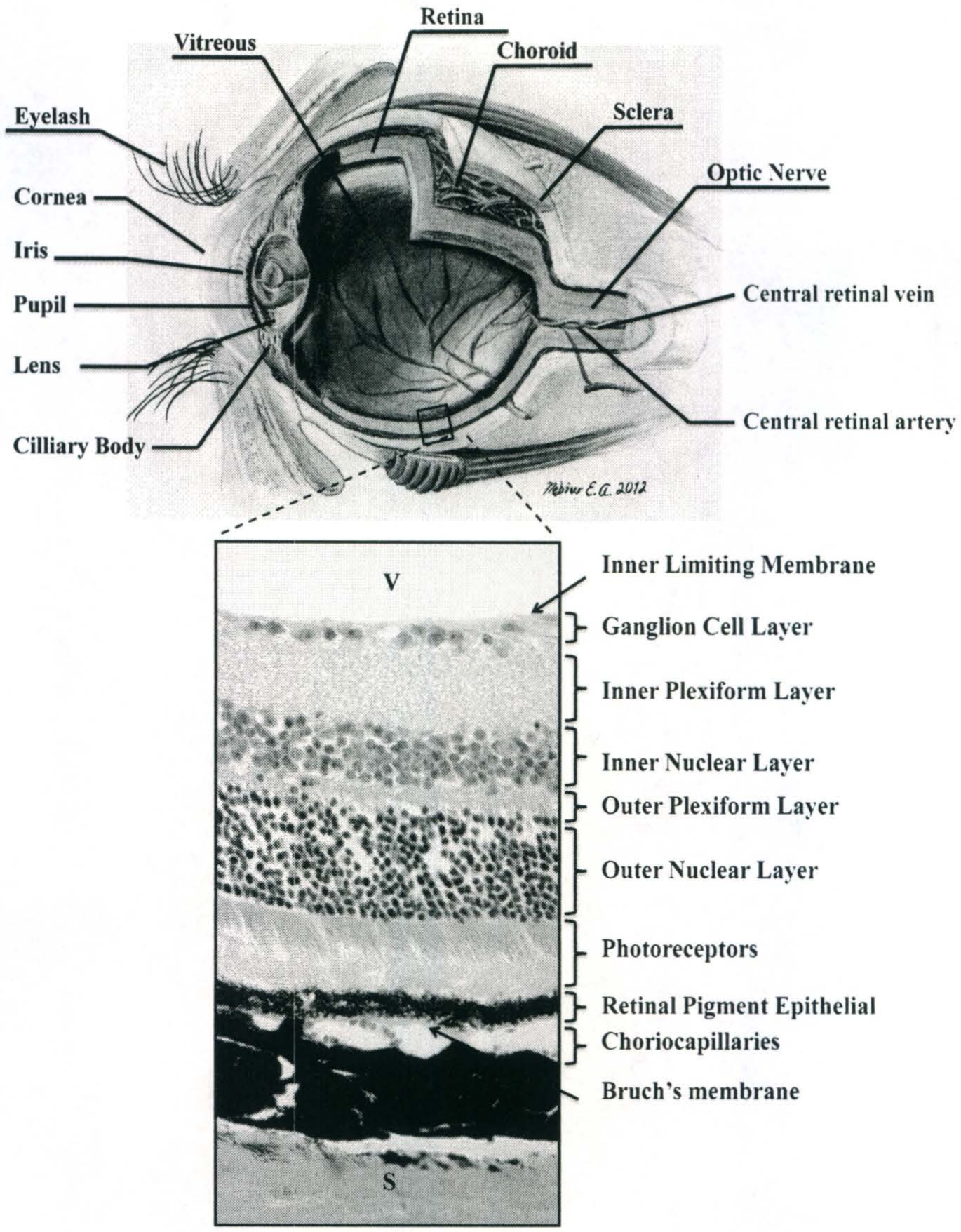
TRE	**Tetracycline responsive element**
(TGF- β)	**Transforming growth factor β**
TBS	**Tris buffer saline**
TBST	**Tris buffer saline with Tween**
Tbdn	**Tubedown**
Tyr	**Tyrosine**
(VEGF)-A	**Vascular endothelial growth factor A**
V	**Vitreous**
WCL	**Whole cell lysate**
N-WASP	**Wiskott-Aldrich syndrome protein**
Zeo	**Zeocin**
ZO-1	**Zonular occludin-1**

1. INTRODUCTION

1.1 The eye and the retina

The eyes are wonderful sensory organs that give us vision, which is arguably the most used of the five senses and one of the primary means we use to gather information from our surroundings. Light waves reflected from an object enter the eye by passing through the cornea and the lens (Figure 1A). The cornea and the lens are the eye's primary refractive structures and both have two key optical properties to this end - refractive power (light refraction) and transparency (light transmission, Kaufman & Alm, 2011). Light rays then continue to traverse a dense, transparent gel-like substance, called the vitreous that fills the globe of the eyeball and helps the eye hold its spherical shape. Eventually, light comes to a sharp focusing point on the retina called the macula which provides the best vision of any location in the retina.

The retina is the fundamental structure involved in visual perception. Retinal tissues line the back two-thirds of the eye and include the neural retina composed of eight distinct layers located between the vitreous body (V) and the retinal pigmented epithelium (RPE). These layers include: inner limiting membrane (INL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), and finally the outer segments photoreceptor cells (Figure 1B). Light stimuli arriving at the retina must travel through all these layers to get to the rods and cones of the photoreceptors where photons are detected and converted into electrical impulses. These impulses, also known as retinal image, are then propagated until they reach the neurons in the optic nerve which carries that image to the brain to be processed.



<u>Figure 1:</u> (A) Structures of the human eye (Used with Artist's permission) **(B) Hematoxylin & Eosin staining of the mouse retina**. The retina comprises of eight distinct layers. Starting from the vitreous (V), these layers are: inner limiting membrane (ILM), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), photoreceptors and the retinal pigment epithelium (RPE). The IPL and OPL contain cell axons establishing synaptic connections while the INL and GCL contain cellular nuclei responsible for vertical 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 choroid is separated from the RPE by Bruch's membrane which contributes to the blood retinal barrier and serves as a guard against vascular intrusion. The outermost structure that covers the eye is the sclera (S). Bruch's Membrane is between the RPE and the Choriocapillaries. Magnification: 400X.

In the brain, these impulses are recreated into the perception of the object (Kaufman & Alm, 2011).

1.2 The vasculature of the retina

The retina is supplied by two major vascular systems: the choroidal and the retinal vasculature. The retinal vessels which are arranged within the inner two thirds of the retina receive 20-30% of the blood flow through the central retinal artery but are vital for maintenance of the inner retinal layers (Henkind et al., 1979). Endothelial cells lining these blood vessels form a tight barrier junction making up the inner component of the blood-retinal barrier (Suburo & D'Amore, 2006). The remaining 70-80% of the blood flow nourishes the outer avascular one third of the retina (particularly the photoreceptors). This high-flow system, called choroidal circulation, is supplied by multiple long and short posterior ciliary arteries, all feeding into an extensive network of fenestrated capillaries called the choriocapillaris. The choriocapillaris are characteristically more permeable than retinal vessels and allow plasma to pool beneath the retinal pigmented epithelium (RPE). Within the RPE is a specialized transport system designed to remove waste from the photoreceptors while providing the cells with essential nutrients. RPE also has tight junctions that regulates the movement of fluids and solutes and constitutes the outer blood retinal barrier (Campochiaro and Hackett, 2003).

In addition to endothelial cells, retinal capillaries also contain pericytes. While endothelial cells line the lumen, pericytes surround the exterior of blood vessels. Retinal vessels have the highest pericyte coverage in the body (Sims, 1986). Pericytes are

vascular mural cells of mesenchymal origin embedded in the basement membrane of microvessels with multiple roles in angiogenesis, maintenance of blood vessel morphology and stability (Sims, 1986; Armulik et al., 20011). Several factors are thought to be involved in pericyte recruitment during vascular development and maintenance, including angiopoietin-1 and its receptor tyrosine kinase Tie-2, vascular endothelial growth factor (VEGF)-A and its receptor flk-1, tissue factor, and platelet-derived growth factor PDGF-B/PDGF-receptor β system and transforming growth factor β (TGF- β; Hammes et al., 2002; D'Amore, 1994).

Under normal physiological conditions, the vasculature of the healthy adult retina is quiescent and vascular leakage is limited. However, some pathological conditions such as diabetes, accumulation of toxic products such as sorbitol or advanced glycation end products (AGEs) and disruption and/or upregulation of VEGF-A and PDGF-B/PDGFRβ signaling pathways can lead to pericyte loss (Fong et al., 2004). Pericyte loss is considered a prerequisite of microaneurysm formation, by focal weakening of the capillary wall (Hammes et al., 2002). This further disrupts the blood retinal barrier and results in angiogenesis. Angiogenesis is characterized by initial vasodilatation of activated blood vessels with increased vascular permeability and degradation of the surrounding matrix, allowing endothelial cell proliferation and migration forming new pathological vessels (Conway et al., 2001). These blood vessels can also grow into other layers of the retina and further disrupt blood retinal barrier by leaking blood, serum, lipids etc. Ultimately, this can lead to neovascular retinopathy resulting in reduced vision and even blindness.

1.3 Neovascular Retinopathies and treatments

As the population ages, vision loss is becoming an increasingly substantial public health problem as it contributes a great burden in terms of functional autonomy, mobility, quality of life and cost. Age-related macular degeneration (AMD) and diabetic retinopathy (DR) are the leading causes of blindness in people over the age of 60 in western populations and working-age patients worldwide (Resnikoff et al., 2004; Klein, 2007; Pascolini et al. 2004). It is predicted that by 2030 , there will be 429 million people with diabetes worldwide (Antonetti, 2012). Nearly all patients with type-1 and more than 60% of those with type-2 diabetes develop some degree of retinopathy after 20 years (Aiello et al. 1994; Mohamed et al. 2007). DR is the most frequent complication of diabetes, afflicting over 90% of persons with diabetes and often progressing to a proliferative disease (Frank, 2004; Yanai et al., 2012). The incidence and prevalence of AMD are more likely to increase with age constituting a very important risk factor along with a combination of environment and genetic factors (Edwards et al., 2007).

Both AMD and proliferative DR (PDR) share the pathological processes of neovascularization and hyperpermeability in one or more of the two main levels of vasculature of the eye, the retinal and/or the choroidal vasculature. AMD is a degenerative disease that is initiated by the thickening of the Bruch's membrane (BM) partly due to accumulation of deposits, changes in retinal pigment and retinal pigment epithelial (RPE) cell loss. This phase of AMD can progress into a neovascular 'wet' form in which proliferative choroidal blood vessels may break through the BM and RPE to nearby layers of the eye. Leakage of blood, serum and lipid from these abnormal vessels

can eventually lead to scarring and detachment of RPE and damage to the retina (Ambati et al., 2003; Bonnel et al., 2003).

Early DR on the other hand, is characterized by increase vascular permeability. As DR advances, it results in closure of the retinal capillaries resulting from ischemia, thrombosis, and non-perfusion. In an effort to compensate for the lack of normal circulation, neovascularization occurs on the retina and optic disc resulting in PDR, which could eventually lead to retinal detachment.

A wide spectrum of therapies ranging from laser photocoagulation to photodynamic therapy (Gehrs et al., 2006; Ambati et al., 2003) have been used to treat PDR and AMD. Although they are effective at slowing disease progression, they rarely result in improved vision (Fong, 2004). On the other hand, treatments with anti-VEGF drugs such as Avastin, Lucentis have seen an improved prognosis of many patients with AMD (Rosenfeld et al., 2006; Brown et al. 2006) and PDR (Nguyen et al., 2006; Chun et al., 2006). However, recent alarming observations raise concerns regarding the efficacy of these treatments beyond 2 years (Ford et al., 2011; Tao, 2010). Additionally, reports of RPE tears after administration of anti-VEGF drugs, with incidence of up to 17%, leading to the suspicion that the anti-VEGF therapy may have adverse effects and even be causative (Chang et al.,2007; Yeh, 2007; Nicolo, 2006). Both AMD and PDR are complex multi-factorial diseases. Thus, further knowledge of the biological mechanisms controlling the growth and permeability of retinal blood vessels is needed to develop either more effective single agents or to identify rational combinations of therapeutic targets that have synergistic effectiveness in preventing and treating neovascular retinopathies

1.4.4 Tubedown

1.4.1 What is Tubedown?

Tubedown (Tbdn, also referred to as Narg1, mNat1, NATH, Naa15) is a 100 kDa protein that was originally isolated from an embryonic endothelial cell line (IEM) as a protein that is significantly downregulated during the formation of capillary-like structures *in vitro* (Gendron et al., 2000). Tbdn protein sequence containing several tetratricopeptide (TPR) motifs, known to mediate protein-protein interactions, suggests that Tbdn might associate with other proteins (Gendron et al., 2002; Willis et al., 2002; Main et al., 2005). Tubedown is homologous to the yeast Nat1 protein (Gendron et al., 2000). Mammalian homologues for Tbdn (mNat2, 70% identity) and Ard1 (Ard2, 81% identity) have also been described (Sugiura et al., 2003; Arnesen et al., 2006). In yeast, Nat1 forms a complex with the acetyltransferase Ard1 (Arrest Defect 1) to make up the essential subunits of the yeast N-terminal acetyltransferase NatA (Park and Szostak, 1992; Gautschi et al., 2003). Additionally, Nat1 mediates stable interaction with the large ribosomal subunit while also contacting nascent polypeptides (when approximately 40 amino acids have emerged from the exit tunnel) and likely directing the growing peptide towards Ard1. Ard1, a catalytic subunit, transfers an acetyl moiety from acetyl-coenzyme A to specific residues in the second position of the N-terminus upon cleavage of the initial methionine by methionine aminopeptidase (Gautschi et al., 2003; Polevoda and Sherman, 2003; Polevoda et al., 2008). While Tubedown has been found to be associated with an acetyltransferase activity (Gendron et al., 2000), acetylation of lysine residues has also been suggested as a function of Ardl and a wide variety of potential substrates for

NatA have been reported (Polevoda et al., 2003; Kimura et al ., 2003; Wang et al., 2004; Geissenhoner et al.. 2004; Lim et al., 2006). Acetylation is one of the most important post-translational modification and is crucial for protein regulation and function. In both yeast and non-human mammalian cell lines, Tbdn and Ard1 complex is involved in the regulation of a broad range of cellular processes varying from cell growth to cellular differentiation (Surgiura et al., 2003; Arnesen et al., 2005; Arnesen et al., 2006; Paradis et al., 2002; Martin et al., 2007, Gautschi et al., 2003; Kimura et al., 2003; Asaumi et al., 2005, Park and Szosatak, 1992). However, in human, this remains to be explored.

1.4.2 Tubedown expression

Tubedown is highly expressed in developing vasculature, and neuronal tissues. However, high levels of Tbdn expression in adults are restricted to a few specific vascular beds, including the ocular endothelium, bone marrow capillaries, blood vessels of regressing ovarian follicles and the choroid plexus endothelium (Gendron et al., 2000; Gendron et al., 2001; Paradis et al., 2002; Paradis et al., 2008; Martin et al., 2007). Tbdn high expression level in the above tissues perhaps suggests that it may play a unique role in these particular areas.

1.4.3 Tubedown functions

While more studies are being done to unveil Tbdn functions, there have been several lines of evidence supporting a crucial role for Tbdn in ocular blood vessels to maintain the homeostasis of the retina, in addition to its role in growth and differentiation. One earlier study, using IEM embryonic endothelial cells, found that Tbdn showed

significant downregulation during capillary growth suggesting a possible role in vascular remodelling (Paradis et al., 2002). In another study, knockdown of Tbdn expression in rhesus RF/6A choroid-retina endothelial cells over-expressing an antisense *Tbdn* cDNA construct (*ASTbdn*) exhibited a significant increase in the formation of capillary-like structures in comparison to controls, indicating an anti-angiogenic regulation mediated by Tbdn (Paradis et al., 2002). To further investigate the effect of Tbdn suppression *in vivo*, Drs. Paradis and Gendron have generated a binary antisense *Tbdn (ASTbdn)* transgenic mouse model (*Tie2/rtTA/Enh-TRE/ASTbdn)* that enables the conditional knockdown of Tbdn protein expression in endothelial cells (Wall et al., 2004).

Endothelial specific Tbdn knockdown mice display pathological features such as retinal and choroidal neovascularization with intra- and pre-retinal fibrovascular lesions, thickening of the retinal tissues and retina–lens adhesions, all of which are characteristics observed in human retinopathies (Wall et al., 2004). The severity of retinal lesions correlated with prolonged Tbdn suppression. Examination of other tissues did not show pathology, perhaps due to the fact that adult Tbdn expression is highly specific to the retinal vasculature. In agreement with the mouse data, studies conducted on human specimens found Tbdn expression to be significantly downregulated in the blood vessels in the retinal lesions in patients with PDR, retinopathy of prematurity (ROP; Gendron et al., 2001; Paradis et al., 2002; Gendron et al., 2006). Likewise, Tbdn expression was also found to be downregulated in older individuals and AMD patients compared to younger individuals. Tbdn expression in the choroidal vasculature was found to be even lower in the AMD specimens than in normal age-matched specimens (Gendron et al., 2010).

Taken together, these studies suggests that retinal and choroidal endothelial Tbdn protein expression is required to maintain retinal homeostasis.

Recently, work from Drs Paradis and Gendron laboratories suggests that Tbdn may perform its homeostatic function by regulating endothelial cells permeability. By conducting permeability assays measuring the amount of FITC-Albumin transported across monolayers of RF/6A cells in which Tbdn levels can be manipulated, Tbdn knockdown in these cells resulted in an increase in transcellular permeability to FITC-Albumin (Paradis et al., 2008). Similarly, Tbdn knockdown in bitransgenic mice results in a significant increase in extravasation of Albumin from abnormal blood vessels to the surrounding tissues in the neural retina (Paradis et al. 2008). These observations are in line with another study done by Asaumi et al. (2005), that found endocytosis of β–amyloid precursor transmembrane protein can be inhibited by transient overexpression of active NatA complex. Furthermore, they noticed secretion of amyloid-β may be dependent on endocytosis, also suppressed by overexpression of both Tbdn and Ard1 (Asaumi et al., 2005). Together, these studies indicate that Tbdn regulates the permeability of endothelial cells.

