A Study of the Protein Phosphatase 1 (PP1) Regulatory Subunit, TIMAP, in Human Glomerular Endothelial Cells

by

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Abstract

TIMAP is a prenylated endothelial-predominant regulatory subunit for the serine/threonine (Ser,S/Thr,T) protein phosphatase 1 catalytic subunit (PP1c). TIMAP was first discovered in glomerular endothelial cells. It is expressed in the developing blood vessels of kidney, and its transcription in cultured glomerular endothelial cells is downregulated by the transforming growth factor beta 1 (TGF1). In turn, TGF1 is a critical regulator of endothelial cell development and differentiation. According to its amino acid sequence, TIMAP belongs to the family of myosin phosphatase regulatory subunits (MYPT), which regulate myosin II dynamics required for various cellular processes including cell proliferation, adhesion, migration and endothelial blood barrier function. In this thesis, we explored the functional role of TIMAP in the regulation in glomerular endothelial cells. We demonstrated for the first time that TIMAP protein depletion attenuates glomerular endothelial cell proliferation, survival and sprouting angiogenesis. We found that TIMAP protein expression is required to maintain the activating phosphorylation of the Ser/Thr kinase Akt, a major positive regulator of cell survival, proliferation, and angiogenesis. Also, we discovered that TIMAP protein depletion reduces the inhibitory Ser 370 phosphorylation of the phospholipid phosphatase and tumor suppressor PTEN, a negative regulator of Akt activity, cell survival, proliferation and angiogenesis. Since TIMAP belongs to the family of myosin phosphatase targeting subunits, we also explored whether it regulates phosphorylation of the myosin II regulatory light chain (MLC2) in vitro and in vivo. We show that the

TIMAP/PP1c holoenzyme can effectively dephosphorylate pMLC2 *in vitro*, but that this function is not observed in living cells, given that TIMAP overexpression strongly augmented MLC2 phosphorylation instead. The TIMAP-induced MLC2 phosphorylation in the glomerular endothelial cells was indirect, and required the TIMAP-PP1c association. The TIMAP-mediated MLC2 hyperphosphorylation was due to a markedly reduced rate of MLC2 dephosphorylation. Since MYPT1 is the predominant MLC2 phosphatase in endothelial cells, we explored whether TIMAP/PP1c inhibits the MYPT1/PP1c phosphatase. TIMAP did not suppress MYPT1 or PP1c expression, it did not bind MYPT1 directly, nor did it alter RhoA- or CP17-dependent MYPT1 regulation. The mechanism whereby TIMAP overexpression reduces MLC2 dephosphorylation therefore remains obscure. Finally, we observed that MLC2 and pMLC2 can bind TIMAP directly, and that this interaction blocks the activating kinase-mediated phosphorylation of MLC2 in vitro. We conclude that TIMAP/PP1c can regulate MLC2 phosphorylation in glomerular endothelial cells, but that this effect is not due to direct myosin phosphatase activity in the living cell.

Dedication

For my first and best friend who is forever missed,
Irina Kabilinski

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List of Abbreviations

Ad	Adenovirus
ADP	Adenosine Diphosphate
ALK	Activin receptor Like Kinase
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
BAD	BCL-2 Agonist of Death
BMP	Bone Morphogenetic Proteins
BrdU	5-bromo-2'-deoxyuridine
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
Cdk2	Cyclin- dependent Kinase 2
cGMP	Cyclic Guanosine MonoPhosphatae
CK2	Casein kinase 2
CKD	Chronic Kidney Disease
CTD	C-Terminal Domain
DiFMUP	6,8-difluoro-4-methylumbelliferyl phosphate
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
ECIS	Electrical Cell-substrate Impedance Sensing
EDTA	Ethylene Diamine Tetraacetic Acid

EdU	5-ethynyl-2'-deoxyuridine
EGF	Epithelial Growth Factor
EGM	Endothelial Cell Growth Medium
EGTA	Ethylene Glycol Tetraacetic Acid
ELC	Essential Light Chain
EMT	Epithelial Mesenchymal Transition
eNOS	Endothelial Nitric Oxide Synthase
ERK	Extracellular signal Regulated Kinase
ERM	Ezrin/Radixin/Moesin
FBS	Fetal Bovine Serum
FCP	TFIIF -associating CTD phosphatase
FGF	Fibroblast Growth Factor
FOXO	Forkhead box O
GSK3	Glycogen Synthase Kinase 3 beta
GST	Glutathione S- Transferase
GLTSCR2	Glioma Tumor Suppressor Candidate Region 2
HCL	Hydrogen Chloride
HFF1	Human Foreskin Fibroblasts
HRP	Horseradish Peroxidase
HPAEC	Human Pulmonary Artery Endothelial Cells
HUVEC	Human Umbilical Vein Endothelial Cells
IGF	Insulin Growth Factor

IF	Immunofluorescence
ILK	Integrin- Linked Kinase
IP	Immunoprecipitation
I-1	Inhibitor 1
I-2	Inhibitor 2
LAMR1	Laminin Receptor 1
MAP2	Microtubule- Associated Protein 2
MAPK	Mitogen Activated Protein Kinase
MBS 85	Myosin- Binding Subunit 85 kDa
MES	2-N-Morpholino ethanesulfonic acid
miRNA	Micro RNA
MLC2	Myosin Light Chain 2
MLCK	Myosin Light Chain Kinase
MOI	Multiplicity of Infection
MOPS	4-Morpholinepropanesulfonic acid
MP	Myosin Phosphatase
MRCKa	Myotonic dystrophy kinase-Related Cdc42 binding Kinase
mTORC2	Mammalian Target of Rapamycin Complex 2
MYPT	Myosin Phosphatase Targeting
NMR	Nuclear Magnetic Resonance
NO	Nitric Oxide
NP-40	Nonidet P 40

PAK	P21-Activated Kinase
PBM	PI(4,5)P2-binding Module
PD	Phosphatase Domain
PDK1	Phosphoinositide Dependent Kinase 1
PH	Pleckstrin Homology
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol 4,5 bisphosphate
PIP3	Phosphatidylinositol 3,4,5 triphosphate
PKA	Protein Kinase A
PKB	Protein Kinase B
PKC	Protein Kinase C
PKG	cGMP- dependent protein kinase
PKGIa	cGMP-dependent Protein Kinase type I alpha
pMLC2	Phosphorylated Myosin Light Chain 2
PP1c	Protein Phosphatase 1 Catalytic subunit
PTEN	Phosphatase and Tensin Homolog
PVDF	Polyvinylidene difluoride
RACK1	Receptor for Activated Protein Kinase C
RLC	Regulatory Light Chain
ROCK	Rho- associated protein Kinase
R-smad	Regulated smad
RTK	Receptor Tyrosine Kinase

SCP	Small CTD phosphatase
SDS-PAGE	Sodium Dodecyl Sulfate- Poly Acrylamide Gel Electrophoresis
Ser, S	Serine
shRNA	Short hairpin RNA
siRNA	Short Interfering RNA
TBST	Tris-Buffered Saline and Tween 20
TCF β	Catenin Transcription Factor beta
TFIIF	Transcription Factor II Family
TGFβ1	Transforming Growth Factor beta 1
TGFβRI	TGFβ receptor type 1
TGFβRII	TGFβ receptor type 2
Thr, T	Threonine
TIMAP	TGFβ-Inhibited Membrane Associated Protein
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
ZIPK	Zipper- Interacting Protein Kinase

CHAPTER 1

General Introduction and Literature Review

1.1 General Introduction

Glomerular capillaries are highly specialized filters that produce a nearly protein-free ultra-filtrate of plasma at a prodigious rate. Glomerular endothelial cells that line the glomerular capillaries are fenestrated and very permeable to water and small solutes. Glomerular endothelial cells respond to and elaborate signals that, in conjunction with the other intrinsic glomerular cells, are responsible for the formation of the intricate three-dimensional glomerular filtration surface that is both highly permeable to water, and nearly impermeable to negatively charged and large globular plasma proteins.

Glomerular capillaries develop through in situ vasculogenesis followed by progressive lengthening, splitting and expansion of capillary loops. Vasculogenic angioblasts first invade the capillary cleft of nascent glomeruli as pre-capillary cords devoid of vascular lumens. This process is stimulated by cues including, vascular endothelial growth factor-A (VEGF-A) and angiopoietin 1. Also, neutralizing VEGF antibodies (Kitamoto, Tokunaga et al. 1997) as well as neutralizing TGF□1 antibodies (Liu, Dardik et al. 1999) impede invasion of the glomerular capillary cleft by angioblasts. Capillary lumen formation requires TGF□1-dependent apoptosis of a subset of immature glomerular endothelial cells (Choi and Ballermann 1995, Fierlbeck, Liu et al. 2003)

Our lab previously reported that TGF□1 plays a key role in glomerular capillary formation and morphogenesis, and since there is strong evidence that TGF□1 is crucial for the development of blood vessels, we sought to identify novel targets downstream of the TGF□ signaling cascade that might be important for endothelial cells function. Subsequently, we identified a novel protein, TIMAP (TGF□1□ inhibited membrane-associated protein), which is predominantly expressed in endothelial cells and strongly downregulated by TGF□1 (Cao, Mattagajasingh et al. 2002) . TIMAP is a regulatory/targeting subunit of PP1c in the MYPT family.

Based on the above, this thesis is aimed at characterizing the role of TIMAP in glomerular endothelial cells from two perspectives, its role in capillary formation and its role as a myosin phosphatase targeting subunit. Hence, I will first review the literature of reversible protein phosphorylation, focusing on the serine/threonine phosphatase, PP1c. Then I will review the significance of reversible protein phosphorylation in the regulation of actin cytoskeleton, in particular the role of MYPT family in myosin II regulation. Lastly, I will review the significance of reversible protein phosphorylation in capillary formation and morphogenesis, and highlight the key pathways that play important role during these processes.

1.2 Reversible Protein Phosphorylation

Protein phosphorylation is a key event in various signaling pathways and regulates diverse physiological processes, including cell proliferation, survival, migration

and differentiation. Thus, disruptive regulation of phosphorylation is associated with numerous diseases such as cancer, diabetes, and cardiovascular diseases. Indeed, the fact that ~70% of the eukaryotic cellular proteins are regulated by phosphorylation (Peti, Nairn et al. 2013) indicates the immense importance of protein phosphorylation in the biological activities of cells. The principle of reversible phosphorylation was conceived by Edmond Fischer and Edwin Krebs when they demonstrated the conversion of phosphorylase *b* to phosphorylase *a* in an *in vitro* reaction that required adenosine triphosphate (ATP) and a converting enzyme (phosphorylase kinase) (Fischer and Krebs 1955). Interestingly, phosphorylase phosphatase, the enzyme that converts phosphorylase *a* back to *b* was discovered a decade earlier (Shi 2009).

Protein phosphorylation is catalyzed by kinases, which transfer phosphate groups from ATP to the protein substrates resulting in their phosphorylation, and by phosphatases that catalyze the removal of phosphate group restoring the un-phosphorylated state of their phospho-protein substrates (Figure 1.1). Protein kinases phosphorylate unique sequences of amino acid residues (consensus sequences) in their various substrates (Aitken 2003). In contrast, protein phosphatases de-phosphorylate phospho-amino acid residues independently of consensus sequences. Protein phosphorylation occurs on three hydroxyl-containing amino acid residues, Tyr, which accounts for only 1.8% of all phosphorylation reactions, and Ser/Thr, which accounts for the majority (~ 98.2%) of all phosphorylation reactions (Olsen, Blagoev et al. 2006). The human genome encodes 90 protein Tyr kinases and 428 protein Ser/Thr kinases (Shi 2009). On the other hand, there are ~100 protein Tyr phosphatases and less than 40 protein Ser/Thr phosphatases (Bollen, Peti et al. 2010, Choy, Page et al. 2012). Whereas

the number of protein Tyr kinases almost matches that for protein Tyr phosphatases, the number of protein Ser/Thr phosphatases is far lower than that for the Ser/Thr kinases. As will be explained below, the exquisite specificity of Ser/Thr phosphatases is not achieved by the number of phosphatases-encoding genes, like in Tyr phosphatases, but by interactions between common catalytic subunits and a large number of diverse regulatory/ targeting subunits.

1.3 Family of Serine/Threonine Protein Phosphatases

The precise regulation of the complex phosphorylation networks is managed by more than 400 Ser/Thr kinases, encoded in the human genome (Manning, Whyte et al. 2002, Peti, Nairn et al. 2012). To achieve precise reversible control, there exists a family of Ser/Thr phosphatases of similar complexity to that of the kinases. However, unlike kinases, the complexity of phosphatases does not lie in the number of genes encoding the phosphatase catalytic subunits, but in the number of regulatory subunits that form multimeric enzymes (holoenzymes) with the catalytic subunits and determine their subcellular and substrate specificity.

There are three families of Ser/Thr phosphatases: phosphoprotein phosphatases, metal-dependent protein phosphatases and aspartate-based phosphatases (Shi 2009). The family of phosphoprotein phosphatases comprises PP1, PP2A, PP2B (calcineurin) and PP4-PP7 (Ceulemans and Bollen 2004). Members of this family interact with diverse regulatory subunits that regulate their substrate specificity. The family of metal-dependent protein phosphatases includes manganese (Mn^{2+}) and magnesium (Mg^{2+})-

dependent protein phosphatases, like PP2C. Unlike phosphoprotein phosphatases, metal-dependent protein phosphatases are not dependent on regulatory subunits, but their substrate specificity is inherent in their amino acid motifs (Shi 2009). Aspartate-based protein phosphatases are represented by the family of transcription factor II F (TFIIF)-associating C-terminal domain (CTD) phosphatase (FCP) and small CTD phosphatase (SCP), and the only known substrate of it is the C-terminal domain of RNA polymerase II (Shi 2009). Lastly, dual- specificity phosphatases can dephosphorylate phospho-Ser/Thr residues as well as phospho-Tyr residues (Patterson, Brummer et al. 2009).

Since the focus of this thesis is the PP1c regulatory subunit, TIMAP, PP1c will be further discussed in the next section.

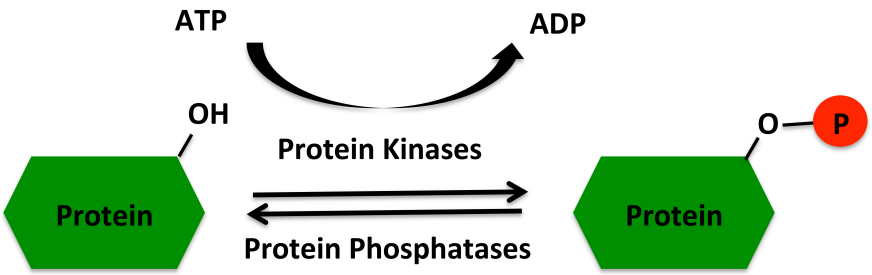


Figure 1.1: Reversible Protein Phosphorylation. Protein kinases phosphorylate proteins by transferring phosphate group (P) from ATP to the hydroxyl group (OH)-containing amino acid residues in protein substrates. Protein phosphatases remove P group from phosphorylated protein substrates.

1.4 Protein Phosphatase 1 Catalytic Subunit (PP1c)

PP1c is a multifunctional Ser/Thr phosphatase that regulates a great variety of cellular processes, including cell proliferation, apoptosis, protein synthesis, actin reorganization, vesicle trafficking, cell polarity, muscle contraction and glycogen metabolism (Cohen 2002, Ceulemans and Bollen 2004). PP1c is highly conserved in eukaryotic evolution with a 72% protein identity between the PP1c isoform of the early eukaryote *Giardia lamblia* and the mammalian PP1c isoforms (Ceulemans, Stalmans et al. 2002). Eukaryotic genomes encode one (*Saccharomyces cerevisiae*) to eight PP1c genes (*Arabidopsis thaliana*) (Ceulemans, Stalmans et al. 2002, Cohen 2002). The catalytic subunit of mammalian PP1 exists as several isoforms encoded by the genes α, β/δ and γ, the later generates two splice- variants γ1 and γ2 (Cohen 1988, Dombradi, Axton et al. 1990, Shima, Haneji et al. 1993). PP1c isoforms possess great sequence identity, ~ 85% between β/δ and γ2 and 93% between γ1 and γ2, with the exception of the amino acid sequence at their N- and C- termini (Ceulemans and Bollen 2004, Fardilha, Esteves et al. 2010, Peti, Nairn et al. 2012).

Mammalian PP1c isoforms display distinct tissue and subcellular distributions (Shima, Haneji et al. 1993, Andreassen, Lacroix et al. 1998). However, while PP1c possesses intuitive potential to dephosphorylate any phospho-Ser/Thr residues, subcellular distribution and substrate specificity are not inherent in the PP1 catalytic subunit due to great amino acid sequence homology among the different isoforms, but determined via its interaction with more than 200 regulatory/targeting subunits (Bollen,

Peti et al. 2010, Choy, Page et al. 2012) (Figure 1.2). For example, the MYPT family of PP1c regulatory subunits regulates myosin/actin function required for muscle contraction and cell motility (Hartshorne 1998, Hartshorne, Ito et al. 1998, Grassie, Moffat et al. 2011). PP1c regulatory/targeting subunits share the PP1c binding motif (RVxF), where x is any amino acid residue. Nonetheless, this binding motif serves only as an anchor to initiate the interaction, which is then followed by low affinity interactions at secondary sites (Wakula, Beullens et al. 2003). Even though the regulatory/targeting subunits of PP1c exhibit a common binding site for PP1c, their structures and regulation are extremely variable (Cohen 2002). They can be regulated at the expression level, by phosphorylation and conformational changes, leading to changes in their interaction with PP1c, and thus, PP1c activity (Cohen 2002). For instance, phosphorylation of the myosin phosphatase targeting (MYPT1) subunit by RhoA- dependent kinase (ROCK) on Thr 696 inhibits its phosphatase activity (Feng, Ito et al. 1999), whereas phosphorylation of the MYPT family members TIMAP and MYPT3 by protein kinase A (PKA)/glycogen-synthase kinase (GSK3β) and PKA, respectively, enhances their phosphatase activity (Yong, Tan et al. 2006, Li, Kozlowski et al. 2007).

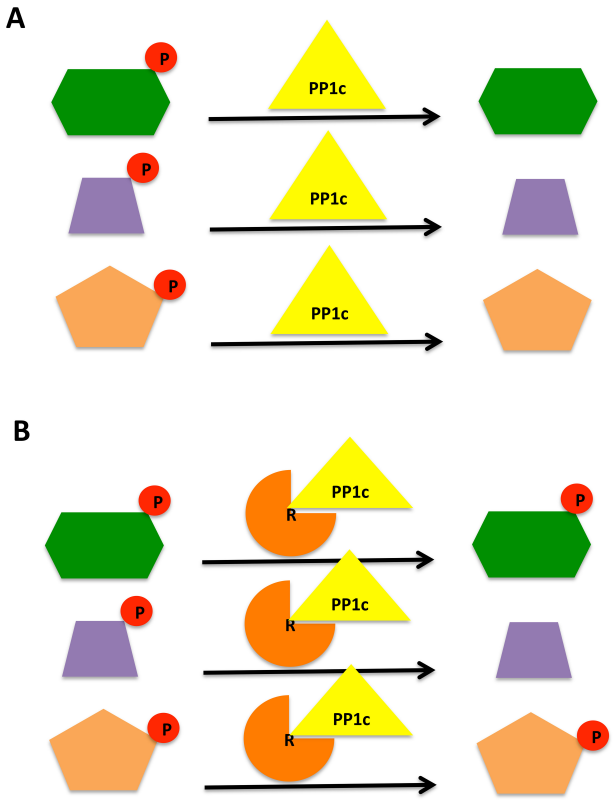


Figure 1.2: Targeting PP1c activity by regulatory subunits. A. Free PP1c exhibits a powerful potential to dephosphorylate any phosphorylated Ser/Thr residues found in phosphorylated protein substrates. **B.** Interaction of PP1c with a regulatory subunit (R) forms a holoenzyme that is only active against a specific phosphorylated protein substrate, but inactive against most phosphorylated proteins.

1.5 Role of Reversible Phosphorylation in Cytoskeleton Regulation

The dynamic reorganization of the cytoskeleton plays a pivotal role in many cellular processes including muscle cell contraction, non-muscle cell motility and cell division. Reversible phosphorylation of various cytoskeletal proteins is a key event that regulates the changes in the cytoskeleton in a spatial and temporal fashion. For example, non-muscle myosin II motor- dependent reorganization of the actin cytoskeleton is regulated by the phosphorylation state of MLC2 (Hartshorne, Ito et al. 2004, Grassie, Moffat et al. 2011). In turn, MLC2 phosphorylation is regulated by the Ca^{2+}- Calmodulin-dependent myosin light chain kinase (MLCK) (Totsukawa, Wu et al. 2004) and a myosin phosphatase (MP) (Hirano, Derkach et al. 2003). In addition, ROCK can enhance MLC2 phosphorylation via inhibition of MP activity (Feng, Ito et al. 1999). Phosphorylation of MLC2 at Ser 19 and Thr 18 increases the extended conformation and ATPase activity of myosin II motor complex (Betapudi 2014) (Figure 1.3), which facilitates the interaction of myosin II with actin filaments, inducing stress fiber formation and actin movement.

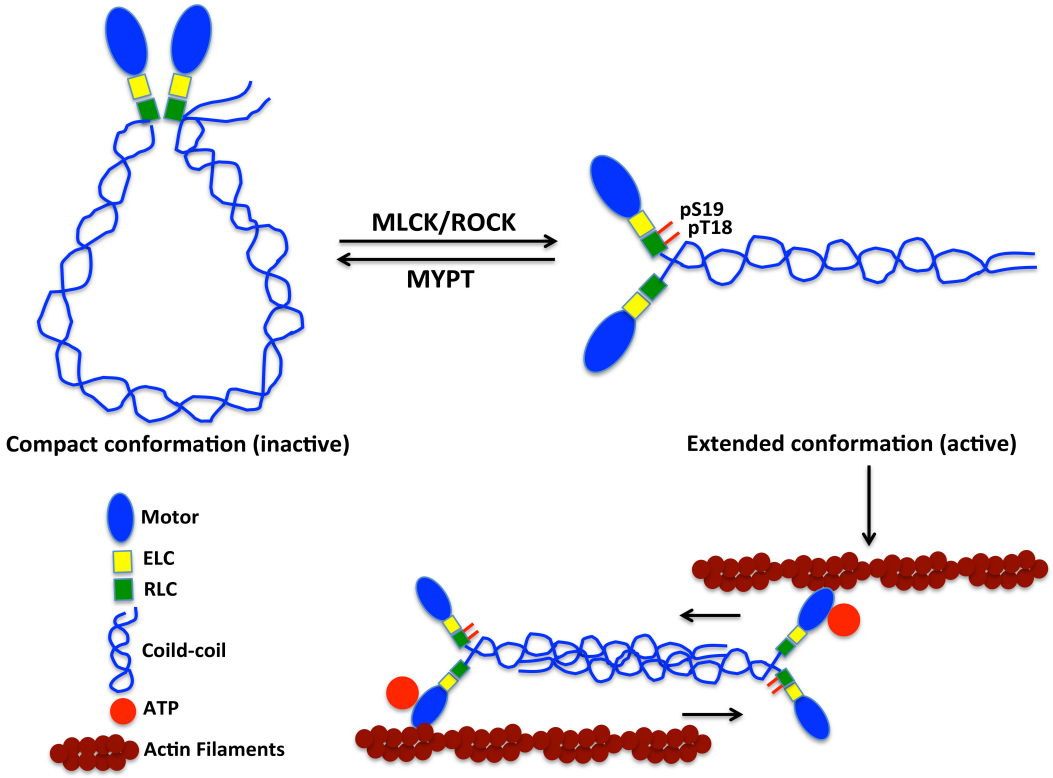


Figure 1.3: Mechanism of activation of non-muscle myosin II motor protein complex. Myosin II complex is composed of double helical coiled-coil domain, two essential light chains (ELC), and two regulatory light chains (RLC), also called MLC2. Phosphorylation of MLC2 (RLC) by MLCK and ROCK or other MLC2 kinases activates myosin II motor protein complex. Activated myosin II protein complex associates with actin filaments and utilizes ATP to generate contractile forces. MYPT dephosphorylates MLC2 and inactivates myosin II. The Figure was modified from Figure 2 and Figure 3 of a review article by (Betapudi 2014) CC-BY copyright licence.

In endothelial cells, nitric oxide (NO), the product of endothelial nitric oxide synthase (eNOS), is a principal stimulator of vasodilation (vessel relaxation) through cyclic GMP (cGMP)- dependent protein kinase (PKG)- mediated decrease in Ca^{2+} concentration and, thus, MLCK inactivation and MP activation, leading to a rapid dephosphorylation of MLC2 and relaxation (Kitazawa, Semba et al. 2009).

Myosin phosphatase is a key regulator of smooth muscle contraction and non-muscle cell motility. For example, it plays a critical role in regulating the endothelial-blood barrier (Verin, Patterson et al. 1995). The holoenzyme of MP was first isolated from chicken gizzard (Alessi, MacDougall et al. 1992), and consists of a 38 kDa PP1c□, a 110 kDa myosin binding subunit (MBS); also known as MYPT1, and a 20 kDa small subunit (M20) of unknown function. MYPT1 subunit belongs to the family of myosin phosphatase targeting (MYPT) proteins (Grassie, Moffat et al. 2011).

1.6 Family of Myosin Targeting (MYPT) Proteins

The MYPT family is comprised of five proteins: MYPT1, MYPT2, MYPT3, 85 kDa myosin binding subunit (MBS 85), and TIMAP (Grassie, Moffat et al. 2011). Figure 1.4 shows a comparison of MYPT family members. According to amino acid sequence, all MYPT members share the RVxF motif located near the N-terminus. This is followed by several ankyrin repeats, each composed of 29-33 amino acid residues, which make a platform for protein-protein interaction, including phosphorylated myosin II (Grassie, Moffat et al. 2011). The central region following the ankyrin repeats contains regulatory domains important for MYPTs function. The main mechanism of regulation of MYPTs

function involves phosphorylation of certain amino acids in the central region by different Ser/Thr kinases. This mechanism is best understood for MYPT1, for which phosphorylation by ROCK at Thr 696 and Thr 853 inhibits the phosphatase activity (Kimura, Ito et al. 1996, Feng, Ito et al. 1999, Muranyi, Derkach et al. 2005). Both of the inhibitory phosphorylation sites are conserved in MYPT2, only the site corresponding to Thr 696 is conserved in MBS 85, and neither site is conserved in TIMAP and MYPT3. The C-terminus of MYPT1, MYPT2 and MBS 85 contains leucine zipper domain, which plays a role in dimerization and protein-protein interaction (Grassie, Moffat et al. 2011). In contrast, TIMAP and MYPT3 lack this domain but contain a C-terminal CAAX motif (prenylation site), which targets the proteins to the plasma membrane (Skinner and Saltiel 2001, Cao, Mattagajasingh et al. 2002). The variations in the sequences at the C-terminus suggest that different MYPTs might target PP1c to distinct subcellular locations and substrates.

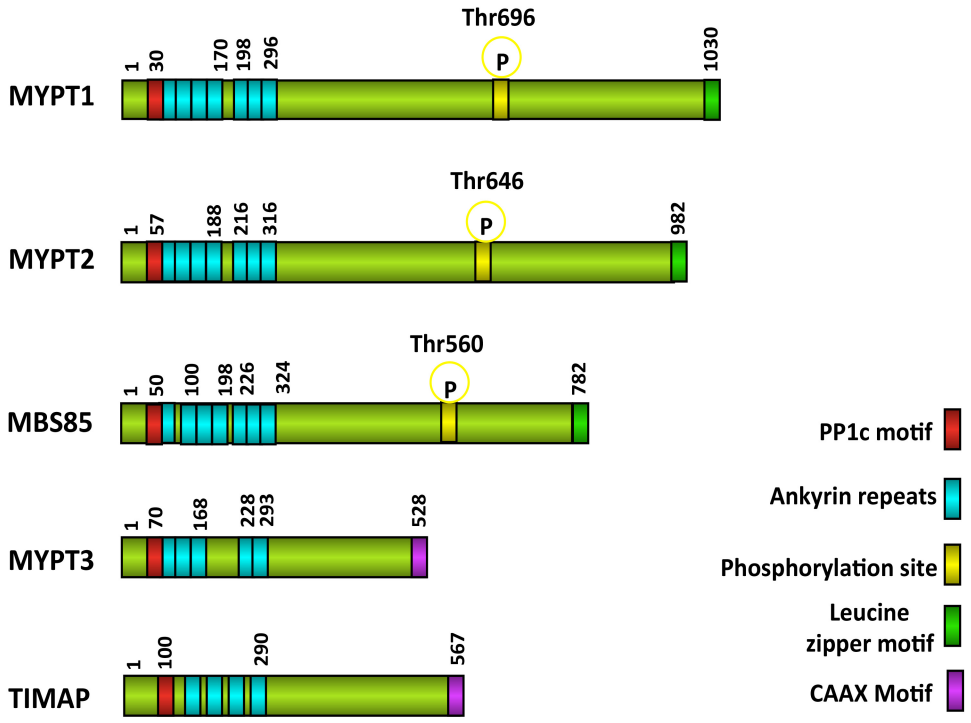


Figure 1.4: The domain structure of MYPT family members. PP1c- binding motif is shown in red. The ankyrin repeats domain is in blue. The ROCK inhibitory phosphorylation sites are in yellow. The leucine zipper motif is in green. The C-terminal CAAX motif is in purple. Figure was made based on information in the review article by (Ito, Nakano et al. 2004).

1.6.1 MYPT1

MYPT1 is the most studied member of the MYPT family, and its mechanism of action and regulation is well characterized. The MYPT1 gene produces two isoforms, 110 kDa and 130 kDa (Shimizu, Ito et al. 1994, Brozovich 2002). Although MYPT1 is expressed in various tissues and cell types, it is present at much higher concentrations in smooth muscle cells (Matsumura and Hartshorne 2008). MYPT1 binds to PP1c via its N- terminal PP1 binding domain (RVxF), and it interacts with the C-terminus of PP1c via the ankyrin repeats region (Hirano, Phan et al. 1997).

As a targeting subunit of PP1c, MYPT1 has the potential to interact with various proteins. The most important binding partner of MYPT1 is myosin II. There are two controversial views in regards to MYPT1-myosin II interaction. One suggests that MYPT1 binds to the phosphorylated MLC2 (pMLC2) via the ankyrin repeats (Hirano, Phan et al. 1997), and that this binding becomes less efficient when MLC2 is dephosphorylated (Hartshorne, Ito et al. 1998). The other view is that dephosphorylated MLC2 binds to the C-terminus of MYPT1 (Johnson, Cohen et al. 1997). Multiple interactions are expected at the ankyrin repeats region. For example, Tau and MAP2 bind to the ankyrin repeats region and are dephosphorylated by MYPT1 (Amano, Kaneko et al. 2003). Furthermore, the C-terminus of MYPT1 has been shown to interact with many molecules, including M20 (Hirano, Phan et al. 1997, Johnson, Cohen et al. 1997), GTP-bound RhoA (active RhoA) (Hartshorne, Ito et al. 2004), acidic phospholipids (Ito, Feng et al. 1997, Hartshorne, Ito et al. 1998) and moesin (Amano, Fukata et al. 2000). Other

partners may interact with MYPT1 via its leucine zipper motifs, such as interleukin-16 precursor and RhoA-interacting protein (Grassie, Moffat et al. 2011). The multiple interactions of MYPT1 explain its various cellular localizations and suggest that MYPT1 exhibits different cellular functions in addition to the dephosphorylation of pMLC2. However, this thesis will focus on the myosin phosphatase function of MYPT1.

The phosphatase activity of MYPT1 is the principal determinant of the MLC2 phosphorylation state. MYPT1 activity is regulated by different mechanisms, including phosphorylation of MYPT1 (Feng, Ito et al. 1999), phosphorylation of an inhibitory protein, CPI-17 (Eto, Ohmori et al. 1995), and translocation and dissociation of the holoenzyme (Gong, Fuglsang et al. 1992, Koga and Ikebe 2008). The first two mechanisms are the most well characterized and frequently cited. MYPT1 displays several phosphorylation sites including Thr 696 and Thr 853 (numbering represents human MYPT1). Phosphorylation on these sites results in inhibition of MYPT1 activity (Feng, Ito et al. 1999, Muranyi, Derkach et al. 2005). Also, Phosphorylation of Thr 853 reduces MYPT1 binding to myosin II (Velasco, Armstrong et al. 2002). Several protein kinases have been shown to phosphorylate MYPT1 at Thr 696 residue including, ROCK (Kimura, Ito et al. 1996, Feng, Ito et al. 1999), zipper-interacting protein kinase (ZIPK) (Haystead 2005), myotonic dystrophy kinase (Muranyi, Zhang et al. 2001) and integrin-linked kinase (ILK) (Kiss, Muranyi et al. 2002, Muranyi, MacDonald et al. 2002). Some of these kinases (ROCK, ZIPK, and ILK) can also directly phosphorylate MLC2 and have been shown to induce a Ca^{2+}- independent contractility in non-muscle cells (Hartshorne, Ito et al. 2004). In contrast, MLCK, which phosphorylates MLC2 preferably at Ser 19 and induces Ca^{2+}- dependent contractility, is not implicated in regulating

MYPT1 phosphorylation. The fact that several protein kinases are involved in the inhibitory phosphorylation of MYPT1 suggests that many cellular pathways that regulate myosin II phosphorylation converge on inhibition of MYPT1 activity.

CPI-17 (17 kDa) is a PP1c inhibitor, first isolated from pig aorta (Eto, Ohmori et al. 1995), expressed in smooth muscle, brain, platelets and endothelial cells (Eto, Senba et al. 1997, Watanabe, Ito et al. 2001, Eto, Bock et al. 2002, Kolosova, Ma et al. 2004). Unlike the PP1c inhibitors (I-1 and I-2) that bind to and specifically inhibit PP1c activity (Connor, Frederick et al. 2000), CPI-17 inhibits both PP1c and PP1 holoenzymes (Hartshorne, Ito et al. 2004). Phosphorylation of CPI-17 at Thr 38 enhances its inhibitory potency by ~1000 fold (Eto, Ohmori et al. 1995) and causes a substantial suppression of MP activity (Senba, Eto et al. 1999), resulting in enhanced myosin II phosphorylation and smooth muscle contraction. Several kinases are implicated in phosphorylation of CPI-17 at Thr 38, such as protein kinase C (PKC□/□) , ROCK, ZIPK and ILK (Koyama, Ito et al. 2000, Eto, Kitazawa et al. 2001, MacDonald, Eto et al. 2001, Deng, Sutherland et al. 2002, Eto 2009), indicating inputs from different signaling pathways. Furthermore, different agonists, such as histamine, endothelin-1, and angiotensin II (Kitazawa, Eto et al. 2000, Eto, Kitazawa et al. 2001, Kitazawa, Eto et al. 2003) have been shown to stimulate phosphorylation of CPI-17 at Thr 38 in smooth muscle. In endothelial cells, PKC- mediated phosphorylation of CPI-17 at Thr 38 enhances MLC2 phosphorylation, induces reorganization of actin cytoskeleton and focal adhesions, and results in histamine- induced barrier dysfunction (Kolosova, Ma et al. 2004). On the other hand, NO-mediated suppression of Ca^{2+} release results in inactivation of PKC and consequent

dephosphorylation of CPI-17, leading to MYPT1 activation and MLC2 dephosphorylation (Kitazawa, Semba et al. 2009).

1.6.2 MYPT2

MYPT2 (110 kDa) was first cloned from the human brain library (Fujioka, Takahashi et al. 1998). The gene of MYPT2 produces two splicing isoforms, MYPT2A and MYPT2B (Arimura, Suematsu et al. 2001). Unlike MYPT1, which is expressed in smooth muscle and most non- muscle cells, MYPT2 is predominantly expressed in cardiac and skeletal muscle and brain (Grassie, Moffat et al. 2011). As mentioned earlier, it retains ROCK inhibitory phosphorylation sites of MYPT1 (Thr 646 and Thr 808), and phosphorylation at Thr 646 inhibits the phosphatase activity (Okamoto, Kato et al. 2006). Similar to MYPT1, MYPT2 binds specifically to PP1cδ via its RVxF (RVRF) motif and enhances the phosphatase activity towards pMLC2 in cardiac and skeletal muscle (Fujioka, Takahashi et al. 1998, Okamoto, Kato et al. 2006), and it binds RhoA at the C-terminus (Okamoto, Kato et al. 2006). The MP holoenzyme in cardiac and skeletal muscle consists of MYPT2, PP1cδ and M21 (Moorhead, Johnson et al. 1998). *In vivo* studies of transgenic heart overexpressing MYPT2 demonstrated that MYPT2 is the main regulatory subunit of cardiac MP, and its primary function is to regulate PP1cδ activity towards pMLC2 (Mizutani, Okamoto et al. 2010). In fact, overexpression of MYPT2 in the mouse heart was associated with an increase in PP1cδ expression and a decrease in MLC2 phosphorylation (Mizutani, Okamoto et al. 2010).

1.6.3 MBS 85

MBS 85 (85 kDa) was first identified as a substrate of myotonic dystrophy kinase-related Cdc42 binding kinase-a (MRCK□), which mediates Cdc42- induced actin reorganization (Tan, Ng et al. 2001). MBS 85 protein is ubiquitously expressed, and its amino acid sequence is most similar to MYPT2 (Grassie, Moffat et al. 2011). Like other MYPTs, it specifically binds to PP1c□ via its modified form of PP1c-binding RVxF motif (RTVRF), which is located at the N-terminus and followed by the ankyrin repeats region (Grassie, Moffat et al. 2011). The C-terminal leucine zipper domain is similar to those of MYPT1 and MYPT2 (Grassie, Moffat et al. 2011). The central region of MBS 85 exhibits a single ROCK phosphorylation inhibitory site (Thr 560), which upon phosphorylation inhibits the phosphatase activity of PP1c□ (Tan, Ng et al. 2001). Similar to MYPT1 and MYPT2, the principal function of MBS 85 is to target PP1c□ to phosphorylated myosin II and dephosphorylate pMLC2. The N-terminal- mediated targeting of PP1c□ to phosphorylated myosin II causes dephosphorylation of pMLC2 (Grassie, Moffat et al. 2011), thus disrupts myosin II/actin assembly and stress fiber formation. In addition, the leucine zipper domain of MBS 85 can regulate actin stress fiber formation by competitive binding to proteins that activate the phosphatase, such as the cGMP- dependent protein kinase type 1- alpha (PKGI□) (Tan, Ng et al. 2001).

1.6.4 MYPT3

MYPT3 (58 kDa) was cloned from an adipocyte cDNA library (Skinner and Saltiel 2001). It is expressed mainly in heart, liver and brain (Skinner and Saltiel 2001). Although MYPT3 shares several structural characteristics with other MYPT family members, such as the N-terminal PP1c binding motif (KQVLF) and the ankyrin repeats region, its C-terminus lacks the leucine zipper domain and the inhibitory phosphorylation sites that are present in MYPT1, MYPT2 and MBS 85. Instead, the C-terminus of MYPT3 contains a CAAX box that targets MYPT3 to the cell membrane (Skinner and Saltiel 2001); therefore MYPT3 localizes predominantly to cell membranes. Unlike the previously described MYPTs, MYPT3 binding to PP1c inhibits the phosphatase activity towards pMLC2 (Skinner and Saltiel 2001). However, phosphorylation of MYPT3 at Ser 340/341/353 residue in the central region by PKA disrupts the inhibitory interaction between the central region and the ankyrin repeats domain of MYPT3, enhancing the phosphatase activity towards pMLC2 (Yong, Tan et al. 2006).

1.6.5 TIMAP, an Endothelial MYPT

TIMAP (63 kDa, **T**GFβ1 **I**nhibited **M**embrane **A**ssociated **P**rotein) was initially identified in glomerular endothelial cells by representational difference analysis under TGFβ1- targeted transcriptional repression (Cao, Mattagajasingh et al. 2002). TIMAP is predominantly expressed in all cultured endothelial and hematopoietic cells, compared to non- endothelial cells (Cao, Mattagajasingh et al. 2002). Immunofluorescence studies of

neonatal rat kidney tissues revealed that TIMAP protein immunoreactivity localizes almost exclusively to the vascular endothelium (Cao, Mattagajasingh et al. 2002). According to its amino acid composition, TIMAP belongs to the MYPT family of PP1c regulatory subunits and its structure most closely resembles that of MYPT3 (Grassie, Moffat et al. 2011). Both proteins exhibit a C-terminal CAAX motif that mediates their prenylation and consequent association with cell membranes. Prenylation of TIMAP is facilitated by farnesyl transferase (Cao, Mattagajasingh et al. 2002). Indeed, TIMAP has been shown to localize to the plasma membrane of glomerular endothelial cell filopodia and regulate filopodia formation, and deletion of its CAAX motif inhibits TIMAP localization to the plasma membrane and promotes its accumulation in the nucleus (Cao, Mattagajasingh et al. 2002, Li, Kozlowski et al. 2007). TIMAP contains a nuclear localization domain; therefore it can be detected in the nucleus of glomerular endothelial cells (Cao, Mattagajasingh et al. 2002), but its nuclear function has not yet been characterized. Like MYPT3, TIMAP lacks the C-terminal inhibitory phosphorylation domain found in other MYPTs which is required for the regulation of their myosin phosphatase function (Grassie, Moffat et al. 2011).