1.4 Regulation of endothelial cell permeability

By regulating tissue fluid and transporting essential nutrients across the vessel wall, endothelial cell permeability is very important for the maintenance of vascular homeostasis (Minshall et al., 2002; Malik & Mehta, 2006). Endothelial cell hyperpermeability is closely associated with angiogenesis and together, characterize the hallmark of proliferative neovascular retinopathy. Transport of plasma proteins and

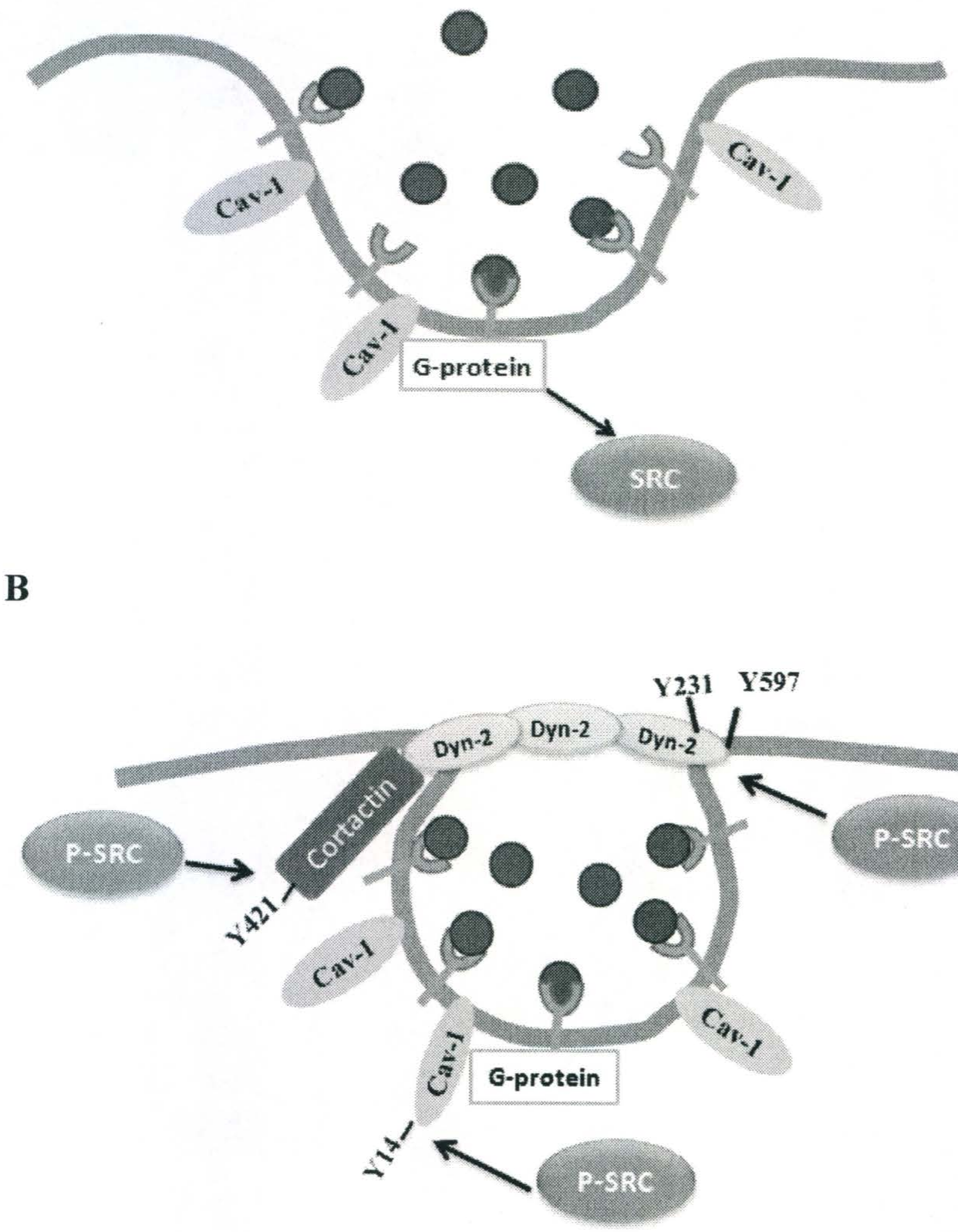
solutes across the endothelium occurs via two different routes: transcellular and paracellular. The transcellular pathway takes place via caveolae-mediated vesicular transport. On the other hand, the paracellular pathway is mediated via interendothelial junctions.

The paracellular permeability of the endothelial barrier is maintained by the interendothelial junctions which restrict the movement of plasma proteins, the size of Albumin and larger, from the vessel lumen by tightly connecting adjacent endothelial cells into a monolayer (Kaufman & Alm, 2003; Komarova and Malik, 2010). Among the different types of interendothelial junction structures in the vascular endothelium, tight junctions and adherens junctions are the best characterised in respect to their function in mediating cell–cell adhesion and thus barrier properties. Adherens junctions composed of the vascular endothelial (VE)-cadherin complexes with catenins, are dominant in most vascular beds. Tight junctions are zipper-like structures formed at the cell–cell contact area by a group of transmembrane proteins including: claudins, occludins and zonular occludins (ZO-1 and ZO-2), all highly expressed in the blood–brain barrier and retinal microvasculature (Komarova and Malik, 2010; Kumar et al., 2010). Several inflammatory mediators such as Thrombin, Bradykinin, Histamine and Vascular endothelial growth factor (VEGF) upon binding to their receptors, can disrupt the organization of interendothelial junctions and Integrin-ECM interactions, thereby causing endothelial hypermeability (Malik & Mehta, 2006; Kumar et al., 2010).

In contrast, the transcellular pathway allows for the transport of macromolecules such as Albumin and Albumin-bound ligands, insulin, lipids, and hormones from vessel lumen to interstitial space (Komarova and Malik, 2010). Transcytosis 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 the human plasma making up for 60% of the total protein content. As for its function in endothelial physiology, Albumin act as a circulating chaperone for hydrophobic substances, fatty acids, and hormones, and many molecules whose transport is crucial for cell functions (Mehta and Malik, 2006). The specific mechanism(s) by which endothelial cells internalize and transport Albumin from the luminal to abluminal cell surface are not completely understood. However, it is thought to be initiated by binding of Albumin to its cell surface binding glycoprotein gp60. This induces clustering of these receptors and recruitment of Caveolin-1 (Minshall et al., 2000). Interaction of gp60 with Caveolin-1 triggers activation of Gαi, eventually leading to phosphorylation of c-Src at tyrosine 416 rendering c-Src active (Komarova & Malik, 2010; Hu et al.,2008). Activated c-Src then tyrosine phosphorylates members of the internalization pathway such as Caveolin-1, Dynamin (Shajahan et al., 2004; Tiruppathi et al., 1997) and Cortactin (Orth et al., 2002). Together, these sequential phosphorylation dependent events facilitate caveolar scission and transcellular vesicular transport of Albumin (Hu et al., 2008; Komarova & Malik., 2010). Given its important function in homeostasis, disruption of this pathway can cause imbalance in the nutrients that could render the endothelium prone to pathogenesis. For example, the presence of extravascular Albumin has been well-documented to associate with an increase in retinal vascular permeability and angiogenesis (Vinores et al., 1998, Erickson et al., 2007; Paradis et al., 2008), all of which underlies the pathology of retinopathy of prematurity, diabetic retinopathy, and age-related macular degeneration



<u>Figure 2:</u> Proposed mechanism of caveolae-mediated albumin transport

(A). Albumin binding to its cell surface receptor gp60 induces recruitment of Caveolin-1 (Cav-1) and activation of G-protein which in turns activate Src. (B) Activated Src (P-SRC) then phosphorylates other components of the pathway such as Cortactin at Tyr421, Cav-1 at Tyr14, and Dynamin-2 (Dyn-2) at Tyr231 and Tyr597. These sequential phosphorylation facilitate vesicular scission and transcellular vesicular transport of Albumin.

(Schlingemann et al., 1999; Leto et al., 2001; Chronopoulos et al., 2011). Studies in Drs Paradis and Gendron laboratories have suggested that Tbdn plays a regulatory role in this pathway due to the fact that Tbdn knockdown both *in vitro* and *in vivo* leads to increased extravasation of Albumin (Paradis et al., 2008). To further elucidate Tbdn mechanism governing transcellular permeability pathway, it is important to look at Tbdn's relationship with other major players of the pathway (i.e., Cortactin and c-Src).

1.5 Cortactin

Another piece of evidence that points to a possible link between Tbdn and regulation of endothelial cell permeability is the interaction of Tbdn with Cortactin. Tbdn was found to interact and co-localize with Cortactin at the cellular cortex (Paradis et al., 2008). Cortactin is a 80-85 kDa filamentous cortical actin-binding protein initially characterized as a tyrosine phosphorylated substrate in v-Src transformed chick embryo fibroblasts (Wu et al., 1991). Since its discovery, Cortactin has emerged as a key protein involved in the coordination of membrane dynamics and cytoskeleton remodeling (Cosen-Binker and Kapus, 2006). Structurally, Cortactin consists of an N-terminal acidic domain (NTA), six-and-a-half tandem repeats (cortactin repeats) at its N-terminus, followed by an α-helix, a proline-rich region (PRR) and a Src Homology-3 (SH3) domain at its C-terminus (Figure 3). Through the NTA domain, Cortactin binds and activates actin-related protein (Arp) 2/3, enhancing N-WASP (Wiskott-Aldrich syndrome protein) - mediated nucleation and actin polymerization steps necessary for dynamic remodeling of the actin cytoskeleton (Ammer and Weed, 2008). While investigation is undergoing to examine how Cortactin's interactions with the above binding partners could impact the

regulation and formation of actin-rich membrane-cytoskeleton structures, recent studies have examined the role of post-translational modifications of Cortactin such as phosphorylation (Lua and Low, 2005; Cao et al., 2010) .The majority of reports indicate that high levels of tyrosine phosphorylation correlate with elevated cell migration and cancer metastasis (Huang et al., 1998, 2003; Daly et al., 2004; Ammer and Weed; 2008). In the case of tyrosine phosphorylation, Cortactin can be phosphorylated by members of the Src family kinases v-Src, c-Src and Fyn on three sites (Tyr421, Tyr466 and Tyr482) with Tyr421 being the most important site, as previous studies have found that phosphorylation at Tyr421 by Src family kinases creates a binding site for the c-Src SH2 domain (Weed and Parsons, 2001; Cosen-Binker and Kapus, 2006). This stable interaction then allows further c-Src phosphorylation at Tyr466 and Tyr481 respectively.

Cortactin phosphorylation by c-Src has been implicated in a variety of functions, one of which is the regulation of endocytosis through actin polymerization (Ammer and Weed, 2008). Since transcellular permeability requires endocytosis as the initial step (Daly et al., 2004) and Cortactin is known to bind and co-localize with Tbdn at the cell cortex (Paradis et al., 2008), further investigation into the relationship between Tbdn and phospho-Cortactin is needed to explore how Tbdn may regulate the transcytosis of Albumin.

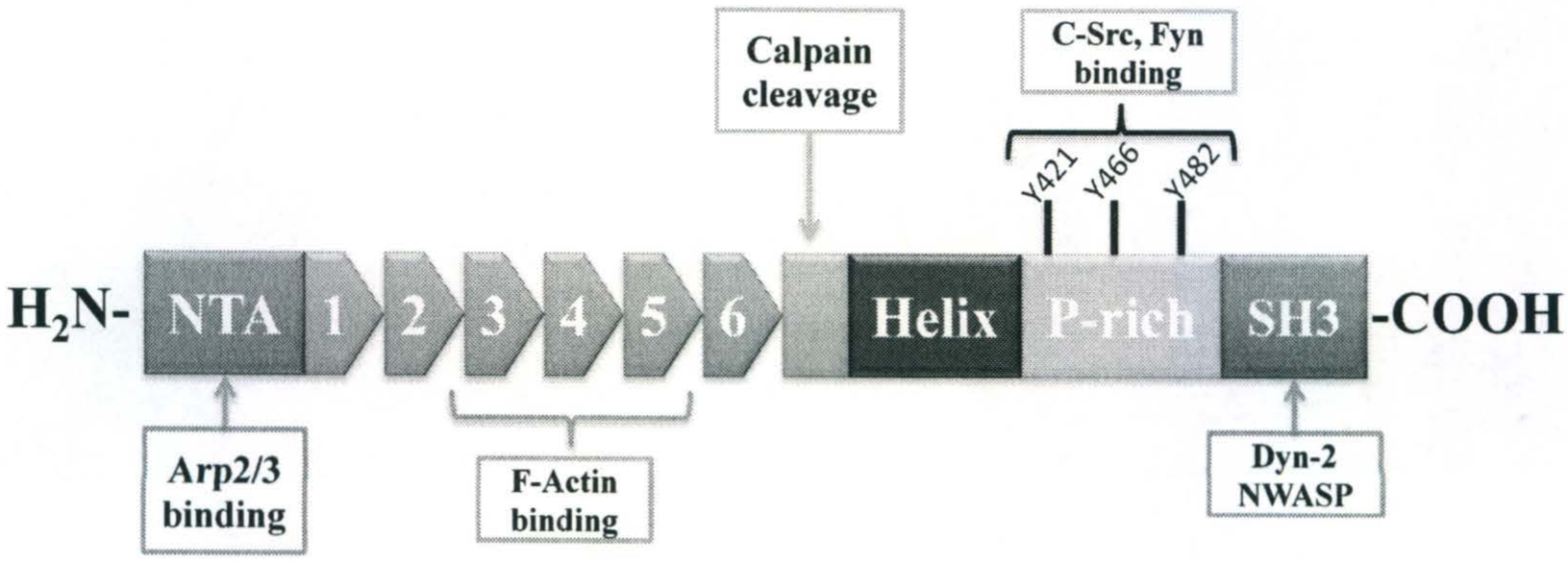


Figure 3: Diagrammatic representation of domain structure of Cortactin and its interacting partner. Binding of Arp2/3 complex occurs through the three amino acid DDW -motif within the NTA domain. The actin-binding domain is located within the repeats region, requiring the fourth repeat and possibly adjacent sequences. Tyrosine phosphorylation by Src family kinases occurs within the proline-rich domain at Y421, Y466 and Y482, creating putative binding sites for SH2 domains.

1.6 Src Family Kinases

The family of Src kinases (SFKs) are comprised of 52-62 kDa nine structurally related molecules: c-Src, Blk, Fyn, Yes, Lyn, Lck, Hck, Fgr, and Yrk, all of which have been recognized to contribute to cellular processes such as proliferation, survival, migration, and specifically in this study, transendothelial permeability (Frame et al., 2002; Summy and Gallick, 2003; Yeatman, 2004). Tyrosine kinases c-Src, Fyn, Yes and Yrk are widely co-expressed in many cell types, including vascular endothelial cells whereas Lyn, Lck, Hck, Fgr and Blk are found primarily in hematopoietic cells (Hu et al., 2008).

From the N- terminus to the C-terminus, c-Src consists of eight functional regions including a myristylated N-terminal site, a Src homology (SH)4 domain, a unique region, a SH3 domain, a SH2 domain, a linker region, a kinase/catalytic domain (SH1 domain),

and a regulatory domain (Figure 4). Myristylation of c-Src along with the SH4 domain, have been shown to be involved in membrane binding (Kim et al., 2009). The SH3 and SH2 domains mediate protein–protein interaction not only with other Src family kinases, but also with many other signaling proteins. The SH2 domain allows interaction with phosphotyrosine containing motifs on proteins, whereas the SH3 domain recognizes pro-x-x-pro motifs, present on a variety of signaling molecules (Cohen et al., 1995; Moarefi et al., 1997; Birukov et al., 2001). The SH1 domain exhibits a tyrosine kinase activity. Tyrosine kinase c-Src can be phosphorylated at two different sites: one at Tyr416, in the catalytic domain, and at Tyr527, in the regulatory domain near the C-terminus. In its inactive state, c-Src assumes a "closed" confirmation stabilized by intramolecular interaction between phospho-Tyr527 and SH2 domain. Phosphorylation at Tyr416 allows c-Src to be in its "open" conformation rendering it active. Tyr416 can be auto-phosphorylated whereas Tyr527 can be phosphorylated and dephosphorylated by Csk (carboxy-terminal Src kinase) and PTP1 (protein tyrosine phosphatase 1) respectively (Martin, 2007).

Regulation through distinct protein domains and post-translational modification accounts for multiple roles c-Src plays in signaling pathways (Kim et al., 2009). One of these roles is coordinating protein complexes that form and internalize caveolae, a process that transports macromolecules, such as Albumin across the endothelial

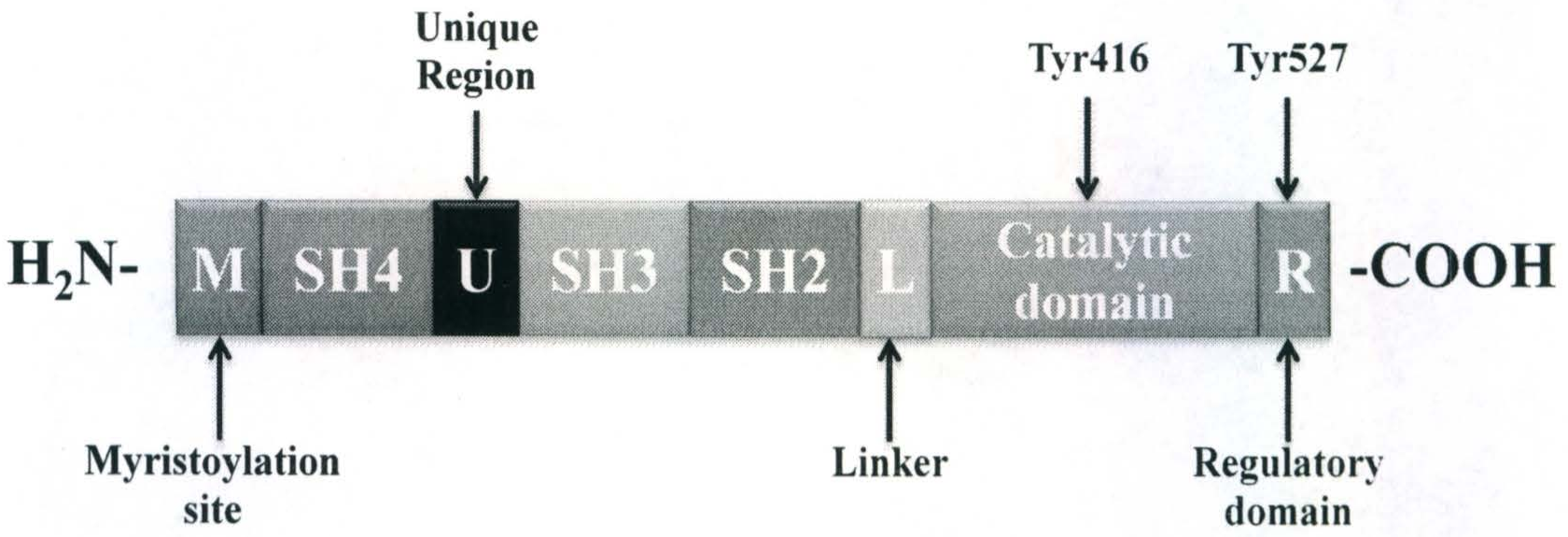


Figure 4: Src family kinase domain structure.

Src consists of eight functional regions including a myristylated site, Src homology (SH)4 domain, unique region, SH3 domain, SH2 domain, linker, the kinase/catalytic domain (SH1 domain), and regulatory domain. The two important phosphorylation sites are Tyr416 and Tyr527 located in the catalytic domain and regulatory domain respectively.

monolayer (Kim et al., 2009). The tyrosine kinase of c-Src becomes activated upon the initial binding of Albumin to its receptor, gp60, at the endothelial surface. Activated c-Src then phosphorylates Caveolin-1 at Tyr14, initiating caveolae fission from the plasma membrane (Shajahan et al., 2004; Kim et al., 2009). Activated c-Src can also regulate the size of the caveolae by phosphorylation of Caveolin-2 through formation of hetero-oligomers with Caveolin-1 (Li et al., 1998). Furthermore, activated c-Src phosphorylates Dynamin-2, a GTPase protein that binds Caveolin-1 and forms a spiral structure at the neck of the caveolae mediating scission from the plasma membrane. Through tyrosine phosphorylation of these proteins, c-Src becomes a major player in obligatory steps of caveolae fission and caveolae-mediated endocytosis. Previously, Tbdn is known to play an important role in this pathway (Paradis et al., 2008). Taken together, the effect of Tbdn knockdown on c-Src activity levels needs to be closely examined as a first step towards further elucidating the mechanism by which Tbdn maintains vascular permeability.

1.7 Rationale for Current Study

Pathological neovascularization is a hallmark of late stage neovascular AMD, PDR, and ROP. There are multiple factors that can pre-dispose individuals to retinal neovascular eye diseases. One of these factors is alteration in the endothelial permeability pathway that induces microvascular leakage in the retinal capillaries. In recent studies, Tbdn has been shown to play a role in regulating endothelial permeability and maintaining homeostasis of retinal vasculature (Gendron et al., 2000; Gendron et al., 2001; Asaumi et al.,2005; Gendron et al., 2006; Paradis et al., 2008; Gendron et al., 2010). The specific mechanism by which Tbdn regulates the transcellular transport of Albumin remains yet to be explored. Given that Cortactin and c-Src are major components of the pathway, this study was undertaken to test the hypothesis that Tubedown regulates the signaling pathway mediating retinal endothelial cell permeability to Albumin by influencing Cortactin and c-Src. In order to do so, this study investigates the effect of Tbdn knockdown on the expression of phospho-Tyr421 Cortactin and phospho-Tyr416 c-Src both *in vitro* and *in vivo* by different molecular techniques. Since Tbdn was found to be much downregulated in the PDR specimens compared to age-matched control (Gendron et al., 2001), this present study also seeks to closely examine the expression of phospho-Tyr416 c-Src when Tbdn is suppressed in human retinopathy. Elucidating Tbdn regulatory pathways is a step needed to better understand its role in retinal homeostasis and ultimately contributing to the discovery of a useful therapy that prevents and treats these devastating neovascular retinopathies causing blindness.

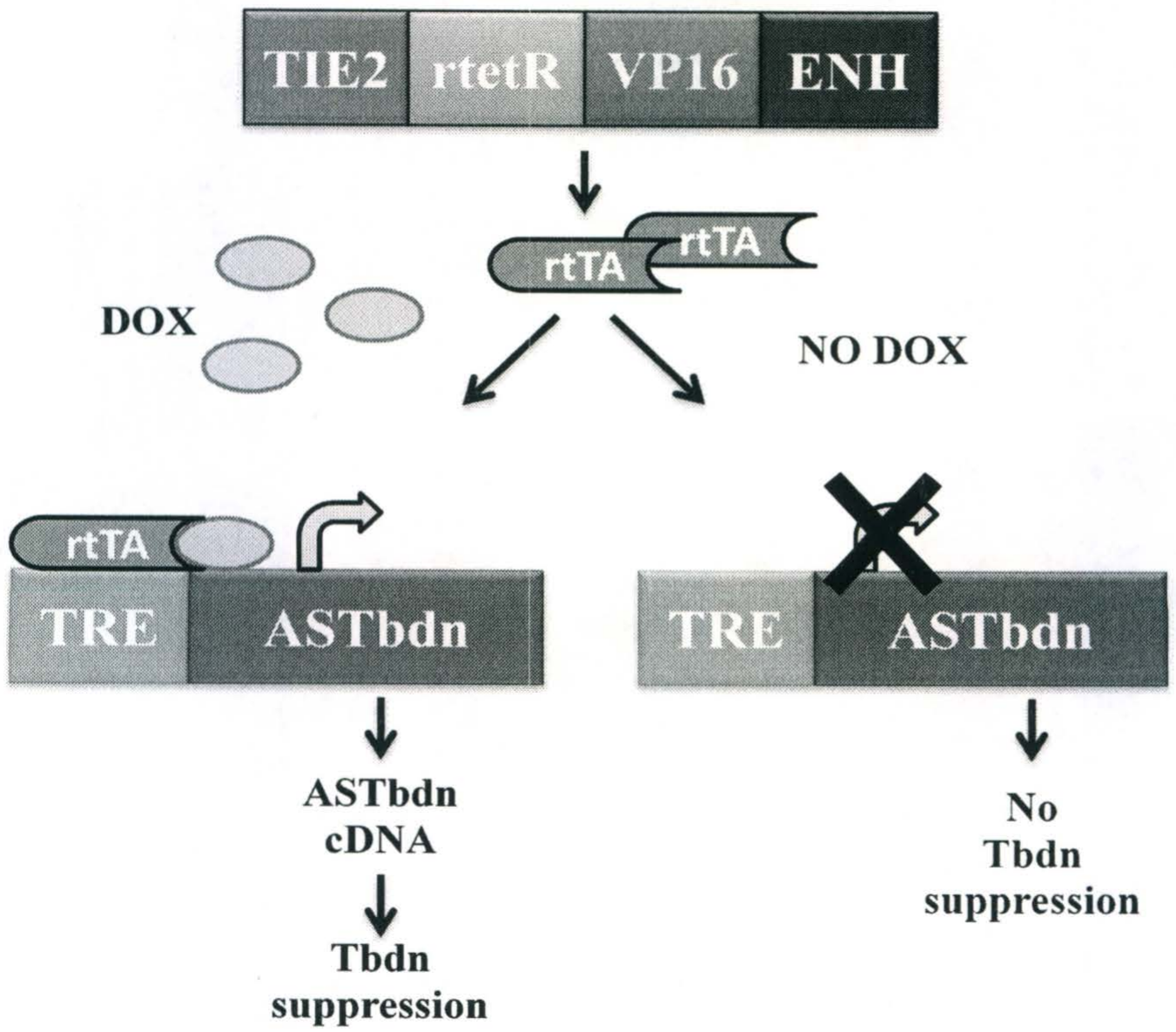
1.8 Overview of Methodology for Current study

To assess the relationship between Tbdn and other known regulators of vascular endothelial permeability in the retina, major components of the Albumin permeability pathway were first investigated in the retinal endothelial cell line RF/6A *in vitro*. RF/6A cell clones stably knocked down for Tbdn expression by expression of an antisense *Tbdn* cDNA fragment [nucleotide sequences 1–1413 in an antisense orientation (*ASTbdn*); Paradis et al; 2002, 2008], as well as RF/6A cells transiently knocked down for Tbdn expression using siRNA, were used to examine the effect of Tbdn levels on components of the Albumin permeability pathway. The activation status of two key components of the Albumin permeability pathway, c-Src and Cortactin, was studied in the above Tbdn knockdown RF/6A cells. The activation status of c-Src was monitored by Western Blotting by using a specific phospho-Tyr416 Src antibody recognizing the activated form of c-Src while the activation status of Cortactin was examined by Western Blotting using a specific phospho-Tyr421 Cortactin antibody recognizing the activated form of Cortactin. In parallel, Tbdn level of expression was measured in all samples to ensure Tbdn downregulation.

As previously mentioned, Tbdn knockdown RF/6A clones exhibit increase in endothelial permeability (Paradis et al., 2008). In order to determine in greater detail whether Tbdn regulation of endothelial permeability requires c-Src activation, we treated RF/6A Tbdn knockdown cell clones with Src inhibitor SKI-606 prior to examining transcellular permeability to FITC-Albumin. Western Blot analysis was done on these cell extracts to measure the level of expression of Tbdn, phospho-Src and phospho-Cortactin.

To describe the relationship between Tbdn and c-Src *in vivo*, an endothelial specific conditional Tbdn knockdown transgenic mouse model (*Tie2/rtTA/Enh-TRE/ASTbdn)* was employed (Wall et al., 2004). This mouse system which enables conditional knockdown of endothelial derived Tbdn, utilizes two separate constructs driven by two distinct promoters: the *Tie2* promoter and the tetracycline response element (*TRE*) promoter which drives the expression of the *ASTbdn* described above. *Tie2* promoter directs endothelial specific expression of the reverse tet transactivator protein (rtTA). Doxycycline (Dox), is introduced in the mouse diet, binds to rtTA and induces a conformational change that allows rtTA to activate the *TRE* promoter, directing the expression of the *ASTbdn*. As a result, endogenous Tbdn protein expression is suppressed. In the absence of Dox, rtTA does not bind the *TRE* promoter and endogenous Tbdn protein expression is not decreased. Analysis of phospho-Tyr416 Src level *in vivo* was accomplished by using histological and immunohistochemical techniques in addition to Western Blotting on mouse retinal extracts.

Similarly we wished to determine if c-Src activation occurred in human PDR patients as part of the pathology of the disease. Therefore, we examined phospho-Tyr416 Src levels by immunohistochemistry on paraffin-embedded human PDR samples compared to normal specimens.



<u>Figure 5:</u> Schematic diagram of the conditional Tbdn knockdown bitransgenic mouse model. *Tie2* promoter directs endothelial specific expression of rtTA protein. In the presence of Dox from the mouse diet. Binding of Dox to rtTA allows it to bind *TRE* which in turns directs expression of *ASTbdn* cDNA construct and suppress the expression of Tbdn. When Dox is absent, rtTA cannot bind *TRE*; as a result, Tbdn is not suppressed.

2. WORK PREPARED FOR PUBLICATION

Signaling pathways involved in Tubedown regulation of retinal endothelial permeability

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2.1 CO-AUTHORSHIP STATEMENT

This project and manuscript was completed under the supervision of Drs. Paradis and Gendron. The bitransgenic mouse model (*TIE2/rtTA/Enh-TRE/ASTBDN-1)* was previously generated by Drs Gendron and Paradis laboratory. Mouse work, including all aspects of colony management, Tbdn induction, etc., was performed and supervised by Drs. Paradis and Gendron with the technical assistance of Danielle Gardiner and Krista Squires. Molecular analysis of tail DNA for mouse genotyping was performed by Danielle Gardiner. The siRNA transfections were done by Kindra Grozinger and Nhu Ho under the supervision and guidance of Dr. Paradis. Western Blots and densitometry on transfected extracts were done by Nhu Ho with the help of Kindra Grozinger. Stimulations of RF/6A cells with BSA were done by Dr. Paradis and Maria Whelan who also helped generate data for part of Figure 6 and the supplementary figure. Dr. Paradis and Nhu Ho generated data for Figure 8. Dr. Paradis and Nhu Ho with the technical assistance of Danielle Gardiner generate data for Figure 9. While Western Blots on mouse retinal extracts were done by Dr. Paradis with technical assistance from Danielle Gardiner, all densitometry analysis was done by Nhu Ho. Paraffin embedded eyes were sectioned by Krista Squires and Histology Services of the Medical Education and Laboratory Services, Faculty of Medicine. Immunohistochemistry and all quantitative analyses were done by Nhu Ho under the supervision of Drs. Gendron and Paradis. Dr. Gendron, Dr. Paradis and Nhu Ho are jointly responsible for writing the text of this manuscript and preparing figures 6-13. Dr. Good provided us with human PDR specimens and valuable clinical insight and feedback on the manuscript.

2.2 ABSTRACT

Vascular hyper-permeability is a well-known factor contributing to pathogenesis of neovascular retinopathies. Tubedown (Tbdn) is an N- terminal acetyltransferase subunit which associates with Cortactin and plays a role in adult retinal blood vessel homeostasis by regulating vascular permeability. The purpose of this study is to describe how Tbdn regulates transcellular permeability through the tyrosine kinase c-Src and its substrate Cortactin. Tbdn knockdown in retinal endothelial cells by stable transfection or by siRNA lead to an increase in the levels of activated phospho-Tyr416 c- Src and phospho-Tyr421 Cortactin. In addition, inhibition of c-Src family of kinases and Cortactin phosphorylation in retinal endothelial cells *in vitro* inhibited the Tbdn knockdown-induced permeability to Albumin. Similarly, *in vivo* conditional endothelial specific knockdown of Tbdn, resulted in fibrovascular lesions characterized by retinal neovascularization and hyper-permeable vessels, increased levels of activated phospho-Tyr416 c-Src were found, compared to normal retina. Previously we linked loss of retinal endothelial Tbdn with proliferative diabetic retinopathy (PDR). Herein, we show that activated phospho-Tyr416 c-Src is expressed at high levels in the vessels of retinal lesions of PDR. Taken together, these results implicate Tbdn as an important regulator of retinal endothelial permeability and homeostasis by mediating a signaling pathway involving c-Src and Cortactin.

2.3 INTRODUCTION

The endothelial barrier is an important functional property of retinal endothelium that usually limits plasma Albumin to intravascular areas in normal retinas (Daly et al., 2004; Vinores et al., 1993, 1998). Neovascular retinopathies are known to involve breakdown of the endothelial barrier and changes in retinal endothelial cell permeability and integrity which associated with extravasation of Albumin (Pino et al., 1983). The neovascularization process is modulated by the combined action of pro-angiogenic growth factors (VEGF, bFGF and IGF-1; Das et al., 2003), integrins and extracellular matrix components (Paques et al., 1997; Campochiaro et al., 2003; Friedlander et al., 1996; Ljubimov et al., 1996). Endothelial cell hyperpermeability has been widely linked to angiogenesis in various diseases including cancer and diabetic retinopathy (Enea et al., 1989; Antonetti et al., 1998; Leto et al., 2001; Mousa et al.,2010; Khamdhadia et al., 2012).

Tubedown (Tbdn, also referred to as Narg1, mNat1, NATH, Naa15) has been defined from previous research in our laboratories as a regulator of vascular permeability in adult retinal blood vessels (Paradis et al., 2008, Gendron et al., 2010). Tbdn is highly homologous to Nat1, an essential subunit of the yeast N-terminal acetyltransferase NatA complex (Park and Szostak, 1992; Gendron et al., 2000). In yeast, Tubedown mediates the stable interaction of NatA with the large ribosomal subunit and directing the polypeptide towards the acetytransferase Ard1. This allows acetylation of amino acid residues in the second position of the N-terminus following cleavage of the initial methionine (Gautschi et al., 2003; Polevoda and Sherman, 2003; Polevoda et al., 2008).

In both yeast and mammals, the Tbdn/Ard1 complex has been shown to play an important role in the regulation of a broad range of cellular processes varying from cell growth to cellular differentiation (Surgiura et al., 2003; Arnesen et al., 2005; Arnesen et al., 2006; Paradis et al., 2002; Martin et al., 2007, Gautschi et al., 2003; Kimura et al., 2003; Asaumi et al., 2005, Park and Szosatak, 1992). In adults, high levels of Tbdn expression are restricted to vascular beds of the eyes, blood vessels of regressing ovarian follicles and the choroid plexus endothelium (Gendron et al., 2000; Gendron et al., 2001; Paradis et al., 2002; Paradis et al., 2008). Previous research has shown that Tbdn protein expression is suppressed in eyes from patients with proliferative diabetic retinopathy (PDR; Gendron et al., 2001). In addition, Tbdn knockdown in retinal endothelial cells *in vitro* and in animal models has been associated with increases in retinal angiogenesis and Albumin permeability, a hallmark to neovascular retinopathy (Paradis et al., 2002, Wall et al., 2004; Paradis et al., 2008).