Similar to the other MYPT family members, TIMAP specifically binds to PP1cδ isoform via its RVxF (KVSF) motif (Csortos, Czikora et al. 2008, Grassie, Moffat et al. 2011, Shopik, Li et al. 2013). Point mutations in the KVSF motif of TIMAP inhibit its interaction with PP1cδ and TIMAP-associated PP1cδ activity (Li, Kozlowski et al. 2007). Structural modeling of the TIMAP-PP1cδ complex predicts a 1:1 stoichiometry of TIMAP: PP1cδ, that the C-terminus of PP1c is buried in the ankyrin region of TIMAP,

and that the active site of PP1cγ remains accessible (Figure 1.5) (Shopik, Li et al. 2013). Consistent with this model, TIMAP interaction with PP1cγ inhibits the C-terminal phosphorylation of PP1cγ by Cyclin A/ cyclin- dependent kinase 2 (cdk2) (Shopik, Li et al. 2013). Furthermore, TIMAP inhibits PP1c activity towards phosphorylase *a* in a concentration dependent manner (Shopik, Li et al. 2013). TIMAP has been shown to regulate PP1cγ activity towards the non-integrin 67 kDa laminin receptor 1 (LAMR1) (Kim, Li et al. 2005, Shopik, Li et al. 2013). LAMR1 is expressed in endothelial cells, at higher levels during endothelial cell proliferation and angiogenesis (McKenna, Simpson et al. 2001), and required for capillary formation and angiogenesis (Iwamoto, Nomizu et al. 1996). Also, LAMR1 expression is greatly elevated in metastatic tumors (Martignone, Menard et al. 1993). In endothelial cells, TIMAP interacts and co-localizes with LAMR1 at the plasma membrane via the ankyrin repeats region of TIMAP, and promotes LAMR1 association with PP1cγ and dephosphorylation (Kim, Li et al. 2005). In contrast, *in vitro*, TIMAP inhibits PP1cγ activity towards LAMR1 and, in the absence of PP1cγ, binds to and attenuates LAMR1 phosphorylation by PKA and PKC (Shopik, Li et al. 2013). Another phosphorylated protein target of TIMAP- PP1cγ is the Ezrin-Radixin-Moesin (ERM) protein family member, moesin. Moesin is the dominant ERM protein in endothelial cells (Berryman, Franck et al. 1993). Like other ERM proteins, it is involved in connecting the actin cytoskeleton to the plasma membrane (Mangeat, Roy et al. 1999), and regulates cell motility, adhesion, filopodia formation and endothelial barrier function (Berryman, Franck et al. 1993, Amieva and Furthmayr 1995, Csortos, Czikora et al. 2008, Fu, Flamini et al. 2008). ERM proteins are regulated by phosphorylation (Matsui,

Maeda et al. 1998, Simons, Pietromonaco et al. 1998, Nakamura, Oshiro et al. 2000). For example, moesin is phosphorylated by ROCK and PKC theta and dephosphorylated by MYPT1 (Fukata, Kimura et al. 1998, Pietromonaco, Simons et al. 1998). In human pulmonary artery endothelial cells (HPAEC), TIMAP and PP1c associate with moesin, and TIMAP co-localizes with moesin at the plasma membrane (Csortos, Czikora et al. 2008). Furthermore, studies in HPAEC depleted of TIMAP revealed that TIMAP augments the barrier- protective effect of cyclic adenosine monophosphate (cAMP)/PKA pathway against the barrier- compromising stimulus, thrombin, and attenuates thrombin-induced phosphorylation of moesin at the cell membrane (Csortos, Czikora et al. 2008). In addition, TIMAP depletion *in vivo* augments lipopolysaccharide - induced vascular leakage in murine lung (Poirier, Gorshkov et al. 2011). Thus, TIMAP is considered to be crucial for the maintenance of endothelial barrier function by reducing moesin phosphorylation. The fact that both MYPT1 and TIMAP target phosphorylated ERM family proteins, and play an important role in protecting the integrity of the endothelial barrier suggests that MYPT family proteins may exhibit redundant cellular and physiological roles, and target different phosphorylated proteins other than myosin II.

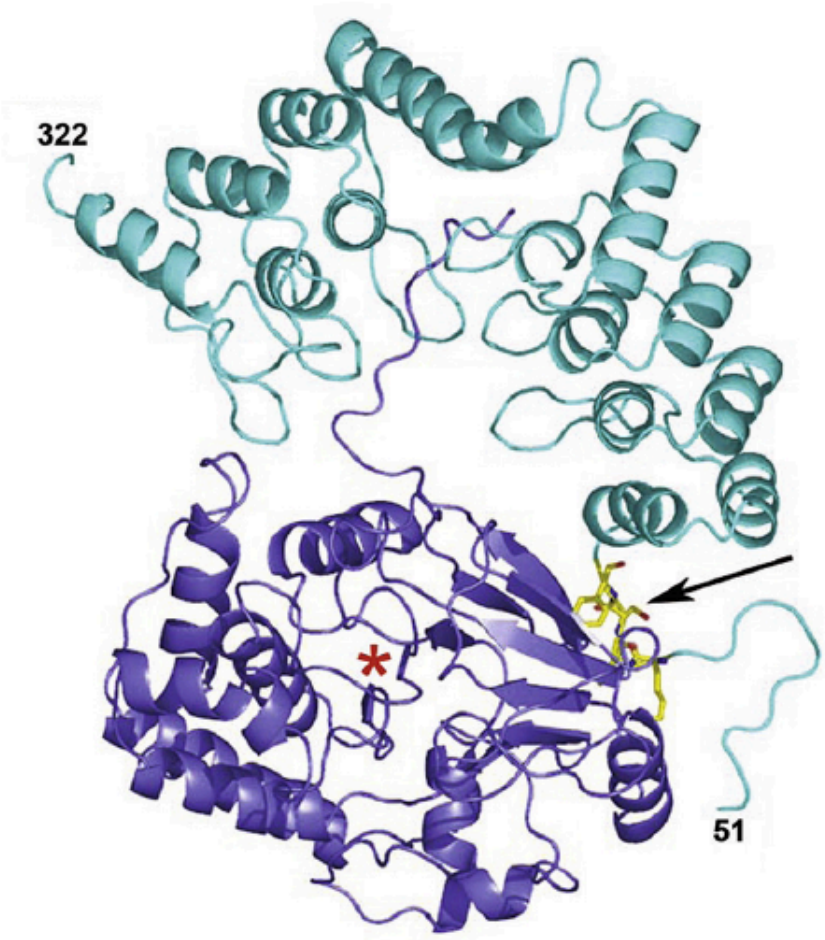


Figure 1.5: **A predicted structural model of TIMAP-PP1c complex.** TIMAP (top, cyan, residues 51-322); PP1c (bottom, purple); Active site of PP1 (Red asterisk); RVSF (yellow sticks). Eight -helices of TIMAP form 4 ankyrin repeats that wrap around the PP1c C-terminus. Adapted from (Shopik, Li et al. 2013). I am an author on the article from which this figure was adapted. According to the publisher copyright policy, I am not required to obtain copyright licence for this figure.

Like MYPT3, TIMAP is regulated by phosphorylation. TIMAP is phosphorylated by GSK3β after a priming phosphorylation by PKA at Ser 333 and Ser 337, respectively (Li, Kozlowski et al. 2007). Phosphorylation at these sites attenuates the interaction of TIMAP with PP1cβ, but enhances its phosphatase activity toward the fluorogenic substrate 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) and phosphorylase *a in vitro,* and LAMR1 in cells (Li, Kozlowski et al. 2007, Shopik, Li et al. 2013). In addition to targeting PP1cβ activity, phosphorylated TIMAP is itself a substrate of its- associated PP1cβ activity, and TIMAP mutant that is unable to bind PP1cβ is hyperphosphorylated (Li, Kozlowski et al. 2007, Shopik, Li et al. 2013). It has been shown that the increase in the degree of thiophosphorylation of TIMAP by PKA or PKA and GSK3β reduces the inhibition of phosphorylated Moesin dephosphorylation by TIMAP-associated PP1cβ (Czikora, Kim et al. 2011).

Several potential binding partners of TIMAP were identified in a study that utilized bacterial two-hybrid screening, including cysteine and glycine rich protein 1 and eukaryotic translation elongation factor 2, which are involved in actin cytoskeleton organization (Adyshev, Kolosova et al. 2006). However, the physiological aspect of these interactions has not been investigated. A recent study identified receptor for activated protein kinase C (RACK1) as a binding partner of TIMAP in vascular endothelial cells (Boratko, Gergely et al. 2013); PKA-mediated phosphorylation of TIMAP attenuated TIMAP-RACK1 complex formation, enhanced TIMAP translocation to the cell membrane, which was associated with enhanced endothelial barrier function. In addition, depletion of RACK1 abolished the association between TIMAP and farnesyl transferase,

indicating that RACK1 is necessary to facilitate TIMAP prenylation.

Although TIMAP is a MYPT family member and regulates endothelial cell cytoskeleton, to date the physiological role of TIMAP-PP1c as a myosin phosphatase has not been characterized.

1.7 TIMAP, an Endothelial-Predominant TGF1- downregulated PP1c Regulatory Subunit

The fact that TGF1 represses TIMAP mRNA synthesis in vascular endothelial cells, and that TGF1 plays an important role in development of vascular endothelium, endothelial cells proliferation, apoptosis, and capillary formation suggest that TIMAP might play an important role in TGF1 signaling pathway in endothelial cells, involving apoptosis, proliferation and capillary formation. Nonetheless, the exact role of TIMAP in these processes has not been uncovered yet.

The next part of the literature review will discuss the role of reversible protein phosphorylation in the development of vascular endothelium, including the major signaling pathways that regulate this process.

1.8 Role of Reversible Protein Phosphorylation in Development of Vascular Endothelium

The vasculature is a highly organized circulatory system that provides all tissues

with sufficient supplies of oxygen and nutrients. The basic constituents of the vasculature, endothelial and blood cells, are determined early during embryogenesis (Balconi, Spagnuolo et al. 2000). The Vascular endothelium lines the inner surface of the entire vascular tree and forms an anticoagulant barrier between blood and tissues with variable permeability depending on location. It regulates various physiological processes, including blood pressure, inflammation, blood clotting, wound healing, permeability and angiogenesis.

The vascular system develops by two processes – vasculogenesis and angiogenesis. Vasculogenesis is the differentiation of endothelial progenitor cells, angioblasts, into blood vessels. Angiogenesis involves migration and proliferation of already differentiated endothelial cells in existing blood vessels to form new vasculature (Flamme, Frolich et al. 1997). During embryogenesis the primary vascular plexus, formed by vasculogenesis, undergoes remodeling, involving sprouting, splitting and regression of branches to form the vascular network.

Mechanisms that regulate angiogenesis have become an area of intensive research since Folkman first proposed that tumor growth is dependent on new blood vessel formation (Folkman 1971). Angiogenesis is crucial for normal blood vessel formation and patterning during embryonic development (Ferrara, Carver-Moore et al. 1996), for post-embryonic blood vessel repair (Banda, Knighton et al. 1982) and remodeling in response to hypoxia (Fong 2009), in the female reproductive cycle (Redmer and Reynolds 1996) and for the establishment of the placental vasculature (Chen and Zheng 2014). Pathological retinal (Crawford, Alfaro et al. 2009) and tumor (Welti, Loges et al. 2013) vascularization also depend on angiogenesis. A great variety of growth factors and

their intracellular signaling pathways regulate angiogenesis, including VEGFs (Connolly, Heuvelman et al. 1989), fibroblast growth factors (FGFs) (Gospodarowicz, Brown et al. 1978), angiopoietins (Suri, Jones et al. 1996), and the TGF-β family (Roberts, Sporn et al. 1986, Pardali, Goumans et al. 2010, Jakobsson and van Meeteren 2013). Interaction of these growth factors with their receptors on endothelial cells stimulates distinct protein phosphorylation cascades involved in the regulation of the different stages of angiogenesis. Upon stimulation of angiogenesis, tissues respond by producing the angiogenic growth factors (Semenza 2012) and inhibiting the TGFβ1 signaling pathway in endothelial cells (Wei, Bedja et al. 2012), which is involved in vessel stabilization and the establishment of perivascular cells (Pardali, Goumans et al. 2010, Jakobsson and van Meeteren 2013). Angiogenic sprouts then form in the direction of the gradient of VEGF and other angiogenic growth factors. This is followed by elongation, stabilization, and specification of the new vessel (Jeltsch, Leppanen et al. 2013).

During angiogenesis, a subgroup of endothelial cells in the existing vessel (tip endothelial cells) initiates the formation of new sprouts; this is followed by lumen formation at the proximal end. Tip endothelial cells respond to the gradient of the angiogenic growth factors and determine the direction of the new blood vessel (Hellstrom, Phng et al. 2007). Elongation of the new vessel depends on proliferation of endothelial cells in the stalk of the developing vessel (Eilken and Adams 2010). Tight regulation of endothelial cell proliferation is crucial for the development of normal vasculature, and defective cell proliferation has been associated with pathological angiogenesis (Jiang, Zheng et al. 2000, Jiang and Liu 2009). Proliferation of stalk endothelial cells is promoted by several angiogenic growth factors, including VEGF

(Connolly, Heuvelman et al. 1989), FGF (Gospodarowicz, Brown et al. 1978), and angiopoietins (Suri, Jones et al. 1996).

1.8.1 PI3K/Akt Pathway and its Role in Angiogenesis

Endothelial cell proliferation during angiogenesis depends on the activation of the intracellular phosphatidylinositol 3-kinase (PI3K)/Akt pathway (Varma, Lal et al. 2005). Akt (also known as protein kinase B, PKB) is a Ser/Thr kinase downstream of PI3K that promotes cell survival (Gerber, McMurtrey et al. 1998), migration (Morales-Ruiz, Fulton et al. 2000), proliferation (Rossig, Jadidi et al. 2001), and angiogenesis (Dimmeler and Zeiher 2000) by phosphorylating a vast range of downstream effector proteins, such as GSK3 (Kim, Skurk et al. 2002), the pro-apoptotic BCL-2 agonist of death (BAD) (Datta, Dudek et al. 1997), Caspase 9 (Casp9) (Cardone, Roy et al. 1998), the forkhead family of transcription factors (FOXOs) (Burgering and Medema 2003, Bouchard, Marquardt et al. 2004) and endothelial nitric oxide synthase (eNOS) (Fulton, Gratton et al. 1999) (Figure 1.6). Akt is also implicated in F-actin remodeling (Qian, Corum et al. 2004). Akt deficient mice display impaired fetal vascularization (Yang, Tschopp et al. 2003, Ackah, Yu et al. 2005), while constitutively active Akt induces pathological angiogenesis (Perry, Banyard et al. 2007).

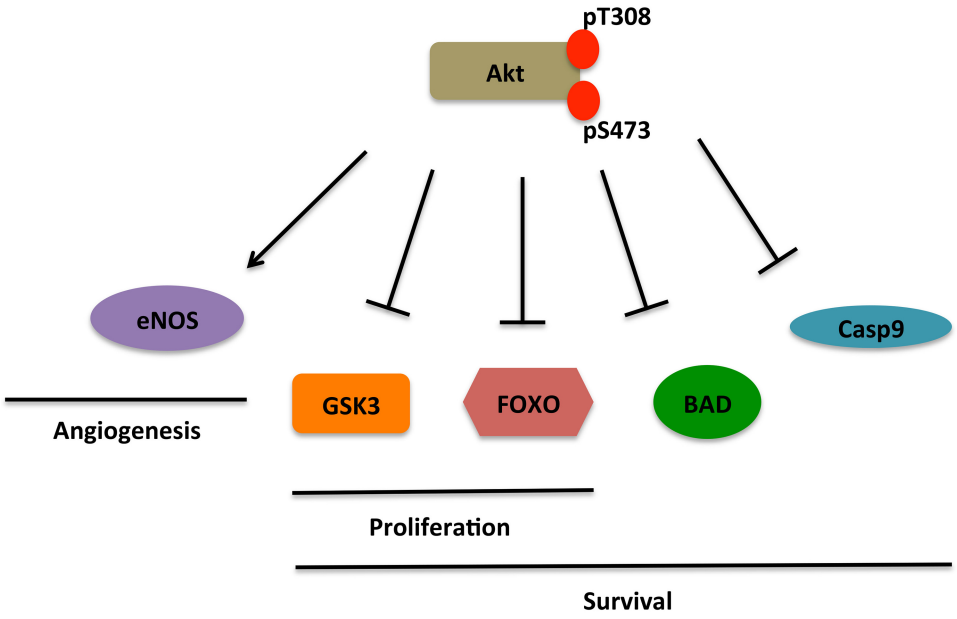


Figure 1.6: **A diagram showing the various downstream effector proteins and physiological processes that are regulated by Akt**. Akt is activated by phosphorylation at its Thr 308 and Ser 473 residues. Inhibitory phosphorylation of GSK3, FOXO, BAD and Casp9 promotes cell survival, inhibitory phosphorylation of GSK3 and FOXO promotes cell proliferation, and activating phosphorylation of eNOS promotes angiogenesis.

Akt is activated by phosphorylation in response to the activation of the receptor tyrosine kinases (RTK) that include VEGF receptor 2 (VEGFR2) and FGF receptors (Figure 1.7) (Alessi, Andjelkovic et al. 1996, Andjelkovic, Jakubowicz et al. 1996, Gerber, McMurtrey et al. 1998). Akt is phosphorylated at Thr 308 in its kinase domain by phosphoinositide- dependent kinase (PDK1); followed by phosphorylation at Ser 473 in its regulatory domain by the mammalian target of rapamycin complex 2 (mTORC2) (Brodbeck, Cron et al. 1999, Wick, Dong et al. 2000, Hresko and Mueckler 2005, Sarbassov, Guertin et al. 2005). Also, ILK has been shown to stimulate Akt phosphorylation at Ser 473 (Persad, Attwell et al. 2001). Phosphorylation at Thr 308 induces the active conformation of Akt, and phosphorylation at Ser 473 stabilizes Akt and exposes the kinase domain to downstream substrates (Yang, Cron et al. 2002, Sarbassov, Guertin et al. 2005). Full activation of Akt occurs when it is phosphorylated at both residues. In order for phosphorylation to occur, Akt and PDK1 must be recruited to the plasma membrane, via their pleckstrin homology (PH) domains, by phosphatidylinositol [3,4,5] triphosphate (PIP3) (Andjelkovic, Alessi et al. 1997). In turn, PIP3 is generated by PI3K- mediated phosphorylation of phosphatidylinositol [4,5] bisphosphate (PIP2) in response to activation of the receptor tyrosine kinases (Franke, Yang et al. 1995, Alessi, Andjelkovic et al. 1996, Andjelkovic, Jakubowicz et al. 1996). The action of PI3K is opposed by the phosphatase and tensin homologue deleted on chromosome 10 (PTEN), which dephosphorylates PIP3 to generate PIP2 (Cantley and Neel 1999, Hill and Hemmings 2002, Gericke, Leslie et al. 2013), resulting in inhibition of Akt phosphorylation. In fact, function-disrupting mutations in PTEN result in hyperactivation of PI3K/Akt, decreased sensitivity to apoptosis, and increased

tumourogenesis (Hamada, Sasaki et al. 2005).

PTEN is a major tumor suppressor and is deleted in many tumors (Li, Yen et al. 1997). Also, it regulates angiogenesis (Suzuki, Hamada et al. 2007), but less is known about this role. Whereas deletion of PTEN is associated with hyperproliferation of endothelial cells and disordered angiogenesis in mice (Hamada, Sasaki et al. 2005), activation of PTEN inhibits angiogenesis, favoring stabilization and differentiation of endothelium or, if excessive, endothelial cell apoptosis (Hoshino, Nishimura et al. 2007). The main function of PTEN is to antagonize the signaling downstream of receptor tyrosine kinase/PI3K and control the concentration of PIP3 via its lipid phosphatase activity, nonetheless, PTEN possesses a protein phosphatase activity as well (Myers, Stolarov et al. 1997). PTEN regulates many cell processes, such as proliferation and apoptosis (Tamguney and Stokoe 2007). Most of these known functions have been attributed to its lipid phosphatase property. PTEN regulates these processes by suppressing Akt activation, as Akt activation depends on the production of PIP3, which is inhibited by PTEN lipid phosphatase activity. However, PTEN can also regulate cellular processes independently of its lipid phosphatase activity, like actin remodeling (Kim, Xu et al. 2011). In addition, studies in neutrophils and *Dictyostelium discoideum* have shown that PTEN regulates directional migration during chemotaxis (Iijima and Devreotes 2002, Iijima, Huang et al. 2004, Billadeau 2008), in which PI3K and PTEN exhibit reciprocal localization at the leading and posterior regions of migrating cells, respectively (Funamoto, Meili et al. 2002).

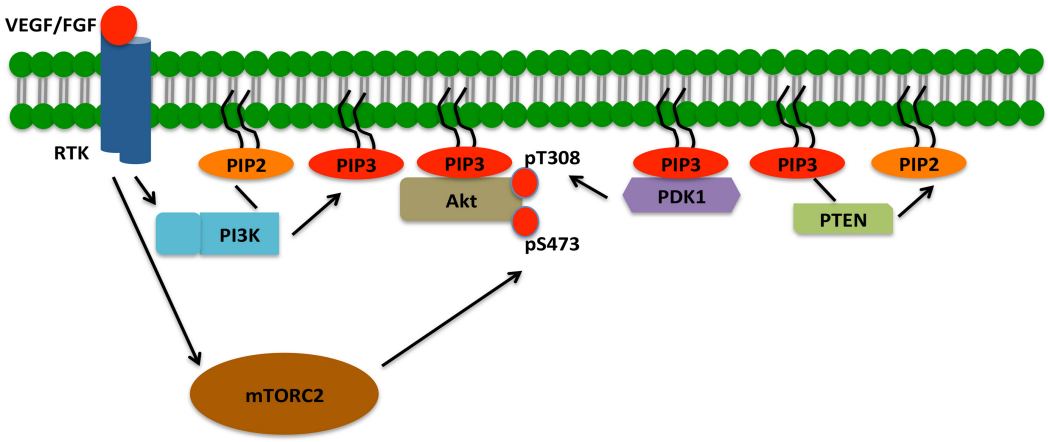


Figure 1.7: Mechanism of PI3K/Akt signaling in endothelial cells. Binding of VEGF or FGF ligand to their cognate RTK on endothelial cells induces dimerization and activation of the RTK. In turn, RTK activates PI3K, which catalyzes the formation of PIP3 from PIP2. PIP3 recruits Akt and PDK1 to the plasma membrane and facilitates Akt phosphorylation at Thr 308 by PDK1. The mTORC2, which is also activated by RTK, phosphorylates Akt at Ser 473. The action of PI3K is reversed by PTEN-mediated dephosphorylation of PIP3 to produce PIP2, limiting the availability of PIP3 for Akt and PDK1 and inhibiting Akt signaling.

Similar to PTEN, myosin II is also localized to the posterior region of migrating neutrophils and *Dictyostelium discoideum* (Uchida, Kitanishi-Yumura et al. 2003). Both PTEN null cells and myosin II phosphorylation mutants have multiple filopodia and impaired chemotaxis and cytokinesis (Heid, Wessels et al. 2004, Wessels, Lusche et al. 2007). Furthermore, PTEN has been found to be important for myosin II subcellular localization during *Dictyostelium* cell migration and cytokinesis (Pramanik, Iijima et al. 2009). These studies suggest that PTEN plays multi-directional roles in regulating the various cell processes, and targets different molecules other than lipid second messengers.

PTEN consists of multiple domains, including the N-terminal PI[4,5]P2-binding module (PBM) and phosphatase domain (PD), a central C2 domain and C-terminal tail. The phosphatase and C2 domains form the catalytic domain that catalyzes the conversion of PIP3 to PIP2, while the C-terminal tail is involved in regulation of PTEN activity (Shi, Paluch et al. 2012). Also, the phosphatase and C2 domains, but not the C-terminal tail, are involved in binding of PTEN to cell membranes (Walker, Leslie et al. 2004). PTEN is mostly cytosolic, and some PTEN is found in the nucleus (Trotman, Wang et al. 2007, Song, Salmena et al. 2008), but for it to be able to regulate cellular functions that require its lipid phosphatase activity it has to be targeted to the cell membranes.

PTEN activity is tightly regulated through multiple mechanisms, including transcriptional regulation by many transcription factors, such as □-catenin/transcription factor (TCF) (Lau, Klausen et al. 2011), and post-transcriptional regulation by a variety of micro RNAs (miRNAs), including miRNA-21, miRNA-22 and miRNA-26 (Meng, Henson et al. 2007, Huse, Brennan et al. 2009, Bar and Dikstein 2010). For example, in

endothelial cells, TGF□□upregulated endothelial miRNA-29a directly targets PTEN, resulting in activation of Akt and stimulation of angiogenesis (Wang, Wang et al. 2013). Furthermore, PTEN is regulated by post- translational modifications that involve acetylation (Ikenoue, Inoki et al. 2008), oxidation (Kwon, Lee et al. 2004), ubiquitylation (Wang, Trotman et al. 2007) and phosphorylation, which regulates its recruitment to the plasma membrane and PTEN stability (Vazquez, Ramaswamy et al. 2000). PTEN is phosphorylated at a cluster of Ser/Thr residues (380-385) in its C-terminal tail (Vazquez, Ramaswamy et al. 2000). Phosphorylation at these residues induces the interaction of the C-terminal tail with the N-terminal C2 and PD domains, which results in inhibition of PTEN membrane recruitment and its lipid phosphatase activity (Figure 1.8), but enhances PTEN stability (Vazquez, Ramaswamy et al. 2000, Odriozola, Singh et al. 2007, Rahdar, Inoue et al. 2009). The substitution to alanine at these phosphorylation residues enhances PTEN membrane affinity, enzymatic activity and degradation (Vazquez, Ramaswamy et al. 2000).

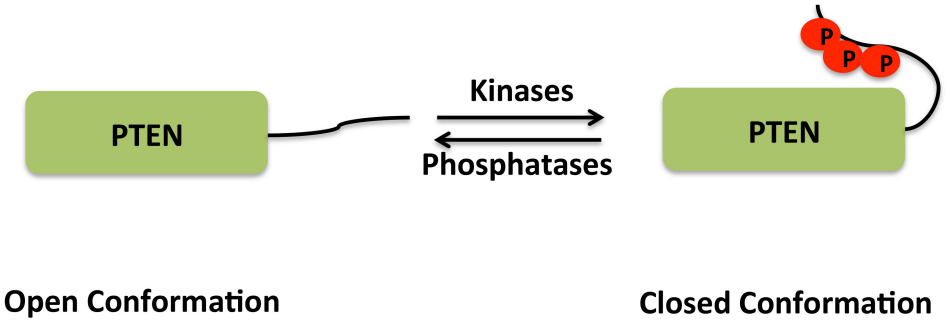


Figure 1.8: Mechanism of PTEN Regulation. Dephosphorylated PTEN exhibits an open conformation that allows PTEN to associate with the plasma membrane. Phosphorylation of PTEN tail induces the closed conformation that prevents PTEN from interaction with the plasma membrane.

Several kinases can phosphorylate PTEN. Casein kinase 2 (CK2) phosphorylates Ser 370 and Ser 385 residues (Torres and Pulido 2001) and GSK3□ phosphorylates Ser 362 and Thr 366 residues in the C-terminal tail (Al-Khouri, Ma et al. 2005); these phosphorylation events regulate PTEN stability to proteasomal degradation and consequent activity. Glioma tumor suppressor candidate region 2 (GLTSCR2) interacts with PTEN and enhances its phosphorylation at Ser 380 and stabilizes the phosphatase (Yim, Kim et al. 2007). Moreover, PTEN can be phosphorylated by ROCK at Ser 220, Thr 232, Thr 319 and Thr 321 in the C2 domain, resulting in increased membrane affinity and PTEN activity in chemoattractant- stimulated leukocytes (Li, Dong et al. 2005).

PTEN phosphatase activity is also regulated by its interaction with other proteins. For example, binding to p85, the regulatory subunit of PI3K stimulates the lipid phosphatase activity of PTEN (Chagpar, Links et al. 2010). Furthermore, interaction of the scaffold protein □□arrestin with PTEN enhances its lipid phosphatase activity and inhibits cell proliferation (Lima-Fernandes, Enslen et al. 2011).

The next signaling pathway that plays an important role in vascular endothelial development is the TGFβ1 pathway.

1.8.2 TGFβ Pathway and its Role in Angiogenesis

TGFβ1 belongs to the TGFβ superfamily of growth factors, which includes TGFβs, bone morphogenetic proteins (BMPs), and activins (Massague 1998). These cytokines are critical players during development, and in physiological and disease states.

They regulate a wide range of cellular processes, such as cell growth, apoptosis, differentiation, migration, and extracellular matrix production (Massague, Blain et al. 2000, Derynck and Akhurst 2007). For example, TGFβ is known to play an important role in tumor growth, epithelial mesenchymal transition (EMT), fibrosis, and vascular development.

TGFβ is essential for normal vascular development (Lebrin, Deckers et al. 2005) and mutations in the proteins involved in TGFβ signaling pathway in endothelial cells cause Hereditary Hemorrhagic Telangictasia (Abdalla and Letarte 2006); a set of genetic disorders characterized by abnormal vessel formation usually due to a disrupted balance between the pro- and anti-angiogenic signals. TGFβ was initially thought to be an inhibitor of angiogenesis and play an important role during the resolution phase of angiogenesis and vascular remodeling (Pepper 1997). However, it has been reported that TGFβ can also stimulate angiogenesis *in vivo* and *in vitro* (Yang and Moses 1990, Fajardo, Prionas et al. 1996, Vinals and Pouyssegur 2001). We have previously shown that TGFβ1 induces glomerular endothelial cells to form capillaries containing lumens *in vitro* (Choi and Ballermann 1995), a process that requires a sub-group of endothelial cells to undergo apoptosis. We also showed that TGFβ1 regulates glomerular capillary formation *in vivo*, and inhibition of TGFβ1 signaling blocks the formation of capillary lumen and endothelial fenestrae (Liu, Dardik et al. 1999).

Members of the TGFβ family elicit their signaling effects via binding to their heteromeric complexes of trans-membrane Ser/Thr kinase receptors (TGFβ receptor type I and type II (TGFβRI and TGFβRII), and then phosphorylation and activation of the intracellular receptor-regulated Smad (R-Smad) proteins (Massague 1998, Dennler,

Goumans et al. 2002). Activated R-Smads then associate with a central Smad4 and translocate to the nucleus to regulate transcription of downstream target genes (Figure 1.9) (Derynck, Zhang et al. 1998). TGFβRII is a constitutively active kinase, whereas TGFβRI needs to be activated by TGFβRII. TGFβRI is expressed as Activin receptor-like kinase 5 (ALK5) or ALK1. Following ligand binding, ALK5 phosphorylates and activates R-Smad 2/3 and ALK1 phosphorylates R-Smad1/5/8 (Figure 1.9).

Endothelial cells express both ALK1 and ALK5 (Goumans, Valdimarsdottir et al. 2002), and thus, respond to TGFβ1 by activation of two distinct TGFβ signaling pathways. Binding of TGFβ1 to TGFβRII induces the formation of the heteromeric complexes, TGFβRII-ALK1 and TGFβRII-ALK5, which activates smad1/5 and smad2/3 pathways, respectively (Miyazawa, Shinozaki et al. 2002). Low levels of TGFβ1 stimulate signaling through the TGFβRII-ALK1-ALK5 complex and subsequent activation of R-Smad1/5/8, which activates transcription of pro-angiogenic target genes such as inhibitor of DNA binding 1 (ID1) (Figure 1.9) (Goumans, Valdimarsdottir et al. 2002). By contrast, high levels of TGFβ1 promotes signaling through the TGFβRII-ALK5 complex and activation of R-Smad2/3, leading to transcription of anti-angiogenic target genes such as plasminogen- activator inhibitor 1 (PAI-1) (Figure 1.9) (Goumans, Valdimarsdottir et al. 2002). Another layer of complexity of TGFβ1 signaling in endothelial cells is added by the expression of a third TGFβ receptor or co-receptor, endoglin. Endoglin recruitment to TGFβRII-ALK1-ALK5 promotes activation of R-Smad1/5/8, and indirectly inhibits activation of R-Smad2/3 (Goumans, Valdimarsdottir et al. 2003, Lebrin, Goumans et al. 2004).

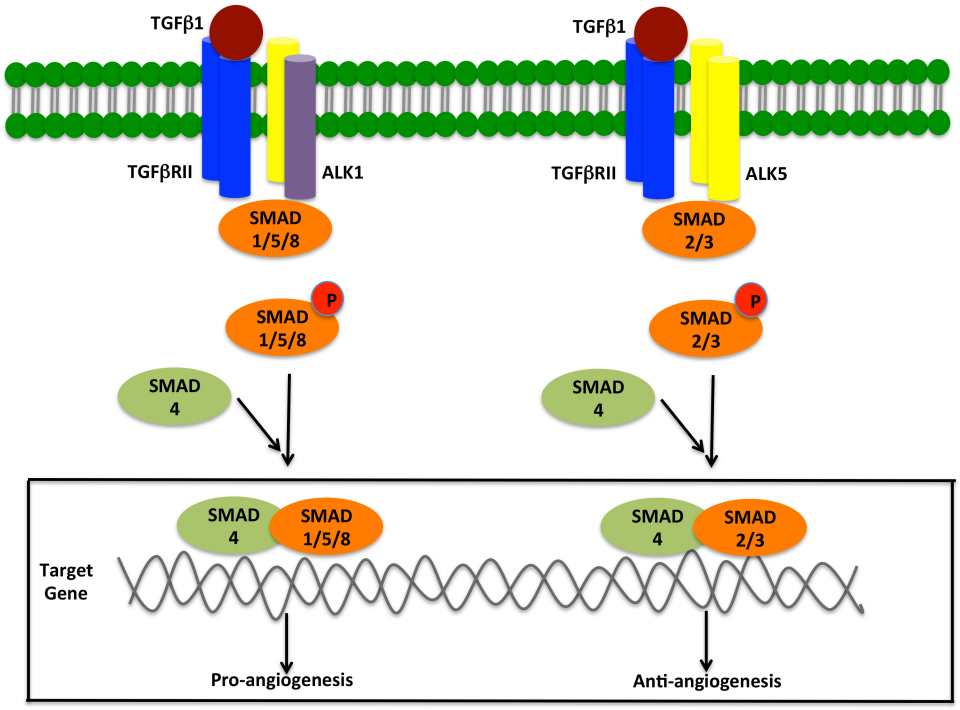


Figure 1.9: Mechanism of TGF□1 signaling in angiogenesis in endothelial cells. TGF□1 signaling depends on the composition of the receptor complex. TGF□1 can stimulate two different pathways in endothelial cells. Low levels of TGF□1 ligand activate signaling through the TGF□RII-ALK1-ALK5 receptor complex and activation of R-Smad1/5/8, resulting in transcription activation of pro-angiogenic target genes. On the contrary, high levels of TGF□1 favors signaling through the TGF□RII-ALK5 receptor complex and activation of R-Smad2/3, which induces transcription activation of anti-angiogenic target genes.

In addition, TGFβ1 signaling during angiogenesis is phase dependent. A study in ALK1 mutant mice demonstrated that during the activation phase of angiogenesis, which involves endothelial cell migration, proliferation and capillary lumen formation, TGFβ1 signals via ALK5, whereas ALK1 is required for the transition to the resolution phase and vascular remodeling by repressing the expression of angiogenic factors (Oh, Seki et al. 2000). Thus, it becomes obvious that the role of TGFβ1 in angiogenesis is dose-dependent and is highly affected by its cellular context and the experimental conditions.

Beside the canonical Smad pathway, TGFβ can activate several non-canonical pathways including, PI3K/Akt, extracellular signal regulated kinase (ERK), mitogen-activated protein kinase (MAPK) and small Rho GTPase (Zhang 2009). Activation of these pathways can occur rapidly in response to TGFβ stimulation, and hence, is considered to be independent of Smad. For instance, rapid activation of RhoA by TGFβ induces stress fiber formation (Edlund, Landstrom et al. 2002). Also, TGFβ can rapidly activate PI3K and consequently stimulate Akt phosphorylation (Bakin, Tomlinson et al. 2000, Shin, Bakin et al. 2001, Wilkes, Mitchell et al. 2005, Lamouille and Derynck 2007), and inhibition of TGFβRI abolishes TGFβ- induced activation of Akt by PI3K (Lamouille and Derynck 2007). It has been shown that PI3K is important for TGFβ-mediated fibroblasts proliferation and morphological changes (Wilkes, Mitchell et al. 2005). Furthermore, studies in pulmonary endothelial cells have shown that TGFβ1 induces permeability in a dose-dependent manner, which is associated with increased MLC2 phosphorylation and stress fibers formation (Birukova, Birukov et al. 2005).

1.8.3 PI3K/Akt Axis is Antagonistic of TGFβ/Smad Axis

In many TGFβ-stimulated cellular responses, PI3K/Akt signaling module antagonizes Smad effects. For example, activation of PI3K/Akt protects cells from TGFβ-induced apoptosis and growth inhibition (Chen, Su et al. 1998, Shin, Bakin et al. 2001, Song, Wang et al. 2006). Part of this protection is attributed to the physical interaction of Akt with Smad3 and preventing its phosphorylation (Conery, Cao et al. 2004, Remy, Montmarquette et al. 2004). However, another study reported that Akt suppresses Smad3 phosphorylation through phosphorylation and activation of its downstream target, mTOR (Song, Wang et al. 2006). In addition, FoxO, which is phosphorylated and inhibited by Akt, is required for TGFβ- induced transcription of inhibitors of cdks and growth inhibition (Seoane, Le et al. 2004).

1.9 Coupling Growth Factor Signals to Actin Cytoskeleton in Regulation of Angiogenesis

During angiogenesis, signals from growth factors, cytokines and extracellular matrix that regulate endothelial cell migration, proliferation, survival and differentiation converge on signaling pathways that regulate cytoskeleton rearrangement. For example, members of Rho family of GTPases are key regulators of actin cytoskeleton and activated by growth factors and extracellular matrix signals (Etienne-Manneville and Hall 2002). It has been shown that VEGF-stimulated angiogenesis in chorioallantoic membrane and

organization of endothelial cells into vessels in skin angiogenesis *in vivo* require activation of Rho (Hoang, Whelan et al. 2004). Also, inhibition of Rho GTPases abrogates endothelial tube formation in three-dimentional matrix *in vitro* (Cascone, Giraudo et al. 2003). As previously mentioned, RhoA regulates MLC2 phosphorylation and consequently myosin contractility and actin assembly via activation of its downstream effector ROCK. Control of myosin contractility and actin assembly is key in cell migration, and ROCK activity determines the net effect on cell migration. In endothelial cells, inhibition of ROCK disrupts vacuole formation and increases cell protrusions, leading to an inhibition of cord formation (Somlyo, Phelps et al. 2003). Furthermore, ERK/MAPK has been shown to promote endothelial cell survival and sprouting by inhibiting ROCK signaling during the active stage of angiogenesis (Mavria, Vercoulen et al. 2006). Increased MLC2 phosphorylation and myosin contractility due to Rho activation is required to suppress VEGFR2- stimulated endothelial cell migration and sprouting (Abraham, Yeo et al. 2009). On the other hand, MLC2 phosphorylation and myosin II activation are the key regulators of cytokinesis during cell proliferation (Matsumura 2005). These studies indicate that signaling modules that regulate myosin contractility and actin cytoskeleton play an important role in the various angiogenesis stages, and are orchestrated with growth factor signaling pathways.

1.10 Summary

Reversible protein phosphorylation is a key player in the various signaling pathways that regulate the biological and physiological processes of endothelial cells including,

survival, proliferation, migration, barrier function, angiogenesis and differentiation. The behavior of endothelial cells during these processes is regulated by a vast array of cues from growth factors, cytokines, and extracellular matrix. These cues usually converge on common signaling pathways that regulate these processes, such as PI3K/Akt, TGF/Smad, ERK-MAPK and pathways that regulate myosin II/actin contractility. Furthermore, endothelial cells undergo morphological changes during these cellular processes that require rearrangement of their cytoskeleton, therefore, growth factor-activated signaling pathways are integrated with pathways that control cytoskeletal rearrangements. The endothelial- predominant PP1c regulatory subunit, TIMAP, is linked to the growth factor and extracellular matrix- activated pathways and the myosin/actin regulatory pathways. Hence, it is conceivable that TIMAP may play an important role in endothelial cell-specific physiological processes, such as angiogenesis and barrier function. Although it has been already shown that TIMAP is crucial for the endothelial barrier function, the role of TIMAP in angiogenesis has not been unraveled. Also, whether TIMAP acts as a MYPT and regulate myosin phosphorylation in endothelial cells is still unknown. Therefore, in this thesis, I sought to study the function of TIMAP in endothelial cell angiogenesis and regulation of myosin phosphorylation.

1.11 Thesis Hypotheses and Objectives

The main objective of this thesis is to characterize the biological and molecular function of the PP1c regulatory subunit, TIMAP, in endothelial cells. The first hypothesis of this thesis is that the TGFβ1– downregulated TIMAP may play a key role in endothelial cell growth and angiogenesis. The second hypothesis is that the endothelial-predominant MYPT family member TIMAP may act as a MYPT in endothelial cells.

The specific aims of this research project are:

1- Determine whether TIMAP is required for endothelial cell growth and angiogenesis.
2- To identify the molecular mechanism of TIMAP-mediated regulation of endothelial cell growth and angiogenesis (Model 1, Figure 1.10).
3- To determine whether TIMAP/PP1c is an active myosin II phosphatase.
4- To investigate whether TIMAP acts as a MYPT in endothelial cells (Model 2, Figure 1.11).

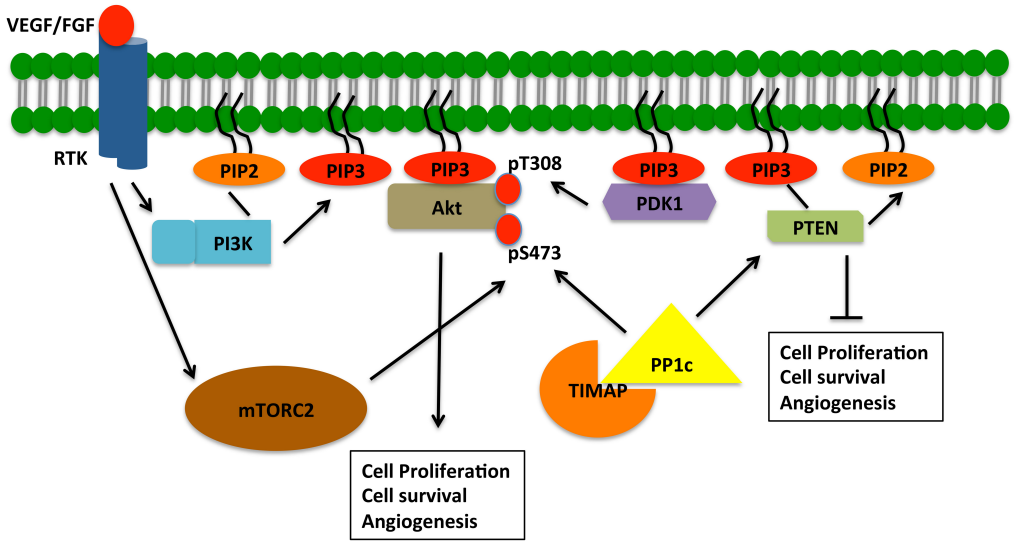


Figure 1.10. Model 1 represents a hypothetical role of TIMAP in endothelial cell growth and angiogenesis. TIMAP/PP1c might dephosphorylate and, hence, inactivate Akt, or it might dephosphorylate and, hence, activate PTEN resulting in inhibition of endothelial cell proliferation, survival and angiogenesis.

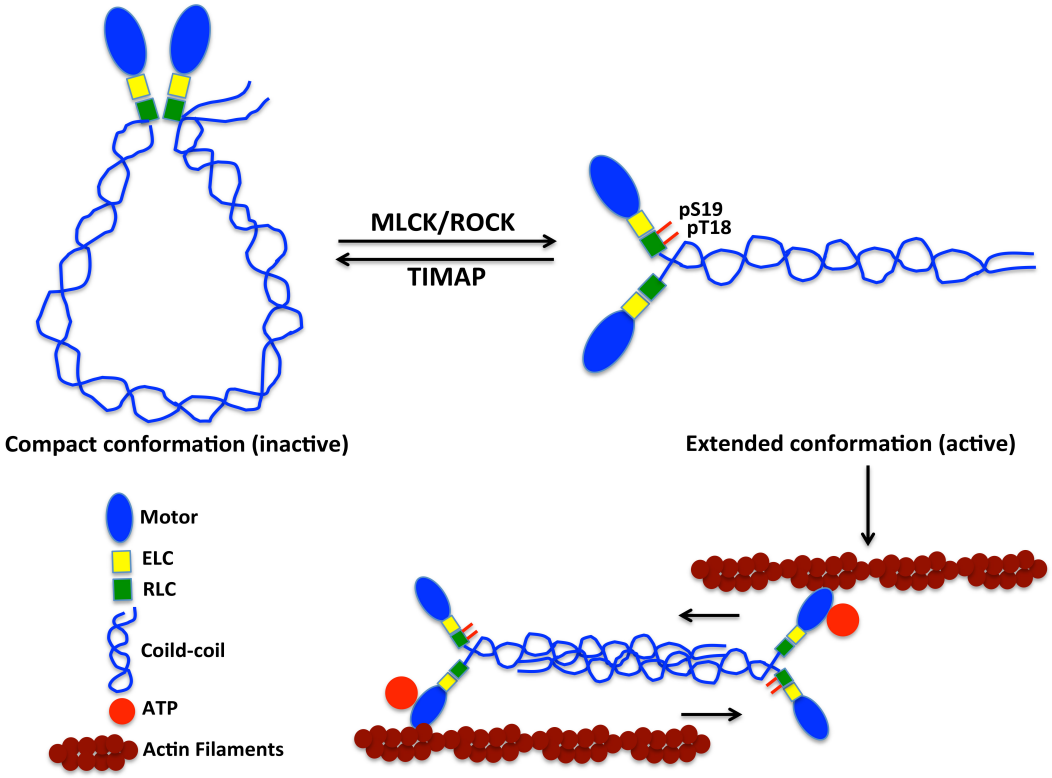


Figure 1.11. Model 2 represents a hypothetical role of TIMAP/PP1c as myosin II phosphatase in endothelial cells. Phosphorylation of MLC2 by MLCK and ROCK or other MLC2 kinases activates myosin II motor protein complex. Activated myosin II protein complex associates with actin filaments and utilizes ATP to generate contractile forces. In endothelial cells, TIMAP may dephosphorylate MLC2 and inactivate myosin II.

CHAPTER 2

Materials and Methods

2.1 Reagents

Reagents were form Sigma-Aldrich (Oakville, ON) unless otherwise specified. Human recombinant proteins VEGF165a (GF094) and basic FGF (GF003) were from Millipore (Billerica, MA, USA). PTEN inhibitor bpV (phen) (catalog#sc-221378) was from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Chicken anti-TIMAP antibody was custom-prepared by Avis laboratories (Tigard, OR) and previously described (Kim, Li et al. 2005). Briefly, affinity purified chicken anti- TIMAP antibody was produced using the synthetic peptide fragment (C)-YNGDIRETRTDQENKDPNP (amino acids 383-401) of TIMAP linked to KLH. This peptide sequence is conserved between human and bovine TIMAP, and is not found in MYPT3 (GenBank Accession No. NM_032902) nor in other known predicted protein sequences. Chicken anti-TIMAP IgY recognizes full length TIMAP, but not its N-terminus (amino acids 1-290). Polyclonal rabbit anti-TIMAP antibodies were previously described (Cao, Mattagajasingh et al. 2002). Monoclonal mouse anti-□Tubulin antibody (AA2, catalog#05-661) was from EMD Millipore and rabbit anti-phospho-Thr696 MYPT1 (catalog#07-251 was from upstate Millipore (Billerica, MA, USA). Monoclonal mouse anti-□actin antibody (AC-15, catalog#A5441), monoclonal mouse anti-FLAG M2 (catalog#F1804), mouse anti-GSK3□ (catalog#G6414) and rabbit anti- phospho-S9 GSK3□ (catalog#9336S) were from Sigma (Canada). Rabbit anti-cleaved caspase 3 (Ab D175, catalog#9661S), mouse anti-caspase

3 (3G2, catalog#9968), rabbit anti- phospho- S473 Akt (193H12, catlog#40585), rabbit anti-phospho-T308 Akt (catalog#92755), monoclonal rabbit anti- phospho-P44/P42 MAPK T202/Y204 (E10, catalog#91065) and rabbit anti-p44/p42 MAPK (catalog#9102), polyclonal rabbit anti-MLC2 (catalog#3672), anti-phospho-Thr18/Ser19 MLC2 (catalog#3674) and anti-phospho-Ser19 MLC2 (catalog#3675), and rabbit anti-phosphoThr850 MYPT1 (catalog#4563P) antibodies were from Cell Signaling Technology (New England Biolabs, Inc. CA). Monoclonal mouse anti-Akt (B-1, catalog#Sc-5298), mouse anti-PTEN (A2B1, catalog#Sc-7974), rabbit anti-phospho-S370 PTEN (catalog#Sc-101787), rabbit anti-phospho-S380/T382/383 PTEN (catalog#sc-101789), rabbit anti-MYPT1 (H-130 catalog#Sc-25618), monoclonal mouse anti-CPI-17 (F-4 catalog# Sc48406) and polyclonal goat anti-phospho-T38 CPI-17 (catalog#Sc17560) antibodies were from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Polyclonal rabbit anti-myosin IIA (catalog#PRB-440P) was from COVANCE, Inc. (Princeton, NJ, USA). Polyclonal rabbit anti-PP1beta (catalog#PA1-12379 Pierce) was from Thermoscientific. Mouse anti-Histidine tag (catalog#MCA1396) was from Serotec and mouse anti-Histidine tag (catalog#OB05) was from CALBIOCHEM. Monoclonal mouse anti-GFP 3E6 was from Invitrogen (molecular probes) and goat anti GFP was a gift from Dr. Luc Berthiaume (University of Alberta). Peroxidase-conjugated donkey anti-rabbit IgG and donkey anti-mouse IgG antibodies were from Jackson Immuno Research laboratories (West Grove, PA). Alexa Fluor 594 or 488-conjugated antibodies were from Life Technologies (Burlington, ON). Alexa Fluor 680 goat anti-mouse antibody for Licor imaging was from Molecular probes (Eugene, OR, USA) and IR Dye 800 conjugated A.P goat anti-rabbit p/n 611-132-122 was from ROCKLAND (Immunochemical, Inc. PA,

USA). Texas red phalloidin (catalog#00033) was from Biotium. Immunoflourescence secondary antibodies; Alexa Fluor 594 goat anti rabbit (H+L catalog#A11037), goat anti mouse (H+L catalog#A11032), goat anti chicken (H+L catalog#A11042) and Alexa Fluor 488 goat anti rabbit (H+L catalog#A11008) and goat anti mouse (H+L catalog#A11029) were from Lifetechnologies (Burlington, ON).

2.2 Cell Culture

-**Primary human glomerular endothelial cells** (the principal cell model used in the studies of this thesis) were purchased from Angio-proteomie (Boston, MA, USA) and maintained in EGM-2 MV Bulletkit growth medium (EBM-2 # CC-3156 basal medium supplemented with 5% fetal bovine serum (FBS), 0.04% Hydrocortisone, 0.4% human hFGF-B, 0.1% VEGF, 0.1% insulin growth factor (IGF), 0.1% Ascorbic acid, 0.1% human epithelial growth factor (hEGF), and 0.1% GA-1000) cat#: CC-4147 (LONZA, Walkersville, MD, USA). Cells were propagated in EGM-2 MV growth medium in tissue culture dishes (Sarstedt) coated with Quick Coating Solution cAP-01 from Angio-proteomie (Boston, MA, USA) in humidified air with 5% CO_2 incubator at 37°C for 72 hours. For experiments that required serum deprivation, EGM-2MV medium was replaced with EBM-2 basal medium for at least 16 hours before performing the experiments. Cell passages 2-7 were used for the experiments; passages 2-4 were used for experiments that were performed with TIMAP knockdown to ensure an optimal level of endogenous TIMAP protein expression at baseline, as TIMAP protein expression level declines with higher passages (data not shown), and passages 5-7 were used for

experiments with ectopic expression of TIMAP to ensure a low level of endogenous TIMAP protein expression at baseline.

-**Primary bovine aortic endothelial cells** were isolated from calf thoracic aorta as described (Arnet, McMillan et al. 1996) and propagated on 0.1% gelatin-coated tissue culture dishes in RPMI-1640 cat# R8758 supplemented with 15% FBS cat# 12483 (GIBCO, life Technologies) and 1% penicillin/ streptomycin in humidified air with 5% CO_2 at 37°C for 72 hours. Aortic endothelial cells were used to perform transient overexpression of ectopic TIMAP.

-Primary human umbilical vein endothelial cells (HUVEC) were obtained from Dr. Allan Murray (University of Alberta), whose laboratory isolates HUVEC routinely. HUVEC were propagated on 0.1% gelatin-coated tissue culture dishes in M199 medium cat# 11150 (GIBCO, life technologies) supplemented with 20% FBS, 1x glutamine, 1x endothelial cell growth supplement (ECGS) cat# 356006 (BD Bioscience) and 1% penicillin streptomycin cat# 14140 (GIBCO, life technologies) in humidified air incubator with 5% CO_2 at 37^{o}C for 72 hours. HUVEC were used to rule out whether the effect of TIMAP on MLC2 phosphorylation or cell growth is specific to glomerular endothelial cells.

-COS-7 cells (African green monkey kidney cells; CRL-1651, ATCC) were propagated on un-coated tissue culture dishes in DMEM medium cat# D5796 (Sigma) supplemented with 10% FBS and 1% penicillin streptomycin in humidified air incubator with 5% CO_2 at 37^{o}C for 72 hours. COS-7 cells were used for ectopic expression of TIMAP, as they do not express endogenous TIMAP protein.

- **Human foreskin fibroblasts (HFF1)** were obtained from Dr. Allan Murray (University of Alberta) and propagated on 0.1% gelatin- coated tissue culture dishes in EGM-2MV growth medium in humidified air incubator with 5% CO_2 at 37^{o}C for 72 hours. HFF1 were used as feeder cells in 3-dimensional angiogenesis assay.

2.3 RNA Interference

Non-targeting siRNA (AllStars Neg) cat#: 1027281 (Qiagen), was used as a non-specific control siRNA. TIMAP-specific siRNA (Hs_PPP1R16B_5) targeting PPP1R16B gene (TIMAP gene) cat#: S103115469 (Qiagen) was used to deplete human glomerular endothelial cells of TIMAP.

siRNA#1 (catalog# SI03115469):

Target sequence: 5'-TCCGGTCAGTGCCTACCAGTA3'.

Sense strand: 5'-CGGUCAGUGCCUACCAGUATT3'.

Antisense strand: 5'-UACUGGUAGGCACUGACCGGA3'.

siRNA#2 (catalog# SI03115469):

Target sequence: 5'-TCCGGTCAGTGCCTACCAGTA-3'.

Sense strand: 5'-CGGUCAGUGCCUACCAGUATT-3'.

Antisense strand: 5'-UACUGGUAGGCACUGACCGGA-3'.

TIMAP- specific siRNA constructs were designed to target the 3' un-translated region (3'UTR) of TIMAP messenger RNA (mRNA); this way TIMAP-specific siRNA will not

target ectopic TIMAP, but endogenous TIMAP only, in experiments were TIMAP knockdown and overexpression are combined. Based on trouble-shooting, two consecutive transfections and 72 hours after the first transfection were optimal to inhibit TIMAP protein expression by TIMAP-targeting siRNA. Human glomerular EC were cultured one day before transfection to achieve 50-60% cell density in EGM-2MV growth medium with no antibiotics (recommended to enhance transfection efficiency and reduce stress on cells caused by the transfection reagent) in p35 tissue culture dishes coated with quick coating solution (see cell culture), and transfected twice on consecutive days with 100nM non-specific control or TIMAP-specific siRNA using HiPerFect transfection reagent (catalog#301705) (QIAGEN) according to manufacturers directions. Transfection medium was prepared for control and TIMAP-specific siRNA and incubated at room temperature for 10-20 minutes before adding it to cells. Each dish received 100 ul EBM-2 basal medium containing 8 ul HiPerFect transfection reagent and 3ul siRNA. Before transfection solution was administered, cells were washed twice with 1 ml EBM-2 basal medium to remove the serum and growth factors, and incubated in 500 ul EBM-2 basal medium for 10-20 minutes in humidified 5% CO_2 incubator to be conditioned for the transfection medium. Master mixes were prepared when needed. Fresh EGM-2MV growth medium was replaced 4-6 hours after each transfection. TIMAP protein expression was optimally inhibited at 72 hours of the first transfection, as determined by western blot. All experiments were started at 72 hours of the first transfection (Figure 2.1).

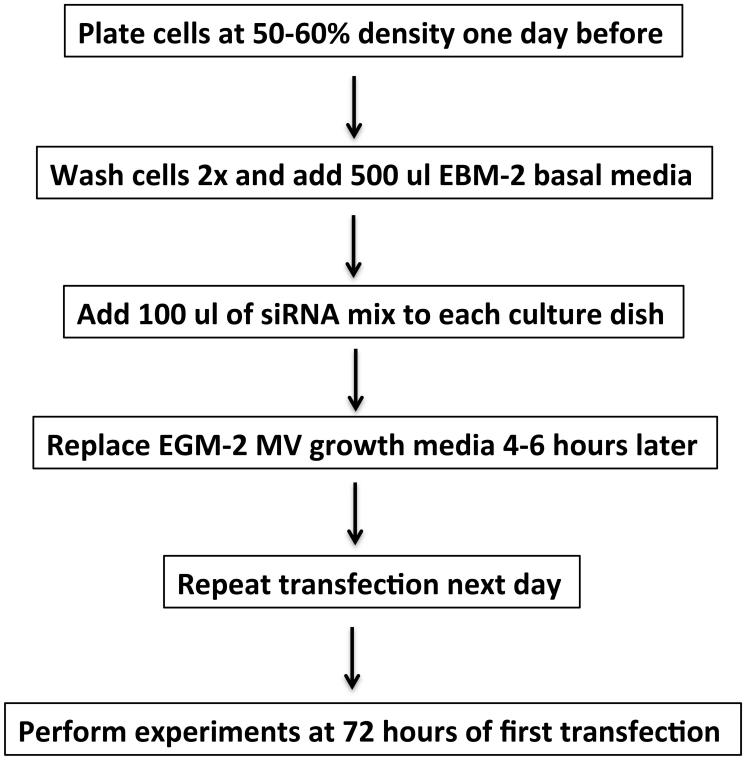


Figure 2.1: A diagram of the workflow for TIMAP depletion with siRNA in glomerular endothelial cells.

2.4 TIMAP- encoding Adenovirus

The DNA fragments of EGFP and EGFP-TIMAP fusion construct were previously described (Cao, Mattagajasingh et al. 2002) and amplified from the corresponding constructs in pEGFP-C1 vector (Figure 2.2) by PCR using the following primers (Figure 2.3):

Forward (both constructs): 5'-CCAGGTACCTACCGGTCGCCACCATGGTGAGCA-3'

Reverse (EGFP): 5'-CGACGTCGACCTAGTTACTTGTACAGCTCGTCCATGCCGAG-3'

Reverse (EGFP-TIMAP): 5'-CGACGTCGACTAGGAGATACGGCAACAGCCATG-3'

The PCR products were sub-cloned into Kpn I/Sal I site of pShuttle-CMV (Gift from Amy Barr, Department of Pediatrics, University of Alberta), originally gift from Bert Vogelstein (He, Zhou et al. 1998) (Figure 2.4). Clones were first screened by restriction enzyme digestion using Kpn I and Sal I (Figure 2.5). Sequence fidelity of the clones was confirmed by sequencing. The pShuttle-CMV–EGFP or –EGFP-TIMAP^{WT} constructs were linearized by digestion with restriction endonuclease PmeI, and then transformed into E. Coli (BJ5183) containing the adenoviral backbone plasmid pAdEasy-1 for homologous recombination. The recombinant plasmids were confirmed by PacI restriction endonuclease analyses. The linearized recombinant plasmid was transfected into the adenovirus packaging cell line HEK293A. The adenoviruses were propagated, harvested, purified and the viral titer was established as previously described (He, Zhou et al. 1998). Trouble- shooting was first performed to determine the optimal conditions of infection. For most experiments, cells were infected with 10 MOI (Multiplicity Of

Infection) of adenovirus for 48 hours unless otherwise specified. Human glomerular endothelial cells were plated one day prior to infection to achieve 70% cell density in EGM-2MV growth medium in p35 tissue culture dishes coated with quick coating solution (see cell culture); the adenovirus constructs were prepared in EGM-2MV growth medium containing 5 μg /ml polybrene reagent to enhance the efficiency of infection. Master mixes were prepared when needed, and each dish received 1 ml of infection solution. Next day another 1 ml of EGM-2MV growth medium was added to each dish and cells were left to grow for another day before the experiments were performed (Figure 2. 9). The level of TIMAP protein expression peaked at 48 hours and started to decline with cells approaching confluent monolayer density at ~72 hours, and the changes in the target phospho-proteins matched this pattern. The level of TIMAP expression was monitored by western blot.

For co-infection with PP1c, adenovirus with open reading frame of protein phosphatase 1 catalytic subunit beta isozyme (PPP1CB), transcript variant 1 with C- terminal Flag and His tag was obtained from (Vigene Biosciences, Parklawn Dr. Rockville, MD 20852 USA).

2.5 Transient Cell Transfection

The GFP-TIMAP fusion constructs were previously described (Cao, Mattagajasingh et al. 2002). For all transient transfections, cells were plated one day prior to transfection to achieve 70% cell density in cell growth medium (see cell culture). Endothelial cells were transfected using FuGENE 6 reagent (Roche, Laval, QE, Canada) and COS7 cells were

transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

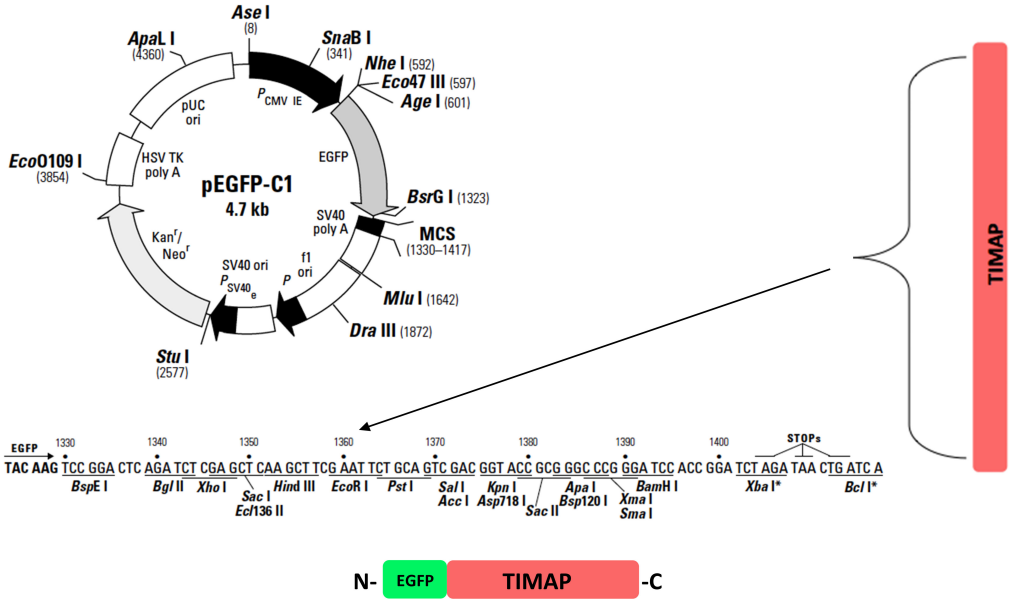


Figure 2.2: pEGFP-C1 vector (Clontech) and EGFP-TIMAP fusion construct. Copyright license from Clontech to publish and distribute the image of pEGFP-C1 vector as part of this thesis is granted.

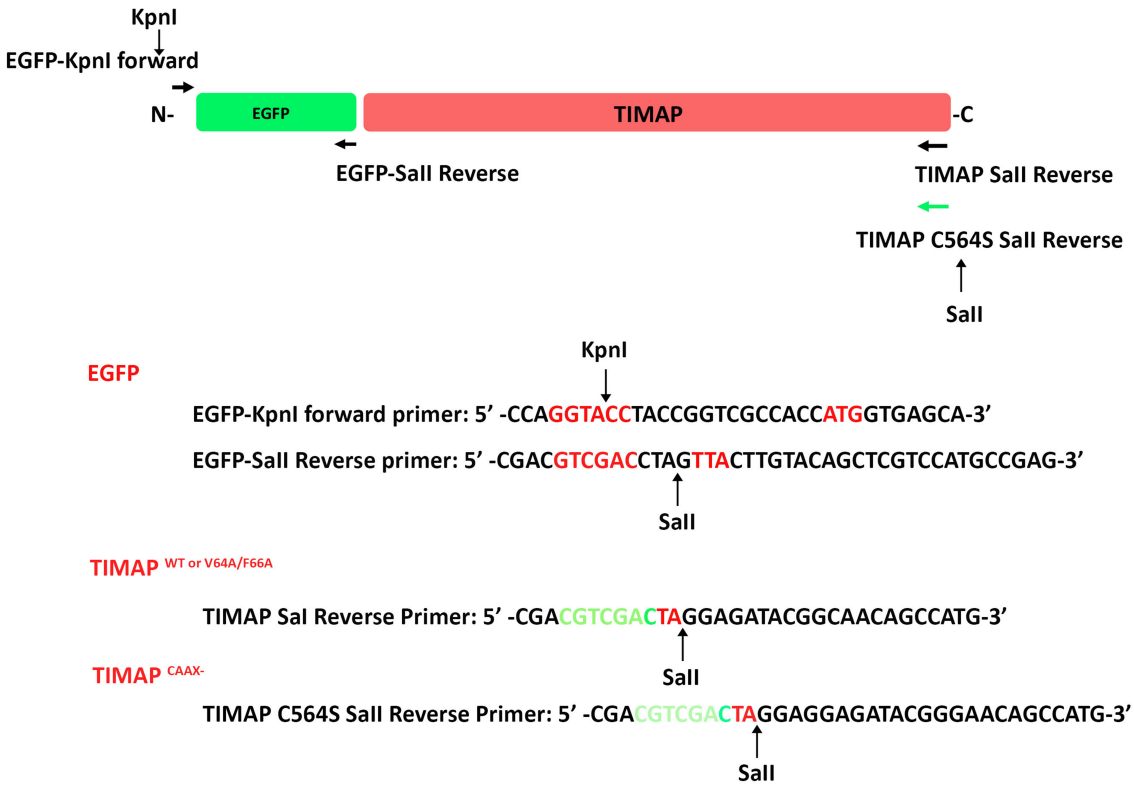


Figure 2.3: Primers used to amplify the EGFP and EGFP-TIMAP coding regions. In order to enhance the subsequent cloning, restriction enzymes Kpn I and Sal I were integrated in the forward and reverse primers, respectively.

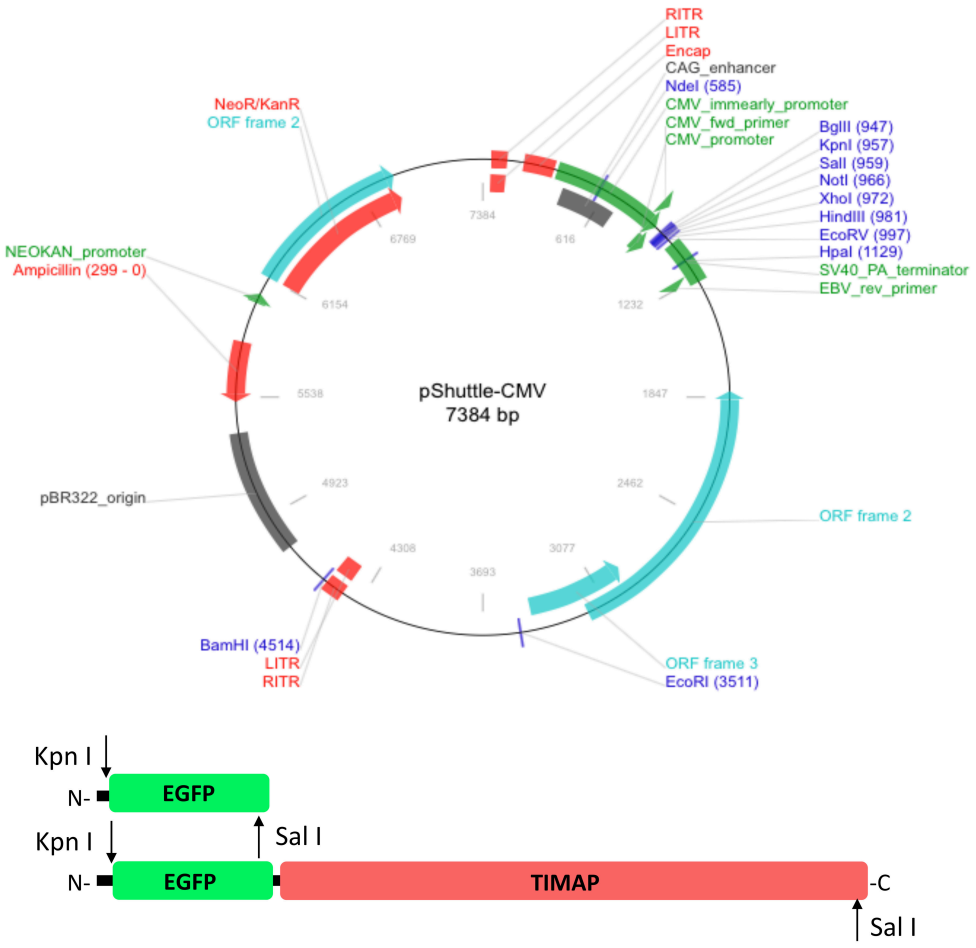


Figure 2.4: Sub-cloning of EGFP or EGFP-TIMAP coding fragments into pShuttle-CMV (Gift from Amy Barr, Department of Pediatrics, University of Alberta), originally a gift from Bert Vogelstein (He, Zhou et al. 1998). Copyright license from Addgene to publish and distribute the image of pShuttle-CMV vector as part of this thesis is granted.

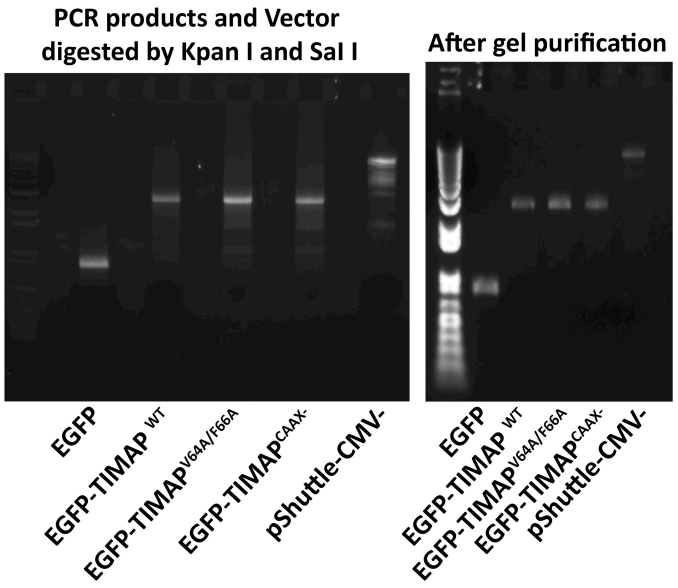


Figure 2.5: Digestion and purification of the PCR products and pShuttle-CMV. PCR products and pShuttle-CMV vector were first digested with Kpn I and Sal I. The plasmid digestion (left panel) and purification of digested DNA samples were performed using agarose gel electrophoresis. The DNA quantity estimation is determined by agarose gel electrophoresis and is shown in the right panel by agarose gel electrophoresis

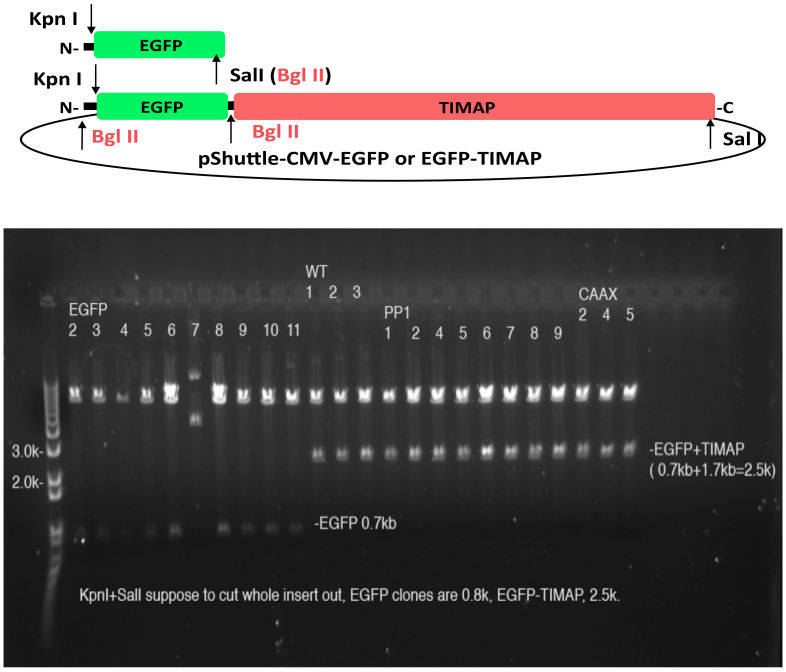


Figure 2.6: Screening for the pShuttle-CMV-EGFP or –EGFP-TIMAP clones by the restriction enzymes, Kpn I and Sal I. A simplified schematic structure of pShuttle-CMV-EGFP or –EGFP-TIMAP is shown in the top panel; the restriction enzyme sites are indicated. The bottom panel shows an agarose gel electrophosresis of the clones digested by Kpn I and Sal I; an approximate of 0.7 kb and 2.5 kb inserts were excised from the expected positive clones of pShuttle-CMV-EGFP and EGFP-TIMAP constructs, respectively.

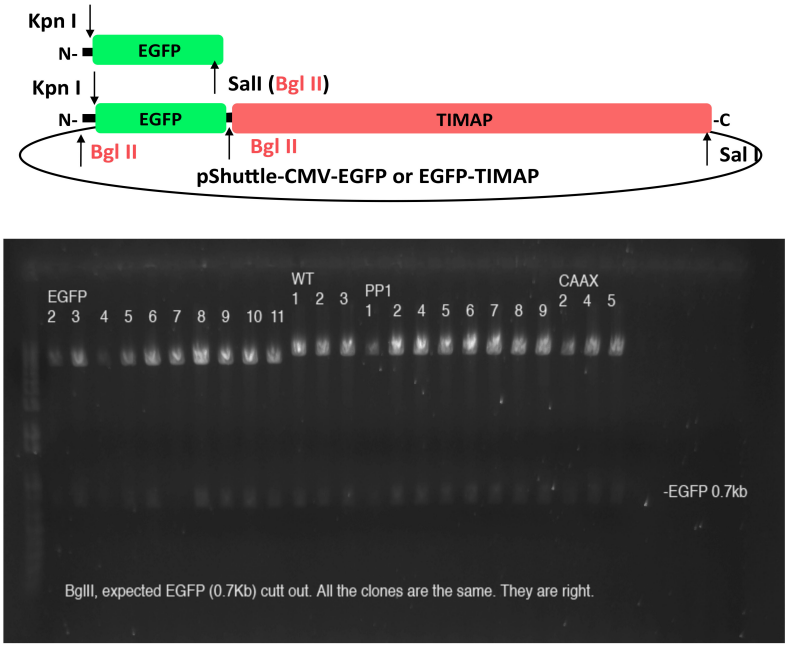
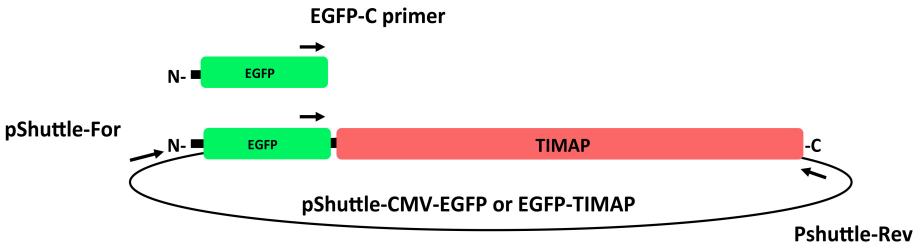


Figure 2.7: Screening for the pShuttle-CMV EGFP-TIMAP clones by the restriction enzyme Bgl II. A simplified schematic structure of pShuttle-CMV-EGFP or –EGFP-TIMAP is shown in the top panel; the restriction enzyme sites are indicated. The bottom panel shows an agarose gel electrophoresis of the clones digested by Bgl II; an approximate of 0.7 kb inserts were excised from the expected clones of both, pShuttle-CMV-EGFP or –EGFP-TIMAP.



sequencing analysis
Jan 10-2012

	pShuttle-For	EGFP-C1	pShuttle-Rev
EGFP-8	**Kpn1, EGFP starting codon (ATG); stop codon (TAA); linker (EcoR1).Right from Start codon ATG to EcoR1 linker and STOP TAA.**		
WT-3	**Kpn1, EGFP starting codon (ATG); linker (EcoR1). Right from beginning to EcoR1 linker**	**PP1 site WT. linker (EcoRI); TIMAP coding start (ATG). Right from EcoR1 linker to TIMAP 830..**	**SalI; TIMAP Stop codon(TAG). Right from 1000-end.**
PP1-6	**Kpn1, EGFP starting codon (ATG); linker (EcoR1). Right from beginning to EcoR1 linker**	**PP1 site mutated. linker (EcoRI); TIMAP coding start (ATG). Right from EcoR1 linker to TIMAP 737.**	**SalI; TIMAP Stop codon(TAG). Right from 1115-end.**
CAAX-2	**Kpn1, EGFP starting codon (ATG); linker (EcoR1). Right from beginning to EcoR1 linker**	**linker (EcoRI); TIMAP coding start (ATG). Right from EcoR1 linker to TIMAP 715.**	**CAAX mutated. SalI; TIMAP Stop codon(TAG). Right from 860-end.**

Figure 2. 8: Summary of the sequencing analyses of pShuttle-CMV- EGFP or -EGFP-TIMAP clones. A simplified schematic structure of pShuttle-CMV- EGFP or -EGFP-TIMAP clones are shown in the top panel; the restriction enzyme sites are indicated. The sequencing primers are shown in the top row; clone name and numbers are indicated in the left column, and the results are shown in the center.

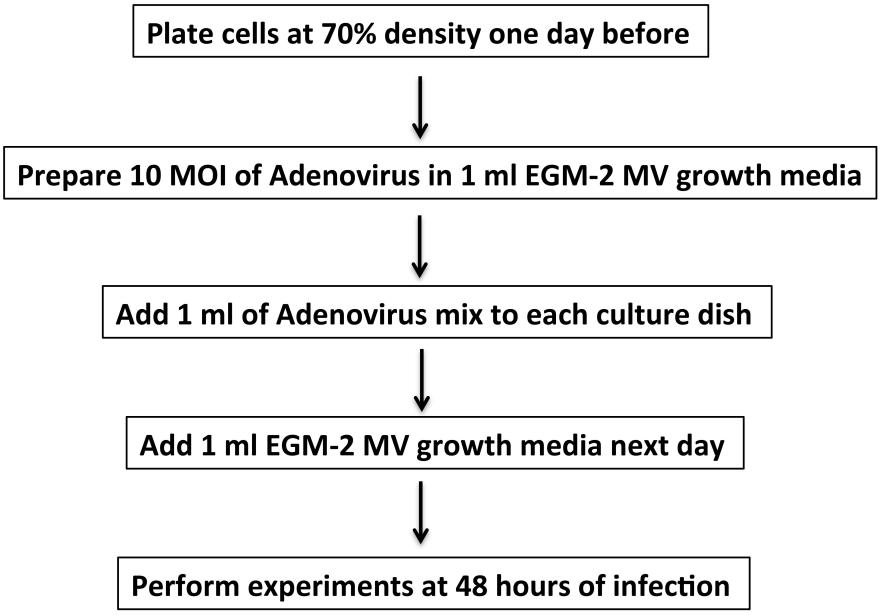


Figure 2.9: A diagram of the workflow for AdGFP-TIMAP infection in glomerular endothelial cells.

2.6 DNA Synthesis Assay

New DNA synthesis, which occurs during the S phase of the cell cycle, was examined to detect cell proliferation using Click-iT EdU Alexa Fluor 488 imaging kit (life Technologies, Burlington, ON, Canada). Click-iT EdU (5-ethynyl-2-deoxyuridine; a nucleoside analog of thymidine) is an alternative to BrdU (bromo-deoxyuridine; also a nucleoside analog) assay. It utilizes mild aldehyde-based fixation and detergent permeabilization to allow access of the small size detection reagent to the DNA, therefore, it does not require the harsh DNA denaturation step (heat, HCL and enzymes) found in the BrdU assay, which can disrupt the DNA quality and the cell morphology and consequently interrupt the ability of co-staining for other cellular molecules and sites. Detection is based on a click reaction, a copper-catalyzed covalent reaction between an azide (in alexa fluor dye) and an alkaline (in EdU). Briefly, Sub-confluent human glomerular endothelial cell monolayers transfected with siRNA for 48 hours were incubated with 10 uM EdU in fresh EGM-2MV growth medium over night. As a negative control of the assay, one plate of human glomerular endothelial cells received EGM-2MV growth medium without EdU. Next day, cell monolayers were washed twice with 1ml 1x PBS to remove the growth medium and excess EdU, detached using 0.05% Trypsin EDTA 1x (cat# 25300-054, GIBCO, Lifetechnologies) for 1 minute, collected and suspended in EGM-2MV growth medium to neutralize the Trypsin effect and spun for 5 minutes at 2000 rpm, and cells were incubated with a photo-stable Alexa fluor dye to fluorescently label the incorporated EdU. 5000 cells were examined for each condition using FACSCalibur, (Figure 2.11).

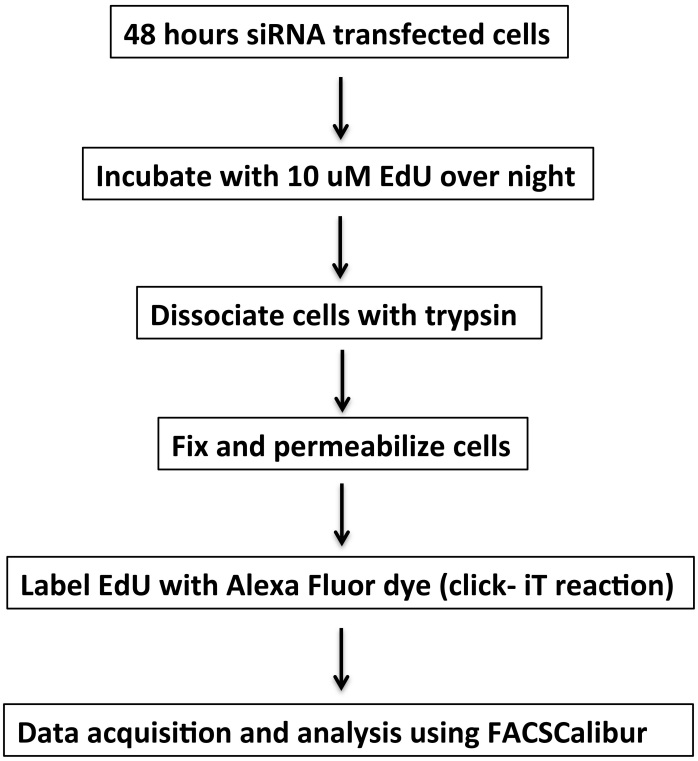


Figure 2.11: A diagram of the workflow for Click-iT EdU imaging assay to detect new DNA synthesis as a measure of cell proliferation.