In addition to Tbdn interacting with Ard1 to form an acetyltransferase complex, Tbdn has also been shown to interact with the actin binding protein, Cortactin (Paradis et al., 2008). Cortactin regulates actin assembly, cytoskeletal remodeling, endothelial barrier integrity and was originally identified as a major substrate of the tyrosine kinase c-Src (Kanner et al., 1990; Wu et al., 1991; Wu et al., 1993; Weed & Parsons, 2001). Cortactin can be phosphorylated by c-Src at residues (Tyr-421, 466, and 482) in a sequential manner. The initial priming phosphorylation at Tyr421 appears to be the most critical for regulating the subsequent phosphorylation (Head et al., 2003). Phosphorylation of Cortactin at Tyr421 by c-Src regulates cytoskeleton remodeling and coordination of

membrane dynamics such as vesicular endocytosis (Cosen-Binker & Kapus, 2006; Ammer & Weed, 2008). The activation of tyrosine kinase c-Src requires phosphorylation at Tyr416 in the catalytic domain, while inactivation requires phosphorylation at Tyr527 in the regulatory domain by Csk (carboxy-terminal Src kinase; Hu et al., 2008). Active Src is known to regulate endothelial permeability to Albumin (Minshall et al., 2000; Shajahan et al., 2004; Tiruppathi et al., 1997; Li et al., 1996); however, the specific mechanism by which endothelial cells transport Albumin from luminal to abluminal sides is not completely understood.

It is also known that binding of Albumin to its cell surface binding protein gp60 induces clustering of these receptors and recruitment of Caveolin-1 (Minshall et al., 2000). Interaction of gp60 with Caveolin-1 triggers activation of Gαi. Activated Gαi, through an as yet unknown mechanism promotes phosphorylation of c-Src at Tyr416 (Komarova & Malik, 2010; Hu et al.,2008). Activated c-Src then tyrosine phosphorylates other members of the pathway such as Caveolin-1, Dynamin (Shajahan et al., 2004; Tiruppathi et al., 1997) and Cortactin (Orth et al., 2002). Studies have demonstrated that phosphorylation of Caveolin-1 by c-Src is a key switch initiating caveolar fission from the plasma membrane (Hu et al., 2008; Li et al., 1996; Conner et al., 2003). Together, these sequential phosphorylation dependent events facilitate caveolar scission and transcellular vesicular transport of Albumin (Hu et al., 2008; Komarova & Malik., 2010) demonstrating that c-Src is a central regulator of transendothelial permeability through its regulation of caveolae formation and endocytosis.

In this study, we tested the hypothesis that Tubedown regulates the signaling pathway mediating retinal endothelial cell permeability to Albumin by influencing Cortactin and/or c-Src. We demonstrate that Tbdn knockdown is associated with activation of c-Src and Cortactin pathways both *in vitro* and *in vivo*. Because c-Src and Cortactin have emerged as potentially crucial players regulating endothelial permeability (Komarova & Malik, 2010; Schnoor et al., 2011), this work further implicates Tbdn as an important regulator of retinal endothelial permeability and is required for the normal maintenance of retinal homeostasis.

2.4 MATERIAL AND METHODS

Antibodies

Purified rabbit anti-Tbdn C755-766 antibody and monoclonal mouse anti-Tbdn antibody (clone OE5) were derived as described previously (Martin et al., 2007; Paradis et al., 2008). Other antibodies used in this study include mouse monoclonal anti-Cortactin 4F11 (Upstate Biotechnology, Lake Placid, NY), mouse monoclonal anti-c-Src clone 327 (Abcam, Cambridge, MA) and anti-α-Tubulin mouse monoclonal antibody (Sigma, St Louis, MO). Rabbit polyclonal anti-phospho-Cortactin Y421 and anti-phospho-Src Y416 were purchased from Cell Signaling Technology (Danvers, MA). Mouse monoclonal anti-Fyn (SC434), anti-Lyn (SC7274) and anti-ERK1 (SC94) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). We also used affinity purified horseradish peroxidase (HRP) conjugated-anti-rabbit IgG and -anti-mouse IgG reagents (Promega, Madison, WI) and AP conjugated-goat-anti-mouse and -goat-anti-rabbit from Jackson ImmunoResearch

(West Grove, PA) as secondary antibodies for Western Blot analyses and immunohistochemistry staining, respectively.

Cell culture

RF/6A, rhesus macaque choroid-retina endothelial cell line (CRL-1780; American Type Culture Collection) were grown as previously described (Gendron et al., 2001). RF/6A clones in which Tbdn expression had been suppressed by stable expression of the antisense *Tbdn* cDNA construct *ASTbdn* (Tbdn knockdown) and negative control RF/6A clones have been described previously (Paradis et al., 2002). RF/6A Tbdn knockdown and parental cell clones were plated at $9.375X10^3$ cells/cm^2 in 6.5 mm wells for permeability assay and at either $9.375X10^3$ cells/cm^2 or $1.364X10^4$ cells/cm^2 in 100 mm tissue culture dishes for Western Blotting. For Albumin stimulation, RF/6A cells were grown in reduced Fetal Bovine Serum (FBS) (Invitrogen, Carlsbad, CA) concentration (0.5 %) for 48 hours followed by 2 hours in serum-free media. The cell monolayer was 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 Tris-buffered saline (TBS).

Animals

Choroid-retinal endothelial Tbdn expression was conditionally knocked down in *TIE2/rtTA/Enh-TRE/ASTBDN* bi-transgenic middle age (7 months) mice as previously described (Wall et al., 2004). Conditional knockdown of Tbdn in endothelial cells was induced by feeding the mice with commercially prepared mouse chow containing Doxycycline (Dox; 600 mg/kg; Bio-Serv, New Jersey), whereas control age-matched

mice were fed with a regular diet not containing Dox. Additional controls included age-matched single transgenic mice (*TIE2/rtTA/Enh* mice or *TRE/ASTBDN* mice) fed with Dox diet for the same length of time. Wild-type or control single transgenic mice aged for periods longer than 16 months were also used to demonstrate the effect of advanced aged. Mice were sacrificed after 1, 2 , and 6 weeks after induction by Dox. For each eye specimen, sections were prepared and analyzed by histology as described previously (Wall et al., 2004) to determine the extent and progression of choroid-retinal pathology. 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 (Protocol 11-01-G).

siRNA Transfections

A pool of siRNA duplexes targeting RF/6A rhesus macaque TBDN (5'-TGCGAGATCTTGAGGGTTA-3'), as well as a control non-silencing siRNA (5'-GATCCGTTCATCGTCACTA-3') were purchased from Dharmacon (Thermo Fischer, Lafayette, CO). PmaxGFP negative control vector was obtained from Lonza (Basel, Switzerland). RF/6A cells were electroporated with 10 and 20nM siRNA using the Neon Transfection system (Invitrogen, Carlsbad, CA) following manufacturer's protocols. Electroporated RF/6A were cultured in DMEM at a density of 9.375 $X10^3$ cells/cm^2 for 72 hrs before harvesting.

Western Blot Analysis

Protein extraction was performed essentially as described previously (Gendron et al.,2000). Cellular monolayers were washed twice with 25 mM Tris-HCl pH 7.6, 150 mM NaCl, harvested and suspended in a cell lysis buffer (50 mM Tris-HCl pH 7.6,150 mM NaCl and 0.5% Brij 96) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.3 U/ml aprotinin and 10 μg/ml leupeptin) andphosphatase inhibitors (1 mM sodium orthovanadate, 25 mM sodium fluoride and 10 mM β-glycerophosphate). Lysates were clarified by centrifugation, protein concentration was determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA) using BSA as the standard. Western Blots were performed by loading equal quantities of protein from each sample onto an SDS-PAGE and transfered to PVDF membrane (Bio-Rad, Hercules, CA). Detection of phosphorylated and unphosphorylated proteins was performed by incubating with specific primary antibodies, horseradish peroxidase conjugated secondary antibody and visualized by using Chemiluminescence detection reagent (Amersham Biosciences, Piscataway, NJ) as previously described (Paradis et. al., 2008). To determine relative protein levels, densitometry analyses were conducted using the Kodak Gel Logic 200 imaging system (Eastman Kodak Company, Rochester, NY) and intensities of each band was analyzed using Kodak Molecular imaging software (Version 4.0, Eastman Kodak Company, Rochester, NY).

Human Eye Specimens

Human eye specimens were obtained from The National Disease Research Interchange (Philadelphia, PA, USA) or from collaborator Dr. William V. Good of Smith Kettlewell

Eye Research Institute, San Francisco, CA. The Aged Normal specimens (n=6) were from patients with an age range of 60-80 years old. The PDR specimens (n=3), some with retinal detachment and developed pre-retinal membrane, were from patients with an age range of 71-91 years old. All research on human specimens followed the tenets of the Declaration of Helsinki and was performed under approval from the Human Investigation Committee of Memorial University.

Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded sections of eye specimens processed as previously described (Paradis et al., 2002). Paraffin-embedded sections (5 μm thick) adhered to glasses slides were deparaffinized in xylene and graded ethanols, post-fixed in 4% paraformaldehyde and washed in Tris Buffered Saline (TBS). Samples were then blocked in 2% ECL Advance Blocking Agent (GE Healthcare, Buckinhamshire UK) for 1 hour at room temperature before incubation with appropriate primary antibody (1:100) or negative control in blocking agent in TBS at room temperature overnight. After washing in TBS 3 times for 10 minutes, slides were incubated with either anti-mouse or anti-rabbit IgG Alkaline Phosphatase (AP). Sections were washed in TBS 3 times for 10 minutes before development using Vector Red AP with Levamisole (Vector Laboratories, Burlingame, CA USA). Sections were then air dried and mounted with Permount (Fisher Scientific, Pittsburg, PA).

Transcellular permeability Assay

For FITC-Albumin transit assays, RF/6A stable ASTbdn clones were seeded onto 1%-gelatin-coated polystyrene filter inserts (Costar Transwell, no. 3470, 6.5-mm diameter, 0.4-μm pore size; Sigma, St Louis, MO) as previously described (Paradis et al., 2002). The cells were pre-treated daily with 0-35 μM of c-Src inhibitor SKI-606 (Bosutinib; Biovision, Mountain View, CA) for 48 hours prior to transcellular permeability assay. Transcellular permeability assay was performed and analyzed following previously published methodology (Paradis et al., 2008).

Data and Statistical Analysis

All immunohistochemically stained sections were viewed and photographed using a Leica DMIRE2 microscope system (Bannockburn, IL, USA) equipped with a QImaging (Surrey BC, Canada), RETIGA Exi camera and Improvision Openlab software (version 5; Coventry, UK) for quantitation of the staining. Tbdn and phospho-src levels in retinal and choroidal blood vessels were expressed as the average staining levels of at least three separate mouse eye specimens. Intensity of Tbdn and phospho-Src staining in blood vessels and background staining (from photoreceptors) were measured by determining the ratio of red color/ green color intensity using HIS Colourspy tool of Openlab software. Negative control antibody produced no background therefore final measurements were calculated by subtracting the background measurements from photoreceptors, which also serve as an internal control. Relative intensities were expressed as the average staining levels ± standard error (SEM). Images were prepared using Photoshop version 4.0 (Adobe Systems, Mountain View, CA).

All quantitative analyses were compared using the two-tailed Student's t-Test with Microsoft Excel (Mississauga, ON, Canada). The data was considered to be statistically significant if the *P* value was less than or equal to 0.05.

2.5 RESULTS

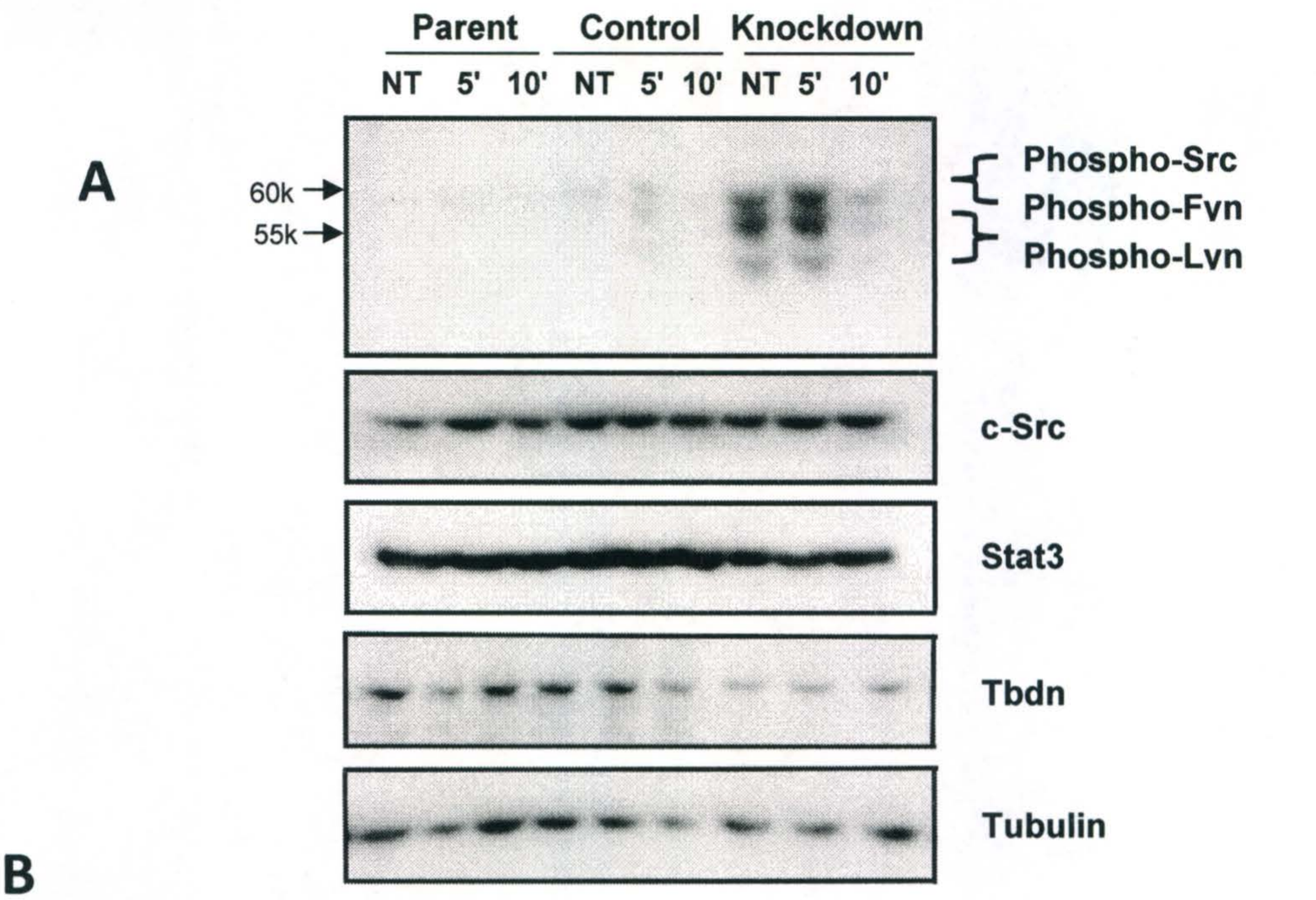
Tbdn knockdown in RF/6A retinal endothelial cells leads to upregulation of Phospho-Tyr416 c-Src levels

Since Tbdn was shown to regulate retinal endothelial permeability to Albumin (Paradis et al., 2008), we assessed the relationship between Tbdn and known regulators of vascular endothelial permeability in the retina. The activation of the tyrosine kinase c-Src plays key roles in the regulation of microvascular barrier function and various endothelial responses including permeability of Albumin (Yuan et al.,2002; Shajahan et al., 2004; Kim et al., 2009; Hu & Minshall, 2009). Moreover, Tbdn was previously found to bind Cortactin, a filamentous-actin binding protein and prominent substrate of c-Src (Wu et al., 1993; Wu et al., 1991). As major components of the Albumin permeability pathway, c-Src and Cortactin were first investigated *in vitro* in the retinal endothelial cell line RF/6A. Both RF/6A cell clones stably knocked down for Tbdn by expression of an antisense *Tbdn* cDNA fragment that exhibit increased transcellular permeability to Albumin (Paradis et al., 2002; 2008) and RF/6A cells transiently knocked down for Tbdn expression using siRNA were used to examine the effect of Tbdn levels on components of the Albumin permeability pathway. The effects of Tbdn knockdown on the levels of

activated c-Src were studied by Western Blot using a phospho-specific (Tyr416) c-Src antibody. Three bands with relative molecular mass of 60 k, 56 k and 53 k were detected by Western Blot analysis of Tbdn knockdown RF/6A clones using the phospho-specific (Tyr416) c-Src antibody (Fig. 6A). To confirm the identity of these bands, immunoprecipitations with antibodies directed against individual Src family members c-Src, Fyn and Lyn followed by Western Blot with activated phospho-Tyr416 c-Src Ab was performed. These analyses confirmed that the activated phospho-Tyr416 c-Src antibody detects c-Src, Fyn, and Lyn in Tbdn knockdown RF/6A clones (Figure 6C). Activated phospho-c-Src and activated phospho-Fyn co-migrated with each other on SDS-PAGE to form the 60 k band while phospho-Lyn corresponded to the two lower molecular weight bands of 53 and 56 k (Figure 6C). These observations are consistent with previously reported molecular weights of Fyn and Lyn (Lannuti et al., 2003; Thorne et al., 2006). Steady state levels of activated phospho-Tyr416 c-Src family detected by Western Blot relative to loading control (Stat3, total c-Src or total Fyn) were upregulated in the Tbdn knockdown RF/6A cell clones, previously reported to exhibit increased transcellular permeability to Albumin (Paradis et al., 2008), compared to the parental and control cellular protein abstract (Fig. 6). Moreover, similar results were obtained when Tbdn was knocked down by siRNA (Fig. 7).