2.7 Electrical Cell-substrate Impedance Sensing (ECIS)

Although DNA synthesis indicates activation of the cell cycle, it does not necessarily reflect the actual cell multiplication achieved by cytokinesis. To obtain an independent measure of cell number that reflects the actual increase in cell division, cell proliferation was studied using ECIS. ECIS is a real- time, label- free, impedance- based method to study the activities of cells in tissue culture, including cell morphological changes, cell locomotion and other behaviors achieved by changes in the cell cytoskeleton. Human glomerular endothelial cell monolayers transfected with siRNA for 72 hours were dissociated by mild trypsinization (as mentioned before) and plated onto 8W10E (W for well and E for electrode) arrays, applied biophysics (NY, USA), in 400ul EGM-2MV growth medium at a cell density of 25,000 cells/well and incubated in humidified 5% CO_2/ Air at 37^{o}C. Measurements of electrical impedance were determined every 5 minutes at 15 KH using ECIS model 1600, applied biophysics (NY, USA).

2.8 Apoptosis

Staurosporine- stimulated apoptosis of endothelial cells was described earlier (Thuret, Chiquet et al. 2003). Human glomerular endothelial cells transfected with control or TIMAP- specific siRNA for 72 hours were washed 2x with EBM-2 basal medium to remove dead cells, then cell monolayers were treated with 200 nM staurosporine in DMSO (0.05%) or DMSO vehicle in EBM-2 basal medium for 4-6 hours. Attached and unattached (dead) cells were harvested by scraping into ice-cold EBM-2 basal medium

followed by sedimentation for 2-5 minutes at 14000g in refrigerated centrifuge (4^{o}C). The pellet was then denatured in 2x Laemmle buffer at 99^{o}C for 10 minutes (Figure 2.13).

The activity of caspase 3/7 was studied using Apo-ONE homogeneous caspase-3/7 assay kit, Promega (Madison, USA), according to manufacturers instructions (Figure 2.13). Cells were treated and cell pellets were prepared as mentioned above. Cell pellets were re-suspended in ice-cold EBM basal medium and 40 ul aliquots of the cell suspension were distributed to individual wells of a 96 well plate. 40 ul of caspase 3/7 reagent mix, containing the fluorogenic substrate, was added and mixed by gently agitating the plate. The assay plate was then covered and allowed to freeze in -80^{o}C for at least 1 hour. Kinetic analysis of Caspase 3/7-mediated substrate cleavage was then detected using Fluoroskan Ascent microplate fluorometer (Thermo Scientific) at wavelength 485/527 nm excitation/emission, with 200 millisecond integration at 30 minutes interval for 20 hours. The assay was done for triplicate wells for each condition. Background activity was determined using the same media: reagent ratios and volumes, but without cells.

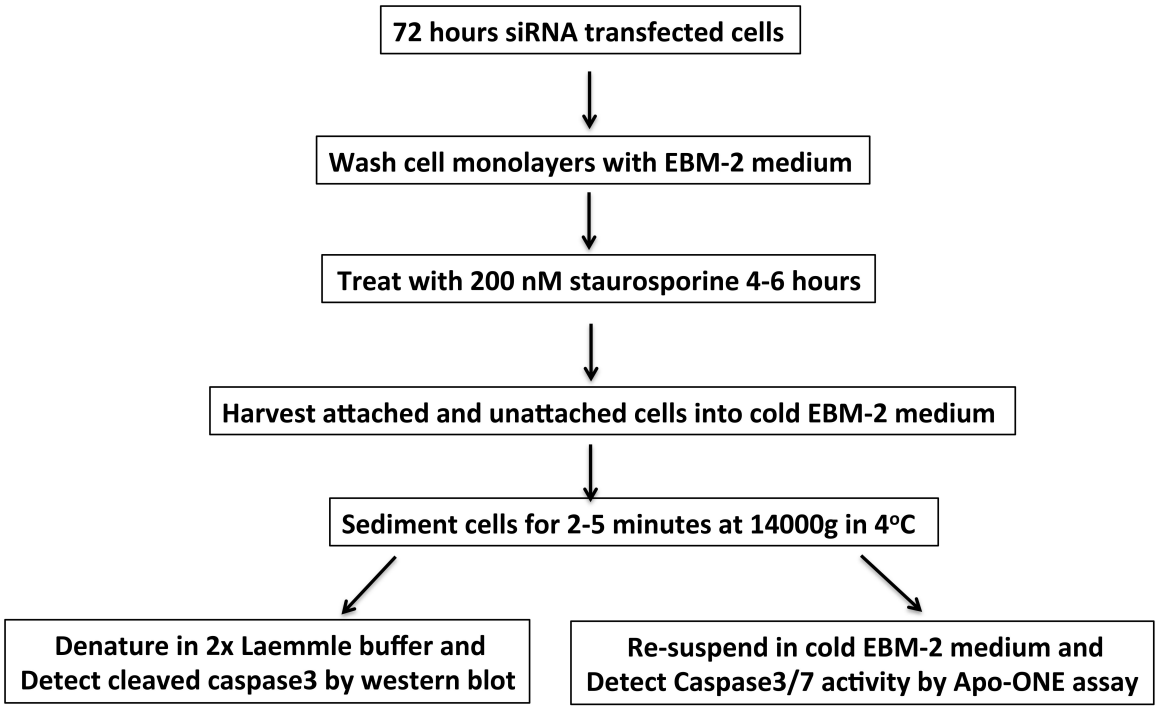


Figure 2.13: A diagram of the workflow to determine the role of TIMAP in apoptosis.

2.9 3-Dimensional Angiogenesis

The role of TIMAP in angiogenesis *in vitro* was evaluated using the 3-dimensional angiogenesis approach as previously described (Nakatsu and Hughes 2008). 3-dimensional angiogenesis assay is a superior alternative of the popular matrigel assay to study angiogenesis *in vitro*. While matrigel assay is excellent in defining homotypic interactions of endothelial cells in the formation of pre-capillary cords on a basement membrane-like matrix, it does not re-capitulate angiogenesis, but rather the differentiation of endothelial cells (Staton, Reed et al. 2009). The assay was performed as follows (Figure 2.14); siRNA transfected glomerular endothelial cells were dissociated by mild trypsinization and harvested into suspension in 1.5 ml EGM-2 MV growth media, then were loaded onto collagen- coated Cytodex 1 beads (GE Healthcare, Canada), pre-washed with EGM-2 MV media, at a density of 400 cells/bead in FACS tubes and incubated for 4 hours, with shaking every 20 minutes, in humidified air with 5% CO2 incubator. Medium containing unattached cells was removed and cells-coated beads were re-suspended in 2mg/ml fibrinogen solution containing 0.15 units/ml aprotinin. 0.625 U/ml Thrombin was added to the center of individual wells of 24 wells plate to initiate fibrin polymerization, followed by addition and gentle mixing with 0.5 ml fibrinogen/bead mixture. Fibrin polymerization was allowed to proceed for 30 minutes before addition of 500 μl EGM-2 MV growth media and incubation in humidified air with 5% CO2 incubator. Angiogenic sprouts were visualized after 18 hours of culture using Leica DMIRB microscope, Microsystem (Ontario, Canada) at 10X magnification. Images for 40 consecutive beads were obtained with a QIMAGING RETIGA EX camera

and evaluated using Openlab improvision software (Lexington, MA). Each data point represents the mean number of sprouts per bead or sprouts length, determined from 40 consecutive beads. Data from 3 independent experiments were used for statistical analysis.

To demonstrate tube formation, fibroblast (HFF1) feeder layers were cultured on the surface of polymerized fibrin to promote longer-term (5 day) endothelial cell survival. Lumen formation was observed after 5 days of culture (Figure 2.14)

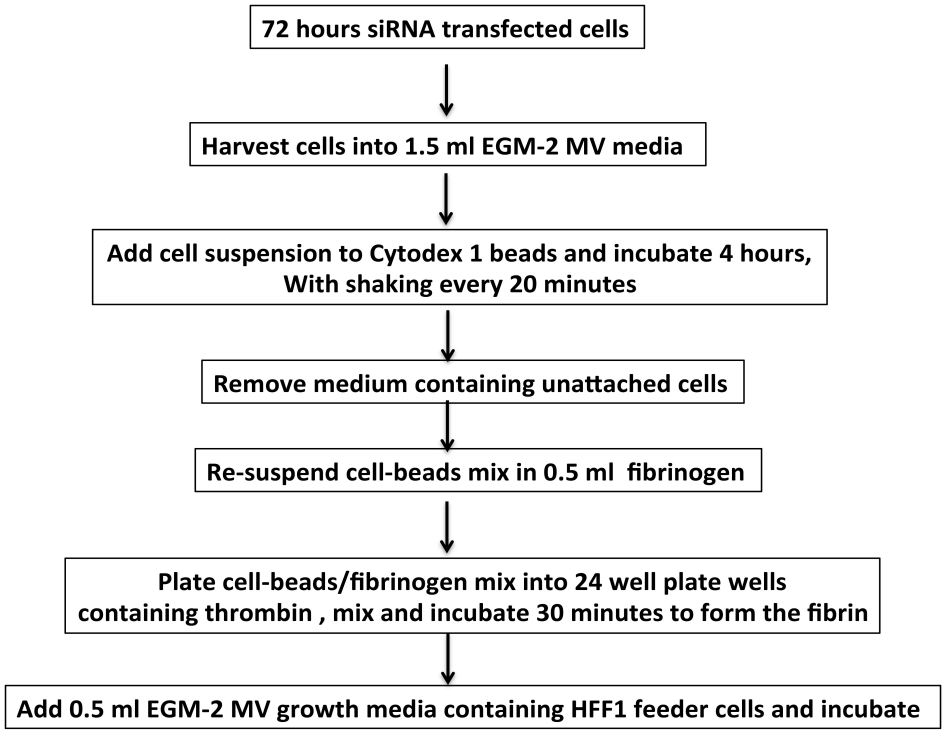


Figure 2.14: A diagram of the workflow for 3-dimensional angiogenesis culture.

2.10 LAMR1 Phosphorylation by PKA and PKC *in vitro*

2 μg His-LAMR1 1-200 was incubated with 2500 unit PKA catalytic subunit (New England Biolabs, Pickering, ON) in 30 μl reaction buffer (50 mM Tris-Cl, pH 7.5, 10 mM MgCl2, 100 μM ATP and 5 μCi □-^{32}P-ATP), or with 12.5 ng PKC catalytic subunit (Enzo Life Sciences, Inc, Farmingdale, NY) in PKC reaction buffer (40 mM MES, pH 6, 1 mM EGTA, 10 mM MgCl2, 100 μM ATP and 5 μCi □-^{32}P-ATP) for 60 minutes at 30 ^{o}C (Shopik, Li et al. 2013).

2.11. MLC2 Phosphorylation by MLCK for TIMAP/PP1c Phosphatase Assay *in vitro*

1 μg MLC2 was incubated with MLCK active fragment (aa 1425-1776) (Sigma, St, Louis, MO) in 25 μl reaction buffer (7mM MOPS, pH 7, 7 mM MgCl2, 1.4 mM EGTA, 0.56 mM EDTA, 10 μM DTT, 0.5 mM CaCl2, 750 ng Calmodulin, 100 μM ATP and 5 μCi □-^{32}P-ATP) for 60 minutes at 30 ^{o}C (Shopik, Li et al. 2013).

2.12 TIMAP and MLC2 Binding Assay *in vitro*.

Recombinant GST-TIMAP^{WT} and GST were prepared and quantified as previously described. Three μg His-MLC$_2$ (Millipore, Billerica, MA) were pre-incubated with 50 ng MLCK or kinase buffer alone at 30°C for 90 min in 25 μl of kinase reaction buffer (7

mM MOPS, pH 7.0, 7 mM $MgCl_2$, 1.4 mM EGTA, 0.56 mM EDTA, 0.5 mM $CaCl_2$, 0.06 μg calmodulin and 200 μM ATP, 0.07 mM DTT, and 1 μM Calyculin A). The reactions were brought to 50 μl with PBS, followed by addition of 7 μg GST-TIMAP and incubation overnight at 4ºC. A 40 μl aliquot of glutathione sepharose 4B beads (GE healthcare) and 350 μl binding buffer (20 mM HEPES, pH 7.5, 100 mM KCl, 10% glycerol, 1 mM EDTA) were added followed by end-over-end rotation for 90 min at 4ºC. The sepharose beads were then sedimented and extensively washed with cold PBS. The proteins were eluted from the sepharose beads by addition of 60 μl 2X SDS-PAGE sample buffer. The interaction between GST-TIMAP and His-MLC2 and phosphorylated His-MLC2 were detected by IB analyses using anti-MLC2 or anti-phospho-MLC2 antibodies, respectively.

2.13 TIMAP-inhibited MLC2 Phosphorylation by MLCK and ROCK *in vitro*

Three μg His-MLC2 were incubated with 7 μg GST-TIMAP or 3 μg GST in PBS overnight, followed by addition of 50 μl glutathione sepharose and 400 μl binding buffer. The reactions were rotated end over end for 90 min at 4ºC. The sepharose beads were then sedimented and extensively washed to remove unbound His-MLC2. Three μg His-MLC2 were added to the same amount of glutathione sepharose beads without GST-TIMAP or GST, and without sedimentation, to serve as free His-MLC2 control. Phosphorylation of TIMAP-associated or free His-MLC2 was initiated by addition of 50 μl of kinase reaction buffer containing 100 ng MLCK, and 1μl ^{32}P-γATP (5 μCi/μl, 3000

Ci/mmol). For ROCK1 phosphorylation, 50ng ROCK1 (Abcam, Boston, MA) was used, and $CaCl_2$ and calmodulin were omitted in the reaction buffer. The phosphorylation reactions were carried out at 30°C for 100 min, and stopped by adding 40 μl of 4X SDS-PAGE sample buffer. The proteins were separated on 10% SDS-PAGE gels. MLC2 phosphorylation was detected by autoradiography and western blot with anti-phospho-MLC2 antibodies. Total His-MLC2 was detected by western blot with anti MLC2 antibody, and served as loading control.

2.14 Cytoskeleton Preparation

Cytoskeletal fraction was prepared as previously described (Berryman, Bruno et al. 2004). Briefly, cell monolayers were washed twice with ice -cold 1X PBS supplemented with 5 nM calyculin A to preserve protein phosphorylation, and then were incubated with 500 μl of 0.5% Triton X-100, 1X complete proteinase inhibitor, 1X PhosStop (Roche) and 100 nM calyculin A in 1X PBS at room temperature. The supernatant was removed and cells were gently washed twice with cold 1X PBS to remove soluble material. The remaining non-soluble cytoskeleton was collected in 200 μl of 2X Laemmle buffer and boiled for 5 minutes.

2.15 Western Blot

Glomerular endothelial cells monolayers were washed once with ice-cold 1x PBS and harvested immediately in 200 µl hot 2X Laemmle buffer, followed by boiling for 10 minutes at 99^{o}C to denature the proteins. Proteins were separated, based on the molecular weight, on 6% (for MYPT1 and myosin IIA), 10% (for TIMAP, Akt, PTEN, PP1c, GSK3) and 12% (for MLC2 and CPI-17) SDS-Polyacrylamide gels in 1x running buffer (25 mMTris base, 192 mM glycerine, and 0.1% SDS in ddH_2O, pH 8.3) at starting electrical current of 25 mAmp/ gel for 20 minuets and finishing current of 35 mAmp/ gel. Proteins were then transferred onto PVDF membranes, Millipore (Billerco, MA, USA) in 1x transfer buffer (25mM Tris base, 192 mM glycine, and 10% methanol in ddH2O) at 100 Voltage for 1 hour. Levels of total and phosphorylated proteins were detected by probing the membranes with primary protein-specific antibodies (diluted at 1:1000) overnight at 4^{0}C, followed with species specific HRP-conjugated or fluorescent secondary antibodies (diluted at 1:10,000), and proteins were detected with ECL chemiluminescent substrate (GE, Healthcare, Baie d'Urfe QE, Canada) or LiCor Odyssey imaging (LI-COR Biotechnology, Lincoin, NE, USA), respectively. For LiCor method, the blocking buffer, which was also used to dilute the antibodies, was 1:1 Odyssey blocking buffer in 1x PBS, and the washes of excess antibodies were done using 1x PBS supplemented with TWEEN. For ECL method, the blocking and antibody dilution buffer was western blocker solution (Sigma) or home made 5% bovine serum albumin (BSA) in 1x Tris Buffered Saline with 0.05% TWEEN 20 (TBST), and washes were done using 1x TBST.

2.16 Immunoprecipitation (IP)

Endogenous TIMAP was immunoprecipitated from human glomerular endothelial cells as previously described (Li, Kozlowski et al. 2007). Briefly, endothelial cell monolayers were washed once with ice-cold 1x PBS and immediately harvested in cold IP lysis buffer (50 nM Tris-HCL, PH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and complete protease inhibitors (Roche Applied Science)). To preserve protein phosphorylation, the IP lysis buffer was supplemented with 30 mM sodium fluoride, 40 mM β-glycerophosphate, 20 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 100 nM calyculin A. Cells were homogenized and centrifuged at 17,000 g for 15 min at 4^{o}C. The soluble cell fraction was incubated with control chicken anti-IgY antibody or chicken anti-TIMAP antibody (1.2 ug/ul) at 4^{0}C for 1 hours to precipitate the target protein, followed by incubation with 50 ul goat anti-chicken IgG-coated beads (Aves) overnight at 4^{o}C to capture the precipitated protein. The beads were centrifuged at 2000 g for 5 minutes at 4^{0}C and washed twice with cold IP lysis buffer, twice with cold IP wash buffer (50 mM Tris-HCL, PH 7.5, 500 mM NaCl, 0.1% Nonidet P-40, 0.05% sodium deoxycholate) and once with cold IP wash buffer (10 mM Tris-HCL, PH 7.5, 0.1% Nonidet P-40, 0.05% sodium deoxycholate). The beads were re-suspended in 60 ul 2x Laemmle buffer and boiled for 10 minutes. To IP GFP-TIMAP, 0.5 ug/ml goat anti GFP antibody and protein A/G beads were used.

2.17 Immunofluorescence (IF)

Endothelial cells were washed once with ice-cold PBS and fixed immediately in 3.7% Paraformaldehyde for 20 min, permeabilized with 0.05% Triton X 100 for 5 min and blocked to reduce non-specific binding with 5% BSA in PBS for 1 hour. Proteins were detected by incubation with the specific primary antibodies at 4ºC overnight, followed by incubation with the species-specific secondary antibodies for 1 hour at room temperature. Cells were mounted with Prolong Gold antifade reagent containing DAPI (Life Technologies). Images were taken at 60X magnification using Zeiss Confocal Microscope, and at 40X magnification using Zeiss Axioplan Microscope.

2.18 Active Rho Pull-Down Assay

To determine changes in Rho small GTPase activation, active Rho pull-down was performed based on the manufacturer's instructions using Active Rho Pull-Down and Detection Kit from Thermo Scientific (part number 16116). Briefly, human glomerular endothelial cells infected with adenovirus for 48 hrs in 60 mm culture dishes were harvested into 500 µl Lysis buffer (25 mM Tris-HCl, pH7.2, 150 mM NaCl, 5 mM MgCl2, 1% NP-40 and 5% glycerol and proteinase inhibitors (Roach), incubated on ice for 5 minutes, centrifuged at 16,000 xg for 15minutes at 4˚C. 60 µl of the supernatant served as INPUT control, and the rest of the supernatant was transferred to spin cup containing 400 µg GST-Rhotekin-RBD and 100 µl glutathione resin. The reaction mixture was incubated at 4˚C for 1 hour with gentle rocking, followed by collection of

glutathione resin using centrifugation at 6000 XG for 15 seconds. Resins were washed with 400 µl Lysis buffer for 3 times. The proteins were eluted with 60 µl of reducing sample buffer (2x SDS Sample Buffer with 5% β-mercaptoethanol). Protein samples were then separated on 12% SDS-PAGE (Rho is ~ 24 kDa), transferred onto PVDF membranes, which were then blotted with rabbit anti-Rho primary antibody (diluted at 1:1000) overnight at 4^{o}C, followed by incubation with HRP-conjugated goat anti-rabbit IgG (H+L) (diluted at 1:500) for 1 hour at room temperature. The protein was detected by ECL chemiluminescent substrate.

2.19 Statistics

Data are shown as the mean ± SEM, and "n" represents the number of independent experiments. Statistical analysis was performed by paired Student *t* test using Excel. Data with multiple variables were analyzed by one-way ANOVA. *P* values < 0.05 were considered significant.

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CHAPTER 3

TIMAP promotes Angiogenesis by suppressing PTEN-mediated Akt inhibition in Human Glomerular Endothelial Cells

3.1 Introduction

Mechanisms that regulate angiogenesis have received massive attention since Folkman first advanced the theory that tumor growth depends on new blood vessel formation (Folkman 1971). Angiogenesis involves the sprouting and elongation of new blood vessels from pre-existing vessels followed by stabilization and vessel specification (Jeltsch, Leppanen et al. 2013). This process is crucial for normal blood vessel formation and patterning during embryonic development (Ferrara, Carver-Moore et al. 1996), for post-embryonic blood vessel repair (Banda, Knighton et al. 1982) and remodeling in response to hypoxia (Fong 2009), in the female reproductive cycle (Redmer and Reynolds 1996) and for the establishment of the placental vasculature (Chen and Zheng 2014). Pathological retinal (Crawford, Alfaro et al. 2009) and tumor (Welti, Loges et al. 2013) vascularization also depend on angiogenesis. While endothelial cells at the tip of the angiogenic sprout determine the direction and pattern of new blood vessel growth (Hellstrom, Phng et al. 2007), elongation of the new vessel stalk requires endothelial cell proliferation (Eilken and Adams 2010). A complex array of growth factors and their intracellular signaling systems regulate angiogenesis, including VEGFs (Connolly, Heuvelman et al. 1989), FGFs (Gospodarowicz, Brown et al. 1978), the angiopoietins

(Suri, Jones et al. 1996), and the transforming growth factor beta (TGF-β) family (Roberts, Sporn et al. 1986, Pardali, Goumans et al. 2010, Jakobsson and van Meeteren 2013).

Precise regulation of endothelial cell proliferation depends, in part, on control over the PI3K/Akt/PTEN signaling cascade (Varma, Lal et al. 2005) and is critical for the establishment of a normal vasculature and inhibition of pathological angiogenesis (Jiang, Zheng et al. 2000, Jiang and Liu 2009). Akt is a Ser/Thr kinase that regulates cell survival (Gerber, McMurtrey et al. 1998), proliferation (Rossig, Jadidi et al. 2001), migration (Morales-Ruiz, Fulton et al. 2000) and angiogenesis (Dimmeler and Zeiher 2000) via phosphorylation of diverse downstream proteins. Constitutively active Akt induces abnormal blood vessel formation (Perry, Banyard et al. 2007), and Akt deficient mice exhibit impaired fetal development and vascularization (Yang, Tschopp et al. 2003, Ackah, Yu et al. 2005). Akt is activated by phosphorylation on Thr 308 in its kinase domain by PDK1 and on Ser 473 in the regulatory domain by the mTOR complex 2 (mTORC2) (Brodbeck, Cron et al. 1999, Wick, Dong et al. 2000, Hresko and Mueckler 2005, Sarbassov, Guertin et al. 2005). These phosphorylation events occur at the plasma membrane after PI[3,4,5]P3-mediated recruitment of Akt and PDK1 (Andjelkovic, Alessi et al. 1997). In turn, PI[3,4,5]P3 is generated by PI3K-mediated phosphorylation of PI[4,5]P2 in response to activation of receptor tyrosine kinases (Franke, Yang et al. 1995, Alessi, Andjelkovic et al. 1996, Andjelkovic, Jakubowicz et al. 1996). Activation of Akt is opposed by the PTEN, which dephosphorylates PI[3,4,5]P3 and consequently blocks Akt activation (Cantley and Neel 1999, Hill and Hemmings 2002, Gericke, Leslie et al.

2013). PTEN deletion in mice results in endothelial cell hyperproliferation and disordered angiogenesis (Hamada, Sasaki et al. 2005).

In patients with chronic kidney disease (CKD), hypoxic stimuli do not result in a sufficiently robust angiogenic response to produce collateral vessels that might mitigate ischemia. Instead, microvascular rarefaction in the peritubular compartment contributes significantly to renal fibrosis in many forms of progressive CKD, in transplant nephropathy and ischemic injury in the aging kidney. It has been postulated that failed angiogenesis may be central to progressive renal fibrosis (Tanaka and Nangaku 2013). Furthermore, while endothelial cell sprouts invade the capillary cleft of developing nephrons during renal development (Liu, Dardik et al. 1999), glomerular endothelial cell injury in glomerulonephritis does not lead to a sprouting angiogenesis response. Instead, glomerular capillary loop number increases through intussusceptive growth (Notoya, Shinosaki et al. 2003, Wnuk, Hlushchuk et al. 2011), proliferating glomerular endothelial cells remain confined within the existing glomerular capillary basement membrane and angiogenic sprouts do not penetrate the mesangium. Finally, even though podocytes continually produce vastly more VEGF than any other differentiated cells *in vivo* (Breier, Albrecht et al. 1992, Simon, Grone et al. 1995), podocyte-derived VEGF does not serve as a stimulus for sprouting angiogenesis in mature glomeruli, but instead is required for the formation and maintenance of a properly differentiated, fenestrated endothelium (Eremina, Sood et al. 2003). Hence, controls over the angiogenic response in the kidney are more complex than those in the hypoxic tumor environment, and may not allow an angiogenic response in the face of hypoxia.

TIMAP was first identified in glomerular endothelial cells, and is highly expressed in peritubular vessels and glomeruli during kidney development. TIMAP is also highly enriched in proliferating endothelial cells in culture, where it localizes to the plasma membrane in endothelial cell projections (Cao, Mattagajasingh et al. 2002). Based on its amino acid sequence and on structural modeling, TIMAP is a PP1c regulatory subunit in the MYPT family (Cao, Mattagajasingh et al. 2002, Grassie, Moffat et al. 2011, Shopik, Li et al. 2013). The C-terminal CAAX motif of TIMAP is modified by prenylation leading to plasma membrane association (Cao, Mattagajasingh et al. 2002, Kim, Xu et al. 2011). In endothelial cells, TIMAP interacts directly with the non-integrin LAMR1 and regulates its phosphorylation (Kim, Xu et al. 2011, Shopik, Li et al. 2013). LAMR1 is up- regulated during endothelial cell proliferation and angiogenesis (Stitt, McKenna et al. 1998, McKenna, Simpson et al. 2001), and is required for capillary formation *in vitro* and angiogenesis *in vivo* (Kubota, Kleinman et al. 1988, Gebarowska, Stitt et al. 2002). TIMAP also regulates Moesin phosphorylation in endothelial cells (Csortos, Czikora et al. 2008).

In this study, we describe a previously unknown function of TIMAP in endothelial cells, namely that TIMAP expression is required for endothelial cell proliferation and survival and for *in vitro* angiogenic sprout formation. Our data suggest that TIMAP supports PI3K- dependent Akt phosphorylation by suppressing PTEN activity in glomerular endothelial cell.

3.2 Results

3.2.1 Inhibition of TIMAP Expression Attenuates Endothelial Cell Proliferation, Survival and Angiogenesis.

To study the function of TIMAP, its expression in human glomerular endothelial cells was inhibited with two distinct TIMAP-specific small interfering RNAs (siRNAs) or non-specific control siRNA. Western blot analysis (Figure 3.1 A) showed that TIMAP-specific siRNA reduced TIMAP protein abundance, on average by 76 ± 7.7 % (mean ± SEM, n= 3), relative to non-specific siRNA. While endothelial cells transfected with control siRNA formed confluent monolayers in complete medium 72 hours after transfection, the cell density of endothelial cells transfected with TIMAP-specific siRNA remained sparse (Figure 3.1 B). Numerous rounded, non-adherent cells were observed in cultures transfected with TIMAP-specific siRNA, but not in cultures transfected with non-specific control siRNA. We also used ECIS, which provides a measure of cell density when monolayers are sub-confluent (Xiao and Luong 2003), to obtain an independent measure of cell number. The human glomerular endothelial cells transfected with control or TIMAP-specific siRNA were plated on 8W10E ECIS arrays at a density of 25,000 cells per well, in complete medium and electrical impedance was monitored for 40 hours. Inhibition of TIMAP expression reduced the rate of electrical impedance development, on average by 93% ± 2% ($p < 0.05$, $n = 4$) (Figure 3.1 C and D). To obtain a direct measure of cell number, human glomerular endothelial cells transfected with control or TIMAP-specific siRNA were plated on 24 well tissue culture plates at a

density of 20.000 cells per well in complete medium, and cell number was counted every day. TIMAP depletion markedly reduced the number of cells (Figure 3.1 E), indicating that inhibition of TIMAP expression suppresses endothelial cell proliferation and/or survival.

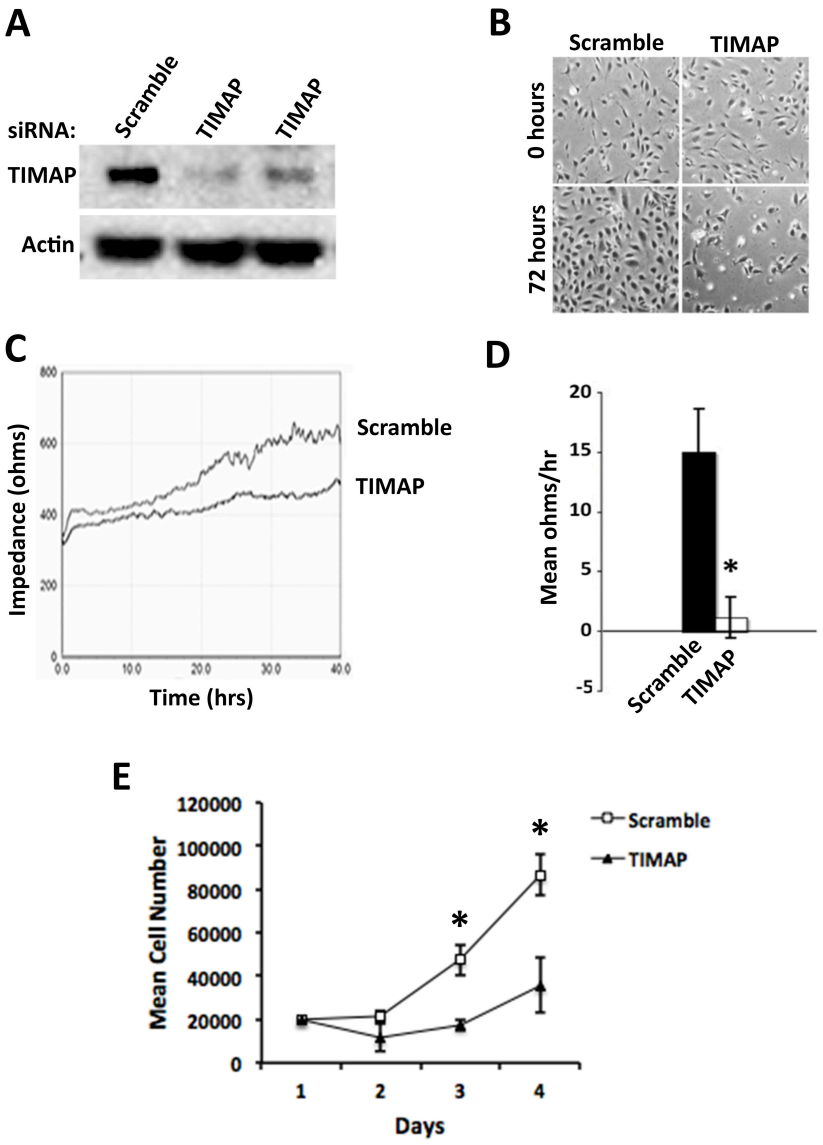

Figure 3.1. TIMAP deficiency impairs endothelial cell growth. A. TIMAP protein expression after transfection with TIMAP targeting siRNA1 (lane 2) and siRNA2 (lane 3), and non-specific control siRNA control (scramble, lane 1). **B.** Bright field images of glomerular endothelial cells transfected with control (scramble) or TIMAP siRNA, taken at 0 and 72 hours after transfection. **C.** ECIS analysis of glomerular endothelial cell growth. The lines show time-dependent changes in electrical

impedance across endothelial cell monolayer transfected with non-specific control siRNA (scramble) or TIMAP siRNA (TIMAP). **D.** Mean rate of electrical impedance development for endothelial cells transfected with nonspecific (scramble) or TIMAP siRNA (TIMAP) (mean ± SEM, n=4 independent experiments, *p < 0.05). **E.** Mean cell count of glomerular endothelial cells transfected with non-specific control siRNA (scramble) or TIMAP siRNA (TIMAP) at 1, 2, 3, and 4 days after plating (mean ± SD of triplicate data points, *p < 0.05. The experiment was repeated two more times with similar results).

To determine whether TIMAP alters sprouting angiogenesis, the human glomerular endothelial cells were transfected with TIMAP-specific or non-specific control siRNA, and seeded on collagen-dextran (Cytodex 1) beads to produce confluent monolayers. The beads were suspended in fibrin gels and cultured in complete medium, and the number of angiogenic sprouts was quantified 24 hours later. Angiogenic sprout formation was significantly lower in endothelial cells transfected with TIMAP-specific siRNA than in endothelial cells transfected with non-specific siRNA, (5.8 ± 1.3 vs. 10.2 ± 0.8 sprouts per bead, $p < 0.05$, $n = 3$) (Figures 3.2 A and B). Essentially identical results were observed with the second TIMAP-specific siRNA2 (Figure 3.4 A). The length of angiogenic sprouts was also significantly reduced in glomerular endothelial cells transfected with TIMAP-specific siRNA (129 pixels ± 6), compared to endothelial cells transfected with control siRNA (161 pixels ± 1, n=3, $P < 0.05$) (Figure 3.2 C). To determine whether TIMAP depletion affects capillary formation, HFF1 feeder cells were cultured on the surface of fibrin gels to enhance the survival of angiogenic sprouts, thus enable the formation of capillaries with lumen. As shown in Figure 3.2 D, at day 5 of angiogenesis glomerular endothelial cells formed capillaries with lumens (arrow). As expected, glomerular endothelial cells transfected with TIMAP-specific siRNA formed less complex network of capillaries. These results are consistent with the reduced number and length of angiogenic sprouts observed at earlier stages of sprouting angiogenesis. These data are consistent with observation observation in cultured endothelial cell monolayers that TIMAP depletion inhibits endothelial cell growth and/or survival and suggest that TIMAP is a positive regulator of angiogenesis.

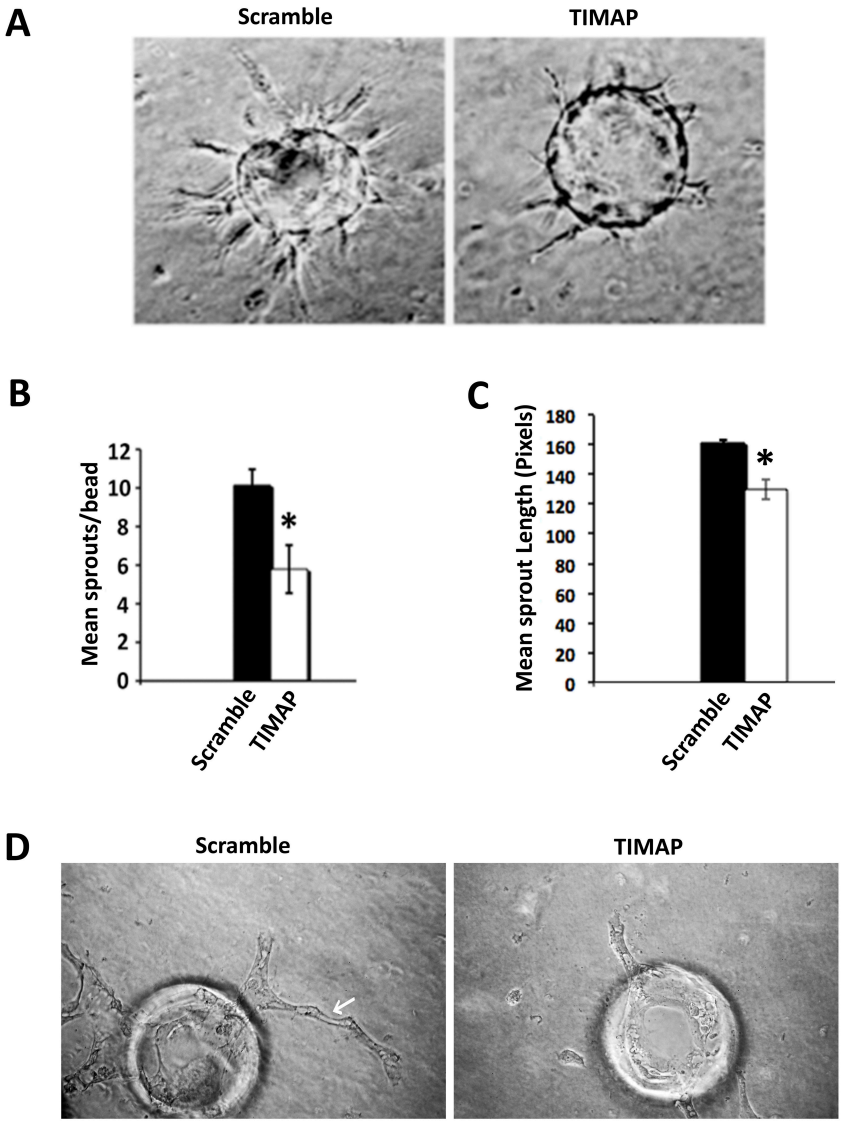

Figure 3.2. TIMAP deficiency impairs angiogenic sprouting. A. Representative bright field images of sprout formation, 24 hours after suspending beads containing glomerular endothelial cells transfected with non-specific siRNA (scramble) or TIMAP siRNA (TIMAP) in fibrin gels without feeder layer. **B.** Mean number of sprouts per bead 24 hours after suspending the beads in fibrin gels, (mean ± SEM, n=3 independent experiments, *p < 0.05). **C.** Mean sprout length 24 hours after suspending the beads in fibrin gels (mean ± SEM, n = 3 independent experiments, *p < 0.05). **D**. Representative bright field images of capillary formation in glomerular endothelial cells transfected with non-specific siRNA (scramble) or TIMAP siRNA

(TIMAP), 5 days after suspending endothelial cells-covered beads in fibrin gels with a fibroblast feeder layer. The arrow in the left panel points to a capillary lumen.

To determine whether reduced TIMAP expression alters cell proliferation, we next quantified DNA synthesis by determining the incorporation of 5-ethynyl-2'-deoxyuridine (EdU) using flow cytometry. In glomerular endothelial cells transfected with TIMAP-specific siRNA, EdU incorporation was 38 ± 2% ($p < 0.05$, $n = 3$) lower than in cells transfected with non-specific control siRNA (Figures 3.3 A and B), indicating that DNA synthesis was attenuated when TIMAP expression was suppressed. Essentially identical results were obtained with the second, distinct TIMAP-specific siRNA2 (Figure 3.4 B).

Given that the effect of TIMAP depletion on DNA synthesis was much less than the effect on electrical impedance development (Figures 3.1 C and D), and since rounded cells were observed in the monolayer of endothelial cells transfected with TIMAP-specific siRNA (Figure 3.1 B), we determined whether TIMAP silencing may also accentuate endothelial cell apoptosis by evaluating the abundance of cleaved caspase 3 and caspase 3/7 activity. To sensitize cells to apoptosis, the endothelial cells were treated with Staurosporine (200 nM, 4 hours) or vehicle. Compared to endothelial cells transfected with non-specific TIMAP siRNA, TIMAP depletion led to a significant increase in cleaved caspase 3 in staurosporine treated cells (3.6 fold ± 0.6), control siRNA (1.6 fold ± 0.8, $n=3$, $p<0.05$) (Figure 3.3 C). Consistent with the findings of caspase 3 cleavage, caspase 3/7 activity was 1.8 fold ± 0.2 higher in endothelial cells transfected with TIMAP-specific siRNA than in endothelial cells transfected with control siRNA ($p < 0.05$, $n = 3$) (Figure 3.3 D). These findings indicate that TIMAP is a survival factor for glomerular endothelial cells *in vitro*.

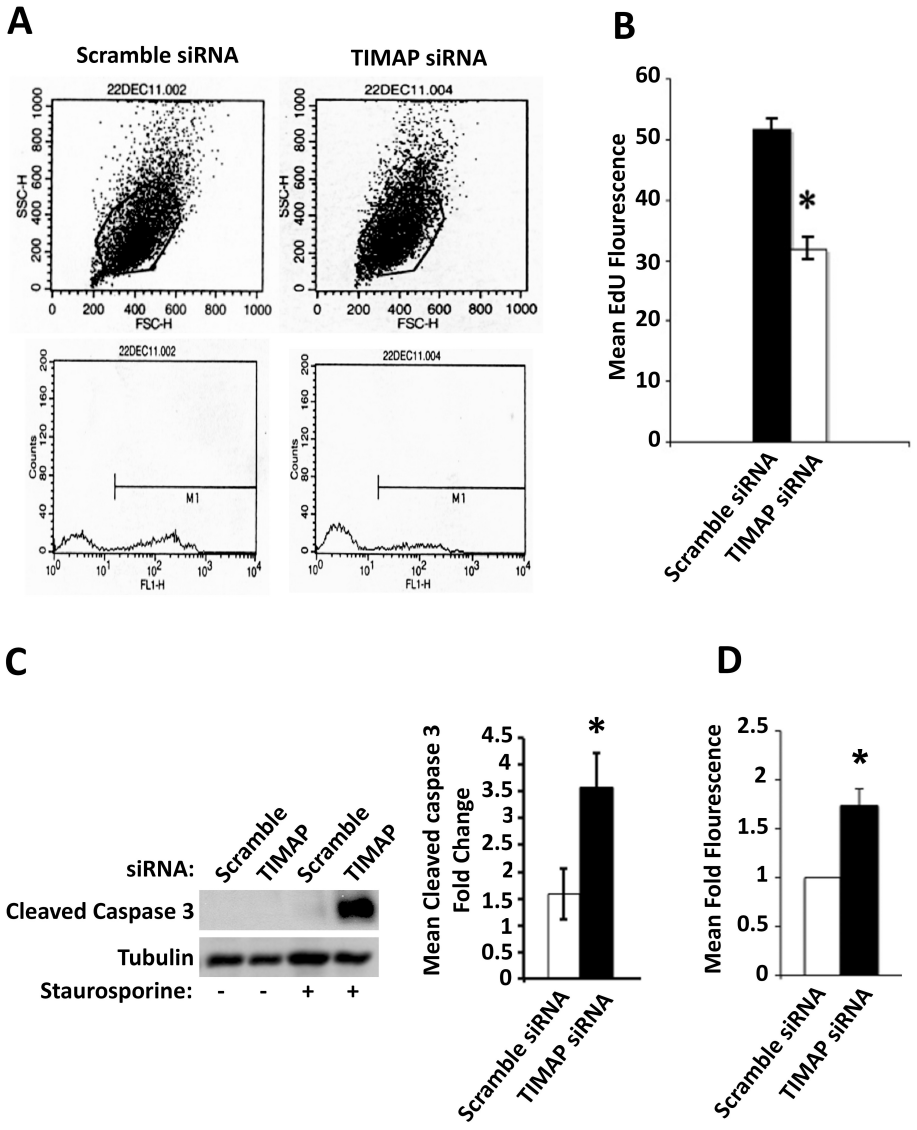

Figure 3.3. TIMAP silencing impairs endothelial cell proliferation and increases Caspase 3 activity. A. Flow cytometry of EdU incorporation (overnight) in glomerular endothelial cells transfected with non-specific siRNA (scramble) or TIMAP siRNA (TIMAP). **B.** Mean EdU incorporation (mean ± SEM, n=3 independent experiments, *p < 0.05). **C.** (Left panel): Western blot of cleaved caspase 3 in glomerular endothelial cells transfected with non-specific siRNA (lanes 1,3) or TIMAP siRNA (lanes 2,4) without (lanes 1,2) or with (lanes 3,4) Staurosporine (200 nM, 4 hours). (Right panel): Quantification of the fold change in cleaved caspase 3

induced by staurosporine (mean ± SEM, n = 3 independent experiments, *p < 0.05). **D.** Quantification of the fold change in caspase 3/7 activity in glomerular endothelial cells transfected with non-specific siRNA (scramble) or TIMAP siRNA (TIMAP) in the presence of 50 nM Staurosporine (4 hours) (mean ± SEM, n=3 independent experiments, *p < 0.05).

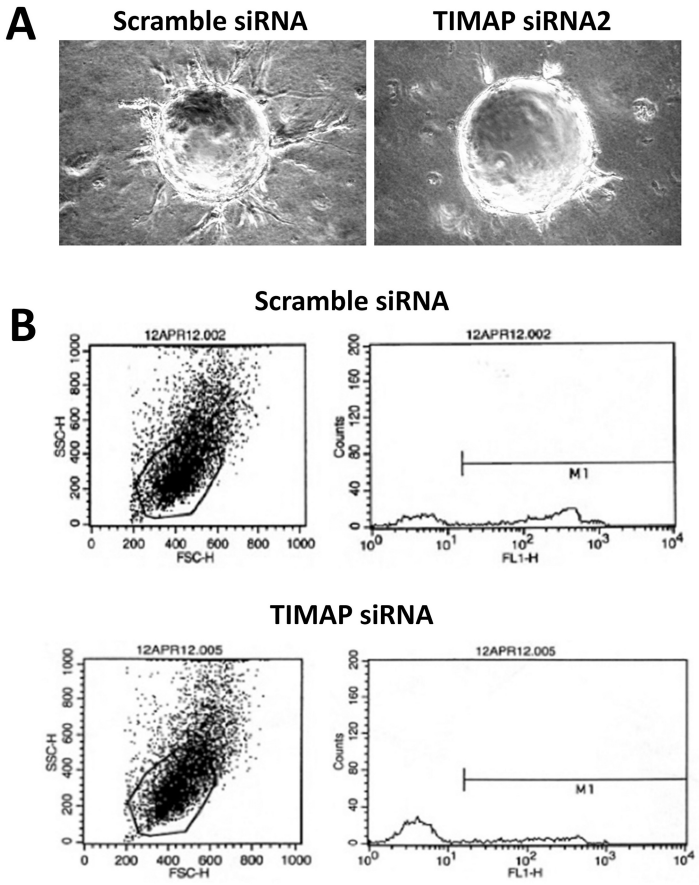

Figure 3.4. The Effects of TIMAP silencing with siRNA 2 on endothelial cell angiogenesis and proliferation. A. Representative bright field images of sprout formation, 24 hours after suspending beads containing glomerular endothelial cells transfected with non-specific scramble siRNA or TIMAP siRNA 2 in fibrin gels. **B.** Flow cytometry of EdU incorporation (overnight) in glomerular endothelial cells transfected with non-specific scramble siRNA or TIMAP siRNA.

3.2.2 TIMAP Depletion Impairs Akt Phosphorylation without Affecting ERK1/2 Phosphorylation.

The findings that TIMAP depletion reduces endothelial cell proliferation and angiogenesis, and accentuates apoptosis suggested an effect on the PI3K/Akt signaling pathway. We therefore evaluated whether TIMAP depletion alters the phosphorylation of Akt in endothelial cells. In human glomerular endothelial cells transfected with TIMAP-specific siRNA and cultured in serum-replete medium, there was a striking reduction of S473 Akt phosphorylation relative to that observed in cells transfected with non-specific control siRNA (Figure 3.5 A). Total Akt abundance did not differ between endothelial cells transfected with TIMAP-specific or non-specific siRNA, and the phosphorylation of ERK1/2 was similar in endothelial cells transfected with TIMAP-specific or non-specific control siRNA (Figure 3.5 A). Conversely, Akt phosphorylation increased when wild-type TIMAP was overexpressed in glomerular endothelial cells using adenoviral gene transfer (Figure 3.5 B). The seemingly different basal pSer 473 Akt between Figure 3.5 A and B reflects the fact that exposure time of the blot in B was reduced to compensate for the massive increase in pSer 473 Akt in adenoviral GFP-TIMAP-transfected cells. Phosphorylation of Akt on Thr 308 was also significantly reduced in endothelial cells transfected with TIMAP-specific siRNA, compared to cells transfected with control siRNA ($n=4$, $P < 0.05$) (Figure 3.5 C). In glomerular endothelial cells transduced with increasing MOI of GFP-TIMAP-adenovirus, a concentration-dependent increase in Ser 473 and T308 Akt phosphorylation proportional to the level of overexpressed TIMAP was observed. No effect on Ser 473 or Thr 308 Akt phosphorylation was observed when

endothelial cells were transduced with GFP-adenovirus (Figure 3.5 D). These results indicate that TIMAP depletion suppresses, and TIMAP overexpression stimulates, the PI3K/Akt pathway in glomerular endothelial cells.

Both, FGF-2 and VEGF-A act on endothelial cell tyrosine kinase receptors, activating the PI3K/Akt and the ERK1/2 MAPK signaling pathways, and both control endothelial cell proliferation and survival. To determine whether TIMAP depletion alters FGF-2 and/or VEGF-A signal transduction in human glomerular endothelial cells, cells were made quiescent by withdrawing serum and were then stimulated with 100 ng/ml FGF-2 or VEGF-A followed by determination of Akt and ERK1/2 phosphorylation. In the human glomerular endothelial cells transfected with TIMAP-specific siRNA, both FGF-2 and VEGF-A stimulated Ser 473 Akt phosphorylation, but Akt phosphorylation was consistently less than that in endothelial cells transfected with non-specific siRNA (Figure 3.5 E and F). Similar to the findings in endothelial cells cultured in serum replete medium (Figure 3.5 A), total Akt abundance as well as ERK1/2 phosphorylation were unaffected by TIMAP silencing. Conversely, glomerular endothelial cells transduced with GFP-TIMAP-adenovirus showed enhanced phosphorylation on Ser 473 in response to FGF, compared to cells transduced with control GFP-adenovirus (Figure 3.6 A). Similar to the findings in TIMAP depletion experiments, the increase in ERK1/2 phosphorylation in response to FGF was similar in cells transduced with either GFP- or GFP-TIMAP- adenovirus. The low levels of pSer 473 and pERK at baseline are due to the serum starvation that preceded FGF stimulation. These results indicate that activation of the PI3K pathway can occur in endothelial cells depleted of TIMAP but that Thr 308

and Ser 473 Akt phosphorylation is blunted when TIMAP is silenced, and that ectopic expression of TIMAP enhances the phosphorylation of Akt.

3.2.3 Akt Phosphorylation is restored by PTEN Inhibition in TIMAP deficient Endothelial Cells.

Since Akt phosphorylation depends on PI3K-mediated production of PIP3, which is reversed by PTEN activation (Cantley and Neel 1999), we next determined whether TIMAP might suppress PTEN activity in endothelial cells. We have previously shown that TIMAP localizes to endothelial cell projections (Cao, Mattagajasingh et al. 2002). In un-transfected human glomerular endothelial cells, endogenous TIMAP and PTEN strongly co-localized at the tip of endothelial cell projections (Figure 3.7 A). Furthermore, endogenous TIMAP and PTEN, but not Akt could be co-immunoprecipitated from human glomerular endothelial cell lysates (Figure 3.7 B). PTEN activity and localization are regulated by phosphorylation on several Ser/Thr residues in the C-terminal tail (Vazquez, Ramaswamy et al. 2000). Since TIMAP is a PP1c regulatory subunit, we determined whether TIMAP silencing alters PTEN phosphorylation.

In human glomerular endothelial cells, TIMAP depletion resulted in significantly less PTEN phosphorylation on S370 (ratio phosphorylated PTEN: total PTEN; 0.56 ± 0.13 in control siRNA vs. 0.12 ± 0.01 in TIMAP siRNA transfected cells ($p < 0.05$, $n = 5$), while phosphorylation on S380/T382/383 remained unchanged (Figure 3.7 C). The S370 residue of PTEN is phosphorylated by casein kinase 2 (CK2), and this

phosphorylation reduces PTEN activity (Torres and Pulido 2001, Miller, Lou et al. 2002). We therefore determined whether bpV(phen), a potent inhibitor of PTEN (Lai, Bao et al. 2009) relieves the effect of TIMAP silencing on Akt phosphorylation. Pilot experiments showed that bpV(phen) increased Ser 473 Akt phosphorylation in a concentration-dependent fashion in un-transfected, TIMAP-depleted and in cells treated with control siRNA, with a maximal effect at a concentration of 20 μM (Figure 3.6 B and C). Treatment of glomerular endothelial cells with 20 μM bpV(phen) eliminated the inhibition of Akt phosphorylation by TIMAP depletion (Figure 3.7 D and E). This finding suggests that impaired Akt phosphorylation in response to TIMAP silencing might be due to increased PTEN activity.

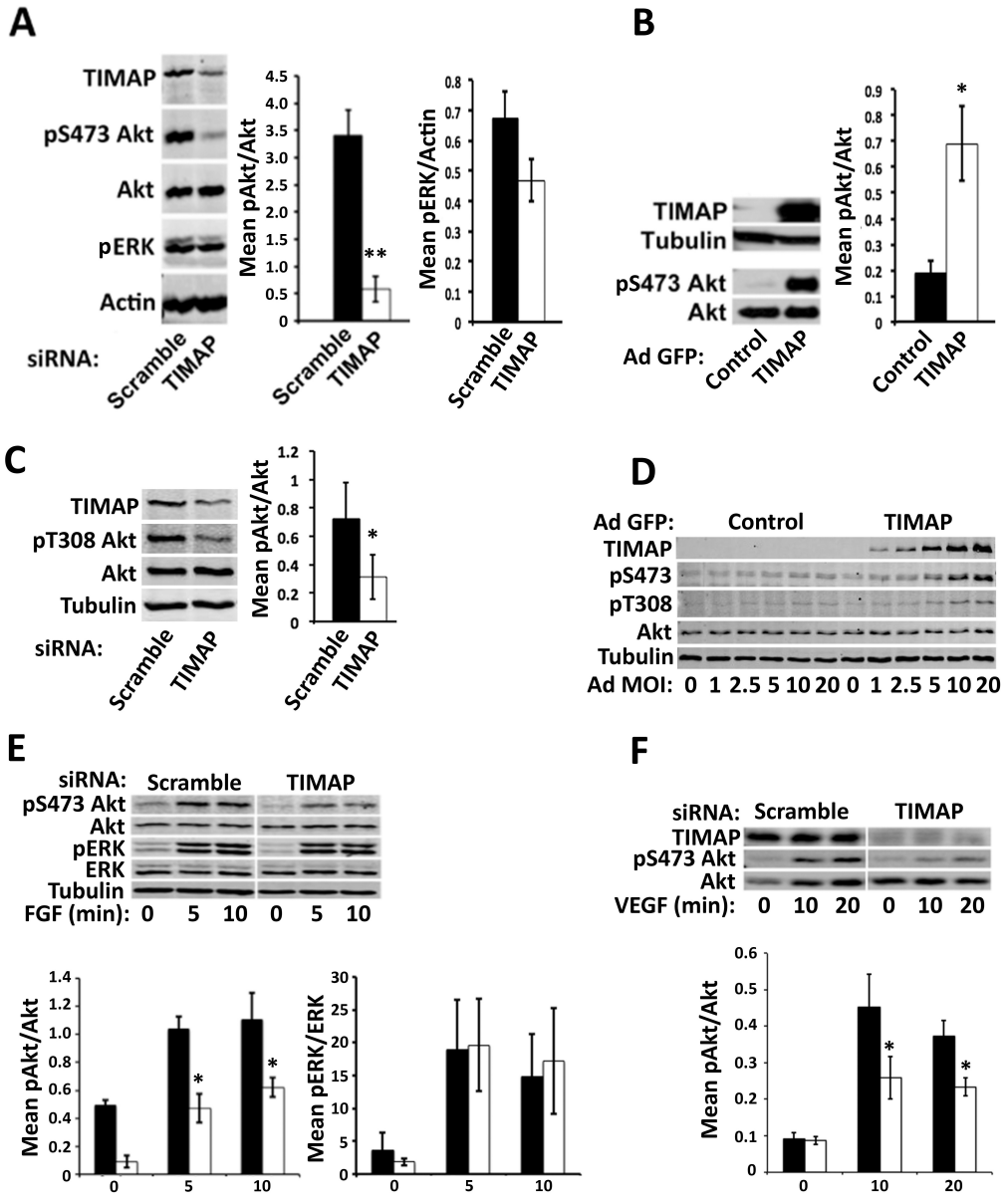

Figure 3.5. TIMAP silencing impairs Akt phosphorylation in endothelial cells. **A.** (Left panel): S473-Akt and ERK1/2 MAPK phosphorylation in glomerular endothelial cells transfected with non-specific control siRNA (scramble) or TIMAP siRNA (TIMAP) and maintained in complete growth medium**.** (Middle Panel): Mean ratio of pS473 Akt : total Akt (mean ± SEM, n=4 experiments, **P < 0.01). (Right Panel): Mean ratio of pERK1/2 : Actin (mean ± SEM, n=4 experiments, P > 0.05). **B.**

(Left Panel): S473 Akt phosphorylation in glomerular endothelial cells expressing AdGFP-TIMAP in complete growth medium (MOI = 20, mean ± SEM, n=4 experiments, *P < 0.05). **C.** (Left panel): T308-Akt phosphorylation in glomerular endothelial cells transfected with non-specific control siRNA (scramble) or TIMAP siRNA (TIMAP) and maintained in complete growth medium**.** (Right Panel): Mean ratio of pT308-Akt : total Akt (mean ± SEM, n=4 experiments, *P < 0.05). **D**. S473 and T308 Akt phosphorylation in glomerular endothelial cells expressing AdGFP control, or AdGFP-TIMAP at increasing MOI (0 – 20) in complete growth medium. A dose-dependent increase in S473- and T308-Akt phosphorylation is observed in endothelial cells transduced with AdGFP-TIMAP, but not AdGFP (representative of 3 experiments). **E.** (Top Panel): S473 Akt and ERK1/2 phosphorylation as a function of time in glomerular endothelial cells transfected with non-specific control siRNA (scramble) or TIMAP siRNA (TIMAP), serum-starved, and treated with 100 ng/ml FGF-2. (Bottom Left Panel): Quantification of the ratio of FGF-2 stimulated S473-Akt : Akt (mean ± SEM, n = 3 independent experiments, *p < 0.05). (Bottom Right Panel): Quantification of the ratio of FGF-2 stimulated pERK1/2 : total ERK (mean ± SEM, n = 3 independent experiments), scramble (black bars) and TIMAP siRNA (white bars). **F.** (Top Panel): S473 Akt phosphorylation as function of time in glomerular endothelial cells transfected with non-specific siRNA (scramble) or TIMAP siRNA (TIMAP), serum-starved, and treated with 100 ng/ml VEGF-A. (Bottom Panel): Quantification of the ratio of pS473-Akt : Akt for 3 independent experiments. (mean ± SEM, n = 3, *p < 0.05)), scramble (black bars) and TIMAP siRNA (white bars).

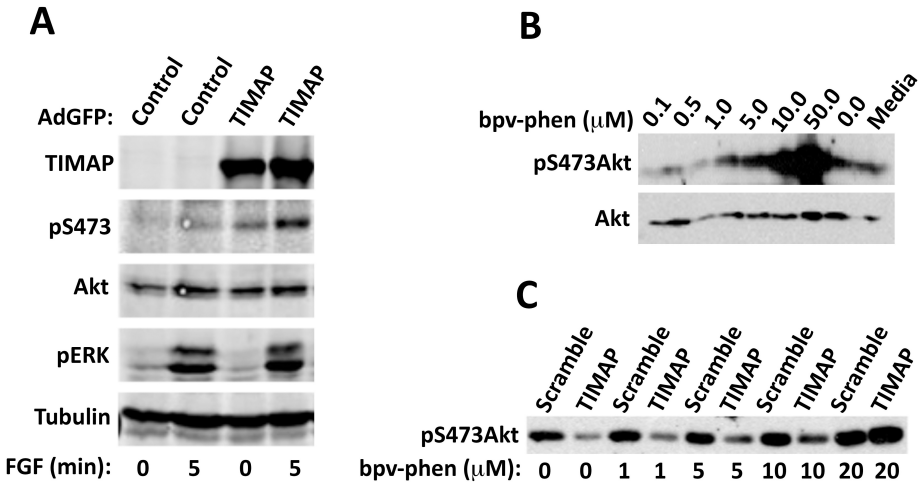

Figure 3.6. FGF-stimulated Akt phosphorylation in infected glomerular endothelial cells, dose-dependent Akt phosphorylation in regular and siRNA transfected glomerular endothelial cells A. S473 Akt and ERK1/2 MAPK phosphorylation in glomerular endothelial cells transduced with GFP (control) or GFP-TIMAP^{WT}, serum-starved and treated with 100ng/ml FGF for 0 or 5 minutes (representative of 2 experiments). **B.** Dose line effect of bpv-phen (30 minutes) on pS473 Akt in un-transfected human glomerular endothelial cells. **C.** Dose line effect of bpv-phen (30 minutes) on pS473 Akt in human glomerular endothelial cells transfected with the none-specific scramble siRNA or TIMAP siRNA (representative of 2 experiments).

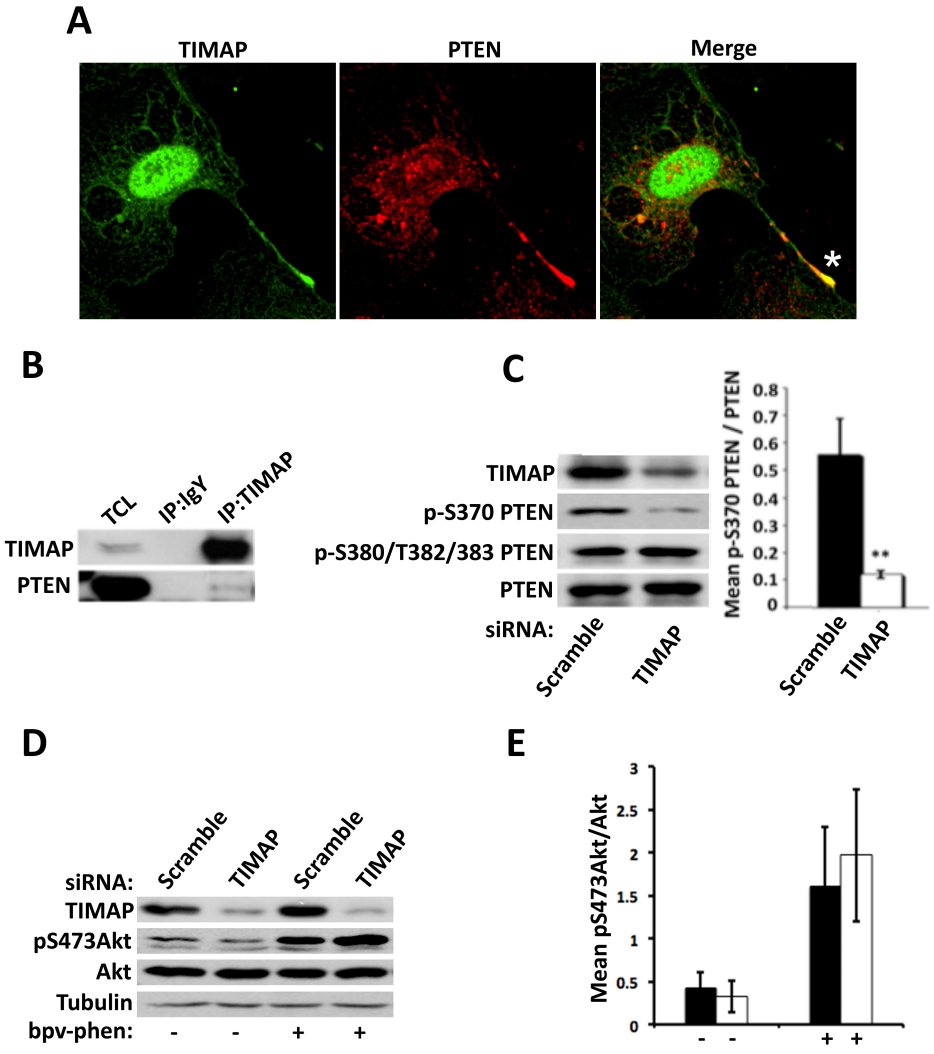

Figure 3.7. Functional interaction of TIMAP and PTEN in glomerular endothelial cells. A. Confocal immunofluorescence images of TIMAP (green) and PTEN (red) co-localization in glomerular endothelial cell protrusions (white asterisk) (representative of 3 experiments). **B.** Western blot of endogenous PTEN and TIMAP (top) or Akt and TIMAP (bottom) in total cell lysates (TCL) and in nonspecific IgY (IP-IgY) or anti-TIMAP IgY (IP-TIMAP) immunoprecipitates (representative of 3 experiments). **C.** (Left panel): Western blot of PTEN phosphorylation on S370, and on the S380/T382/T383 cluster in glomerular endothelial cells transfected with non-specific control siRNA (scramble) or TIMAP

siRNA (TIMAP) and maintained in serum-complete medium. (Right panel): Mean ratio of S370 PTEN: total PTEN (mean ± SEM, n = 5 independent experiments, **p < 0.01). **D.** Western blot of S473 Akt phosphorylation in glomerular endothelial cells transfected with non-specific control siRNA (lanes 1,3) or TIMAP siRNA (lanes 2,4) and treated (lanes 3,4) or not (lanes 1,2) with 20 μM of bpV(phen) for 30 minutes. **E.** Mean ratio of pS473-Akt: total Akt in glomerular endothelial cells transfected with non-specific control siRNA (black bars) or TIMAP siRNA (white bars) treated with (+) or without (-) bpV(phen) (20 μM, 30 minutes); (mean ± SEM, n = 5 independent experiments).

3.3 Discussion

This chapter describes a previously unknown function of TIMAP in endothelial cells. TIMAP silencing in glomerular endothelial cells impairs angiogenic sprout formation *in vitro*, an effect associated with reduced endothelial cell proliferation and increased caspase 3 activation. The physiological consequences of TIMAP silencing in endothelial cells are correlated with impaired Akt activation while the MEK/ERK cascade remains intact. We observed that TIMAP co- localizes with PTEN in endothelial cell protrusions, and that TIMAP silencing suppresses the inhibitory Ser 370 PTEN phosphorylation. Given that PTEN inhibition during TIMAP depletion rescues Akt phosphorylation, we postulate that TIMAP might regulate PTEN activity in endothelial cells and that suppression of TIMAP synthesis might dis-inhibit PTEN, in turn blocking Akt-driven endothelial cell proliferation and survival pathways.

The possibility that TIMAP supports endothelial cell proliferation *in vitro* was initially entertained because glomerular endothelial cells consistently failed to produce confluent monolayers *in vitro* after TIMAP silencing (Figure 3.1 B). That this effect is biologically significant is suggested by the observation that angiogenic sprout formation *in vitro* was also strongly inhibited by TIMAP silencing in glomerular endothelial cells (Figure 3.2), and is consistent with our previous observations that TIMAP is expressed in developing blood vessels (Cao, Mattagajasingh et al. 2002).

Angiogenic sprouting requires formation of guiding endothelial tip cells and elongation due to endothelial cell proliferation in the new vessel stalk (Gerhardt, Golding et al. 2003). Stabilization of the newly formed vessel and inhibition of regression

depends critically on endothelial cell survival (Alon, Hemo et al. 1995). TIMAP silencing inhibits both endothelial cell proliferation and survival, shown by the findings that TIMAP depletion reduced DNA synthesis and increased Caspase 3 cleavage as well as Caspase 3/7 activity (Figure 3.3). These findings are in agreement with a genomic study that was conducted in mice sensitized to c-Myc-induced apoptosis, which identified TIMAP as a novel oncogene that promotes cell survival (Mendrysa, Akagi et al. 2010). Also, the findings are consistent with our previous data that TIMAP is expressed in several leukemia cell lines including, human acute myelogenous leukemia, lymphoblastic T cell leukemia (Cao, Mattagajasingh et al. 2002), which are known to exhibit high survival and proliferation profiles.

Binding of VEGF, FGF-2 and other angiogenic agonists to their specific receptor tyrosine kinases results in activation of the Raf/MEK/ERK and the PI3K-Akt signaling cascades (Nakashio, Fujita et al. 2002, Yang, Wang et al. 2008). Both cascades are required for angiogenic sprout formation and survival of endothelial cells in the newly formed vessel (Shen, Ji et al. 2012) and inhibited by VEGFR-2 antagonists (Alon, Hemo et al. 1995, Hu and Fan 1995, Zhu and Witte 1999). However, we observe that ERK1/2 phosphorylation is essentially unaffected by TIMAP depletion or overexpression, whereas phosphorylation of Akt on Ser 473 and Thr 308 is profoundly reduced by TIMAP silencing and enhanced by TIMAP overexpression in endothelial cells grown in serum-replete medium (Figure 3.5). This observation suggests that only the PI3K/Akt pathway is suppressed by TIMAP silencing while signaling via the Raf/MEK/ERK pathway remains intact. The findings that VEGF-A or FGF-2 fully activates ERK1/2 phosphorylation when TIMAP is silenced (Figure 3.5) also imply that tyrosine kinase

receptor activation by FGF-2 and VEGF-A remains intact when TIMAP expression is inhibited.

Akt activation requires its recruitment to the plasma membrane by PI[3,4,5]P3, which is generated by receptor tyrosine kinase-stimulated PI3K (Sarbassov, Guertin et al. 2005). PI[3,4,5]P3 is dephosphorylated by the powerful tumor suppressor PTEN, in turn inhibiting Akt recruitment and activation (Cantley and Neel 1999). Overexpression of PTEN in endothelial cells was previously shown to inhibit angiogenic sprout formation, and disordered angiogenesis due to hyperproliferation is observed when PTEN is deleted or mutated to reduce activity in endothelial cells (Hamada, Sasaki et al. 2005). We found that TIMAP co-localizes with PTEN in endothelial cell projections and that some PTEN is co-immunoprecipitated with TIMAP from endothelial cell lysates. These observations suggest that TIMAP and PTEN can closely associate, and that this association is localized to the membranes of cell projections and cell tips, though further studies will have to be done to determine whether there is any direct interaction. Nonetheless, a functional interaction between PTEN and TIMAP is evident as TIMAP silencing strongly reduced S370 phosphorylation of PTEN without altering phosphorylation of the S380/T382/383 cluster or PTEN protein abundance. Also, that Akt is not observed in TIMAP immunoprecipitates suggests that TIMAP might not act directly on Akt.

PTEN activity is regulated at several levels including, protein expression, subcellular distribution and phosphorylation on several Ser/Thr residues, especially in its C-terminus (Shi, Paluch et al. 2012). CK2 constitutively phosphorylates the C- terminus of PTEN on residues Ser 370, Ser 380, Thr 382 and Thr 383 (Torres and Pulido 2001). Phosphorylation of these sites regulates PTEN activity by modulating intramolecular

interactions (Rahdar, Inoue et al. 2009), membrane localization and proteasome-mediated degradation (Ross and Gericke 2009). Our results indicate that TIMAP does not alter PTEN protein abundance, but that it specifically regulates PTEN phosphorylation on Ser 370. The fact that the remaining CK2-dependent sites on PTEN are phosphorylated in TIMAP-deficient endothelial cells also implies that TIMAP is unlikely to interfere with CK2 kinase activity directly. A recent nuclear magnetic resonance (NMR)-based study suggests that two temporally independent phosphorylation events, both mediated by CK2 serve to phosphorylate PTEN on the S380-S385 and S361-S370 clusters (Cordier, Chaffotte et al. 2012). Our findings suggest that TIMAP plays a role in the phosphorylation of the S361-S370 cluster, but not the S380-S385 cluster. That Akt phosphorylation is strongly induced by the PTEN inhibitor bpV(phen) (Figure 3.7 D) is in keeping with constitutive PTEN activity in the cultured glomerular endothelial cells. Furthermore, since PTEN inhibition fully restores Akt phosphorylation in endothelial cells in which TIMAP was silenced, it seems plausible that TIMAP may repress PTEN activity in endothelial cells and that depletion of TIMAP dis-inhibits PTEN. Although the degree of Akt phosphorylation is considered indicative of the activation of PI3K and PTEN, the reduction in Akt phosphorylation due to TIMAP depletion is not a direct evidence of increased PTEN activity. A better way to assess the activity of PTEN would be by measuring the PI[4,5]P2 versus PI[3,4,5]P3 production, however, the main finding in this article is the biological role of TIMAP in angiogenesis.

TIMAP is a member of the MYPT family of PP1c targeting subunits (Li, Kozlowski et al. 2007, Csortos, Czikora et al. 2008, Shopik, Li et al. 2013). The question therefore arises whether the effects of TIMAP silencing observed here can be attributed

to reduced TIMAP/PP1c phosphatase activity. If TIMAP/PP1c acted as a phosphatase toward Akt and/or PTEN, TIMAP silencing would have been expected to increase phosphorylation of these targets. However, Thr 308 and Ser 473 Akt as well as Ser 370 PTEN phosphorylation were inhibited when TIMAP was silenced, indicating that these residues are not TIMAP/PP1c phosphatase targets. A limitation of this study is that we have not established whether TIMAP/PP1c is acting as a phosphatase on an unknown target that can suppress PTEN action. More work will be required to define the direct molecular target(s) of TIMAP in the PI3K/Akt/PTEN cascade.

Finally, in *Dictyostelium* cells undergoing chemotaxis, PI3K and Akt are found at the leading edge, segregated from PTEN, which is localized to the trailing edge (Funamoto, Meili et al. 2002). Also, during cytokinesis of dividing *D. discoideum cells*, PTEN and PI3K/AKT segregate (Janetopoulos, Borleis et al. 2005), ensuring continuous, localized Akt activation. Furthermore, PTEN and myosin II co-localize under conditions of chemotaxis and cytokinesis in these cells (Pramanik, Iijima et al. 2009). Since TIMAP is a membrane-associated MYPT family member that suppresses the action of PTEN on Akt in endothelial cells, it is conceivable that TIMAP, by controlling myosin II function, could spatially segregate PTEN from the PI3K/Akt in endothelial cells. Clearly, much more work will be needed to investigate this possibility.

TIMAP is an endothelial cell-predominant protein, expressed in cultured glomerular and aortic endothelial cells (Cao, Mattagajasingh et al. 2002), in proliferating HUVEC (Obeidat M, unpublished observations), and in developing glomerular and in renal peritubular vascular endothelial cells *in vivo* (Cao, Mattagajasingh et al. 2002). During renal development, angiogenic sprouts invade the capillary cleft of developing

nephrons in response to cues from podocyte-derived VEGF. Taken together with the findings in this study, it is therefore plausible that TIMAP, by inhibiting PTEN, promotes endothelial cell proliferation and survival during glomerular capillary development. Since TIMAP is endothelial cell-predominant, but not glomerular endothelial cell-specific, and since PTEN is known to regulate angiogenesis (Hamada, Sasaki et al. 2005), TIMAP may also inhibit PTEN during tumor angiogenesis. It is furthermore tempting to speculate that the limited angiogenic response to VEGF in glomeruli, and during progressive renal fibrosis in the renal interstitium, may be due, in part, to unopposed PTEN action in glomerular and peritubular endothelial cells.

In summary, the endothelial–predominant PP1c regulatory subunit TIMAP is required for angiogenesis, at least *in vitro*. TIMAP is permissive for S473/T308 Akt and Ser 370 PTEN phosphorylation in endothelial cells. Since TIMAP is expressed predominantly in endothelial cells, these findings raise the intriguing possibility that TIMAP might serve as a new target for anti-angiogenic therapy in tumors. Conversely, if a mechanism could be found to activate TIMAP, it could enhance endothelial-cell specific proliferation in chronically hypoxic tissues, for instance during progressive renal fibrosis. Nonetheless, a limitation of this study is that we were not able to demonstrate that ectopic expression of TIMAP in TIMAP-depleted endothelial cells can rescue the impaired cell survival, proliferation and angiogenesis. The reason behind this limitation is that the combination of siRNA transfection and adenovirus infection reduced endothelial cell viability, supposedly due to the incompatibility of these approaches. To overcome this, we would need to co-infect endothelial cells with adenovirus that carries short hairpin RNA (shRNA) targeting the 3'-untranslated region of TIMAP gene and

adenovirus that carries the wild type TIMAP. This approach should ensure simultaneous depletion of endogenous TIMAP and ectopic expression of wild type TIMAP, which cannot be targeted by the Ad-shRNA. Furthermore, we would need to investigate whether TIMAP plays a role in angiogenesis *in vivo* in TIMAP knockout or transgenic mice.

Figure 1 of chapter 4 has been performed by Dr. Laiji Li, a research associate in our lab, and has been published in: Shopik, M.J., Luu, H.A., Li, L., Obeidat, M., Holmes, C.F.B., and Ballermann, B.J. 2013. Multi-directional Function of the Protein Phosphatase 1 regulatory subunit TIMAP. BBRC. 435: 567-73

CHAPTER 4

Phosphatase- dependent and independent Regulation of MLC2 Phosphorylation by the PP1c Regulatory Subunit TIMAP

4.1 Introduction

Myosin motor-dependent muscle and non-muscle cell contraction, and reorganization of the actin cytoskeleton during cell motility and cell morphology changes are governed, in part, by the phosphorylation state of MLC2 (Hartshorne, Ito et al. 2004, Grassie, Moffat et al. 2011). The balance between MLC2 phosphorylation and de-phosphorylation is regulated by MLC2 kinases, including MLCK and ROCK (Totsukawa, Wu et al. 2004) on the one hand, and MLC2 phosphatases (Hirano, Derkach et al. 2003) on the other. MLC2 phosphatases are multimeric enzymes consisting of a PP1c□ subunit, a MYPT subunit, and in some cases a small M20 subunit (Fujioka, Takahashi et al. 1998, Bouchard, Marquardt et al. 2004, Grassie, Moffat et al. 2011). Members of the family of PP1c Ser/Thr phosphatases, PP1c□, PP1c□/□ and PP1c□(□1 and □2) are highly conserved and depend on their many diverse regulatory subunits which define substrate specificity, sub-cellular localization and regulated Ser/Thr phosphatase activity (Cohen 2002). Accordingly, PP1c□ bound to MYPT1 dephosphorylates pMLC2, and cellular phosphatase activity towards pMLC2 is significantly reduced in the absence of MYPT1 (Gailly, Wu et al. 1996).

TIMAP is a prenylated endothelial cell-predominant PP1c regulatory subunit in the MYPT family that was first identified in glomerular endothelial cells as a target of transcriptional repression by TGF1 (Cao, Mattagajasingh et al. 2002). TIMAP shares significant domain homology with MYPT3 (Skinner and Saltiel 2001, Bouchard, Marquardt et al. 2004). The MYPTs share similar N-termini where the KVxF motif is followed by several ankyrin repeats that wrap around PP1c burying the PP1c C-terminus but leaving its active site open (Cohen 2002, Terrak, Kerff et al. 2004, Shopik, Li et al. 2013).

Both, TIMAP and MYPT3 are prenylated at their C-terminus, resulting in plasma membrane association (Skinner and Saltiel 2001, Cao, Mattagajasingh et al. 2002, Kim, Xu et al. 2011). MYPT3 was initially shown to inhibit PP1c activity towards MLC2 *in vitro* (Skinner and Saltiel 2001), but Yong *et al* (Yong, Tan et al. 2006) found that the MYPT3/PP1c holoenzyme has basal MLC2 phosphatase activity *in vitro* that is further stimulated by PKA-mediated Ser340/Ser341/Ser353 phosphorylation. Furthermore, elimination of MYPT3 membrane localization resulted in profound MLC2 dephosphorylation when cells were transfected with CAAX-box deficient MYPT3 (Yong, Tan et al. 2006). Similarly, the Drosophila melanogaster TIMAP/MYPT3 ortholog, Mypt-75D has MLC2 phosphatase activity *in vitro* (Vereshchagina, Bennett et al. 2004).

Elimination of PP1c binding, and thus TIMAP-associated phosphatase activity, due to V64A/F66A substitutions in the KVxF motif of TIMAP induces lengthening of endothelial cell protrusions (Li, Kozlowski et al. 2007). Since formation and retraction of cellular protrusions depends on myosin II- mediated reorganization of actin cytoskeleton,

these data would be consistent with the hypothesis that TIMAP regulates PP1c activity against the non-muscle MLC2. Nonetheless, it was not known whether TIMAP functions as a myosin II phosphatase.

4.2 Results

4.2.1. The TIMAP/PP1cδ holoenzyme dephosphorylates MLC2 *in vitro*.

Since TIMAP is a member of the MYPT family and regulates endothelial cell projections (Li, Kozlowski et al. 2007, Grassie, Moffat et al. 2011), we sought to determine whether TIMAP/PP1cδ holoenzyme is an active myosin phosphatase. To remove the confounding effect of MYPT1, we first investigated the TIMAP-associated phosphatase activity toward MLC2 *in vitro*. MLC2 was pre-phosphorylated with γ-^{32}P-ATP by MLCK and then subjected to de-phosphorylation by PP1cδ in the presence or absence of GST-TIMAP or GST at the molar ratio 1:1 and 1:3 (GST or GST-TIMAP: PP1cδ). Autoradiography shows that free PP1cδ dephosphorylates ^{32}P-MLC2 in vitro, and this activity was not inhibited by GST or GST-TIMAP (Figure 4.1 A). In this experiment, de-phosphorylation of pMLC2 could be due to free PP1cδ. Therefore, we pre-assembled the TIMAP/PP1cδ complex with increasing concentrations of PP1cδ (75, 300 and 1200 ng) on beads, followed by extensive washing to remove free PP1cδ; beads containing TIMAP without PP1cδ served as the negative control. When PP1cδ was bound to TIMAP it strongly dephosphorylated PKA-phosphorylated TIMAP (Figure 4.1 B, TIMAP blot). This observation is consistent with our previously reported

findings that TIMAP itself is a substrate of its associated PP1c (Li, Kozlowski et al. 2007), and shows that the PP1c associated with TIMAP is active under these conditions. However, under the same conditions the TIMAP/PP1c complex did not dephosphorylate PKC or PKA-phosphorylated LAMR1 (Figure 4.1 B, LAMR1 blots for PKC and PKA), a previously identified TIMAP/PP1c binding partner in cells (Kim, Li et al. 2005). We observed that TIMAP-associated PP1c effectively dephosphorylated pMLC2 in a concentration dependent manner (Figure 4.1 B, MLC2 blot, lane 2). Thus, these data indicate that TIMAP/PP1c can dephosphorylate pMLC2 *in vitro*, suggesting that it might also function as a myosin II phosphatase in cells.