To further evaluate the activation of c-Src in response to Tbdn knockdown, RF/6A endothelial cells were stimulated with serum Albumin (BSA) for 5 and 10 minutes to induce the Albumin permeability pathway. Albumin stimulation of Tbdn knockdown, parental and control clones resulted in a transient increase of activated-Src, with highest

increase of 7 fold activation of c-Src/Fyn observed in the Tbdn knockdown clones at 5 minutes compared to parental cells ($p < 0.01$; Fig. 6). Densitometry analysis of Western Blots showed no significant changes in the levels of total c-Src or total Fyn relative to the loading control (Stat3) between the different clones (Fig.6 and Supplementary data).



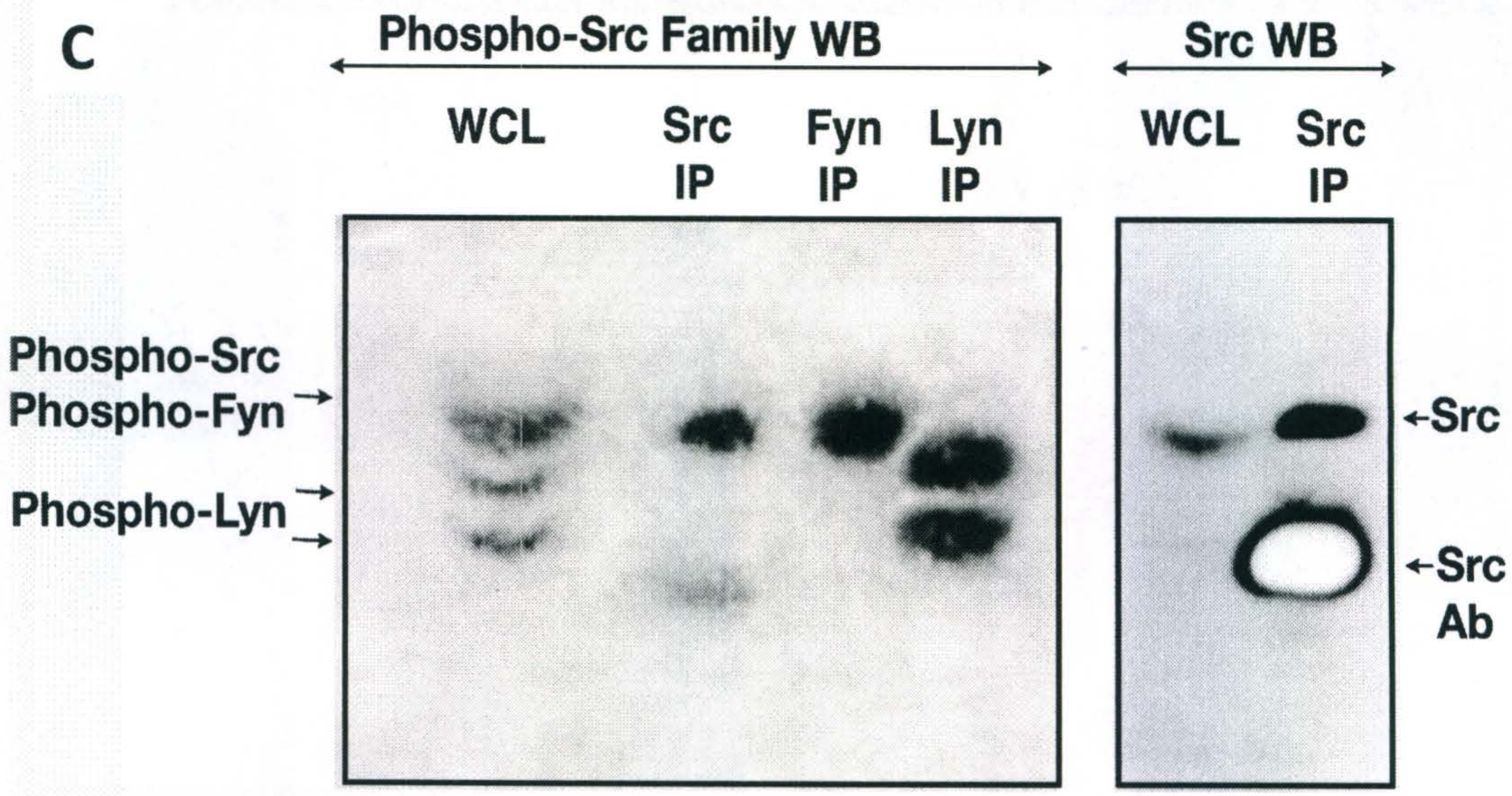


Figure 6: Tbdn knockdown in retinal endothelial cells *in vitro* leads to activation of Src family kinases. Parental RF/6A retinal endothelial cells, Tbdn knockdown RF/6A and control RF/6A cell clones were grown in reduced serum conditions for 48hrs followed by no serum for 2 hours. Cells were next either left untreated (NT) or stimulated with serum Albumin for 5 (5') and 10 (10') minutes. (A) Western Blot analyses of activated Src family kinase and total c-Src kinase levels showed a higher constitutive activation of Src family kinase (identified as c-Src, Fyn and Lyn in Fig. 9A) in Tbdn knockdown clone (Knockdown) which expresses reduced levels of Tbdn compared to the parental and control cells. (B) Quantitation of WB analyses of phospho-Tyr416 Src for activated c-Src/Fyn, total c-Src, total Fyn and loading control (Stat3) for which representatives are shown in previous panels and Supplementary Figure. Levels of activated c-Src/Fyn kinase (60 k band) over loading control are expressed as fold relative to non-treated parental cells. Similar results were obtained for activated Src/Fyn over total c-Src or total Fyn (*$P < 0.05$, n=3). (C) Western blot analysis using phospho-Src antibody revealed the presence of three bands in the whole cell lysate (WCL) of RF/6A clone. To determine exactly which Src family kinases are present in these bands 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. Representative experiments are shown.

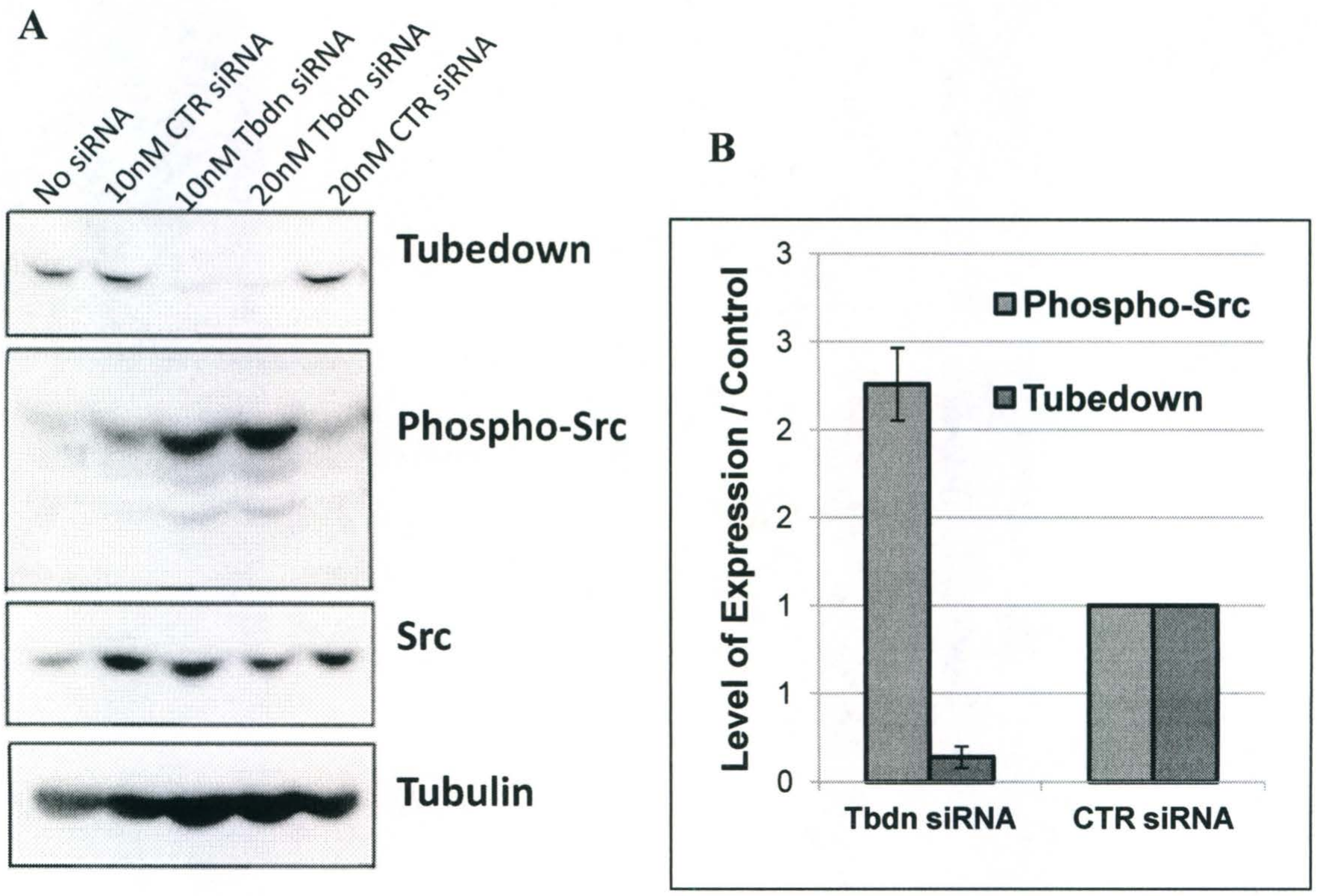
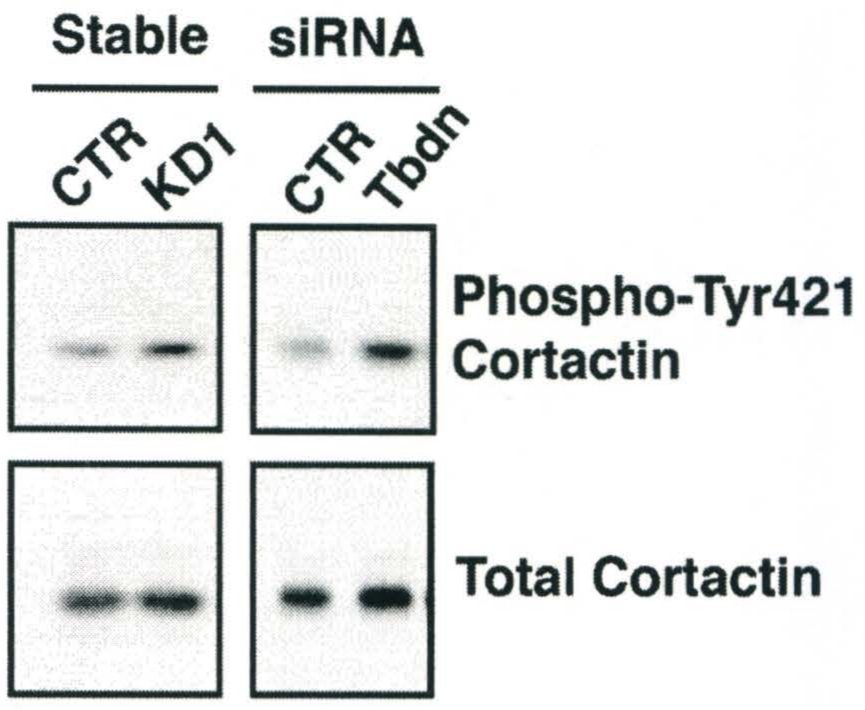


Figure 7: Tbdn knockdown by siRNA in retinal endothelial cells *in vitro* leads to increased phospho-Tyr416 Src (A) Tbdn expression was knocked down by siRNA in RF/6A retinal endothelial cells *in vitro*. Cell extracts from control preparations (No siRNA, 10nM Ctrl siRNA, 20nM CTR siRNA) or Tbdn knockdown at different concentration of siRNA (10nM Tbdn siRNA, 20nM Tbdn siRNA) were analyzed for levels of Tbdn, phospho-Tyr416 Src and total c-Src. Tubulin was also assessed for loading control and sample integrity. (B) Quantitative analysis of the Western Blots. Levels of phospho-Tyr416 Src over total c-Src and levels of Tbdn over loading control (Tubulin) were quantified in controls (CTR siRNA) or Tbdn knockdown (Tbdn siRNA) preparations. Data is expressed as mean +/- S.E.M. in each group. Representative experiments are shown (n=3).

A



B

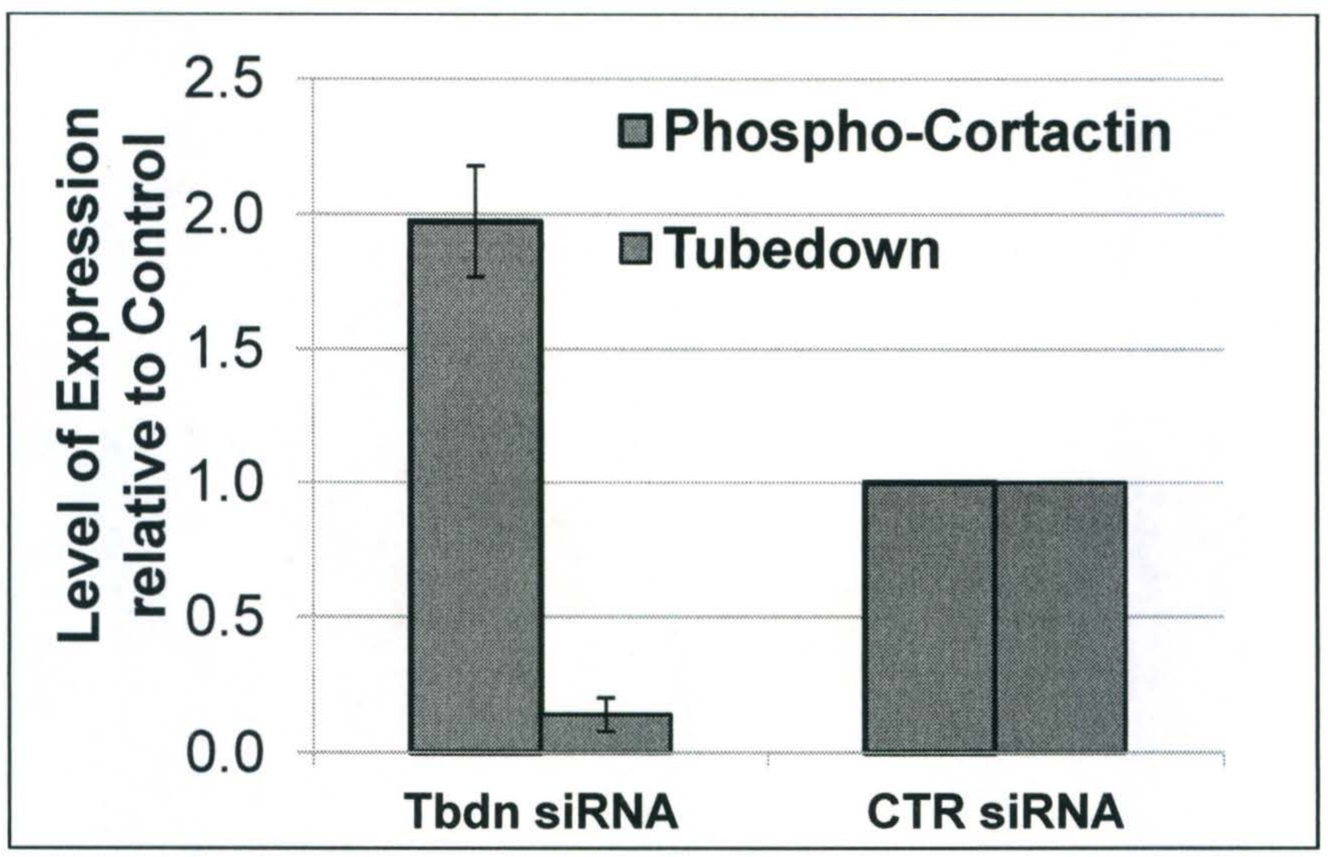


Figure 8: Tbdn knockdown in retinal endothelial cells *in vitro* leads to increase in phosphorylation of Cortactin on Tyr421. (A) Tbdn expression was knocked down by either stable transfection (Stable) of an antisense *Tbdn* construct or siRNA (siRNA) in RF/6A retinal endothelial cells *in vitro*. Cell extracts from controls (CTR) or Tbdn knockdown (Stable KD1 or siRNA Tbdn) were analyzed for levels of phospho-Tyr421 Cortactin versus total Cortactin by WB. Representative results are shown. Representative experiment is shown. (B) Levels of phospho-Cortactin over total Cortactin and levels of Tbdn over loading control were quantified in controls (CTR siRNA) or Tbdn knockdown (Tbdn siRNA). Data is expressed as mean +/- S.E.M. in each group (n=3).

Tbdn knockdown in retinal endothelial cells leads to increased levels of phospho-Tyr421 Cortactin

To further study the mechanism by which Tbdn regulates the Albumin transcellular pathway, we examined the effect of Tbdn knockdown on the levels of phospho-Tyr421 Cortactin. RF/6A cells were knocked down for Tbdn expression as above either transiently by siRNA or stably by transfection with an antisense Tbdn cDNA construct *ASTbdn* (Fig.6 and 7; Paradis et al., 2002; 2008). Tbdn knockdown by either stable transfection or transient transfection was associated with an increase in phospho-Cortactin at tyrosine 421 whereas the level of total Cortactin remained the same for both knockdown approaches (Fig. 8A). Tbdn when knockdowned by 90% (Fig. 7) resulted in a 2 fold increase in phospho-Cortactin over the level of total Cortactin (Fig. 8B).