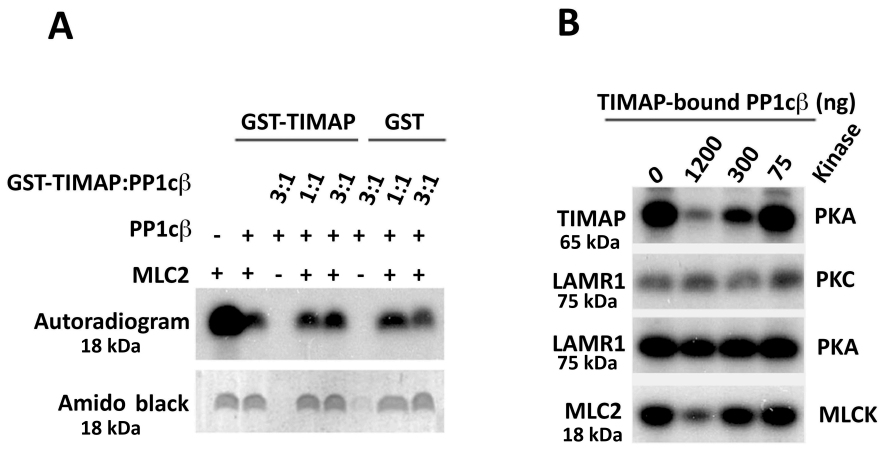

Figure 4.1: GST-TIMAP does not inhibit PP1cb activity toward pMLC2. A. Effect of TIMAP on MLC2-directed PP1c phosphatase activity. MLCK pre-phosphorylated MLC2 was incubated with PP1c without TIMAP, or in the presence of GST-TIMAP or GST, each at molar ratios of 1:1 and 3:1. The ^{32}P-autoradiogram is shown at the top and the amido black stained gel, showing MLC2 loading is at the bottom. **B**. Concentration- dependent activity of TIMAP pre-bound PP1c toward TIMAP, LAMR1 and MLC2. PP1c and immobilized TIMAP were pre-bound, followed by extensive washing. MLC2 or LAMR1 in their kinase reaction buffers were then added to the TIMAP/ PP1c complex. TIMAP phosphorylation shown here occurred after addition of LAMR1/PKA.

4.2.2. TIMAP binds MLC2 directly and blocks its phosphorylation *in vitro*.

The structural model of TIMAP/PP1cγ predicts that the TIMAP ankyrin repeats wrap around the PP1cγ C-terminus making it inaccessible to kinases (Shopik, Li et al. 2013). Indeed, we have previously observed that the direct interaction of TIMAP with PP1cγ inhibits the phosphorylation of PP1cγ by cyclinA/cdk2 (Shopik, Li et al. 2013). Similarly, we also showed that the phosphorylation of LAMR1 by PKA and PKC is inhibited when LAMR1 is bound to TIMAP (Shopik, Li et al. 2013). Since we observed that TIMAP, similar to MYPT1, acts as an MLC2 phosphatase *in vitro* (Figure 4.1), and since MYPT1 interacts with myosin (Johnson, Cohen et al. 1997), we determined whether TIMAP directly interacts with MLC2 *in vitro*. Purified recombinant His-MLC2 was therefore incubated with GST-TIMAP immobilized on glutathione-sepharose beads. GST sepharose served as the negative control. We observed that His-MLC2 was effectively precipitated with GST-TIMAP (Figure 4.2. A, lane 1), but not with the GST-loaded beads (Figure 4.2. A, lane 2), indicating a direct interaction between TIMAP and MLC2.

It has been shown that MYPT1 binds phosphorylated myosin with much higher affinity than un-phosphorylated myosin (Fujioka, Takahashi et al. 1998, Hartshorne, Ito et al. 1998). To determine whether this is the case for TIMAP, we studied the interaction between TIMAP and pMLC2. His-MLC2 was therefore pre- phosphorylated *in vitro* by MLCK using γ-^{32}P-ATP, or left un-phosphorylated, followed by GST-TIMAP pull-down. His-MLC2 was effectively phosphorylated by MLCK *in vitro* (Figure 4.2 B, lanes 2 and

3), and both, pMLC2 and un-phosphorylated MLC2 readily precipitated with GST-TIMAP (Figure 4.2.B, lanes 2-5). This indicates that TIMAP can directly bind MLC2 and pMLC2.

Since TIMAP binds MLC2 directly, we examined whether the direct interaction of TIMAP with MLC2 blocks its phosphorylation, similarly to PP1c and LAMR1 (Shopik, Li et al. 2013). One μg His-MLC2 was pre-incubated with GST-TIMAP or GST at molar ratios of 0.5, 1.0 and 2.0 (GST or GST-TIMAP: His-MLC2). The autoradiogram shows that His-MLC2 is readily phosphorylated by MLCK (Figure 4.2 C, lane 5), and this phosphorylation is inhibited at molar ratio GST-TIMAP: His-MLC2 of 2.0 (Figure 4.2 C, lane 8), while GST alone was without effect (Figure 4.2 C, lanes 9-11). In order to ascertain whether inhibition of MLC2 phosphorylation by TIMAP in the absence of PP1c occurs when MLC2 is bound to TIMAP, His-MLC2 was incubated with GST-TIMAP or GST immobilized on glutathione-sepharose beads followed by extensive washing of the beads to remove unbound His-MLC2. The beads were then incubated with MLCK and □-^{32}P-ATP in kinase buffer to stimulate MLC2 phosphorylation. Free His-MLC2 added to sepharose beads without GST or GST-TIMAP served as positive control (Figure 4.2 D, lane 1). His-MLC2 was observed on beads containing GST-TIMAP (Figure 4.2 D, lane 5) but not on control GST-loaded beads (Figure 4.2 D, lane 2). While free His-MLC2 was readily phosphorylated by MLCK (Figure 4.2 D, lanes 7 and 9), autoradiography revealed little, if any phosphorylation of GST-TIMAP-associated His-MLC2 (Figure 4.2 D, lane 5).

Since autoradiography only detects the total phosphorylation level of proteins, we determined whether TIMAP masks the activating phosphorylation sites on MLC2 that are known to regulate myosin activity under physiological conditions in cells. *In vitro* phosphorylation of GST-TIMAP associated His-MLC2 was performed with ROCK or MLCK as described above and evaluated using anti-pS19 MLC2 and anti-pT18/S19 MLC2 antibodies. Free His-MLC2 was phosphorylated on Ser19 and Thr18 by ROCK (Figure 4.2 E, left blot, lane 4) and MLCK (Figure 4.2 E, right blot, lane 4). This phosphorylation was substantially inhibited when His-MLC2 was bound to GST-TIMAP (Figure 4.2 E, lanes 2 and 3), even though PP1c was absent.

These experiments demonstrate that TIMAP inhibits MLC2 phosphorylation at the activating sites Thr18 and Ser19 in a PP1c-independent fashion. Thus, while TIMAP/PP1c can act as a pMLC2 phosphatase (Figure 4.1), TIMAP can also bind to MLC2 and mask the Thr18/Ser19 phosphorylation sites, preventing their phosphorylation by ROCK and MLCK.

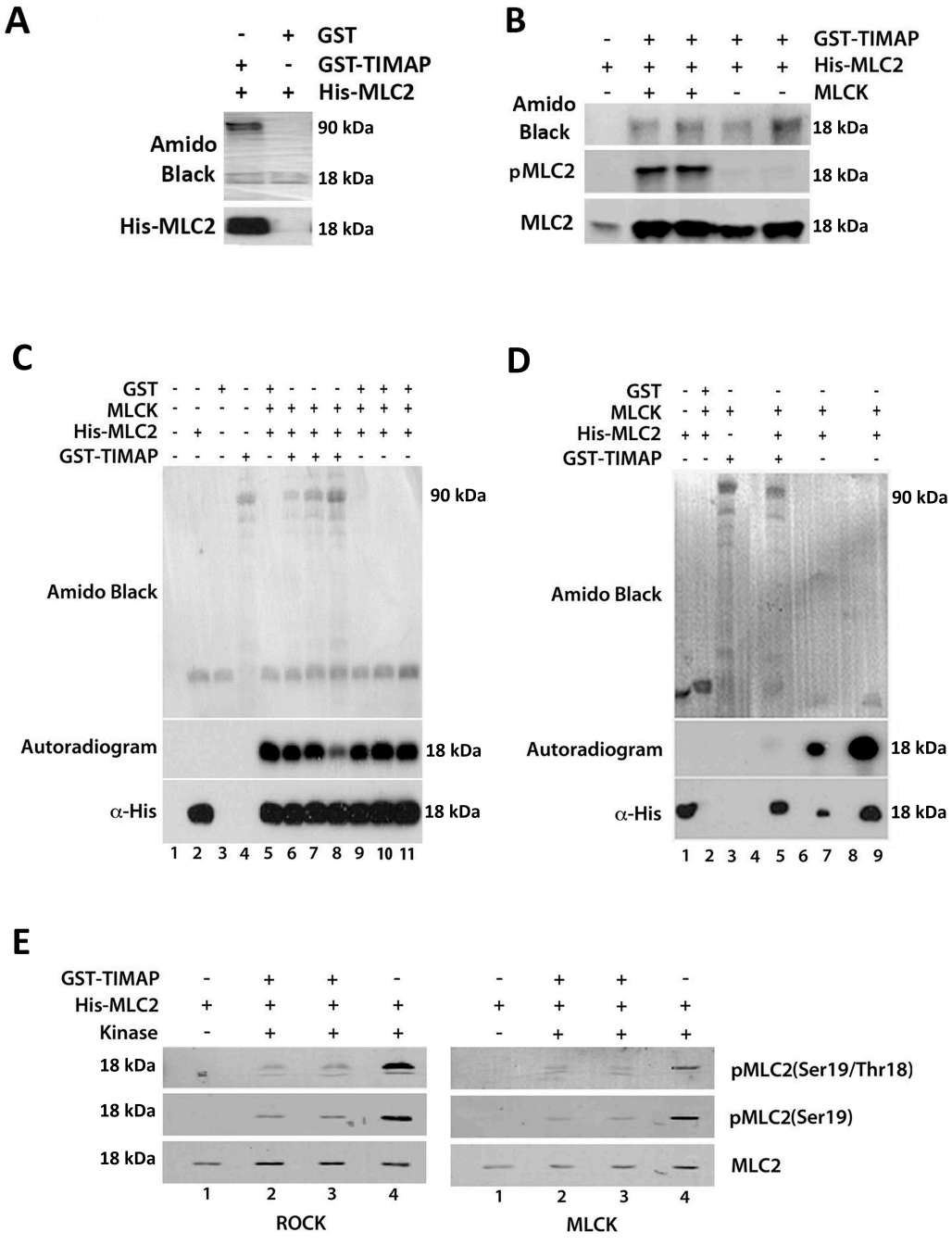

Figure 4.2: TIMAP blocks MLCK- and ROCK-dependent MLC2 phosphorylation.

A. Recombinant GST-TIMAP^{WT} or GST immobilized on glutathione-sepharose was

incubated with recombinant His-MLC2 *in vitro*. Bead associated proteins were resolved by SDS-PAGE. Amido black (top panel) and anti-MLC2 (bottom panel). **B.** His-MLC2 was phosphorylated *in vitro* by MLCK, or left un-phosphorylated, followed by GST-TIMAP pulldown. Control beads were loaded with GST. **C.** His-MLC2 (lanes 2 and 5-11) was incubated in the absence (lanes 2,3) or presence (lanes 5-11) of MLCK, after pre-incubation without (lane 5) or with GST-TIMAP^{WT} at molar ratios (GST-TIMAP^{WT}: MLC2) of 0.5 (lane 6), 1.0 (lane 7) or 2.0 (lane 8) or GST at molar ratios (GST: MLC2) of 0.5 (lane 9), 1.0 (lane 10) and 2.0 (lane 11). Amido Black stained gel (upper panel), His-MLC2 autoradiography (middle panel) and anti-His immunoblot (lower panel). **D.** His-MLCK was pre-incubated with GST (Lane 2) or GST-TIMAP^{WT} (lane 5) on glutathione sepharose beads. Unbound MLC2 was removed. Free MLC2 without MLCK (lane 1) served as loading control. Free MLC2 added to glutathione beads (50 ng, lane 7 and 125 ng, lane 9) served as positive control for MLC2 phosphorylation in the presence of glutathione beads. Lanes 4, 6, and 8 are empty. Amido black stained gel (upper panel), His-MLC2 autoradiography (middle panel) and anti-His immunoblot (lower panel). **E.** MLC2 phosphorylation *in vitro* by ROCK (left panel) or MLCK (right panel) in the presence or absence of GST-TIMAP^{WT}. Immunoblots with phospho- specific anti-MLC2 Thr18/Ser19 (upper panels), anti-MLC2 Ser19 (middle panels) antibodies and anti-MLC2 antibodies (lower panels). Results in each panel represent 2 independent experiments.

4.2.3. Endogenous TIMAP Associates with MLC2 in Endothelial Cells

Since TIMAP is an endothelial- predominant MYPT and since our data demonstrate that TIMAP/PP1c can dephosphorylate MLC2 *in vitro*, that it directly interacts with MLC2, and that the interaction can block MLCK-and ROCK-mediated phosphorylation of MLC2, we next sought to determine whether the interaction between TIMAP and MLC2 occurs in glomerular endothelial cells. Endogenous TIMAP was immunoprecipitated from glomerular endothelial cells with chicken anti-TIMAP IgY. Western blot analysis of the precipitate revealed that MLC2 readily co-immunoprecipitated with anti-TIMAP (Figure 4.3 A, lane 2), but not with non-specific IgY antibody (Figure 4.3 A, lane 1). This suggests that the endogenous TIMAP and MLC2 proteins can exist in the same complex in endothelial cells and that the association between them is specific. In cells, MLC2 and myosin II heavy chain exist as a myosin II complex, therefore we determined whether MLC2 and TIMAP associate in a complex that also contains myosin II heavy chain, and found that myosin II heavy chain was detected with anti- myosin IIA antibody in anti-TIMAP (Figure 4.3 A, lane 2), but not IgY immunoprecipitates (Figure 4.3 A, lane 1). This predicts that the interaction between TIMAP and MLC2 has a physiological relevance.

We next sought to examine whether the interaction between TIMAP and myosin II is dependent on PP1c activity. Glomerular endothelial cells were pre-treated with 100 nM calyculin A, a potent inhibitor of PP1c activity (Favre, Turowski et al. 1997) for 15 minutes prior to immunoprecipitation of endogenous TIMAP. Phosphorylated MLC2 was detected in total cell lysates (Figure 4.3 B, TCL lane 2) and anti-TIMAP

immunoprecipitates (Figure 4.3 B, IP lane 2) when PP1c activity was inhibited, suggesting that TIMAP-associated pMLC2 is not dephosphorylated when the catalytic activity of PP1c is inhibited. In contrast un-phosphorylated MLC2 and Myosin IIA heavy chain were detected in anti- TIMAP immunoprecipitates only when PP1c activity was maintained (Figure 4.3 B, IP lane 1), although they were equally detected in total cell lysates in the absence and presence of calyculin A treatment (Figure 4.3 B, TCL lanes 1 and 2). This indicates that the association between TIMAP and myosin II complex is dependent on PP1c activity. We previously reported that PP1c dissociates from TIMAP when TIMAP is phosphorylated on Ser333/Ser337, causing the affinity of PP1c for TIMAP to be reduced (Li, Kozlowski et al. 2007). Here, we observed a marked electrophoretic mobility shift of TIMAP in the presence of calyculin A, consistent with TIMAP phosphorylation (Figure 4.3 B, TCL lane 2 and IP lane 2). We also observed less PP1c in anti- TIMAP immunoprecipitates upon calyculin A treatment (Figure 4.3 B, IP lane 2). This finding is consistent with reduced PP1c binding to phosphorylated TIMAP.

Hence, TIMAP interacts directly with MLC2. This direct interaction is not altered by MLC2 phosphorylation, and TIMAP co-immunoprecipitates MLC2 and Myosin II heavy chain from endothelial cells. However, inhibition of PP1c activity significantly inhibits this association.

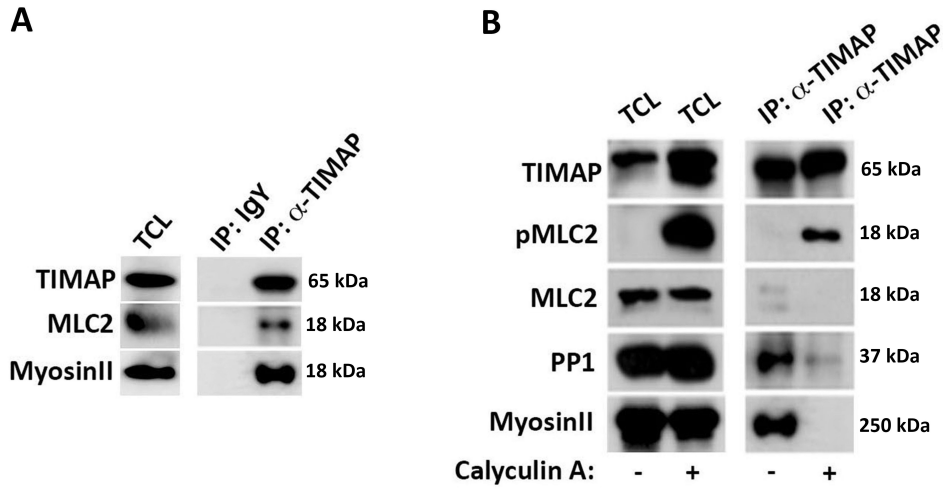

Figure 4.3: Endogenous TIMAP co-immunoprecipitates un-phosphorylated myosin. A. Immunoprecipitates prepared from glomerular endothelial cell lysates with non-immune chicken IgY (control) or anti-TIMAP IgY. Blots were probed with rabbit anti-TIMAP (top panel), rabbit anti-MLC2 (middle panel) and rabbit anti-myosin IIA (bottom panel) to determine whether TIMAP, MLC2 and myosin II exist in one complex (results represent 2 independent experiments) **B.** Glomerular endothelial cells were pre-treated with or without the PP1c inhibitor calyculin A (CA, 100 nM) followed by immunoprecipitation with control chicken IgY or anti-TIMAP IgY. Blots were probed with rabbit anti-TIMAP (top panel), rabbit anti-MLC2 (second panel), rabbit anti-PP1c (third panel) and rabbit anti-myosin IIA (bottom panel). Results represent 2 independent experiments).

Phosphorylated MLC2 is associated with the cortical actin and stress fibers in cells, and TIMAP localizes to the endothelial cell membranes and projections (Cao, Mattagajasingh et al. 2002, Li, Kozlowski et al. 2007). Hence, we sought to determine the subcellular location of TIMAP-MLC2 interaction. In sub-confluent glomerular endothelial cells, confocal immunofluorescence (40X) showed that endogenous TIMAP is found predominantly in cellular projections (Figure 4.4, 40X, red), where it co-localizes with MLC2 (Figure 4.4, 40X, top, green) and pMLC2 (Figure 4.4, 40X, bottom, green). By contrast, while MLC2 and pMLC2 were observed in glomerular endothelial cell stress fibers (Figure 4.4, 60X, green), endogenous TIMAP (Figure 4.4, 60X, red) did not co-localize with the stress fibers-associated MLC2 or pMLC2, suggesting that the association between the two proteins occurs predominantly at the cell cortex.

4.2.4. PP1c-deficient TIMAP (TIMAP$^{V64A/F66A}$) Inhibits MLC2 Phosphorylation in Endothelial Cells

Since TIMAP/PP1c dephosphorylates MLC2 *in vitro* and that TIMAP interacts with MLC2 in endothelial cells, we designed experiments to determine whether TIMAP/PP1c acts as an MLC2 phosphatase in cells. We reasoned that the KVxF PP1c binding motif mutant TIMAP$^{V64A/F66A}$, which cannot interact with PP1c (Li, Kozlowski et al. 2007), should block TIMAP associated phosphatase activity toward pMLC2 in cells in a dominant negative fashion. We transiently transfected endothelial cells with GFP-TIMAP$^{V64A/F66A}$ and first examined whether GFP-TIMAP$^{V64A/F66A}$ co-localizes with MLC2 in theses cells. Confocal immunofluorescence (40X) analysis of fixed cells

immune-labeled with anti-MLC2 antibody shows that GFP-TIMAP$^{V64A/F66A}$ co-localizes with endogenous MLC2 at the cell membrane and protrusions (Figure 4.5 A). This finding is similar to the co-localization data of endogenous TIMAP and MLC2 (Figure 4.4). We next examined MLC2 phosphorylation in fixed cells by immunofluorescence analysis using anti- pT18/S19 MLC2 antibody (Figure 4.5 B). Since the transient transfection efficiency is <50%, cells that remained un-transfected served as controls. TIMAP$^{V64A/F66A}$ was observed in a fibrillar pattern throughout the transfected cells, with more localized accumulation along, and at the tip of endothelial cell projections (arrow, Figure 4.5 B). Substantially less pMLC2 was observed in TIMAP$^{V64A/F66A}$ transfected cells compared to controls. In the transfected cells pMLC2 was preserved only at the tip of the endothelial cell projections (arrow, Figure 4.5 B). Therefore, expression of TIMAP devoid of PP1c profoundly inhibits MLC2 phosphorylation. Immunofluorescence images of TIMAP$^{V64A/F66A}$ transiently transfected cells were then scored semi-quantitatively for the presence or absence of cortical pMLC2 by a blinded observer. Reduced cortical pMLC2 was consistently observed in TIMAP$^{V64A/F66A}$ transfected cells compared to control cells, in three independent experiments (Figure 4.5 C). This observation suggests that TIMAP modifies MLC2 phosphorylation through mechanisms not mediated by TIMAP/PP1c holoenzyme activity, and would be consistent with the inhibition of kinase-mediated MLC2 phosphorylation by PP1c-deficient TIMAP.

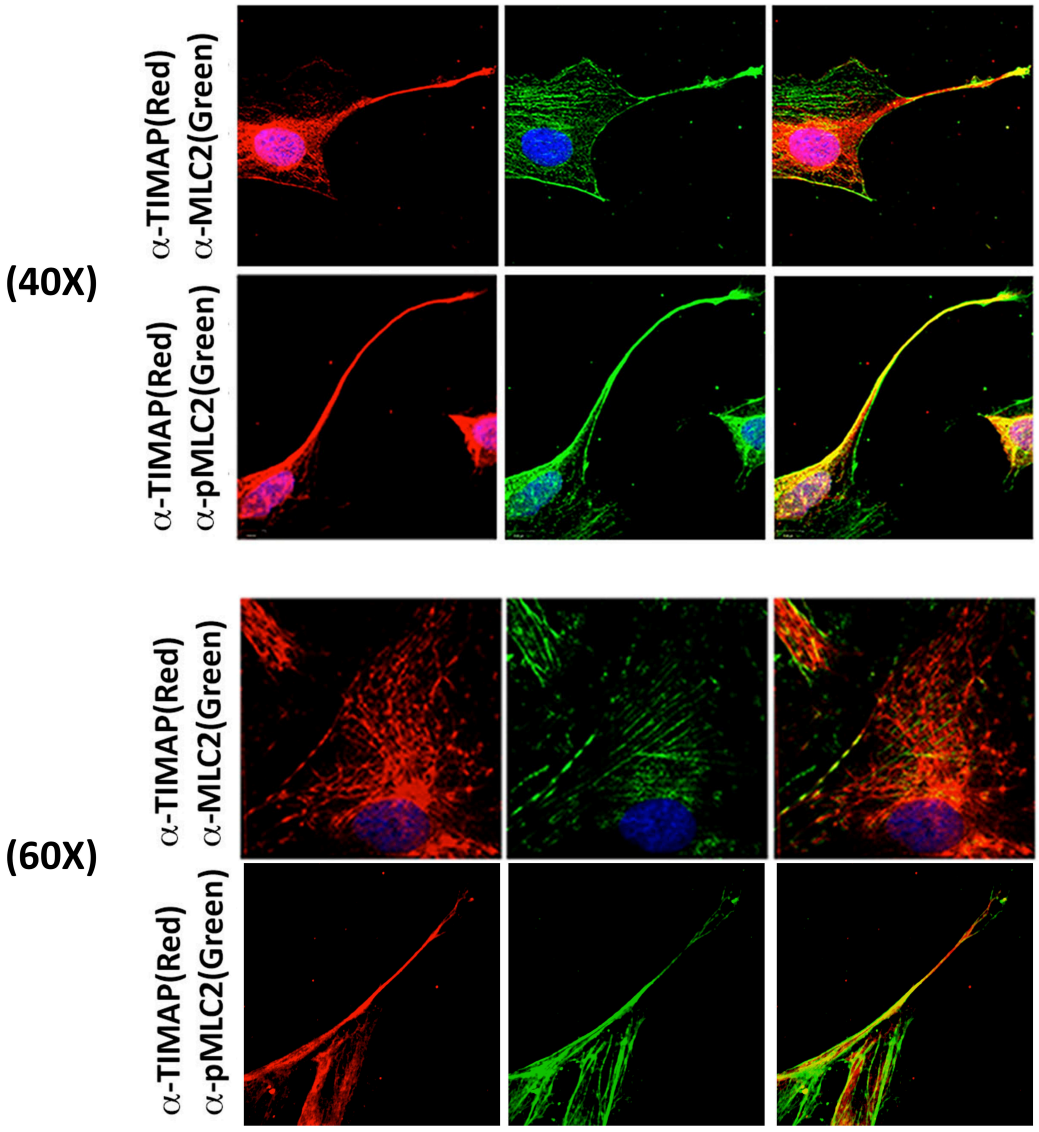

Figure 4.4: Polarized co-localization of endogenous TIMAP with endogenous MLC2 and pMLC2. Sub-confluent glomerular endothelial cells were labeled with chicken anti-TIMAP antibody (red), and rabbit anti-MLC2 antibody (green) (upper panel) or rabbit anti-pT18/S19 MLC2 antibody (lower panel). Images were obtained by confocal microscopy at 40X and 60X and representative of 3 independent experiments.

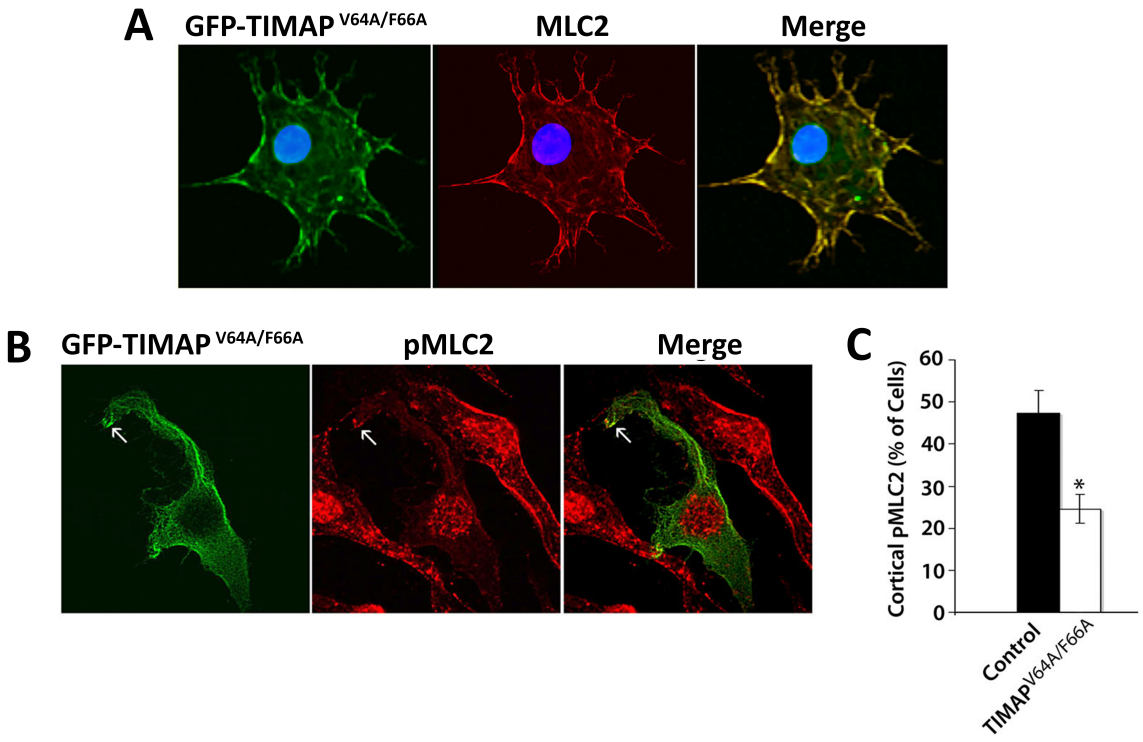

Figure 4.5: PP1c-deficient TIMAP (TIMAP$^{V64A/F66A}$) inhibits cortical MLC2 phosphorylation in endothelial cells. A. Confocal immunofluorescence analysis (40X) for GFP-TIMAP$^{V64A/F66A}$ (left) and mouse anti-MLC2 (middle) of endothelial cells transiently transfected with GFP-TIMAP$^{V64A/F66A}$; cortical MLC2 co-localizes with GFP-TIMAP$^{V64A/F66A}$ (merge) **B.** Confocal immunofluorescence analysis (40X) for GFP-TIMAP$^{V64A/F66A}$ (left) and rabbit anti-pT18/S19 MLC2 (middle) of endothelial cells transiently transfected with GFP-TIMAP$^{V64A/F66A}$. Un-transfected cells in the same monolayer serve as control. **C**. Quantification of cortical MLC2 phosphorylation in endothelial cell cultures transiently transfected with GFP-TIMAP$^{V64A/F66A}$ (mean ± SEM, n = 3 independent experiments).

4.3 Discussion

This study has uncovered a previously unknown mechanism by which the endothelial cell- predominant MYPT family member TIMAP can regulate MLC2 phosphorylation. Like all MYPT family PP1c regulatory subunits, TIMAP preferentially binds PP1cδ (Cao, Mattagajasingh et al. 2002, Li, Kozlowski et al. 2007, Csortos, Czikora et al. 2008, Shopik, Li et al. 2013). Here we show that the TIMAP/PP1cδ holoenzyme effectively dephosphorylates MLC2 *in vitro*. Similarly, TIMAP-associated PP1cδ dephosphorylates TIMAP itself, under the same conditions, but fails to dephosphorylate LAMR1, previously reported to be a TIMAP/PP1c substrate (Kim, Li et al. 2005). Also, MLCK- and ROCK-mediated Thr18/Ser19 phosphorylation of MLC2 is blocked by TIMAP. This TIMAP effect is independent of PP1c activity. Furthermore, we demonstrate that TIMAP binds MLC2 and pMLC2 directly, TIMAP associates with MLC2 and myosin II heavy chain and co-localizes with MLC2 and pMLC2 in endothelial cell periphery and projections. We also found that inhibition of PP1c activity enhances the phosphorylation level of MLC2, but reduces the total protein levels of myosin II heavy chain and MLC2 in TIMAP immunoprecipitates.

It is well known that PP1c is not free in cells, but always exists in association with its diverse regulatory subunits. Based on the structure of TIMAP and its association with PP1c, it was therefore logical to postulate that TIMAP/PP1c represent a phosphatase holoenzyme. Since TIMAP is a member of the MYPT family of PP1c regulatory subunits, it was furthermore plausible that TIMAP functions as a myosin phosphatase,

similar to the other members of this family. We therefore set out to first determine whether TIMAP can dephosphorylate pMLC2 *in vitro*, under fully controlled conditions. We found that TIMAP does not inhibit PP1c activity against MLCK- and ROCK-phosphorylated MLC2 *in vitro,* while, under the same experimental conditions, it inhibits dephosphorylation of PKA and PKC-phosphorylated LAMR1 by PP1c (Figure 4.1). The fact that the TIMAP-pre-associated PP1c can readily dephosphorylate pMLC2 further confirms the finding and rules out the possibility of pMLC2 being dephosphorylated by free PP1c. The findings *in vitro* strongly suggest that TIMAP does not inhibit PP1c towards pMLC2, which would be expected if pMLC2 were not a target for the putative TIMAP/PP1c holoenzyme. The limitation of this *in vitro* finding is that we did not demonstrate activation of the holoenzyme. For instance, MYPT1 is known to be more active against pMLC2 when dephosphorylated on Thr 696 and Thr 853 residues, and its activity is inhibited when these residues are phosphorylated (Kimura, Ito et al. 1996, Feng, Ito et al. 1999, Muranyi, Derkach et al. 2005). On the other hand, MYPT3 phosphatase activity against MLC2 is enhanced by its PKA-mediated Ser353/Ser340/Ser341 phosphorylation (Yong, Tan et al. 2006). Here, we did not attempt to determine whether alteration in the TIMAP phosphorylation status could further augment its pMLC2 phosphatase activity. Also, since pMLC2 *in vivo* usually exists in association with the myosin II heavy chain, it is not clear whether TIMAP would have access to MLC2 when it is bound to the heavy chain of myosin. Nonetheless, the data support a putative role for TIMAP/PP1c as a myosin phosphatase.

The main cellular myosin phosphatase regulatory subunit, MYPT1 interacts

directly with its target, the 20 kDa MLC2 (Ichikawa, Hirano et al. 1996, Toth, Kiss et al. 2000). To determine whether TIMAP similarly interacts with MLC2, we studied the direct interaction between His-MLC2 and GST-TIMAP recombinant proteins *in vitro*. We observed that purified, recombinant His-MLC2 interacts directly with purified immobilized GST-TIMAP *in vitro* (Figure 4.2 A).

One might have expected that TIMAP would preferentially target phosphorylated MLC2, similar to the finding that MYPT1 binds phosphorylated myosin with much higher affinity than un-phosphorylated myosin (Fujioka, Takahashi et al. 1998), but we observed that the direct interaction of TIMAP with MLC2 was independent of the MLC2 phosphorylation state (Figure 4.2 B). This finding predicts that de-phosphorylation of pMLC2 by TIMAP/PP1c would, itself, not lead to dissociation of MLC2 from TIMAP.

We previously reported that the interaction of PP1c and LAMR1 with TIMAP prevents their phosphorylation by kinases (Shopik, Li et al. 2013). In this regard, the model of the PP1c/TIMAP interaction predicts that the C-terminal tail of PP1c, which can be phosphorylated by cyclinA/cdk2 resulting in PP1c inactivation, is buried in the cage-like structure formed by the N-terminal TIMAP ankyrin domains (Shopik, Li et al. 2013). This configuration would predict that TIMAP-associated PP1c should not be phosphorylated by cyclinA/cdk2. Indeed, we showed that the phosphorylation of TIMAP-pre-bound PP1c by cyclinA/cdk2 was blocked (Shopik, Li et al. 2013). Furthermore, the phosphorylation of the TIMAP binding partner LAMR1 by PKA and PKC is also blocked when LAMR1 is bound to TIMAP (Shopik, Li et al. 2013). We therefore investigated whether the interaction of TIMAP with MLC2 might similarly mask the activating phosphorylation sites on MLC2. Indeed, we observed that in the

absence of PP1c, TIMAP inhibits the activating Thr18/Ser19 MLC2 phosphorylation by MLCK and ROCK *in vitro* (Figure 4.2 C), and MLCK-mediated phosphorylation was completely blocked when MLC2 was pre-associated with TIMAP (Figure 4.2 D). This is similar to the inhibitory effect of TIMAP on PKA/PKC mediated LAMR1 phosphorylation, and cyclinA/cdk2 mediated PP1c phosphorylation (Shopik, Li et al. 2013).

Having demonstrated that TIMAP/PP1c can dephosphorylate pMLC2, that TIMAP and MLC2 interact directly, and that the interaction can block MLCK- and ROCK-mediated phosphorylation of MLC2 as well, we next sought to determine whether TIMAP also interacts with the myosin complex in endothelial cells. We observed that MLC2 and myosin II heavy chain co-immunoprecipitated with endogenous TIMAP from endothelial cell lysates (Figure 4.3 A). Co-immunoprecipitation of TIMAP and MLC2 could have been due to an indirect association, given that MYPT1 binds myosin and regulates PP1c activity towards MLC2 (Johnson, Cohen et al. 1997). However, we observed that purified, recombinant His-MLC2 interacts directly with purified immobilized GST-TIMAP *in vitro*, in the absence of PP1c or other proteins that could have been part of a complex (Figure 4.2 A). We also, showed that inhibition of PP1c activity significantly enhances TIMAP-associated MLC2 phosphorylation, but disrupts the interaction between TIMAP and the myosin II complex (Figure 4.3 B). This finding could be due to the dissociation of the complex when PP1c is inhibited or that most of the phosphorylated myosin II is in the non-soluble fraction attached to the actin cytoskeleton (Figure 1.1), which makes the IP no longer possible.

In glomerular endothelial cells, we first determined whether TIMAP and MLC2 co-localize. We observed that TIMAP predominantly co-localized with MLC2 and pMLC2 in cell projections (Figure 4.4, 40X). Although MLC2 and pMLC2 staining was observed on stress fibers, it did not co-localize with TIMAP staining there (Figure 4.4, 60X). Since TIMAP is prenylated MYPT family member, this finding is not unexpected, and would predict that TIMAP only regulates MLC2 associated with cortical actin.

Lastly, we asked whether TIMAP regulates MLC2 phosphorylation in endothelial cells. We reasoned that if TIMAP/PP1c holoenzyme were a myosin II phosphatase, it would be plausible that PP1c-deficient TIMAP might act as a dominant negative and augment MLC2 phosphorylation. Surprisingly, we observed that the endothelial cells transiently transfected with the PP1c-deficient TIMAP and cultured in serum-replete medium exhibit a significantly reduced pMLC2 immunoreactivity compared to untransfected control cells (Figure 4.5 B and C). This finding is unexpected and suggests that TIMAP does not act as a myosin II phosphatase in cells. Since the PP1c-deficient TIMAP co-localizes with endogenous MLC2 (Figure 4.5 A), and the finding that TIMAP binds to MLC2 and inhibits its phosphorylation by MLCK and ROCK in the absence of PP1c (Figure 4.2), we can speculate that the decrease in MLC2 phosphorylation caused by the PP1c-unbound TIMAP is consistent with the kinase inhibition function of TIMAP (Figure 4.2). However, whether the endogenous TIMAP, whose ankyrin pocket would be occupied by PP1c, can directly bind MLC2 and inhibit its kinase-mediated phosphorylation is unknown.

TIMAP differs from other MYPT family members, including MYPT3, in that the X amino acid in the KVxF motif of TIMAP is a serine residue, which could be

phosphorylated and, thus, could block PP1c binding. We have made a TIMAP mutant by replacing the serine to alanine in the KVSF domain (KVAF), which did not change TIMAP binding to PP1c. But, we did not yet make the phosphomimetic mutant TIMAP by replacing the serine in its KVSF motif to aspartic or glutamic acid (KVEF or KVDF). So, we postulate that the phosphorylation of the serine residue in the KVSF PP1c binding motif of TIMAP would potentially block PP1c binding. Nonetheless, using the KVAF mutant TIMAP we have, we could first test whether the KVSF to KVAF mutation would affect TIMAP phosphorylation in cells using □-^{32}P-ATP, since PP1c binding to TIMAP reduces its phosphorylation (Li, Kozlowski et al. 2007). If this is true, then we will produce the phosphomimetic mutant TIMAP (KVEF or KVDF) and examine whether it could co-immunoprecipitate PP1c from cells. Hence, we would be able to determine whether TIMAP phosphorylation on the serine in its KVSF motif would inhibit its binding to PP1c and, therefore, allows its binding to and inhibition of MLC2 phosphorylation. On the other hand, when TIMAP is not phosphorylated on the serine in its KVSF motif it would bind to PP1c, which would then prevent its interaction with MLC2.

Alternatively, we could also examine whether the interaction of PP1c with TIMAP inhibits TIMAP interaction with MLC2 by loading PP1c onto microcystin beads (available at Dr. Holmes lab), then adding TIMAP^{WT} to the PP1c on microcystin beads, as previously reported (Shopik, Li et al. 2013), and lastly adding MLC2 to the complex and determine whether it could bind to PP1c- bound TIMAP. Furthermore, we could investigate whether association of PP1c with TIMAP alters TIMAP association with MLC2 *in vitro* by determining whether free PP1c and free MLC2 compete for binding to

TIMAP. Increasing concentration of PP1c would be added to a fixed concentration of MLC2, then GST-TIMAP would be added to the mixture and the GST would be pulled down to determine the level of TIMAP- bound MLC2. If with increasing the concentration of PP1c less MLC2 would bind to TIMAP, then this would suggest that PP1c and MLC2 compete to bind to TIMAP or, that the binding of PP1c to TIMAP changes the conformation of TIMAP, hence, reducing its affinity to MLC2.

Determining that TIMAP can differentially regulate MLC2 phosphorylation depending on whether its bound or un-bound to PP1c or, that TIMAP phosphorylation on serine in its KVSF motif plays a role in its binding to PP1c and regulation of MLC2 phosphorylation would provide a novel mechanism by which TIMAP/PP1c regulates myosin activity.

CHAPTER 5

TIMAP Induces Phosphorylation of MLC2 by virtue of its Interaction with PP1c

5.1 Introduction

In chapter 4, we have provided evidence that TIMAP-PP1c holoenzyme is an active MLC2 phosphatase *in vitro* (Figure 4.1). We also showed that TIMAP interacts with MLC2 and pMLC2 *in vitro* and that TIMAP co-localizes with both MLC2 and pMLC2 in glomerular endothelial cell projections at, or near the cell membrane (Figure 4.4). Taken together with the fact that TIMAP is a member of the myosin phosphatase targeting subunits family, the findings predict that TIMAP should also act as a MLC2 phosphatase in cells.

We therefore determined whether overexpression of TIMAP replicates MLC2 phosphatase action in living glomerular endothelial cells. Contrary to expectations, we find that adenoviral infection with GFP-TIMAP^{WT} significantly induces MLC2 phosphorylation in endothelial cells compared to GFP controls. The PKA/GSK3β phosphorylation-defective site mutant (GFP-TIMAP$^{S333A/S337A}$) and the PKA/GSK3β phosphomimic (GFP-TIMAP$^{S333E/S337E}$) mutant similarly induced MLC2 phosphorylation, but the RVXF TIMAP mutant (GFP-TIMAP$^{V64A/F66A}$) which cannot bind PP1c, did not. We also find that GFP-TIMAP^{WT} attenuates the rate of dephosphorylation of pMLC2, suggesting an inhibitory effect on MYPT1 activity, but mechanism causing MYPT1 inhibition was not identified. Nonetheless, the data

demonstrate that in living glomerular endothelial cells, overexpressed TIMAP does not act as a myosin phosphatase.