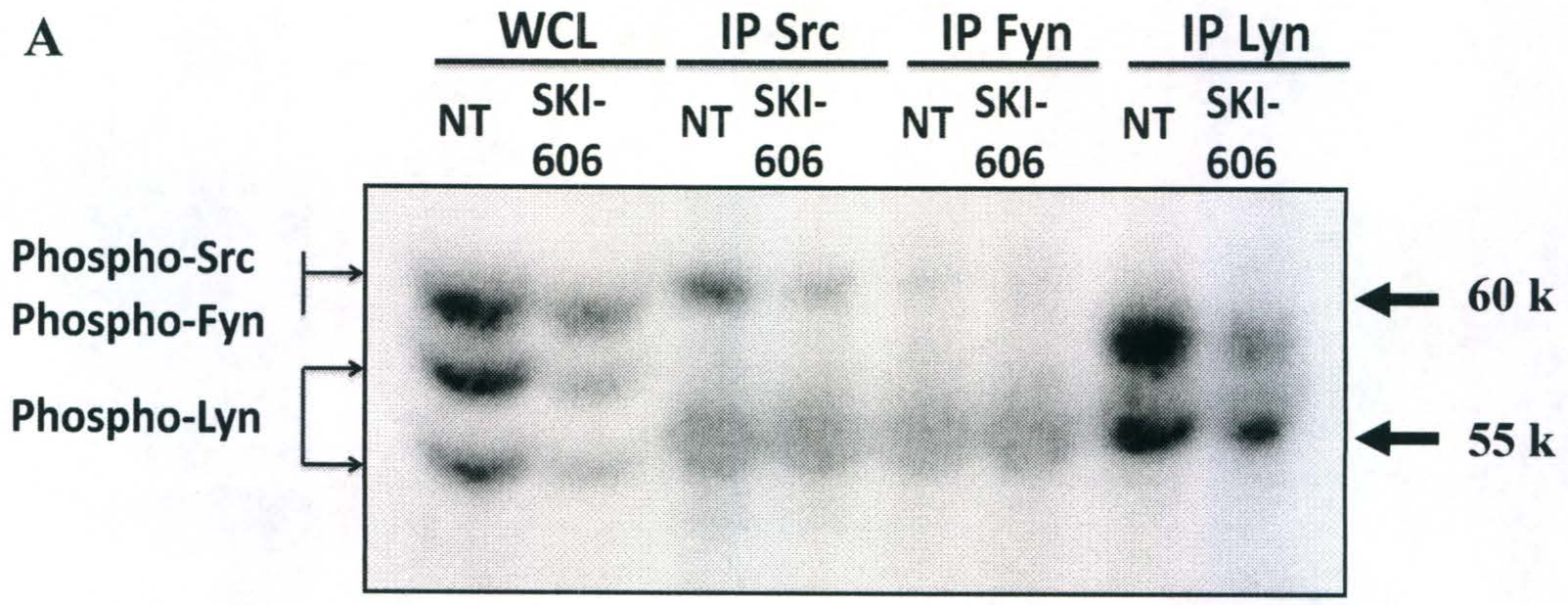
c-Src inhibition in Tbdn knockdown RF/6A cell clones leads to decreases in Albumin permeability

Tbdn loss has been previously shown to lead to an increase in permeability of retinal endothelial cells to Albumin (Paradis et al., 2008). To determine in greater detail whether or not Tbdn regulates endothelial permeability through c-Src, we used the c-Src inhibitor SKI-606. As expected, Western Blot analysis of cell extracts of Tbdn knockdown RF/6A cells treated with 1.5 μM of SKI-606 showed a decrease of approximately 60% in phospho-Tyr416 c-Src/Fyn levels compared to non-treated cells. Moreover, this reduction in the levels of active c-Src/Fyn correlated with a decrease in phospho-Tyr421 Cortactin, while expression levels of both Cortactin or c-Src in the

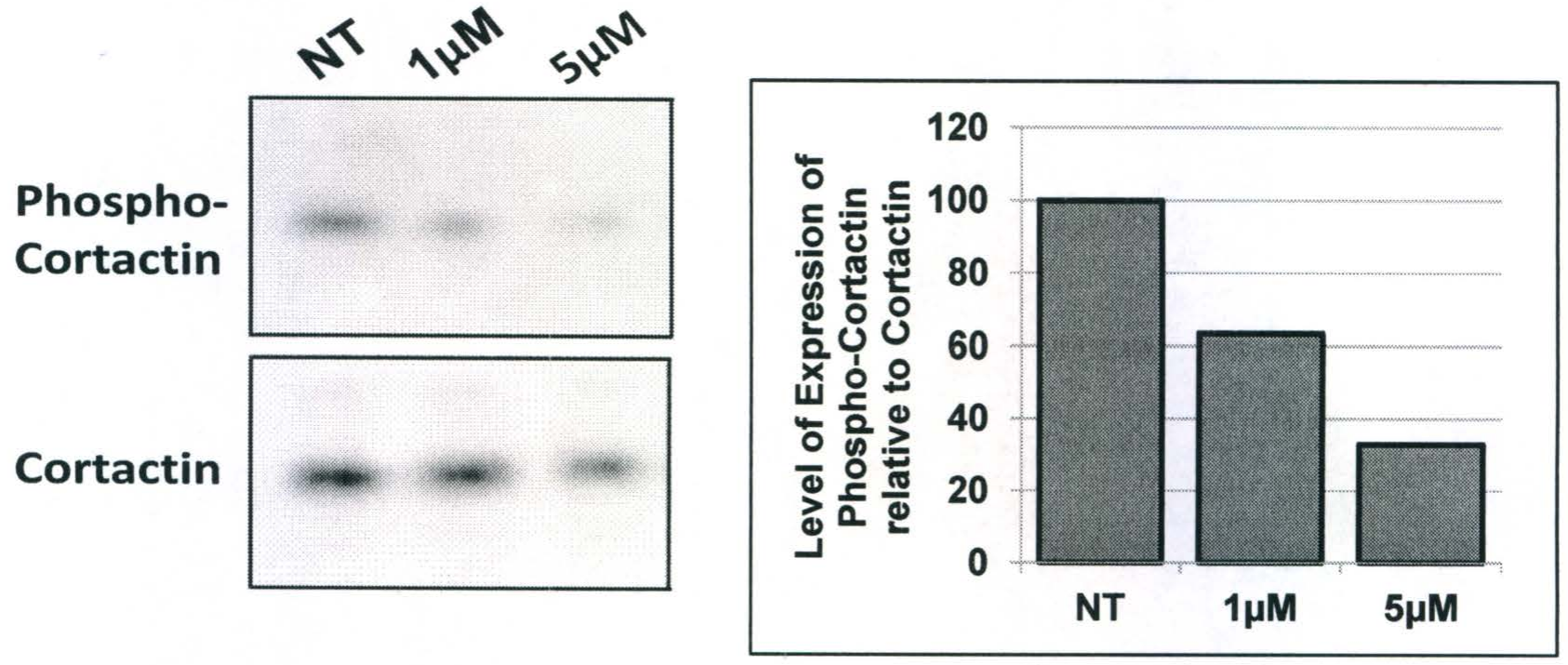
treated versus non-treated RF/6A cells extracts were not different (Fig. 9 A, B). To further examine which Src family kinase was inhibited by the drug, immunoprecipitations with antibodies specific to c-Src, Fyn and Lyn were performed. We found that in RF/6A Tbdn knockdown cells, SKI-606 inhibited the activation of c-Src, Lyn and Fyn. However, Fyn kinase was less affected by the drug in comparison to the c-Src and Lyn kinases (Fig. 9A).

Next, the effect of SKI-606 on endothelial cell permeability to Albumin of RF/6A Tbdn knockdown cells was examined. Cellular permeability assays performed on Tbdn knockdown RF/6A cells pre-treated with various concentrations of SKI-606 daily for 48 hours revealed a decrease of approximately 10 and 20 % in the rate of FITC-Albumin transit across the cell monolayer at 1.5 uM and 3.6 uM of inhibitor, respectively (Fig. 9C). However, at concentrations of 5 uM and above of SKI-606, the permeability of the endothelial cells knockdown for Tbdn was no longer inhibited by the c-Src inhibitor. Moreover, at concentration of 35 uM of SKI-606, the rate of FITC-Albumin transit across the cell monolayer was significantly higher than non-treated control and independent of time suggesting that the cell monolayers had become leaky. Interestingly, when levels of inhibition of c-Src activation by SKI-606 were examined under the same culture conditions of the permeability assay, which requires a 7 fold higher cell density than normal culture conditions, approximately only 15% reduction of active c-Src/Fyn levels were observed at 1.5 μM of SKI-606 compared to control (Fig. 9D). Under these high cell density culture conditions, 5 μM and 10 μM of SKI-606 were necessary to reduce the

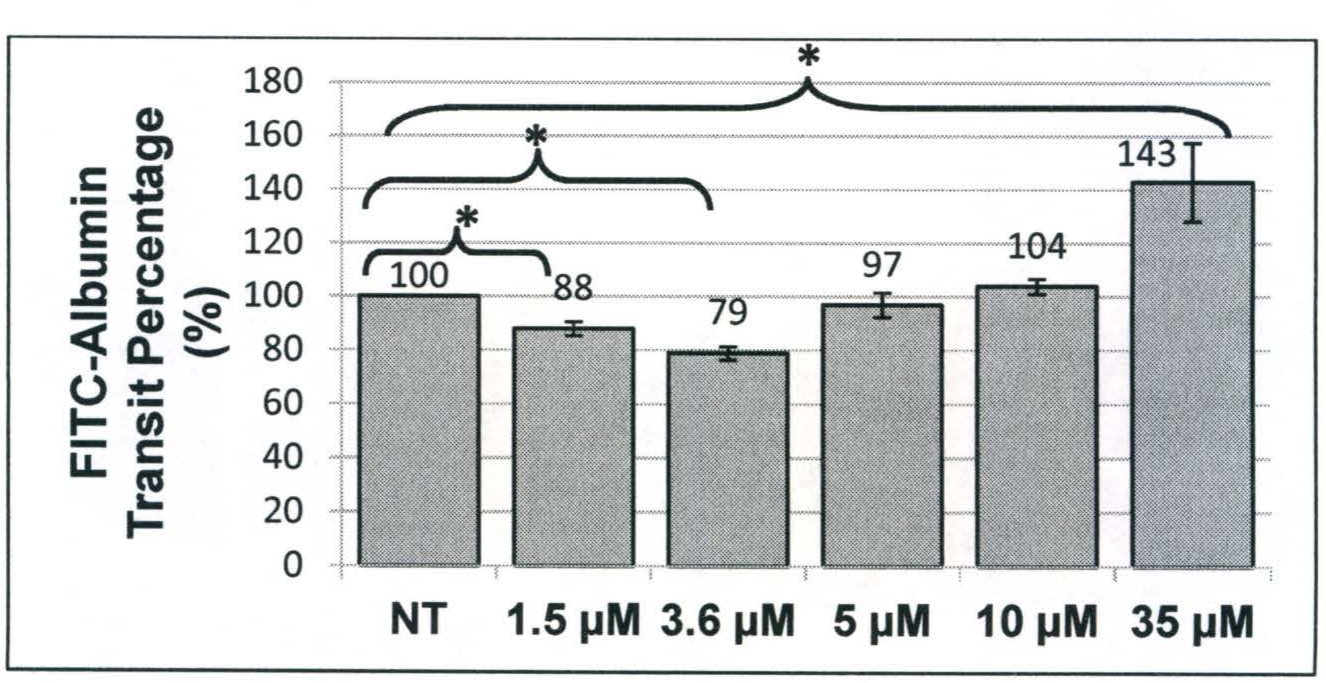
A


WCL
IP Src
IP Fyn
IP Lyn
NT
SKI-606
Phospho-Src
Phospho-Fyn
Phospho-Lyn
60 k
55 k

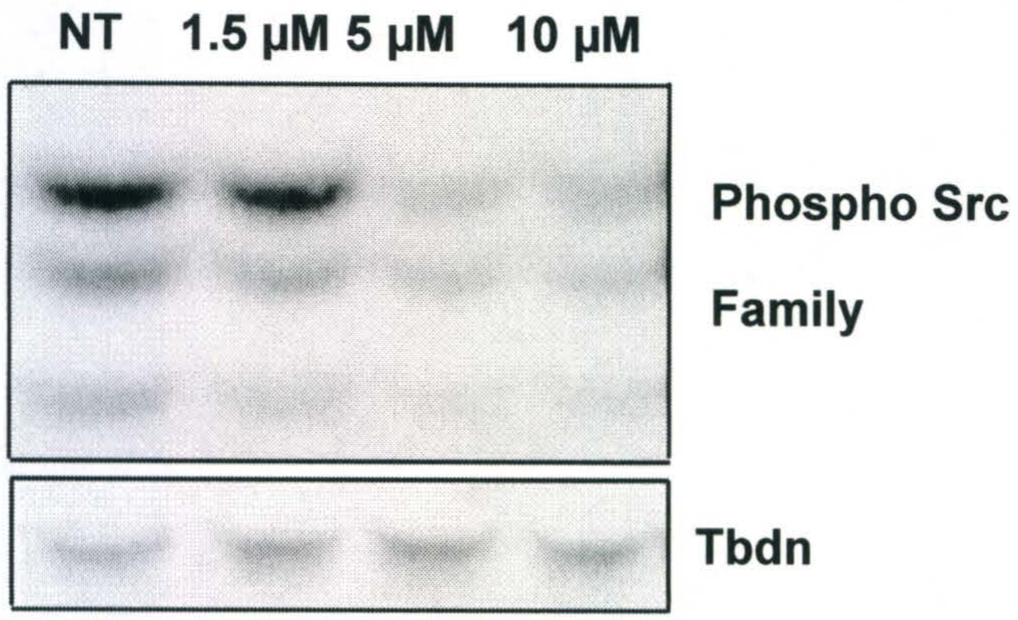
B


NT
1μM
5μM
Phospho-Cortactin
Cortactin
Level of Expression of Phospho-Cortactin relative to Cortactin
120
100
80
60
40
20
0
NT
1μM
5μM

C


FITC-Albumin Transit Percentage (%)
180
160
140
120
100
80
60
40
20
0
100
88
79
97
104
143
*
*
*
NT
1.5 μM
3.6 μM
5 μM
10 μM
35 μM

D



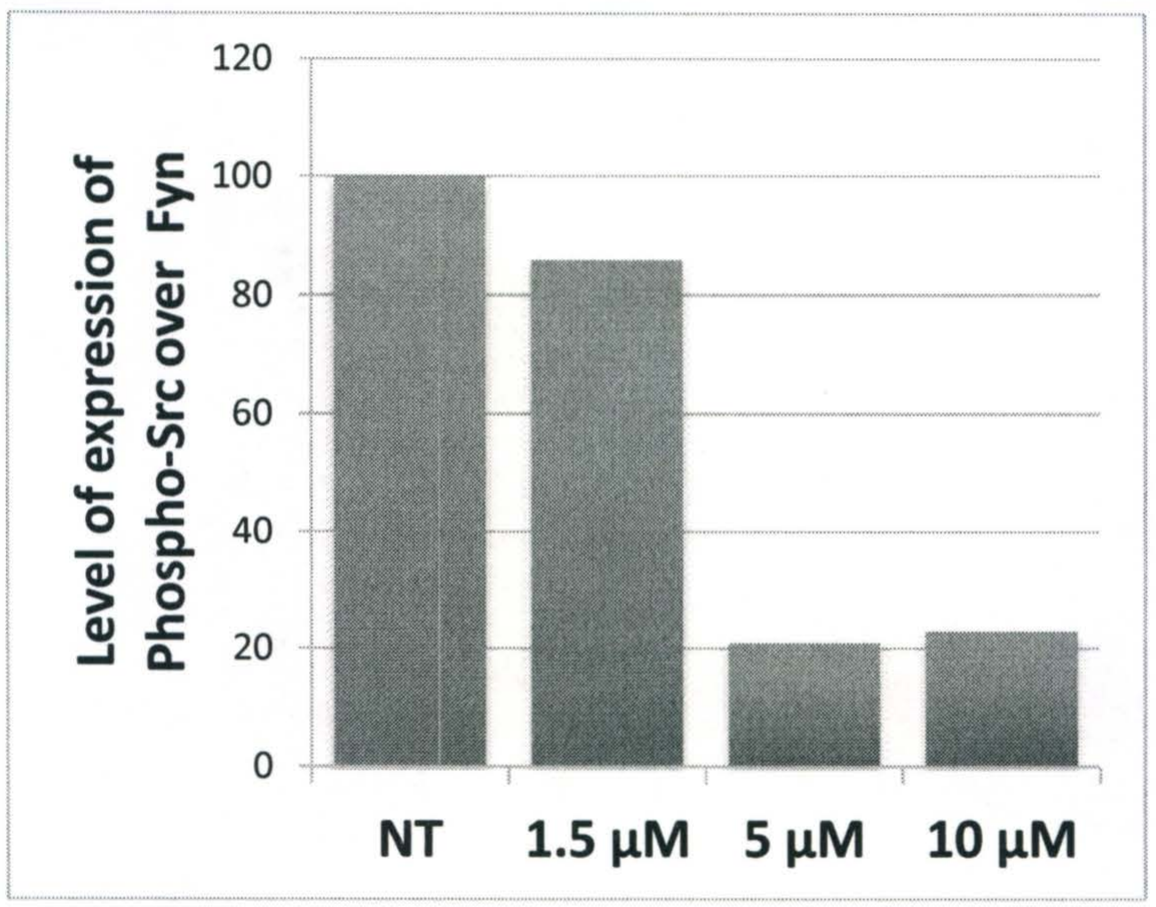


Figure 9: Phospho-Tyr 416 Src inhibitor SKI-606 (Bosutinib) decreases cellular permeability in Tbdn knockdown cell clones. (A) WB with a phospho-Tyr416 Src family antibody (activated Src family kinase) of whole cell lysate (WCL) of RF/6A retinal endothelial cells knocked down for Tbdn revealed three bands. Immunoprecipitations (IP) were performed using antibodies against three Src kinase family members: c-Src, Fyn and Lyn and IPs were analyzed by WB with phospho-Tyr416 Src family antibody. Phospho-Tyr416 Src inhibitor SKI-606 (Bosutinib, 1.5 µM) inhibits c-Src Lyn and Fyn. Fyn and c-Src antibodies used for IP produced a band at 55 k. (B) Inhibition of phospho-Src by Bosutinib results in decreases in levels of phospho-Tyr 421 Cortactin over total Cortactin. (C) Percentages of FITC-albumin transit across cellular monolayer of Tbdn-knockdown (ASTbdn) cells treated with various concentrations of SKI-606 as indicated or with vehicle only (NT= 0 µM, n=3). (D) Western Blot and densitometry showing activated Src family kinases and Tbdn in RF/6A Tbdn knockdown cells treated with SKI-606 under optimal permeability assay conditions at high cell density (*$P<0.05$). Representative experiments are shown

levels of activated c-Src/Fyn significantly in contrast to the marked inhibition seen at 1.5 μM under lower cell culture density described in panel A (Fig. 9A).

Tbdn knockdown in blood vessels leads to increased levels of activated phospho-Tyr416 Src.

Using a conditional-endothelial specific bitransgenic Tbdn knockdown mouse model (Wall et al., 2004), we next examined the expression level of activated phospho-Tyr416 c-Src to determine if it plays a role in the increase in permeability to Albumin previously observed in this mouse model (Paradis et al., 2008). In comparison to control age-matched mice, increased immunostaining for activated phospho-Tyr416 c-Src was observed in retinal lesions of both 2 and 6 week Tbdn knockdown bitransgenic mice (Fig. 10). Quantitation of the immunostaining in retinal blood vessels revealed a maximal 10 fold increase in activated phospho-Tyr416 c-Src at 6 weeks Tbdn knockdown compared to control (Fig. 10B).

To confirm our immunohistochemical findings that blood vessels of retinal lesions of Tbdn knockdown mice show upregulation of activated c-Src levels, Western Blot analysis for activated phospho-Tyr416 c-Src was performed on isolated retinal tissues from 6 week Tbdn knockdown mice and control mice. As shown in Figure 11A, retinal tissue from 6-week Tbdn knockdown mice showed a significant increase (3.4 fold) in the 60 k phospho-Tyr416 c-Src protein band ($p < 0.01$) while total c-Src remained unchanged in all extracts (Fig. 11). Interestingly, in comparison to RF/6A, phospho-Tyr416 specific c-Src antibody only detected one band at approximately 60 k in mouse retina (Fig. 11B)

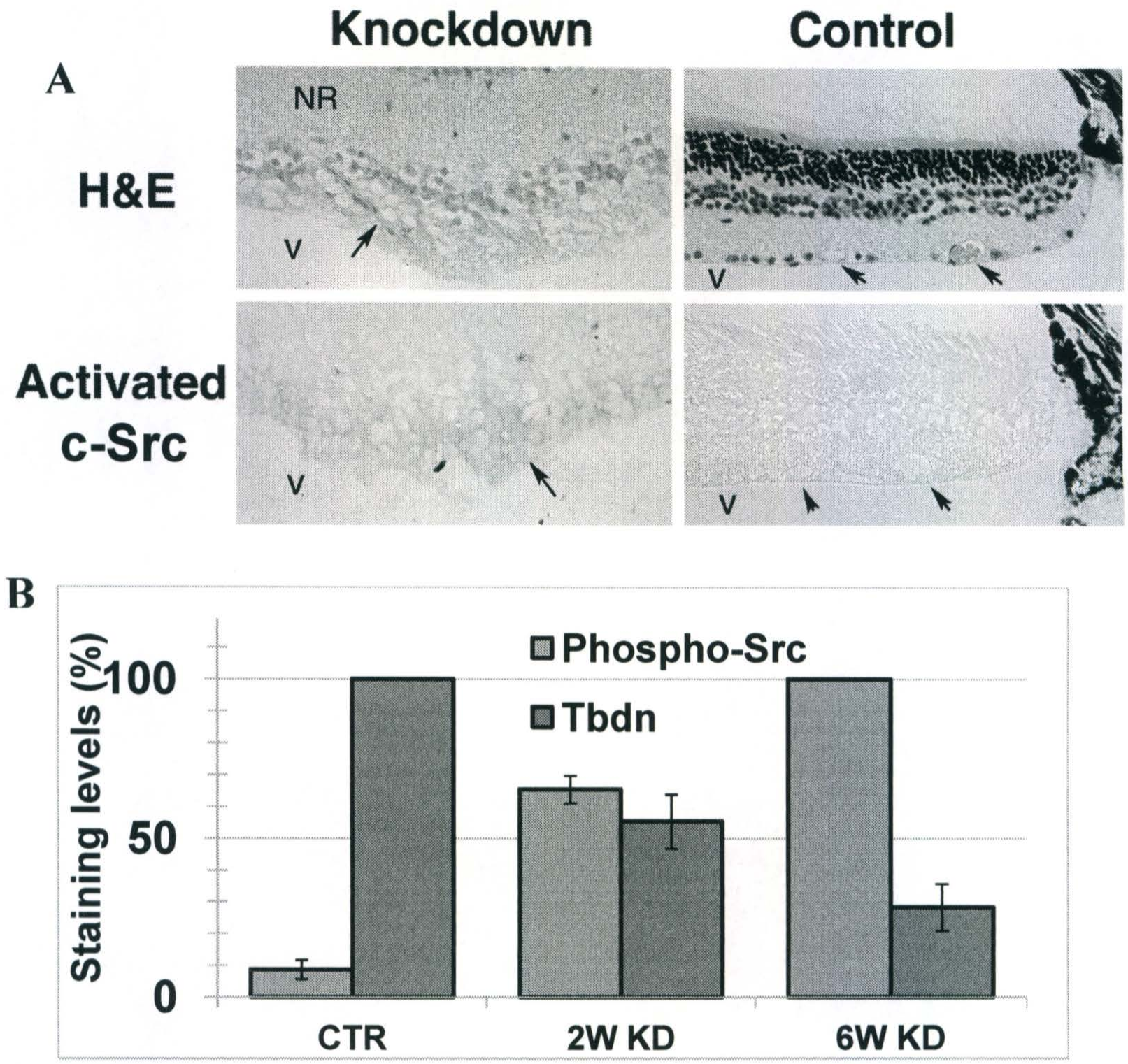


Figure 10: Expression of activated phospho-Src in retinal lesions Tbdn knockdown mice compared to control. (A) Top panels, hematoxylin/eosin (H&E) staining shows abnormal blood vessels and retinal thickening observed in Dox-induced Tbdn knockdown mice (left panels, Knockdown) compared to control middle age mice (right panels, Control). Lower panels, Immunohistochemical analysis of phospho-Tyr416 Src (Activated c-Src) in retinal lesions of Dox-induced Tbdn knockdown versus age-matched middle age mice revealing intense staining in knockdown mice (red stain as shown by arrow). Retinal section stained with no primary antibody showed no staining (not shown). All images are oriented with the vitreous cavity of the eye at the bottom of the panels. 400X. Representative experiment is shown. (B) Quantitation of expression of activated phospho-Tyr416 Src (Activated Src) in retinal blood vessels of lesions Dox-induced Tbdn knockdown (2W KD, 6W KD) compared to normal retinal blood vessels of age-matched control (CTR). Data shown in B is expressed as mean +/- S.E.M. of at least 3 duplicate experiments in each group. (n=3)

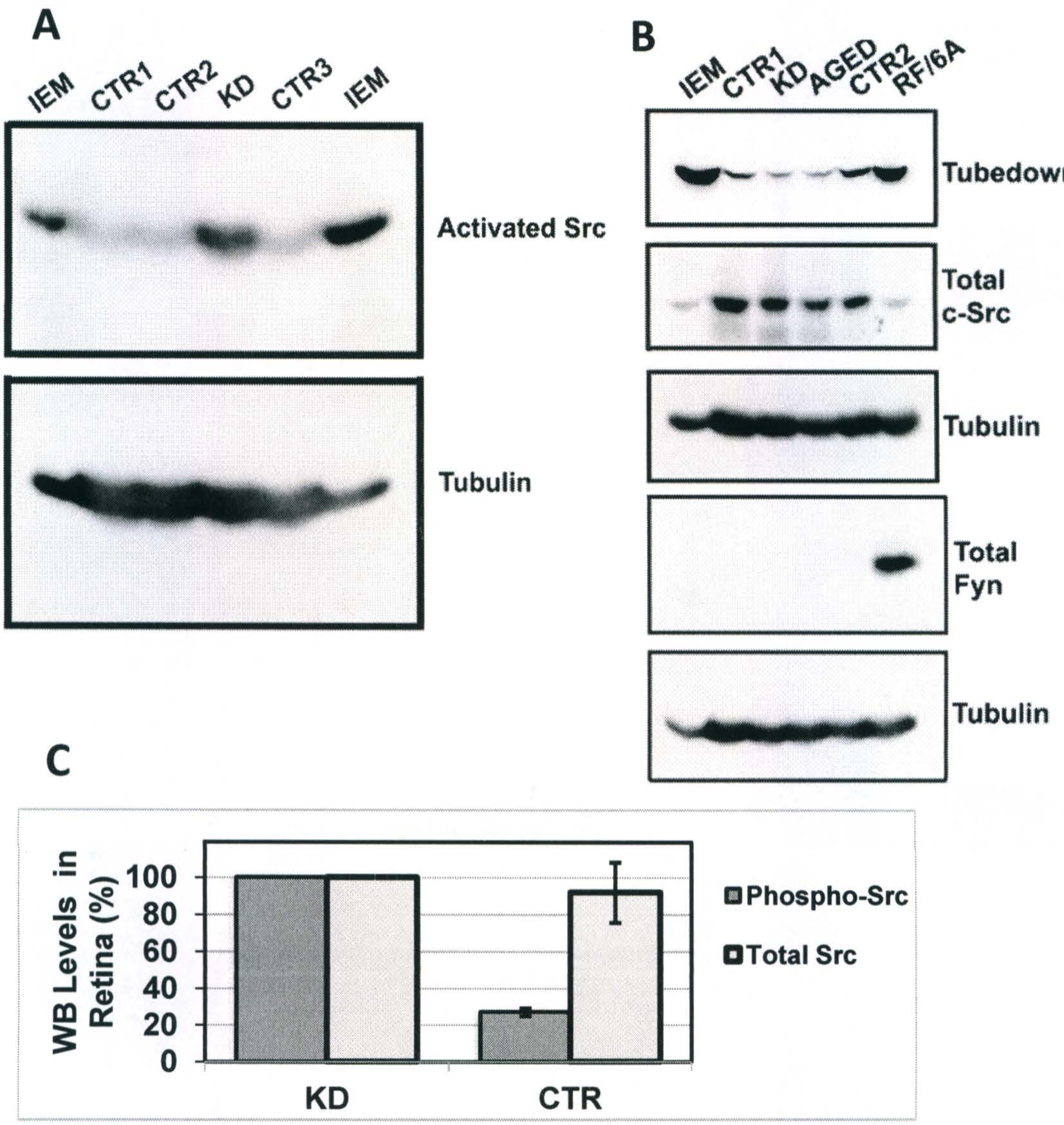


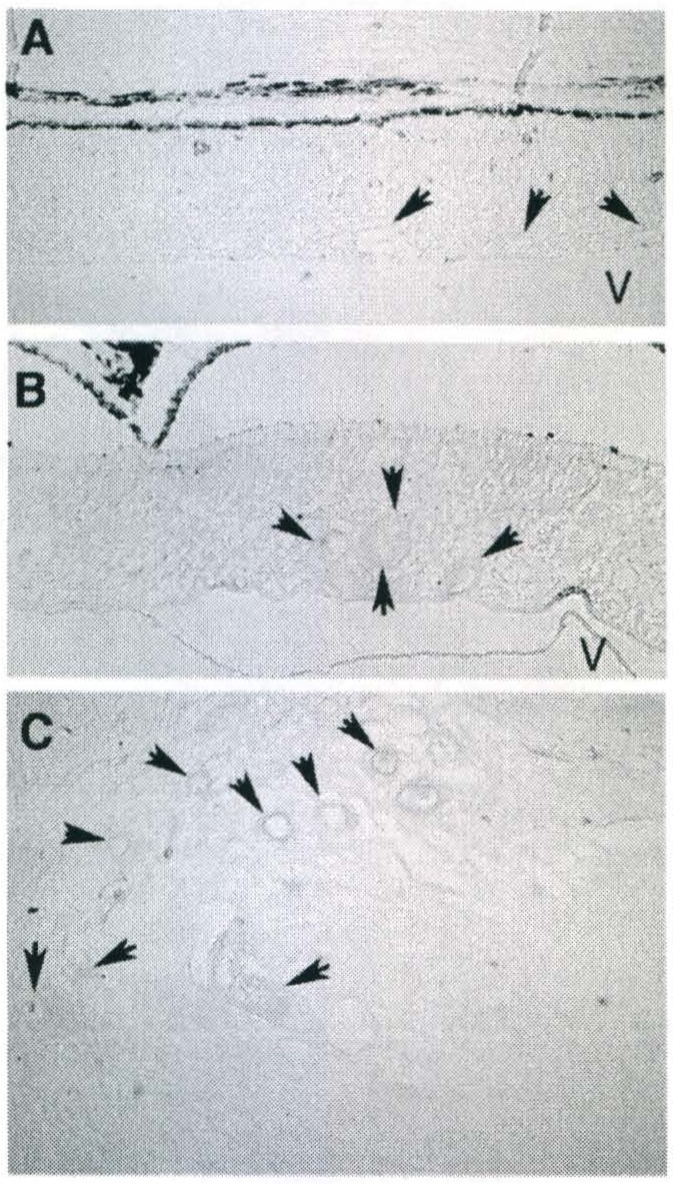
Figure 11: Increased phospho-Tyr 416 Src expression in retinal lesions of Tbdn knockdown mouse model. (A, B) Western Blots of cell lysate from IEM embryonic endothelial cells, RF/6A retinal endothelial cells as well as retinal lysates from controls (CTR1, CTR2, CTR3), Tbdn knockdown (KD) and aged mice (AGED) were performed using the antibodies indicated at right. Tubulin was used for loading control and sample integrity. (C) Quantitations were performed on WB analyses of phospho-Tyr416 Src and total c-Src for which representatives are shown in two previous panels. Levels of activated Src are expressed as percent of the maximal levels observed in Dox-induced Tbdn knockdown animals. Means +/- S.E.M. are indicated.Representative experiments are shown. (n=3)

instead of 3 bands as previously observed in RF/6A cells (Fig. 6A, 9A) indicating that Lyn is not detectable by Western Blot in mouse retinal tissues *in vivo*. We next asked if the increase in the 60 k phospho-Tyr416 c-Src protein band resulted solely from the increase in the levels of phospho-Tyr416 c-Src or included activated phospho-Fyn as well. Western Blot analysis using an antibody for Fyn revealed no detectable expression of Fyn in mouse retinal tissues as compared to RF/6A cells (Fig. 11B).

Immunoprecipitation of c-Src followed by Western Blot with phospho-Tyr416 c-Src antibody confirmed that the 60 k band detected in the mouse retina extract corresponded to c-Src (data not shown).

Neovascular retinopathy specimens exhibit increased levels of phospho-Tyr416 c-Src

Since the retinal lesions of endothelial Tbdn knockdown mice display increases in retinal angiogenesis, hyperpermeability to Albumin and thickening of the retinal tissues, all of which are features observed in PDR (Wall et al., 2004; Paradis et al., 2008), we next investigated if the levels of expression of activated phosphos-Tyr416 c-Src are altered in human eye specimens with PDR. Immunohistological analyses revealed increased levels in the blood vessels of fibrovascular lesions of human neovascular retinopathy specimens (Fig. 12 B,C) compared to a normal aged human specimen (Fig. 12A). Further quantitative analysis showed that neovascular retinopathy specimens had a significant increase (8 fold) in active Src family kinases in blood vessels of retinal lesions compared to age-matched normal specimens ($p < 0.0001$).