5.2 Results

5.2.1 Overexpression of TIMAP^{WT} Enhances MLC2 Phosphorylation in Glomerular Endothelial Cells.

To examine whether TIMAP acts as a myosin phosphatase in glomerular endothelial cells, the cells were infected with AdGFP-TIMAP^{WT} or the RVXF mutant AdGFP-TIMAP$^{V64A/F66A}$, which is incapable of binding PP1c (Li, Kozlowski et al. 2007). For immunofluorescence microscopy, uninfected control cells were mixed with infected cells before plating them on cover slips. Surprisingly, there was a dramatic induction of MLC2 phosphorylation and increased stress fiber formation in glomerular endothelial cells overexpressing GFP-TIMAP^{WT}, compared to uninfected control cells in the same monolayer (Figure 5.1 A). This effect of GFP-TIMAP was observed under otherwise un-stimulated conditions. We also observed that overexpression of GFP-TIMAP$^{V64A/F66A}$ using adenoviral gene transfer increases the number and length of glomerular endothelial cell projections consistent with our previous findings in transiently transfected endothelial cells (Li et al., 2007b). However, unlike the effect of GFP-TIMAP^{WT}, overexpression of the PP1c-deficient GFP-TIMAP $^{V64A/F66A}$ did not induce MLC2 phosphorylation or stress fiber formation (Figure 5.1 A). These findings are contrary to expectations, which predicted that if TIMAP functions as a bona fide myosin phosphatase

targeting subunit in cells, GFP-TIMAP$^{V64A/F66A}$, which is devoid of PP1c activity (Li, Kozlowski et al. 2007), should have enhanced, and GFP-TIMAP^{WT} should have reduced the level of MLC2 phosphorylation.

To rule out that the dramatic increase in MLC2 phosphorylation caused by the overexpression of GFP-TIMAP^{WT} is not due to an enhanced MLC2 protein expression, glomerular endothelial cells were infected with AdGFP (control), AdGFP-TIMAP^{WT}. The levels of pMLC2 and total MLC2 were determined by western blot analyses with anti-pT18/S19 MLC2 and anti-MLC2 antibodies, respectively (Figure 5.1 B). GFP-TIMAP was not detected in control AdGFP infected cells. Consistent with the immunofluorescence data (Figure 5.1 A), MLC2 phosphorylation was increased in cells infected with AdGFP-TIMAP^{WT}, compared to control infected cells (Figure 5.1 B). However, total MLC2 protein level remained similar to control infected cells (Figure 5.1 B). This finding suggests that the change in MLC2 phosphorylation is not caused by a change in total MLC2 protein expression.

To quantify the changes in MLC2 phosphorylation, glomerular endothelial cells were infected with AdGFP (control), AdGFP-TIMAP^{WT} and AdGFP-TIMAP$^{V64A/F66A}$ at an increasing MOI of 10, 20 and 40. The level of pMLC2 was determined by western blot analysis with anti-pT18/S19 MLC2 antibody, and normalized to the tubulin loading control (Figure 5.1 C). The TIMAP protein level increased appropriately with increasing AdGFP-TIMAP^{WT} or AdGFP-TIMAP$^{V64A/F66A}$ MOI. GFP-TIMAP protein was not detected in control AdGFP infected cells. Even at 10 MOI, MLC2 phosphorylation was significantly higher in cells infected with AdGFP-TIMAP^{WT}, compared to control and AdGFP-TIMAP$^{V64A/F66A}$ infected cells (Figure 5.1 C). Quantification of 3 independent

experiments (Figure 5.1 D) shows a highly significant increase in pMLC2 in AdGFP-TIMAP^{WT} infected endothelial cells, while the level of pMLC2 remained similar to control in AdGFP-TIMAP$^{V64A/F66A}$ infected cells.

Phosphorylated moesin, a member of the ERM protein family, is known to be dephosphorylated by PP1c, and was previously reported to be a substrate for the TIMAP/PP1c phosphatase activity in human pulmonary artery endothelial cells (Csortos, Czikora et al. 2008). We therefore determined whether TIMAP/PP1c functions as an ERM phosphatase in glomerular endothelial cells. However, overexpression of AdGFP-TIMAP^{WT} or AdGFP-TIMAP$^{V64A/F66A}$ did not alter ERM phosphorylation in glomerular endothelial cells, even at an MOI of 40 (Figure 5.1 C). This result suggests that TIMAP, at least when overexpressed as GFP-TIMAP^{WT}, does not act as an ERM phosphatase in glomerular endothelial cells. More importantly, the findings also indicate that enhanced MLC2 phosphorylation in response to AdGFP- TIMAP^{WT} infection is specific for MLC2, and not a general effect on all targets of PP1c.

It could be argued that AdGFP-TIMAP^{WT}–mediated stimulation of MLC2 phosphorylation is an off- target effect produced by extremely high levels of TIMAP protein expression, since protein overexpression can interfere with proper co- and post-translational processing. We therefore infected the cells with much lower MOI of AdGFP and AdGFP-TIMAP^{WT}, starting at 2.5 MOI with a gradual increase up to 40 MOI. The level of pMLC2 remained unchanged when cells were infected with increasing MOI of control AdGFP, and GFP-TIMAP was not detected in these cells. In AdGFP-TIMAP^{WT} infected cells, we observed increased MLC2 phosphorylation even at an MOI as low as 2.5, with relatively lower levels of GFP-TIMAP protein (Figure 5.1 E).

These results suggest that an artifact due to massive protein overexpression probably does not explain the findings that GFP-TIMAP^{WT} induces MLC2 phosphorylation in glomerular endothelial cells.

It could also be argued that GFP might interfere with the myosin phosphatase activity of TIMAP/PP1c. In this regard, it has been shown that MYPT3 forms a dimer that binds to PP1cβ in a hetero-tetrameric complex (Yong, Tan, Lim, & Leung, 2006), probably involving the MYPT3 N-terminal coil-coil domain. Although dimerization has not been shown for TIMAP, MYPT3 and TIMAP are structurally closely related, so if TIMAP also dimerizes, the GFP tag fused to the TIMAP N-terminus could interfere with dimerization. It was therefore important to determine whether TIMAP without the GFP tag can also induce MLC2 phosphorylation. However, an AdTIMAP vector without GFP tag was not available, and the transfection efficiency of glomerular endothelial cells with cDNA constructs is extremely low. We therefore used COS7 cells transiently transfected with plasmid cDNA constructs to determine whether TIMAP without GFP tag also induces MLC2 phosphorylation. COS7 cells were transfected transiently with GFP, GFP-TIMAP^{WT} and GFP-TIMAP$^{V64A/F66A}$ or alternatively, with empty vector, TIMAP^{WT} and TIMAP$^{V64A/F66A}$, followed by evaluation of MLC2 phosphorylation examined by western blot analysis (Figure 5.1 F). Similar to the observation in endothelial cells, overexpression of both GFP-TIMAP^{WT} and tag-free TIMAP^{WT} enhanced endogenous MLC2 phosphorylation in otherwise unstimulated COS7 cells (Figure 5.1 F) when compared to the GFP or empty vector controls. Overexpression of GFP-TIMAP$^{V64A/F66A}$ and TIMAP$^{V64A/F66A}$ did not induce MLC2 phosphorylation relative to control COS7 cells. This finding suggests that the effect of TIMAP on pMLC2 is not due to an artifact

introduced by the GFP tag. Furthermore, since TIMAP is expressed in endothelial cells but not in COS7 cells (Cao et al., 2002), the findings that $TIMAP^{WT}$ also induces MLC2 phosphorylation in COS7 cells implies that this effect of TIMAP is not due to interference by overexpressed TIMAP with the activity of endogenous TIMAP.

It is well known that phosphorylated MLC2 associates with the actin cytoskeleton (Betapudi 2014). We therefore determined whether overexpression of GFP-$TIMAP^{WT}$ increases the level of pMLC2 in the cytoskeletal fraction (Figure 5.1). Glomerular endothelial cells were infected with AdGFP (control), AdGFP-$TIMAP^{WT}$ and AdGFP-$TIMAP^{V64A/F66A}$. Cytoskeletal fractions were prepared, as described in 2.12, by lysing adherent cells with Triton-X100, then harvesting the detergent insoluble fractions that remained attached to the cell culture dishes. Soluble fractions (sup) were prepared by lysing adherent cells with NP40 lysis buffer, sedimentation at 14000 x g for 15 minutes followed by separation of the soluble supernatants from the insoluble pellets. Total cell lysates (TCL) were prepared by harvesting adherent cells immediately in hot 2X Laemmle buffer, and served as the positive control. The level of pMLC2 was determined by western blot analysis with anti-pT18/S19 MLC2 antibody. GFP-TIMAP was detected in AdGFP-$TIMAP^{WT}$ and AdGFP-$TIMAP^{V64A/F66A}$, but not control AdGFP infected cells, and was relatively enriched in the cytoskeleton fractions, compared to the supernatants (Figure 5.1 G). The PP1c□ protein level was higher in the cytoskeletal fraction of AdGFP-$TIMAP^{WT}$ infected cells, compared to control and AdGFP-$TIMAP^{V64A/F66A}$ infected cells (Figure 5.1 G). This finding is further explained in Figure 5.5. Also, PP1c□ was enriched in the cytoskeletal fraction of AdGFP-$TIMAP^{WT}$ infected cells, but not in

the supernatant, and it remained similar in the cytoskeletal fractions and supernatants of control and AdGFP-TIMAP$^{V64A/F66A}$ infected cells (Figure 5.1 G). Similar to findings in (Figure 5.1 A, C, D and F), the level of pMLC2 was increased in cells overexpressing GFP-TIMAP^{WT}, compared to cells overexpressing control and GFP-TIMAP$^{V64A/F66A}$ (Figure 5.1 G). Furthermore, and similar to PP1cβ, the level of pMLC2 was markedly higher in the cytoskeletal fraction of AdGFP-TIMAP^{WT} infected cells, compared to the supernatant, while it remained similar in the cytoskeletal fractions and supernatants of control and AdGFP-TIMAP$^{V64A/F66A}$ infected cells. This finding is consistent with the fact that overexpressed GFP-TIMAP^{WT} stimulates MLC2 phosphorylation, which results in enhanced association of pMLC2 with actin. Furthermore, these data indicate that association of GFP-TIMAP^{WT}, PP1cβ and pMLC2 proteins with the cytoskeleton fraction is enhanced in AdGFP-TIMAP^{WT} infected cells. Taken together, our results demonstrate that GFP-TIMAP^{WT} does not act as a phosphatase toward MLC2 in living cells, but strongly enhances global MLC2 phosphorylation without the requirement for agonist stimulation. As predicted, phosphorylation of MLC2 in response to GFP-TIMAP^{WT} overexpression leads to its association with cytoskeletal actin.

TIMAP is a plasma membrane associated MYPT family member (Cao et al., 2002; Grassie, Moffat, Walsh, & MacDonald, 2011), and predominantly co-localizes with cortical, but not stress fiber-associated MLC2 and pMLC2 (Figure 4.4). Since our findings indicate that overexpressed GFP-TIMAP^{WT} enhances global MLC2 phosphorylation, and not only cortical MLC2 phosphorylation (Figures 5.1), we examined whether this effect is due to co-localization of overexpressed GFP-TIMAP^{WT} with MLC2 at cellular locations other than the cortex of the cell. Glomerular endothelial

cells were infected with AdGFP-TIMAP^{WT} and immuno-stained with anti-MLC2 and anti-pT18/S19 MLC2 antibodies. Confocal immunofluorescence analysis of the infected cells revealed that, similar to endogenous TIMAP, overexpressed GFP-TIMAP^{WT} predominantly co-localized with MLC2 and pMLC2 at the cell cortex (Figure 5.2, middle top for MLC2, and middle bottom for pMLC2). Although TIMAP and pMLC2 were detected in the center of the cell, no co-localization was observed between the two proteins in that location. Thus, although overexpressed GFP-TIMAP^{WT} is associated with the cell cytoskeleton and stimulates global MLC2 phosphorylation, this effect is not due to co-localization of TIMAP and MLC2 at cellular locations other than the cell membrane.

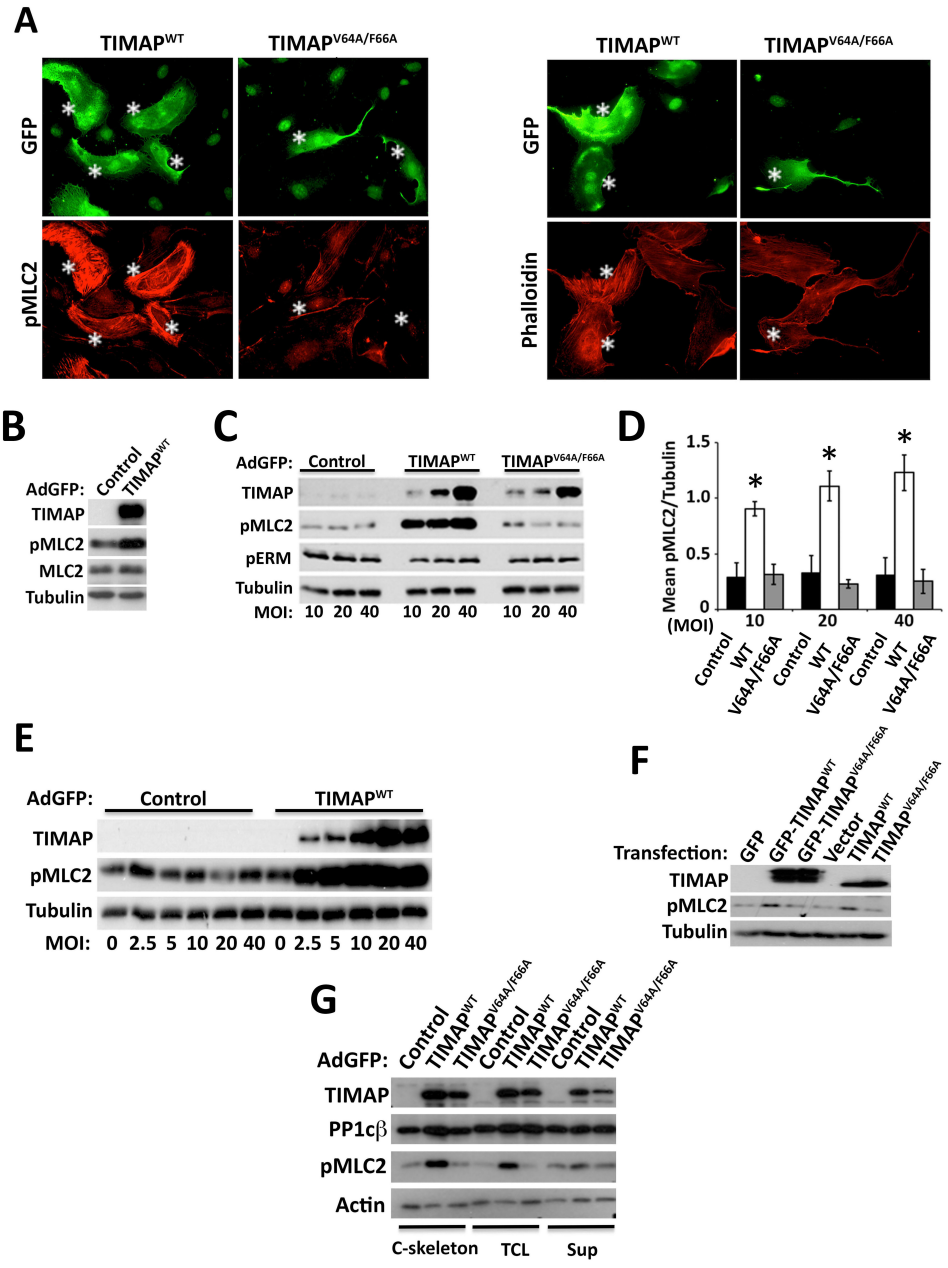

Figure 5.1: Overexpression of GFP-TIMAP^{WT} enhances MLC2 phosphorylation in glomerular endothelial cells. A. Immunofluorescence images (40X) of overexpressed GFP-TIMAP^{WT} (green, top left, white asterisks), overexpressed GFP-TIMAP$^{V64A/F66A}$ (green, top right, white asterisks), endogenous pMLC2 (left panel) and Phalloidin (right panel) (red) in glomerular endothelial cells infected for 48 hours and re-plated on glass cover slips coated with quick coat overnight in serum-

replete medium (representative of 3 independent experiments). **B.** Western blot of TIMAP, pMLC2, total MLC2 and Tubulin in total cell lysates of glomerular endothelial cells infected with AdGFP-control or AdGFP-TIMAP^{WT} for 48 hours and maintained in serum-replete medium (representative of 3 independent experiments). **C.** Western blot of TIMAP, pMLC2, pERM and Tubulin in total cell lysates of glomerular endothelial cells infected with AdGFP-control or AdGFP-TIMAP^{WT} or AdGFP-TIMAP$^{V64A/F66A}$ at MOI 10, 20, and 40 for 48 hours and maintained in serum-replete medium. **D.** Mean ratio of pMLC2: Tubulin (mean ± SEM, n=3 independent experiments, *p< 0.001). **E.** Western blot of TIMAP, pMLC2 and Tubulin in total cell lysates of glomerular endothelial cells infected with AdGFP-control or AdGFP-TIMAP^{WT} at MOI 0, 2.5, 5, 10, 20, and 40 for 48 hours and maintained in serum-replete medium (representative of 2 independent experiments). **F**. Western blot of TIMAP, pMLC2 and Tubulin in total cell lysates of COS-7 cells transfected with AdGFP-control or AdGFP-TIMAP^{WT} or AdGFP-TIMAP$^{V64A/F66A}$ (lanes 1,2 and 3, respectively) and with vector control, TIMAP^{WT} or TIMAP$^{V64A/F66A}$ (lanes 4,5 and 6, respectively) for 48 hours and maintained in serum- replete medium (representative of 2 independent experiments). **G.** Western blot of TIMAP, PP1c, pMLC2 and Actin (loading control) in the cytoskeleton fraction (C-skeleton), total cell lysates (TCL) and soluble fraction (sup) of glomerular endothelial cells infected with AdGFP-control or AdGFP-TIMAP^{WT} or AdGFP-TIMAP$^{V64A/F66A}$ for 48 hours and maintained in serum-replete medium (representative of 2 independent experiments).

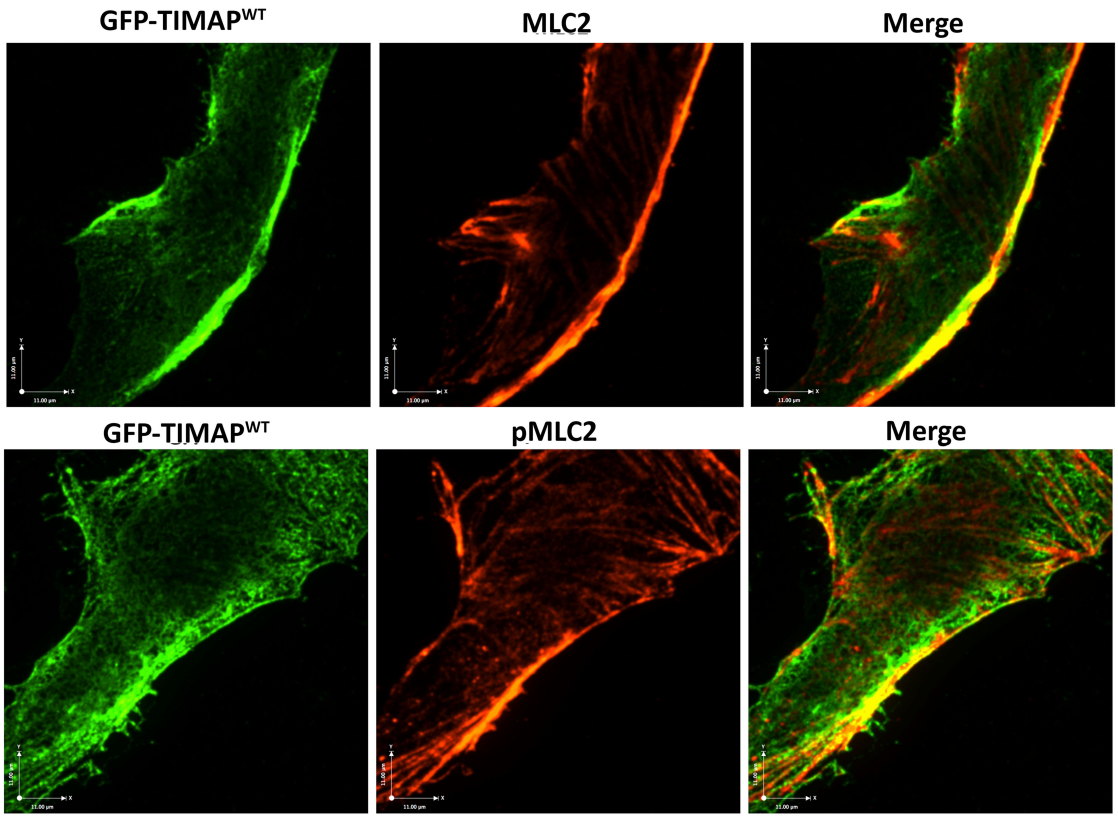

Figure 5.2: AdGFP-TIMAP^{WT} co-localizes with cortical MLC2 and pMLC2 in glomerular endothelial cells. A. Confocal immunofluorescence images (60X) of glomerular endothelial cells overexpressing GFP-TIMAP^{WT} (GFP, left) immune-stained with anti- MLC2 (Middle top) or pMLC2 (middle bottom) antibody (red). Cells were infected for 48 hours and re-plated overnight on glass coverslips coated with quick coat in serum-replete medium. Merge (right) shows co-localization between GFP and MLC2 or pMLC2 (representative of 2 independent experiments).

5.2.2 PKA/GSK3β Phosphorylation site Mutants of TIMAP also Enhance MLC2 Phosphorylation.

We previously reported that TIMAP is itself phosphorylated at Ser333 and Ser337 by GSK3β and PKA, respectively. This phosphorylation enhances the activity of the TIMAP/PP1c holoenzyme immuno-precipitated from cell lysates, toward the small artificial fluorogenic substrate 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) (Li et al., 2007b). The phosphomimic mutant ($TIMAP^{S333E/S337E}$) showed enhanced activity of its associated PP1c toward DiFMUP, whereas the $TIMAP^{S333A/S337A}$ mutant, which cannot be phosphorylated by PKA/GSK3β, showed reduced activity even though it bound PP1c perfectly well (Li et al., 2007b). It is similarly well-known that activation of MYPT1 requires dephosphorylation of two specific residues (Thr 696 and Thr 853) in the C-terminal region of the molecule (Feng, Ito et al. 1999). In both cases, it is thought that the C-terminus of the regulatory subunit auto-inhibits the bound PP1c subunit, and that phosphorylation regulates this auto-inhibition, producing an active enzyme when MYPT1 is dephosphorylated at Thr 696 and Thr 853, and conversely when TIMAP is phosphorylated at Ser 333 and Ser 337.

We therefore reasoned that overexpressed GFP-$TIMAP^{WT}$ could be inactive when introduced into cells, and that PKA/ GSK3β phosphorylation at Ser 333 and Ser 337 might be required to demonstrate activity against pMLC2 in cells. To test this possibility glomerular endothelial cells were infected with adenoviral constructs encoding the phosphorylation-defective GFP-$TIMAP^{S333A/S337A}$ mutant or the phosphorylation

mimic GFP-TIMAP$^{S333E/S337E}$, and MLC2 phosphorylation was examined by immunofluorescence and western blot analysis. Immunofluorescence microscopy revealed that overexpression of both, GFP-TIMAP$^{S333A/S337A}$ and GFP-TIMAP$^{S333E/S337E}$, enhanced MLC2 phosphorylation compared to uninfected control cells (Figure 5.3 A), and that overexpression of GFP-TIMAP$^{S333A/S337A}$ or GFP-TIMAP$^{S333E/S337E}$ increased stress fibers formation (Figure 5.3 A). Similar to the findings by immunofluorescence microscopy, western blot analyses of glomerular endothelial cells infected with the increasing MOI (10, 20 and 40) of AdGFP-TIMAP$^{S333A/S337A}$ or AdGFP-TIMAP$^{S333E/S337E}$ revealed that MLC2 phosphorylation was strongly enhanced at 10 MOI compared to AdGFP control infected cells, and it reached a maximum at 20 MOI (Figure 5.3 B). Neither GFP-TIMAP nor pMLC2 were detected in AdGFP infected control cells. Furthermore, the effect of GFP-TIMAP$^{S333A/S337A}$ and GFP¬TIMAP$^{S333E/S337E}$ on MLC2 phosphorylation was essentially identical.

To determine whether the different AdGFP-TIMAP constructs used in this study form a complex with endogenous PP1c, we infected glomerular endothelial cells with the different AdGFP constructs and immunoprecipitated TIMAP. We observed that GFP-TIMAP was detected in total cell lysates and in TIMAP immunoprecipitates of cells infected with the different AdGFP-TIMAP constructs, but not in lysates or immunoprecipitates from AdGFP infected control cells. Endogenous PP1c was detected in all total cell lysates and co-immunoprecipitated with GFP-TIMAP^{WT}, TIMAP$^{S333A/S337A}$ and GFP-TIMAP$^{S333E/S337E}$, but not with GFP-TIMAP$^{V64A/F66A}$. These findings are consistent with our previous work (Li et al., 2007b) and indicate that only

TIMAP capable of forming a complex with PP1c can enhance MLC2 phosphorylation in endothelial cells under otherwise unstimulated conditions.

Taken together, the results in this section indicate that, like GFP-TIMAP^{WT}, GFP-TIMAP$^{S333A/S337A}$ and GFP-TIMAP$^{S333E/S337E}$ enhance MLC2 phosphorylation in otherwise unstimulated cells, and therefore suggest that the state of PKA/GSK3β phosphorylation of TIMAP at Ser333/Ser337 does not play a role in augmenting MLC2 phosphorylation. Since we know that Ser333/Ser337 phosphorylation of TIMAP regulates its associated phosphatase activity (Li, Kozlowski et al. 2007, Shopik, Li et al. 2013), the results are consistent with the emerging interpretation that TIMAP-PP1c does not display phosphatase activity toward pMLC2 under these experimental conditions. Furthermore, the GFP-TIMAP-stimulated MLC2 phosphorylation is dependent on the ability of TIMAP to form a complex with PP1c. The data so far indicate that TIMAP-PP1c does not act as a myosin phosphatase in endothelial cells, but instead enhances MLC2 phosphorylation through an as yet undetermined mechanism.

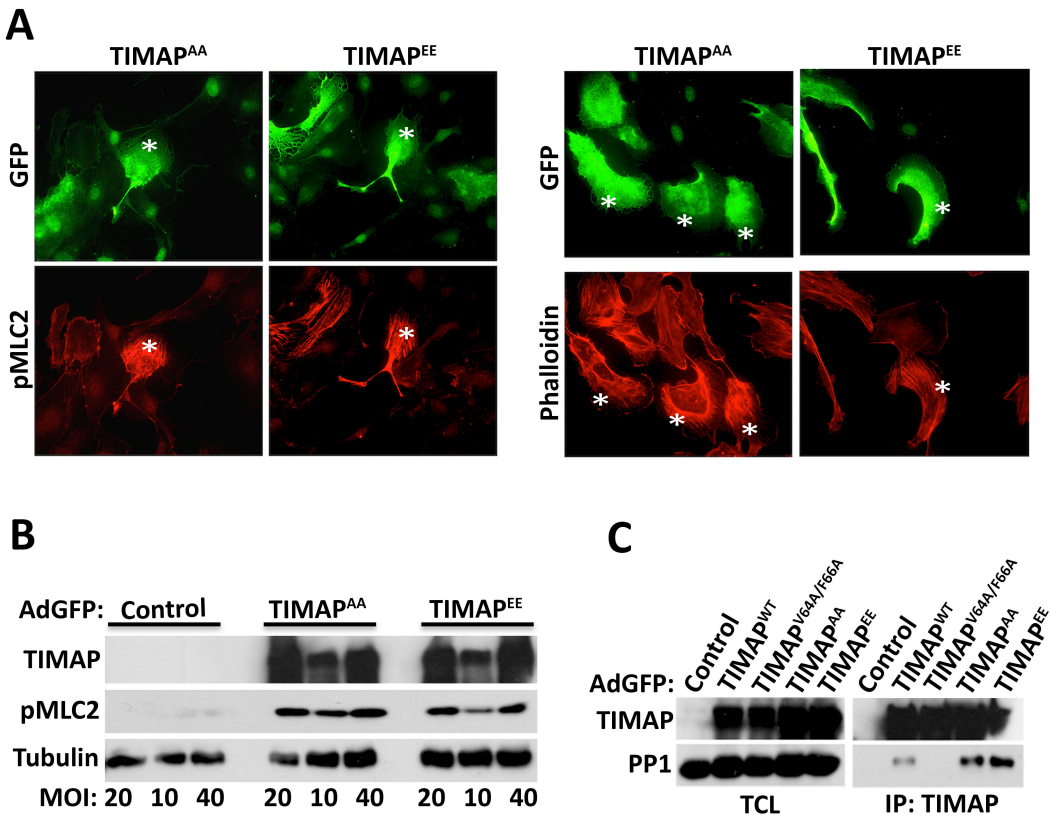

Figure 5.3: Overexpression of GFP-TIMAP^{AA} and GFP-TIMAP^{EE} enhances MLC2 Phosphorylation in glomerular endothelial cells. A. Immunofluorescence images (40X) of overexpressed GFP-TIMAP^{AA} (green, top left, white asterisks), overexpressed GFP-TIMAP^{EE} (green, top right, white asterisks), endogenous pMLC2 (left panel) and Phalloidin (right panel) (red) in glomerular endothelial cells infected for 48 hours and re-plated on glass coverslips coated with quick coat overnight in serum-replete medium (representative of 3 independent experiments). **B.** Western blot of TIMAP, pMLC2 and Tubulin in total cell lysates of glomerular endothelial cells infected with AdGFP-control, AdGFP-TIMAP^{AA} or AdGFP-TIMAP^{EE} at MOI 20, 10, and 40 for 48 hours and maintained in serum-replete medium (representative of 2 independent experiments). **C.** Western blot of TIMAP and PP1c in total cell lysates (TCL) and anti-TIMAP IgY (IP-TIMAP) immunoprecipitates of glomerular endothelial cells infected with AdGFP-control, AdGFP-TIMAP^{WT}, AdGFP-

TIMAP$^{V64A/F66A}$, AdGFP-TIMAP^{AA} or AdGFP-TIMAP^{EE} for 48 hours and maintained in serum-replete medium (representative of 2 independent experiments).

5.2.3 Overexpression of TIMAP^{WT} Reduces the Rate of Dephosphorylation of pMLC2.

Phosphorylation of MLC2 is regulated by the actions of myosin kinases and MYPTs (Hirano, Derkach, Hirano, Nishimura, & Kanaide, 2003; Totsukawa et al., 2004). MLCK and ROCK phosphorylate MLC2 on both Ser19 and Thr18 (Ikebe 1989, Itoh, Hara et al. 1992, Getz, Dangelmaier et al. 2010). ROCK simultaneously phosphorylates the principal myosin phosphatase MYPT1 on Thr696 and Thr853, inactivating it (Feng, Ito et al. 1999, Birukova, Smurova et al. 2004). The fact that overexpressed TIMAP/PP1c induces total MLC2 phosphorylation suggests an inhibitory effect on the myosin phosphatase or an activating effect on the myosin kinase(s). To determine whether the increase in pMLC2 in response to overexpression of GFP-TIMAP^{WT} is due to attenuated myosin phosphatase activity, glomerular endothelial cells were infected with AdGFP, AdGFP-TIMAP^{WT} or AdGFP-TIMAP$^{V64A/F66A}$. Cells were then treated with Y27632, an inhibitor of ROCK or ML7, an inhibitor of MLCK. The remaining Thr18/Ser19 phosphorylation of MLC2 then determined as function of time by western blot analysis with anti-pT18/pS19 MLC2 antibody. The western blots show that at time = 0, the level of pMLC2 was higher in cells infected with AdGFP-TIMAP^{WT} compared to the AdGFP control and AdGFP-TIMAP$^{V64A/F66A}$ infected cells (Figure 5.4 A). In AdGFP and AdGFP-TIMAP$^{V64A/F66A}$ infected cells, inhibition of MLCK with ML7 resulted in a time-dependent decrease of pMLC2, with a maximum inhibition of MLC2 phosphorylation at 30 - 60 minutes after addition of the inhibitor. By contrast, in AdGFP-TIMAP^{WT} infected cells, ML7 also reduced MLC2 phosphorylation, but substantial pMLC2 remained 60 minutes after addition of the inhibitor (Figure 5.4 A).

To determine whether the difference at 30 and 60 minutes is accounted for by the difference in the initial higher level of pMLC2, vs. a difference in the rate of dephosphorylation, the ratio of pMLC2 at each time point: pMLC2 at t =0 was determined from 3 independent experiments (Figure 5.4 B). The rate of MLC2 dephosphorylation was significantly reduced in cells infected with AdGFP-TIMAP^{WT} (white bars), compared to AdGFP (black bars) and AdGFP-TIMAP$^{V64A/F66A}$ (grey bars) infected cells.

While MLCK directly phosphorylates MLC2 in cells (Ishikawa & Hidaka, 1990; Totsukawa et al., 2004), the rapid ROCK-mediated increase in MLC2 phosphorylation is mainly due to inhibition of MYPT1 activity toward pMLC2 (Feng et al., 1999). Therefore, inhibition of ROCK results in activation of MYPT1, and consequently the dephosphorylation of pMLC2. Consistent with previous results, we observed that the level of pMLC2 at t = 0 was higher in cells infected with AdGFP-TIMAP^{WT} compared to AdGFP control and AdGFP-TIMAP$^{V64A/F66A}$ infected cells (Figure 5.4 C). Inhibition of ROCK with Y27632 resulted in a very rapid, time-dependent dephosphorylation of pMLC2 in all infected cells (Figure 5.4 C). However, whereas pMLC2 was nearly undetectable in control AdGFP- and AdGFP- TIMAP$^{V64A/F66A}$ infected cells 5 min after addition of Y27632, substantially more pMLC2 remained detectible in the AdGFP-TIMAP^{WT}-infected at the same time-point (Figure 5.4 C). To determine whether the difference is accounted for by the difference in the initial higher level of pMLC2, or a difference in the rate of dephosphorylation, the ratio of pMLC2 at each time point: pMLC2 at t =0 was determined (Figure 5.4 D). Similar to the findings with ML7, the rate of pMLC2 dephosphorylation was significantly reduced in cells infected with AdGFP-

TIMAP^{WT} (white bars), compared to AdGFP (black bars) and AdGFP-TIMAP$^{V64A/F66A}$ (grey bars) infected cells (Figure 5.4 D). The findings that dephosphorylation during ROCK inhibition is much more rapid than that observed with ML7 suggests strongly that MYPT1, which is known to be inhibited by ROCK is the phosphatase acting to dephosphorylate pMLC2 in these glomerular endothelial cells. Even though the time course of Y27632 treatment was much shorter than that for ML7, presumably due to the activation of MYPT1, the results still suggest that myosin phosphatase activity was significantly reduced in AdGFP-TIMAP^{WT} infected cells compared to AdGFP control and AdGFP¬TIMAP$^{V64A/F66A}$ infected cells. The results in this experiment also suggest that the pool of MLC2 that is hyperphosphorylated in the presence of GFP-TIMAP^{WT} is sensitive to ROCK inhibition and therefore to MYPT1 activation.

We therefore conclude that overexpression of GFP-TIMAP^{WT}, but not GFP-TIMAP$^{V64A/F66A}$ inhibits the myosin phosphatase activity in glomerular endothelial cells, implying that overexpressed GFP-TIMAP^{WT} does not act as a typical myosin phosphatase in endothelial cells. The fact that GFP-TIMAP^{WT} strongly enhances global MLC2 phosphorylation in otherwise unstimulated cells, that GFP-TIMAP^{WT} attenuates the rate of pMLC2 dephosphorylation, and that the pMLC2 is sensitive to ROCK inhibition all suggest that overexpressed AdGFP-TIMAP^{WT} interferes with MYPT1 action against pMLC2. Nonetheless, since ERM proteins have been reported to be substrates of MYPT1 phosphatase activity (Eto, Kirkbride et al. 2005), but GFP-TIMAP^{WT} does not augment ERM protein phosphorylation, the findings imply that the effect of GFP-TIMAP^{WT} to enhance phosphorylation is somehow restricted to pMLC2.

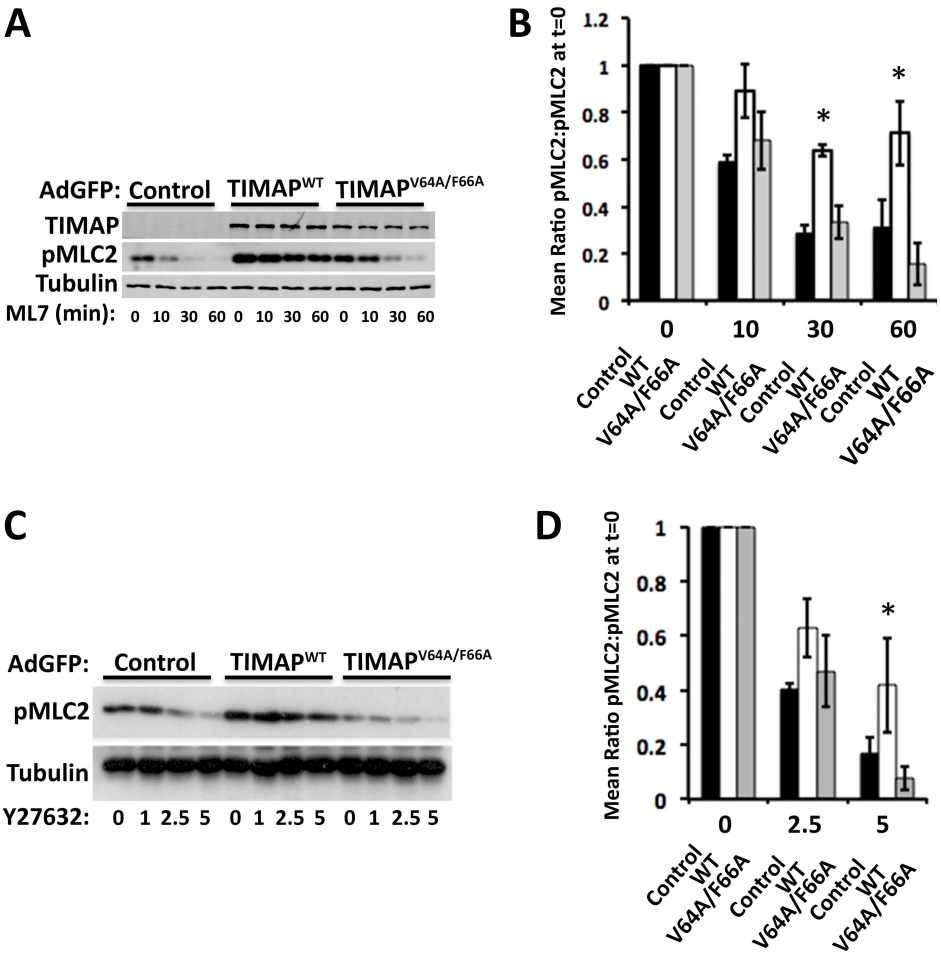

Figure 5.4: Overexpression of GFP-TIMAP^{WT} reduces the rate of dephosphorylation of pMLC2 in glomerular endothelial cells. A. Western blot of TIMAP, pMLC2 and Tubulin in total cell lysates of glomerular endothelial cells infected with AdGFP-control, AdGFP-TIMAP^{WT} or AdGFP-TIMAP$^{V64A/F66A}$ for 48 hours and treated with ML7 in serum-replete medium for 0, 10, 30 and 60 minutes. **B**. Fold change of pMLC2 (mean ± SEM, n=3 independent experiments, *p< 0.001). **C**. Western blot of pMLC2 and Tubulin in total cell lysates of glomerular endothelial cells infected with AdGFP-control, AdGFP-TIMAP^{WT} or AdGFP-TIMAP$^{V64A/F66A}$ for 48 hours and treated with Y27632 in serum-replete medium for 0, 1, 2,5 and 5 minutes. **D**. Fold change of pMLC2 (mean ± SEM, n=4 independent experiments, *p< 0.05).

5.2.4 GFP-TIMAP^{WT} does not Reduce MYPT1 Protein Abundance

MYPT1 is the dominant MYPT in most non-skeletal muscle cells, and changes in MYPT1 activity determine the state of MLC2 phosphorylation and stress fibers formation (Totsukawa et al., 2004). Similar to our findings with GFP-TIMAP^{WT} overexpression (Figures 5.1), silencing of MYPT1 strongly enhances MLC2 phosphorylation, and stress fiber formation in endothelial cells (Xia, Stull et al. 2005). Furthermore, given that the rate of pMLC2 dephosphorylation is markedly slower in glomerular endothelial cells overexpressing GFP-TIMAP^{WT} than in control cells (Figures 5.4), and that dephosphorylation of stress-fiber associated MLC2, which does not co-localize with AdGFP-TIMAP^{WT} (Figure 4.2) is nevertheless inhibited by GFP-TIMAP^{WT}, an indirect mechanism involving inhibition of MYPT1 seems most plausible. Since silencing of MYPT1 strongly induces stress fibers formation and contractility in smooth muscle cells (Cheng, Cheng et al. 2013), we first determined whether GFP-TIMAP^{WT} alters MYPT1 protein abundance. Glomerular endothelial cells were infected with AdGFP control, AdGFP-TIMAP^{WT} or AdGFP-TIMAP$^{V64A/F66A}$ (Figure 5.5 A). No change in the MYPT1 protein abundance was observed. We also determined the localization of MYPT1 in glomerular endothelial cells expressing either the AdGFP-TIMAP^{WT} or AdGFP-TIMAP$^{V64A/F66A}$ in order to define whether MYPT1 localization changes under conditions of AdGFP-TIMAP^{WT}-induced MLC2 phosphorylation. MYPT1 immunoreactivity in the glomerular endothelial cells was fibrillar and did not localize to the periphery of cells (Figure 5.5 B). The localization of MYPT1 immunoreactivity was similar whether in cells expressing GFP-TIMAP^{WT} or GFP-TIMAP$^{V64A/F66A}$. Taken together, the findings

indicate that the enhanced MLC2 phosphorylation caused by AdGFP-TIMAP^{WT} is not due to a change in MYPT1 protein abundance or MYPT1 protein re-localization.