D

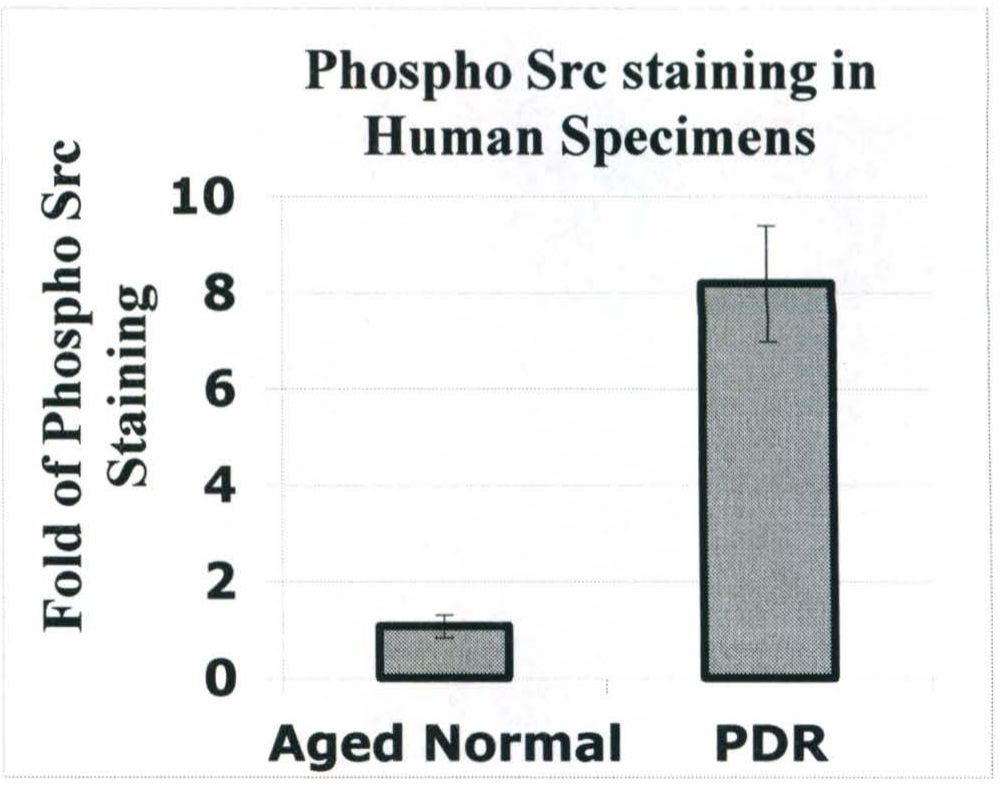
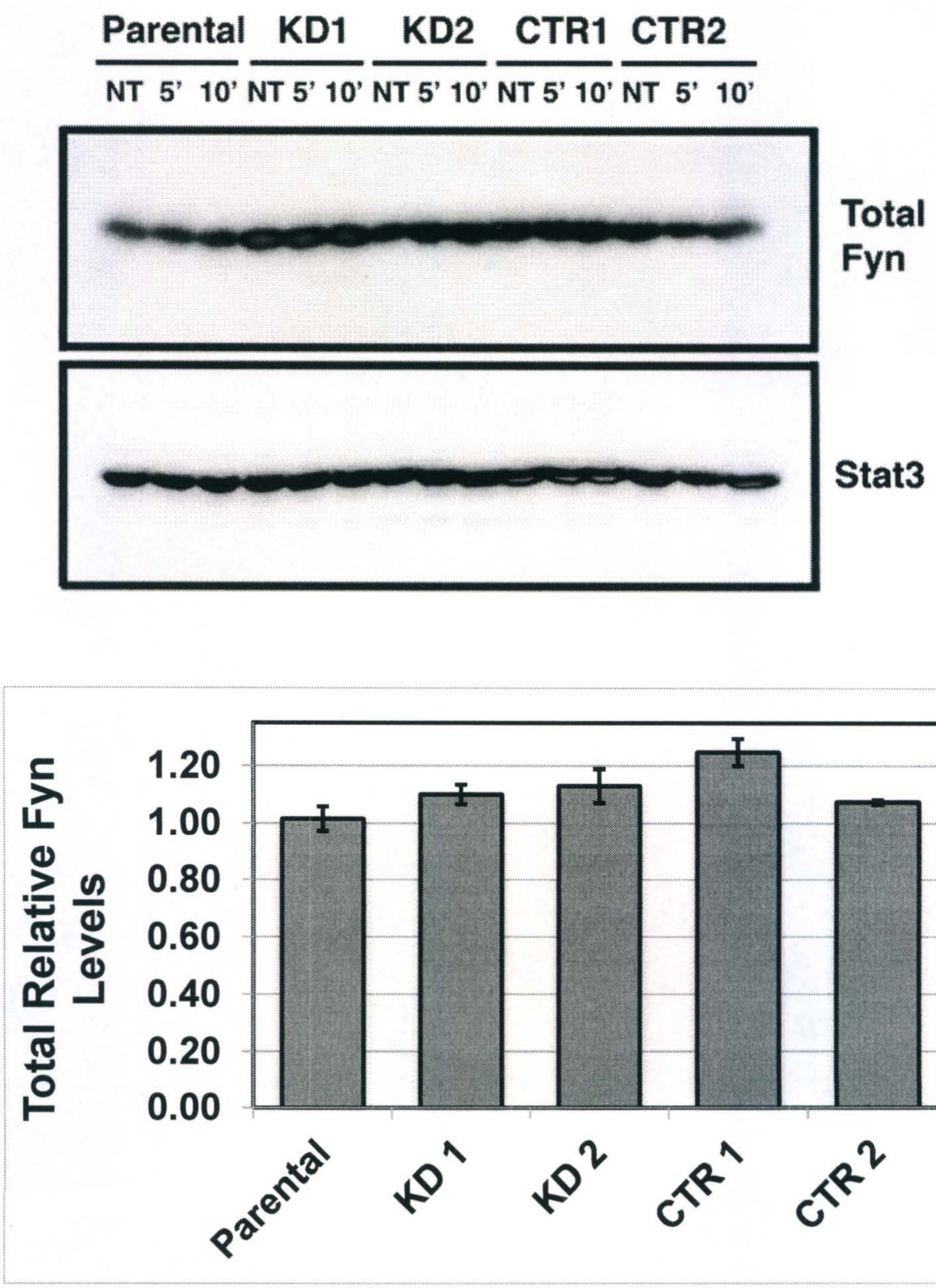


Figure 12: Immunohistochemical analysis of phospho-Tyr416 Src in retinal lesions in neovascular retinopathy in human. (A) shows stain for phospho-Tyr416 Src (Activated Src) revealing no staining in blood vessels (arrowed) of a normal aged human specimen (Aged Normal). (B) and (C) both represent human PDR specimens. (C) has more severe pathology than (B) and both show staining for phospho-Tyr416 Src revealing intense staining in blood vessels of fibrovascular lesion areas (red stain as shown by arrows) of PDR. Images are oriented with the vitreous cavity of the eye at the bottom of the panels although the fibrovascular lesional area in the neovascular retinopathy image (panel C) takes up the whole panel. 200X. Representative experiments shown. (D) Levels of activated Src family kinase in retinal blood vessels are expressed as fold of staining over a reference normal aged specimen. Values for age-matched normal are significantly different than the neovascular values ($p<0.0001$). Data shown in D is expressed as mean +/- S.E.M. of 3 duplicate experiments in PDR group and 6 duplicates in the Aged Normal group.



Supplementary figure: RF/6A parental endothelial cells, Tbdn knockdown RF/6A cell clones and control cell clones were grown in reduced serum conditions for 48hrs followed by no serum for 2 hours. Next cells were either non-treated (NT) or stimulated with serum Albumin for 5 (5') and 10 (10') minutes. Samples were analyzed by WB for total Fyn levels in parental retinal endothelial cells (Parental), Tbdn knockdown (KD 1, KD 2) and control clones (CTR 1, CTR 2). STAT3 WB was performed for loading control and sample integrity. (C) Quantitative analysis showed no significant changes in the levels of total Fyn over Stat3 among the different clones. (n=3)

2.6 DISCUSSION

Endothelial cell permeability, which regulates tissue fluid and the transport of essential nutrients across the vessel wall, is important for maintenance of tissue homeostasis (Minshall et al., 2002; Malik & Mehta, 2006). Transport of plasma proteins and solutes across the endothelium occurs via two different routes: transcellular, via caveolae-mediated vesicular transport and paracellular, through interendothelial junctions (Komarova and Malik, 2010). While both pathways contribute to the transport of molecules across the membrane, Albumin transport from the luminal to abluminal side is mediated strictly via the transellular pathway (Hu and Minshall, 2009). Endothelial cell hyperpermeability is closely associated with angiogenesis and both are hallmarks of neovascular retinopathy. Increased retinal vascular permeability is one of the early pathophysiological mechanisms underlying retinal neovascular diseases such as PDR (Leto et al., 2001; Kumar et al., 2010). Therefore, it is imperative to define in detail the mechanisms mediating vascular permeability and discover how these mechanisms are regulated. As previous studies in our lab have shown that Tbdn is an important regulator of endothelial permeability in the retina (Paradis et al., 2008; Paradis et al., 2002), in this study, we further investigated the interaction of Tbdn with other key components of endothelial permeability signalling pathway regulation.

Our present results provide new evidence that Tbdn regulation of retinal endothelial function is dependent on the regulation of c-Src activation and Cortactin phosphorylation. Members of the Src family of tyrosine kinases [SFK (c-Src, Blk, Fgr, Fyn, Hck, Lck, Lyn, Yes and Yrk)] are involved in a wide variety of cellular processes

(Kim et al., 2009). Aberrant activation of c-Src leads to pathologies including cancer and leaky blood vessels (Kim et al., 2009). SFKs play an important role in mediating the phosphorylation of Cortactin and Dynamin and stimulating receptor-mediated endocytosis (Cao et al., 2010). When Tbdn is knocked down in retinal endothelial cells, we observed an increase in the levels of phospho-Tyr416 c-Src and phospho-Tyr421 Cortactin (Fig 6, 7, 8). Upon stimulation with Albumin for 5 minutes, the Tbdn knockdown cell clones exhibited an even greater and more significant increase in the levels of activated c-Src compared to the parental and control clones. It is known that once activated, c-Src interacts with Csk binding protein (Cbp) through its SH2 domain (Ingley, 2008). Adaptor protein Cbp then recruits Csk (C-terminal Src kinase) to the plasma membrane to mediate phosphorylation of c-Src at Y527 rendering c-Src inactive (Ingley, 2008; Hu et al., 2008). This negative feedback loop might underline the return of c-Src activation levels to baseline at 10 minutes post Albumin treatment in RF/6A cells (Fig 6).

Moreover, we showed that Tbdn regulation of endothelial permeability requires c-Src activation (Fig. 9). When inhibiting c-Src activation with SKI-606, we observed a decrease in transit of FITC-Albumin across the RF/6A Tbdn knockdown cells monolayer at 1.5 μM and 3.6 μM (Fig. 9C). However, as the concentration of inhibitor was increased, a significant increase of FITC-Albumin transit which was also independent of time was observed, suggesting that the cell monolayer might have become leaky as a result of the drug treatment. This may be due to c-Src involvement in actin assembly and focal adhesion formation through its phosphorylation of focal adhesion kinase (FAK) at

Y861 (Kim et al., 2009). In fact, this is in line with another observation by Elliott and co-workers who found that SKI-606 inhibition of c-Src activation lead to a decrease in adhesion of renal epithelial cells to extracellular matrix (Elliott et al., 2011).

Activated c-Src phosphorylates other components of the pathway such as Cortactin, Caveolin-1 and Dynamin-2 to facilitate caveolar scission and transcellular vesicular transport of Albumin (Kim et al., 2009). Cortactin phosphorylation by c-Src has also been shown to be involved in a range of cellular processes including transmigration of leukocytes (Yang et al., 2006) and endocytosis of Transferrin (Cao et al., 2010). Phosphorylation of Cortactin by c-Src enhances Cortactin binding affinity to Dynamin by up to 5 fold (Zhu et al., 2007) and this binding is essential for vesicle formation at the plasma membrane (Cao et al., 2003). Here we showed that phosphorylation of Cortactin is linked to c-Src activation in retinal endothelial cells (Fig. 9B). By inhibiting c-Src activation using inhibitor SKI-606, levels of phospho-Tyr421 Cortactin were found to be downregulated (Fig. 9B). More importantly, we showed that Tbdn knockdown leads to an increase in the levels of activated c-Src and Tyr421-phosphorylated Cortactin (Fig 6-8).

There are two scenarios that may explain the increase in activated c-Src and phosphorylated Cortactin as a result of Tbdn knockdown. While it is unlikely that Tbdn itself can acetylate substrates directly, Tbdn associates with Ard1, which exhibits acetytransferase activity (Gendron et al., 2000; Arnesen et al., 2005). The functional consequences of acetylation are diverse and include modification of protein-protein interactions and regulation of phosphorylation of proteins in both positive and negative manners. For example, acetylation of p53 by p300 and PCAF triggers p53 recruitment to DNA allowing it to activate or repress specific genes (Sakaguchi et al., 1998). On the

other hand, acetylation of FOXO1 promotes its phosphorylation at Ser-253 through the PI3K-PKB signaling pathway (Matsuzaki et al., 2005). We speculate that Tbdn/Ard1 complex might bind c-Src directly and promote its binding to Csk which phosphorylates c-Src at Y527 and inactivates it. Another possibility is that the Tbdn/Ard1 complex binds c-Src and further promotes its dephosphorylation at Tyr416 to decrease its activity. As a result, Tbdn knockdown would lead to an increase in level of activated c-Src and subsequent increased phosphorylation of downstream proteins such as Cortactin. Cortactin itself can be acetylated by PCAF (Ammer and Weed, 2008). The fact that there are no significant differences in Cortactin acetylation in PCAF -/- compared to PCAF +/+ fibroblast cells may indicate that besides PCAF, additional acetyltransferase(s) may acetylate Cortactin (Zhang et al., 2007). We previously found that Tbdn interacts with Cortactin *in vivo* (Paradis et al., 2008). Though the effect of acetylation still remains unclear, it is tempting to speculate that Tbdn might bind and sequester Cortactin from phosphorylation of c-Src. Tbdn knockdown thus leads to more Cortactin being phosphorylated by activated c-Src. Phosphorylated Tyr421 on Cortactin has been shown to form a stable interaction with the SH2 domain of c-Src preventing c-Src from being inactivated resulting in an increase in c-Src activation (Weed and Parsons, 2001).

In addition, our data provide evidence that loss of Tbdn expression leading to retinal hyperpermeability and retinopathy in mice *in vivo* involves c-Src activation. It is possible that increases in activation of c-Src in the retinal endothelium and surrounding tissues may be a contributing factor that predisposes healthy retina to the development of neovascularization and retinal pathology. Proliferative diabetic retinopathy is

characterized by neovascularization originating from the retina and/or optic disk in patients with diabetes mellitus (Gunduz and Bakri, 2007). Previously, Tbdn protein expression was found to be downregulated in PDR specimens compared to control (Gendron et al., 2001). Furthermore, Tbdn suppression in mouse endothelium resulted in similar pathological characteristics to those observed in human PDR (Wall et al. 2004). In this study, Western Blot and immunohistochemistry analyses of the conditional endothelial Tbdn knockdown mouse model confirmed the upregulation in activated c-Src which reaches levels up to 10 fold above control in blood vessels of retinal lesions (Fig 10, 11). Interestingly, our results herein also indicate a significant increase in c-Src activation in PDR specimens (Fig. 12 B,C). These observations are consistent with our previous *in vitro* and *in vivo* studies. High level of c-Src activation has been widely linked to vascular hyperpermeability (Mehta and Malik, 2006). Increased endothelial permeability usually causes abnormal extravasation of blood components such as Albumin and accumulation of fluid in the extravascular space (Hu et al., 2008). Furthermore, all of these lead to inflammation and recruitment of stimuli such as cytokines and growth factors that bind their cognate receptors in the tissue (Kim et al., 2009; Kumar et al., 2009). Such receptor binding results in activation of Src family kinases in the tissue (Kim et al., 2009). This may explain why in addition to elevated staining levels of phospho-Tyr416 c-Src in the blood vessels of retinal lesions, there is also light staining for activated c-Src in the surrounding retinal tissues supporting a hypothesis that leakiness of the vessels induced by c-Src activation would lead to collateral tissue damage and c-Src activation in bystander cells.

In summary, our present studies provide evidence that Tbdn regulation of retinal endothelial permeability is dependent on c-Src activation and Cortactin phosphorylation. Whether this regulation is dependent on c-Src activation *in vivo* is currently under investigation by our laboratory.

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3. SUMMARY

Worldwide, there are 194 million individuals who suffer from diabetes. This number is expected to more than double by 2030 due to the increasing frequency of obesity, life expectancy, and improved detection of the disease (Wild et al., 2004; Al-Rubeaan et al., 2010; Antonetti et al., 2012). Approximately 70% of those who have diabetes for 10 years or more will develop some form of diabetic retinopathy, such as proliferative diabetic retinopathy (PDR). PDR is caused by neovascularization and hyperpermeability in the vasculature of the retina. Previous studies in the laboratory of Drs Paradis and Gendron have implicated Tbdn as an important regulator of endothelial permeability in the retina (Paradis et al., 2008). Further studies elucidating the Tbdn signaling pathway can provide us with insights into developing better molecular treatments targeting this cause of blindness.

Previous studies have indicated that Tbdn is suppressed in neovascular areas of retinal endothelium in patients with diabetic retinopathy (Gendron et al., 2001). Additionally in a recent study, Tbdn has been implicated as a regulator of retinal endothelial permeability (Paradis et al., 2008). To examine the mechanisms by which Tbdn performs its regulatory role, we investigated the effect of Tbdn knockdown on components of the permeability pathway, Cortactin and c-Src.

This study reinforces the hypothesis that Tbdn regulation of retinal endothelial permeability is dependent on c-Src activation and phosphorylation of Cortactin as Tbdn knockdown in both *in vitro* and *in vivo* systems lead to a significant increase in phosphorylation of c-Src at Tyr416 and Cortactin at Tyr421. In addition to these findings,

we also found that human neovascular retinopathy is associated with the activation of Src family of kinases. It is possible that Tbdn loss predisposes the retina to neovascularization and hyperpermeability through activation of a c-Src dependent pathway. While the specific mechanism by which Tbdn governs this pathway is unknown, it is appealing to speculate that Tbdn through its association with Ard1 which has an acetyltransferase activity, binds to c-Src and either prevents its phosphorylation at Tyr416 or increases the rate binding to Csk which inactivates c-Src. Another speculation is that Tbdn might bind and sequester Cortactin from being phophosphorylated by c-Src and from forming a stable complex with c-Src SH2 domain. This complex would prevent c-Src from being dephosphorylated and becoming inactive. In both cases, Tbdn knockdown would result in an increase in c-Src activation and correspondingly, an increase in phosphorylation of Cortactin as observed.

In conclusion, Tbdn displays a tremendous potential of being the central regulator in the development of ocular neovascular diseases. Nevertheless, Tbdn's involvement in the signaling pathway of retinal endothelial permeability involving c-Src and Cortactin warrants further investigation. While we have demonstrated here that Tbdn's role in retinal permeability is mediated by regulating c-Src activation, we must further investigate if loss of Tbdn expression leading to retinal hyperpermeability and retinopathy in mice *in vivo* requires c-Src activity through the use of c-Src knockout mice. Early data using c-Src inhibitor showed that c-Src activation is required for transendothelial permeability of Albumin. Further study into c-Src regulated hypermeability in the Tbdn/c-Src regulation of retinal permeability is still required. However, preliminary data

on the Tbdn/c-Src regulation of retinal permeability provides evidence for a potential new target to treat and/or prevent this detrimental disease.

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