5.2.5 GFP-TIMAP^{WT} does not reduce PP1cβ protein abundance or MYPT1 association

Since GFP-TIMAP^{WT}, GFP-TIMAP$^{S333A/S337A}$ and GFP-TIMAP$^{S333E/S337E}$ all strongly stimulate MLC2 phosphorylation (Figure 5.1 and 5.3) and all bind PP1cβ (Figure 5.3 C), it can be concluded that enhanced MLC2 phosphorylation in response to TIMAP overexpression is PP1c-dependent. Since the PP1cβ catalytic subunit is shared by TIMAP and MYPT1 (Grassie, Moffat et al. 2011), we considered the possibility that GFP-TIMAP^{WT} competes with MYPT1 for PP1cβ, sequestering and inhibiting PP1cβ, and leaving a pool of MYPT1 without associated PP1cβ, reducing MYPT1-dependent phosphatase activity against specific MYPT1 substrate(s). We therefore first determined the abundance of endogenous PP1cβ in glomerular endothelial cells infected with AdGFP control, AdGFP-TIMAP^{WT}, and GFP-TIMAP$^{V64A/F66A}$ by western blot analysis of whole cell lysates using anti-PP1cβ antibodies. Expression of endogenous PP1c protein was higher in cells infected with 10 MOI AdGFP-TIMAP^{WT}, compared to control and AdGFP-TIMAP$^{V64A/F66A}$ infected cells. Increasing the MOI did not further increase the expression of endogenous PP1cβ in our cells (Figure 5.5 C). This finding indicates that endothelial cells respond to the overexpression of GFP-TIMAP^{WT} with enhanced expression (or reduced degradation) of the endogenous PP1cβ subunit,

whereas overexpression of the PP1c-deficient TIMAP$^{V64A/F66A}$ does not augment endogenous PP1cβ abundance. This finding indicates that the enhanced MLC2 phosphorylation in our cells upon infection with AdGFP-TIMAP^{WT} cannot be attributed to inhibition of PP1cβ protein expression. The data furthermore are consistent with the conclusion that the effect of AdGFP-TIMAP^{WT} on MLC2 phosphorylation is PP1cβ-dependent, and with the possibility that the cells are compensating for the lack of sufficient PP1cβ for partnering targeting subunits by making more of it. The finding that an increasing AdGFP-TIMAP^{WT} MOI above 10 is associated with a dose-dependent increase in MLC2 phosphorylation (Figure 5.1 C-E), whereas PP1cβ expression is maximal at an AdGFP-TIMAP^{WT} MOI of 10 and does not increase further, would still leave open the possibility that competition of AdGFP-TIMAP^{WT}, but not AdGFP-TIMAP$^{V64A/F66A}$ for PP1cβ reduces the availability of PP1cβ for MYPT1, thereby reducing MYPT1/PP1cβ activity. We reasoned that if PP1cβ is the rate-limiting step for MLC2 dephosphorylation and sequestered and inhibited by GFP-TIMAP^{WT}, then further overexpression of PP1cβ might reduce MLC2 phosphorylation. We therefore co-infected endothelial cells with AdFlag-PP1cβ, to investigate this possibility. While we observed strong expression of the Flag-PP1cβ protein, the total pool of PP1cβ did not change, suggesting that the cells regulate endogenous PP1cβ expression not only in response to overexpression of a regulatory subunit that can bind it (Figure 5.5 C), but also in response to exogenous expression of PP1cβ (Figure 5.5 D). Furthermore, overexpression of AdFlag-PP1c did not reduce MLC2 phosphorylation in response to GFP-TIMAP^{WT} overexpression. Taken together, these findings indicate that PP1cβ protein abundance is coordinately regulated with TIMAP expression. We postulate that the increase in PP1cβ

abundance is sufficient to mitigate competition between TIMAP and MYPT1 for PP1cβ, and that the effect of GFP-TIMAP^{WT} on MLC2 phosphorylation is not due to reduced association of PP1cβ with MYPT1. Nonetheless, since GFP-TIMAP^{WT} strongly induces MLC2 phosphorylation, since the rate of pMLC2 dephosphorylation is reduced in GFP-TIMAP^{WT} expressing cells (Figure 5.4), since the GFP-TIMAP^{WT} induced pool of pMLC2 is sensitive to ROCK inhibition (Figure 5.4 C), and since MYPT1 is the principal myosin phosphatase in endothelial cells, it still is highly likely that TIMAP, in some way, regulates MYPT1 activity.

5.2.6 GFP-TIMAP^{WT} does not Alter ROCK-dependent MYPT1 Phosphorylation.

MYPT1 activity is strongly inhibited by phosphorylation at Thr 696 and Thr 853 in the C-terminal domain (Feng, Ito et al. 1999, Muranyi, Derkach et al. 2005). We already know that ROCK inhibition with Y27632 results in almost complete MLC2 dephosphorylation by 5 minutes in AdGFP and AdGFP-TIMAP$^{V64A/F66A}$ infected cells (Figure 5.4 C and D). By contrast, in AdGFP-TIMAP^{WT} infected cells the rate of dephosphorylation in the presence of the ROCK inhibitor was significantly slower (Figure 5.4 C and D). This finding, together with the massive increase in MLC2 phosphorylation could be consistent with increased RhoA/ROCK activity in the AdGFP-TIMAP^{WT} infected cells, causing inhibition of MYPT1 activity. We therefore used western blot analysis with anti-pT696 and anti p-T853 specific antibodies to evaluate inhibitory MYPT1 phosphorylation in total cell lysates of AdGFP, AdGFP-

TIMAP$^{V64A/F66A}$ and AdGFP-TIMAP^{WT} infected glomerular endothelial cells. We observed that the ratio of pT696 MYPT1: total MYPT1 and of pT853: total MYPT1 were essentially the same, whether the cells were infected with AdGFP-TIMAP^{WT}, AdGFP control or AdGFP-TIMAP$^{V64A/F66A}$ (Figure 5.6 A and B). To further resolve whether RhoA activation could be responsible for MYPT1 inhibition by AdGFP-TIMAP^{WT}, we also determined the abundance of GTP-Rho (active Rho) in cells expressing GFP-TIMAP^{WT}, GFP or GFP-TIMAP$^{V64A/F66A}$ using the Rhotekin -GTP-Rho pulldown assay. Rho was detected in all cells, and the abundance of Rho-GTP pulled from the lysates did not differ between GFP-TIMAP^{WT}, GFP and GFP-TIMAP $^{V64A/F66A}$ expressing cells. These findings suggest that the activity of Rho is not significantly changed by AdGFP-TIMAP^{WT} infection, and is consistent with the observation that overexpression of GFP-TIMAP^{WT} does not alter MYPT1 phosphorylation on the ROCK-sensitive sites Thr 696 and Thr 853 in the MYPT 1 C-terminus (Figure 5.6 A and B).

As discussed in chapter 1, MYPT1 activity can also be inhibited by CPI-17 when it is phosphorylated on Thr 38 (Eto, Ohmori, Suzuki, Furuya, & Morita, 1995; Senba, Eto, & Yazawa, 1999). Since we know that overexpressed GFP-TIMAP^{WT} inhibits pMLC2 dephosphorylation, we also evaluated whether total and/or phosphorylated CPI-17 increased in cells expressing GFP-TIMAP^{WT}. Western blot analysis showed no significant difference in the levels of pT38 CPI-17 or total CPI-17, relative to the -tubulin loading control, whether cells were infected with AdGFP-TIMAP^{WT}, AdGFP or AdGFP-TIMAP$^{V64A/F66A}$. Therefore, changes in CPI-17 abundance or phosphorylation cannot explain the apparent inhibitory effect of GFP-TIMAP^{WT} on MYPT1. The data

furthermore rule out the possibility that GFP-TIMAP^{WT} acts as a phosphatase toward CPI-17.

5.2.7 GFP-TIMAP^{WT} does not Inhibit MYPT1 through a Direct Interaction

The phosphatase activity of all members of the MYPT family of PP1c regulatory subunits is regulated through phosphorylation of their C-terminal domain (Figure 1.2). It has also been shown that expression of C-terminal fragments of MYPT1 and MYPT3 can inhibit MYPT1/PP1c and MYPT3/PP1c activity (Yong, Tan et al. 2006), respectively, implying that the C-terminal domain of the regulatory subunit could flip into the active site of the associated PP1c, blocking the phosphatase activity. Since overexpression of GFP-TIMAP^{WT} seems to inhibit MYPT1 activity, we considered the possibility that TIMAP^{WT} interacts directly with MYPT1, with the C-terminal domain of TIMAP blocking the active site of MYPT1-associated PP1c. We reasoned that if TIMAP were to directly affect MYPT1 activity, the two proteins should be in the same complex. To examine this, glomerular endothelial cells were infected with AdGFP control AdGFP-TIMAP^{WT} and AdGFP-TIMAP$^{V64A/F66A}$, GFP-TIMAP was immunoprecipitated with anti-GFP antibody and MYPT1 was detected with anti-MYPT1 antibody. GFP-TIMAP^{WT} and GFP-TIMAP$^{V64A/F66A}$ were detected in the total cell lysates (Figure 5.7 A left) and in the GFP immunoprecipitates (Figure 5.7 A right) of the infected cells, but not in AdGFP control infected cells. MYPT1 was detected in the total cell lysates of all infected cells (Figure 5.6 A left), but MYPT1 was not detected in the GFP immunoprecipitates of any of the infected cells (Figure 5.6 B). As expected, PP1c was readily detected only in the

GFP immunoprecipitates of GFP-TIMAP^{WT} infected cells, and served as the positive control. These data suggest that GFP-TIMAP^{WT} and MYPT1 are not in the same complex in our infected cells.

We also evaluated the cellular distribution of MYPT1 and GFP-TIMAP^{WT} using confocal immunofluorescence. Glomerular endothelial cells were infected with AdGFP-TIMAP^{WT} or GFP-TIMAP $^{V64A/F66A}$ and followed by immuno-labeling with anti-MYPT1 antibody. As shown in (Figure 5.6 B), GFP-TIMAP^{WT} and GFP-TIMAP$^{V64A/F66A}$ were predominantly localized at the cell membrane/cell projections, with some cytoplasmic distribution. This is consistent with our previous findings of endogenous TIMAP (Figure 4.4) and (Cao et al., 2002). In contrast, MYPT1 was predominantly observed in the cytoplasm, with minimal cell membrane staining where it co-localized with TIMAP. This finding indicates that GFP-TIMAP and MYPT1 can co-localize in glomerular endothelial cells, but this co-localization is restricted to only a few areas at or near the plasma membrane. The vast majority of MYPT1 immunoreactivity did not co-localize with GFP-TIMAP^{WT} or GFP-TIMAP$^{V64A/F66A}$

Thus, findings that MYPT1 and GFP-TIMAP^{WT} fail to co-immunoprecipitate and that the majority of MYPT1 fails to co-localize with GFP-TIMAP^{WT} in glomerular endothelial cells essentially rules out the possibility that TIMAP and MYPT1 form heterodimers, and also rules out the possibility that the TIMAP C-terminus could directly inhibit MYPT1-associated PP1c.

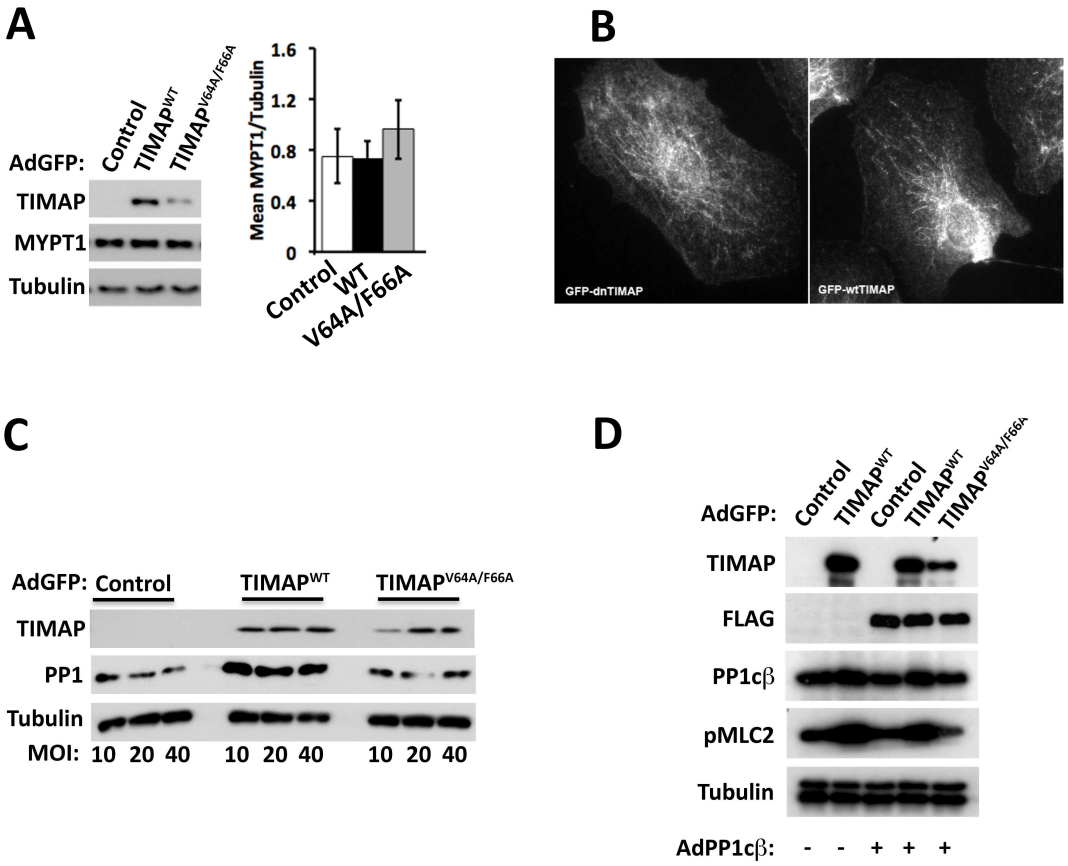

Figure 5.5: Overexpression of GFP-TIMAP^{WT} increases endogenous PP1c protein expression without affecting endogenous MYPT1 protein expression. **A**. (Left panel): Western blot of TIMAP, MYPT1 and Tubulin in total cell lysates of glomerular endothelial infected with AdGFP-control, AdGFP-TIMAP^{WT} or AdGFP-TIMAP$^{V64A/F66A}$ for 48 hours and maintained in serum-replete medium. (Right panel): Ratio of MYPT1: Tubulin (mean ± SEM, n=3 independent experiments). **B.** Immunofluorescence images (40X) of glomerular endothelial cells overexpressing GFP-TIMAP^{WT} (left) or GFP-TIMAP$^{V64A/F66A}$ (right) immuno-stained with anti-MYPT1 antibody. Cells were infected for 48 hours and re-plated on glass coverslips coated with quick coat overnight in serum-replete medium (representative of 2 independent experiments). **C**. Western blot of TIMAP, PP1c and Tubulin in total cell lysates of glomerular endothelial cells infected with AdGFP-control, AdGFP-TIMAP^{WT} or AdGFP-TIMAP$^{V64A/F66A}$ at MOI 10, 20, and 40 for 48 hours and

maintained in serum-replete medium (representative of 2-3 independent experiments). **E.** Western blot of TIMAP, FLAG, PP1c pMLC2 and Tubulin in total cell lysates of glomerular endothelial infected with AdGFP-control, AdGFP-TIMAP^{WT} or AdGFP-TIMAP$^{V64A/F66A}$ co-infected (+) or not (-) with AdPP1c for 48 hours and maintained in serum-replete medium (representative of 2-3 independent experiments).

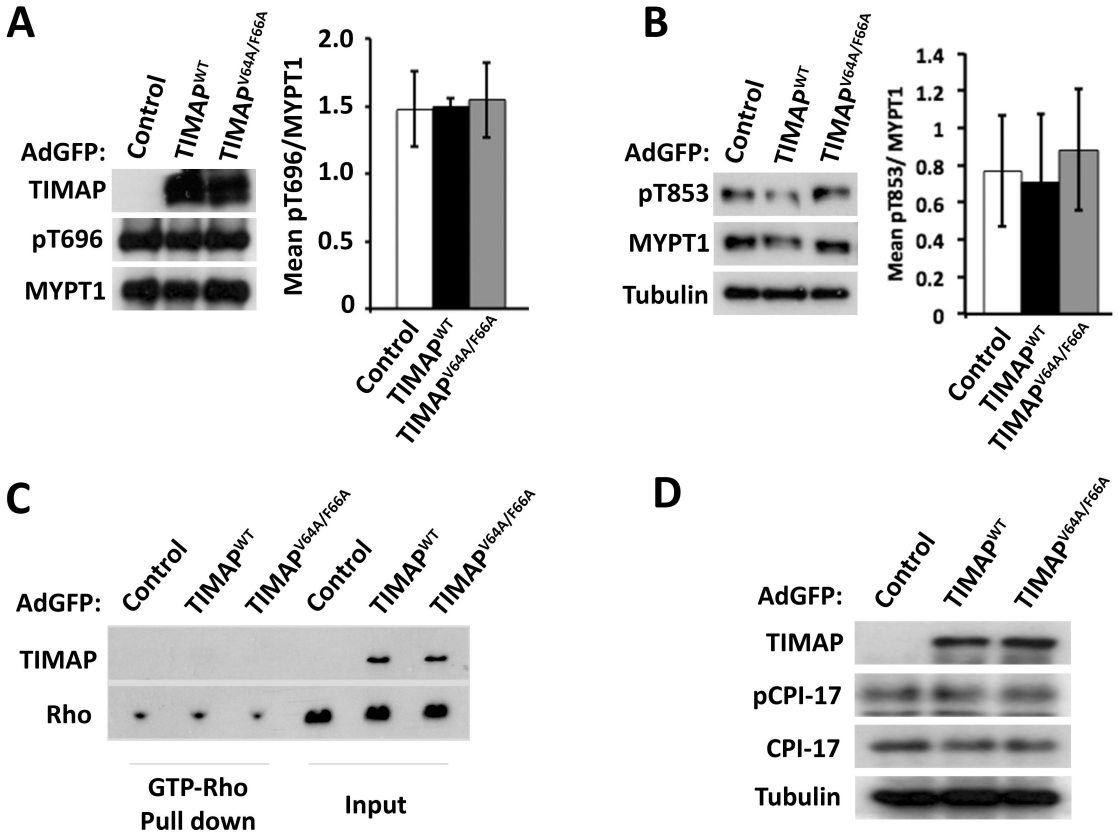

Figure 5.6: Overexpressed GFP-TIMAP^{WT} does not change MYPT1 or MYPT1 inhibitor (CPI-17) phosphorylation. **A.** (Left panel): Western blot of TIMAP, pT696 MYPT1 and MYPT1 in total cell lysates of glomerular endothelial cells infected with AdGFP-control, AdGFP-TIMAP^{WT} or AdGFP-TIMAP$^{V64A/F66A}$ for 48 hours and maintained in serum-replete medium. (Right panel): Ratio of pMYPT1: total MYPT1 (mean ± SEM, n=3 independent experiments). **B**. (Left panel): Western blot of TIMAP, pT853 MYPT1 and MYPT1 in total cell lysates of glomerular endothelial cells infected with AdGFP-control, AdGFP-TIMAP^{WT} or AdGFP-TIMAP$^{V64A/F66A}$ for 48 hours and maintained in serum-replete medium. (Right panel): Ratio of pMYPT1: total MYPT1 (mean ± SEM, n=3 independent experiments). **C.** Western blot of TIMAP and Rho in GTP-Rho immunoprecipitates or input of glomerular endothelial cells infected with AdGFP-control, AdGFP-TIMAP^{WT} or AdGFP-TIMAP$^{V64A/F66A}$ for 48 hours and maintained in serum-replete medium

(representative of 2 independent experiments). **D**. Western blot of TIMAP, pT38 CPI-17, total CPI-17 and Tubulin in total cell lysates of glomerular endothelial cells infected with AdGFP-control, AdGFP-TIMAP^{WT} or AdGFP-TIMAP$^{V64A/F66A}$ for 48 hours and maintained in serum-replete medium.

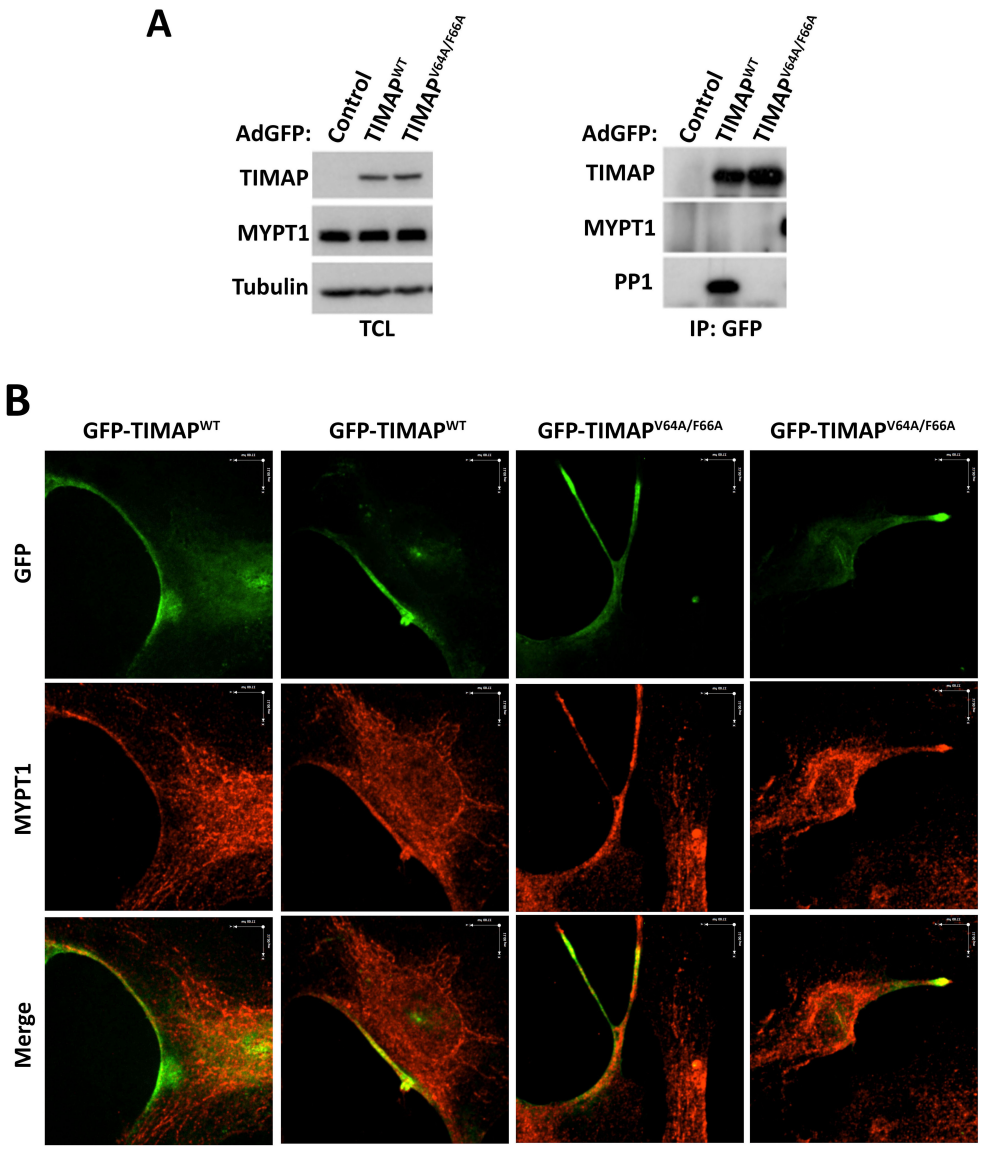

Figure 5.7: Overexpressed GFP-TIMAP does not associate with MYPT1 in glomerular endothelial cells. A. Western blot of TIMAP, MYPT1 and Tubulin in total cell lysates (TCL) and anti-GFP IgG (IP-GFP) immunoprecipitates of glomerular endothelial cells infected with AdGFP-control, AdGFP-TIMAP^{WT} or AdGFP-TIMAP$^{V64A/F66A}$ for 48 hours and maintained in serum-replete medium

(representative of 2 independent experiments). **B.** Confocal immunofluorescence images (60X) of glomerular endothelial cells overexpressing GFP-TIMAP^{WT} (Panel 1 and 2) or TIMAP$^{V64A/F66A}$ (Panel 2 and 3) (GFP, left) immunostained with anti-MYPT1 antibody (red, middle). Merge (right) represents colocalization (representative of 2 independent experiments).

5.3 Discussion

This study has revealed an un-expected role of TIMAP in terms of its regulation of MLC2 phosphorylation in endothelial cells. Contrary to our findings in chapter 4 that $TIMAP^{WT}$/PP1c holoenzyme can dephosphorylate pMLC2 *in vitro,* overexpression of $TIMAP^{WT}$, but not $TIMAP^{V64A/F66A}$ strongly augments total MLC2 phosphorylation in endothelial cells. Similar results are obtained by overexpression of the GSK3 phosphorylation site mutants, $TIMAP^{S333A/S337A}$ and $TIMAP^{S333E/S337E}$. This enhanced MLC2 phosphorylation depends on the ability of TIMAP to bind PP1c, as the only TIMAP construct for which overexpression did not increase MLC2 phosphorylation is the PP1c-binding deficient TIMAP, $TIMAP^{V64A/F66A}$. Furthermore, the degree of MLC2 phosphorylation is proportionally increased with the MOI value. Since this finding of TIMAP was not predicted, we designed experiments to rule out whether the overexpressed TIMAP interferes with the function of the endogenous myosin phosphatase. We found that overexpressed $TIMAP^{WT}$ attenuates the rate of baseline pMLC2 dephosphorylation after inhibition of MLCK and ROCK in endothelial cells. Although MYPT1 is the main MYPT in most cells and changes in its activity directly affect total MLC2 phosphorylation, we show that $TIMAP^{WT}$ does not interfere with MYPT1 expression under the experimental conditions examined in this study. Based on the evidence provided in this study, we think that TIMAP does not act as a myosin phosphatase in cells.

TIMAP is an endothelial- predominant plasma- membrane associated MYPT (Cao, Mattagajasingh et al. 2002, Grassie, Moffat et al. 2011). Like other MYPT family

members, it preferentially binds PP1c (Cao, Mattagajasingh et al. 2002, Li, Kozlowski et al. 2007, Csortos, Czikora et al. 2008, Shopik, Li et al. 2013), and the TIMAP/PP1c holoenzyme effectively dephosphorylates pMLC2 *in vitro* (Figure 4.1) and (Shopik, Li et al. 2013). We examined whether TIMAP functions as a MYPT in living endothelial cells. We used a $TIMAP^{WT}$ construct and a TIMAP construct in which dual V64A/F66A point mutations in the KVSF motif prevent PP1c binding (Li, Kozlowski et al. 2007), similar to mutations in the nuclear PP1c regulatory subunit NIPP1 (Trinkle-Mulcahy, Ajuh et al. 1999) that lead to dominant negative activity. Contrary to expectations, $TIMAP^{WT}$, but not $TIMAP^{V64A/F66A}$ profoundly increased global MLC2 phosphorylation and stress fibers formation in glomerular endothelial cells under otherwise un-stimulated conditions (Figure 5.1 A, C and D). To our knowledge, this finding has not been reported for other MYPT family members, and suggests that $TIMAP^{WT}$ might not act as a myosin phosphatase in living cells. For example, heart-specific overexpression of MYPT2 in transgenic mice results in a decrease in MLC2 phosphorylation (Mizutani, Okamoto et al. 2010), consistent with a myosin phosphatase function. Similarly, overexpression of MBS 85 in HeLa cells has been shown to decrease MLC2 phosphorylation and stress fibers formation (Tan, Ng et al. 2001). Furthermore, overexpression of wild type MYPT3 in HeLa cells does not affect MLC2 phosphorylation, but overexpression of the CAAX-motif deleted MYPT3 results in its cytoplasmic translocation, a decrease in global MLC2 phosphorylation, and a profound loss of stress fibers across the cell (Yong, Tan et al. 2006), indicating that disruption of MYPT3-membrane association strongly enhances its associated phosphatase activity

toward phosphorylated myosin II. Since TIMAP is structurally closely related with MYPT3, it would be possible that overexpression of CAAX-deleted TIMAP similarly enhances its associated phosphatase activity toward pMLC2, however we did not attempt to investigate this here. Nevertheless, this still suggests that the overexpressed wild type TIMAP does not act as a myosin II phosphatase in cells.

We also observed that the level of MLC2 phosphorylation determined by immunofluorescence analysis was reduced in individual cells infected with AdGFP-TIMAP$^{V64A/F66A}$, compared to control un-infected cells (Figure 5.1 A, asterisks). This finding is consistent with our previous observation of reduced MLC2 phosphorylation in endothelial cells transiently transfected with GFP-TIMAP$^{V64A/F66A}$ plasmid (Figure 4.5 B and C), and with the kinase inhibition function of TIMAP against MLC2 when its not bound to PP1c (Figure 4.2 C-E). However, the level of pMLC2 determined by western blot analysis remained unchanged in cells infected with AdGFP-TIMAP$^{V64A/F66A}$, compared to AdGFP control cells (Figure 5.1 B and C). A plausible explanation for this could be that the baseline level of pMLC2 in AdGFP infected cells is relatively low (Figure 5.1 B and C). Western blot analysis is more sensitive for detection of increased levels of pMLC2 than decreased levels. One way to resolve this question is to infect cells with AdGFP or AdGFP-TIMAP$^{V64A/F66A}$ and stimulate MLC2 phosphorylation with well-known agonists, like thrombin, which is known to disrupt endothelial barrier function by activating ROCK-mediated MLC2 phosphorylation (Essler, Amano et al. 1998, van Nieuw Amerongen, Musters et al. 2008). Alternatively, we can also stimulate MLC2 phosphorylation by serum, which we (Obeidat M, unpublished data) and others (Giuliano, Kolega et al. 1992) have shown can significantly stimulate MLC2

phosphorylation. Nevertheless, this finding further indicates that TIMAP does not act as a typical MYPT in endothelial cells otherwise the overexpressed GFP-TIMAP$^{V64A/F66A}$ should have enhanced MLC2 phosphorylation. For instance, overexpression of the *Drosophila* isoform of MYPT (MYPT-75D) that is unable to bind to PP1cδ, similar to TIMAP$^{V64A/F66A}$, enhances MLC2 phosphorylation *in vivo* (Vereshchagina, Bennett et al. 2004), consistent with a myosin phosphatase function.

It has been reported that silencing TIMAP in cultured HPAEC blocks inhibition by forskolin/PKA of thrombin-stimulated ERM phosphorylation and endothelial monolayer permeability, suggesting that ERM is a substrate of PKA-activated TIMAP/PP1cδ holoenzyme (Csortos, Czikora et al. 2008, Czikora, Kim et al. 2011). In this regard, myosin phosphatase is considered an ERM phosphatase as well (Eto, Kirkbride et al. 2005), and the MYPT family member MBS 85 has been shown to associate with and dephosphorylate the ERM family member moesin (Fukata, Kimura et al. 1998). However, we did not observe changes in ERM phosphorylation in glomerular endothelial cells when GFP-TIMAP^{WT} or GFP-TIMAP$^{V64A/F66A}$ was overexpressed (Figure 5.1. C). Although it could be argued that the experimental conditions in our study are different from those in the TIMAP/ERM study mentioned above (Csortos, Czikora et al. 2008), we can still conclude that under the same experimental conditions in our study, the effect of GFP-TIMAP^{WT} is specific to MLC2 and does not involve all PP1cδ substrates, at least, not ERM protein.

The phosphatase activity of MYPT family members is regulated by phosphorylation on several Serine and Threonine residues in their C-terminal regions

(Grassie, Moffat et al. 2011). For example, MYPT1 phosphorylation on Thr696 and Thr853 by ROCK strongly inhibits MYPT1-associated phosphatase activity toward pMLC2 (Feng, Ito et al. 1999). On the other hand, PKA-mediated phosphorylation of Ser340/341/353 on MYPT3, the most structurally related MYPT to TIMAP, enhances the basal phosphatase activity of MYPT3/PP1c holoenzyme toward pMLC2 (Yong, Tan et al. 2006). Similarly, we reported that TIMAP-associated PP1c activity toward DiFUMP, LAMR1 and phosphorylase (a) is enhanced by PKA/GSK3 -mediated phosphorylation of TIMAP on Ser333 and Ser337 (Li, Kozlowski et al. 2007, Shopik, Li et al. 2013). Here, we found that similar to the wild type TIMAP, the PKA/GSK3 phosphorylation mutants GFP-TIMAP$^{S333A/S337A}$ and GFP-TIMAP$^{S333E/S337E}$ strongly augment MLC2 phosphorylation and induce stress fibers formation (Figure 5.3 A and B), indicating that the enhanced MLC2 phosphorylation is not regulated by the PKA/GSK3 phosphorylation of TIMAP. It could still be possible that the phosphatase activity of TIMAP/PP1c is regulated by phosphorylation of TIMAP, but this would have to involve other unidentified sites. As discussed in chapter 4, one possible regulatory phosphorylation site would be the serine residue in the PP1c binding KVSF motif of TIMAP. The fact that all the GFP-TIMAP constructs that can bind to PP1c are able to induce MLC2 phosphorylation further implies that phosphorylation of this serine residue might play an important role in regulating TIMAP/PP1c interaction and activity.

MLC2 phosphorylation is regulated by myosin kinases and myosin phosphatases. While MLCK directly phosphorylates MLC2 on the activating phosphorylation sites Thr18/Ser19, ROCK enhances MLC2 phosphorylation by its inhibitory Thr696 and

Thr853 phosphorylation of MYPT1 (Feng, Ito et al. 1999). We observed that the rate of pMLC2 dephosphorylation after inhibition of MLCK or ROCK was significantly decreased in cells overexpressing GFP-TIMAP^{WT}, compared to control GFP and the PP1c-deficient mutant GFP-TIMAP$^{V64A/F66A}$ overexpressing cells (Figure 5.4 B and D). Since MYPT1 activity is the principal determinant of the phosphorylation state of MLC2 in cells, and since the pool of hyperphosphorylated MLC2 in cells overexpressing GFP-TIMAP^{WT} is sensitive to ROCK inhibition and therefore to MYPT1 activation (Figure 5.4 C), we conclude that the activity of MYPT1 is attenuated by the overexpressed GFP-TIMAP^{WT}. Also, this indicates that the GFP-TIMAP^{WT} does not act as a myosin phosphatase in cells. Otherwise it would have enhanced the rate of pMLC2 dephosphorylation.

In addition to MLCK and ROCK, several other kinases, including ILK (Kiss, Muranyi et al. 2002, Muranyi, MacDonald et al. 2002, Wilson, Sutherland et al. 2005), ZIPK (MacDonald, Borman et al. 2001, Moffat, Brown et al. 2011) and P21-activated kinase (PAK) (Chew, Masaracchia et al. 1998, Takizawa, Koga et al. 2002, Chu, Pham et al. 2013), have been reported to enhance MLC2 phosphorylation directly by phosphorylating MLC2 or indirectly by inhibiting MYPT1. Hence, it would be possible that the increase in MLC2 phosphorylation by the overexpressed GFP-TIMAP^{WT} could result from activation of other myosin kinases that we did not investigate here. Nonetheless, the fact that the rate of MLC2 dephosphorylation, indicative of myosin phosphatase activity, is reduced by the overexpression of GFP-TIMAP^{WT}, it becomes reasonable to conclude that the overexpressed GFP-TIMAP^{WT} has an inhibitory effect on the myosin phosphatase rather than an activating effect on a myosin kinase.

Silencing of MYPT1 expression strongly enhances MLC2 phosphorylation and stress fiber formation (Xia, Stull et al. 2005), similar to our findings with the overexpressed GFP-TIMAP^{WT}. Therefore, we determined whether GFP-TIMAP^{WT} interferes with MYPT1 protein expression. However, we observed no difference in MYPT1 protein abundance in GFP-TIMAP^{WT} overexpressing cells, compared to control GFP and GFP-TIMAP$^{V64A/F66A}$ overexpressing cells (Figure 5.5 A).

Furthermore, MYPT1 exhibits various sub-cellular distributions depending on the biological activities of the cell, its interaction with PP1c and its activation state. For example, in growing endothelial cells, MYPT1 localizes on stress fibers, but in resting cells it localizes to the cell periphery (Hirano, Niiro et al. 1999). Also, when MYPT1 is overexpressed alone it predominantly localizes to the nucleus, whereas when co-expressed with PP1c it re-localizes to the actin filaments (Eto, Kirkbride et al. 2005). By comparing MYPT1 immunoreactivity in AdGFP- TIMAP^{WT} to that in AdGFP-TIMAP$^{V64A/F66A}$ infected cells, we observed very identical distribution of MYPT1 (Figure 5.5 B). Hence, the enhanced MLC2 phosphorylation caused by overexpression of GFP-TIMAP^{WT} is not due to changes in MYPT1 protein expression or sub-cellular distribution.

It has been shown that loss of *Drosophila* PP1c results in increased MLC2 phosphorylation and actin reorganization (Vereshchagina, Bennett et al. 2004), similar to our findings of GFP-TIMAP^{WT} overexpression. However, we found that the PP1c protein abundance was elevated in GFP-TIMAP^{WT} overexpressing cells, compared to control GFP and GFP-TIMAP$^{V64A/F66A}$ overexpressing cells (Figure 5.5 C). This

finding is similar to the effect of MYPT2 overexpression in the heart in transgenic mice (Mizutani, Okamoto et al. 2010), which also increases PP1cβ protein expression, but, unlike GFP-TIMAP^{WT}, overexpressed MYPT2 leads to decreased MLC2 phosphorylation.

The observation that only the GFP-TIMAP constructs that bind PP1cβ can enhance MLC2 phosphorylation indicates that this effect is PP1c-dependent. Since the PP1cβ catalytic subunit is shared by TIMAP and MYPT1, and since an increasing AdGFP-TIMAP^{WT} MOI above 10 is associated with a dose-dependent increase in MLC2 phosphorylation (Figure 5.1 C, D and E), whereas PP1cβ expression is maximal at an AdGFP-TIMAP^{WT} MOI of 10 and does not increase further (Figure 5.5 C), it could be argued that GFP-TIMAP^{WT} competes with MYPT1 for binding to PP1cβ, reducing MYPT1-dependent phosphatase activity against pMLC2. However, overexpression of exogenous PP1cβ does not reverse the enhanced MLC2 phosphorylation caused by the overexpression of GFP-TIMAP^{WT} (Figure 5.5 D). This effect is different from the effect of the co-expression of MYPT2 with PP1cβ in the heart, which leads to a decrease in MLC2 phosphorylation (Okamoto, Kato et al. 2006), further suggesting that GFP-TIMAP^{WT} is not a typical myosin phosphatase in cells.

Moreover, the fact that when MYPT1 is co-expressed with PP1cβ re-localizes to the actin filaments (Eto, Kirkbride et al. 2005) suggests that the decreased availability of endogenous PP1cβ for binding to MYPT1 can inhibit its phosphatase activity toward pMLC2. Also, the phosphatase activity of PP1c toward pMLC2 is enhanced when it is bound to MYPT1 and decreased when the holoenzyme dissociates or translocates from

the actin filaments (Gong, Fuglsang et al. 1992, Koga and Ikebe 2008). Therefore, it still could be argued that overexpression of GFP-TIMAP that can bind PP1c sequesters PP1c away from MYPT1, resulting in holoenzyme dissociation or translocation, therefore enhancing MLC2 phosphorylation. Since MYPT1 staining is very similar in GFP-TIMAP^{WT} and GFP-TIMAP$^{V64A/F66A}$ overexpressing cells (Figure 5.5 B), together with the finding that endothelial cells express more PP1c in response to overexpression of GFP-TIMAP^{WT} (Figure 5.5 C), we can conclude that the overexpressed GFP-TIMAP^{WT} is unlikely to sequester PP1c away from MYPT1.

Since GFP-TIMAP^{WT} reduces the rate of pMLC2 dephosphorylation (Figure 5.4), and since the GFP-TIMAP^{WT}-induced pool of pMLC2 is sensitive to ROCK inhibition (Figure 5.5 C), it is likely that TIMAP might interfere with MYPT1 activity. MYPT1 activity is regulated by various mechanisms; the most common among them are inhibitory Thr696 and Thr853 phosphorylation of MYPT1 by ROCK (Feng, Ito et al. 1999) and activating Thr38 phosphorylation of its inhibitor CPI-17 (Eto, Ohmori et al. 1995). However, we observed no changes in MYPT1 Thr696 and Thr853 phosphorylation (Figure 5.6 A and B), or the abundance of GTP-RhoA required for ROCK activation (Kimura, Ito et al. 1996). Although this experiment is missing a positive control in which the activity of RhoA is stimulated, taken together the findings that ROCK-dependent phosphorylation sites on MYPT1 are not affected by the overexpression of GFP-TIMAP^{WT}, and that overexpressed GFP-TIMAP^{WT} augments MLC2 phosphorylation under otherwise un-stimulated conditions, similar to those under which the GTP-Rho pull down assay was done, we can conclude that overexpression of

GFP-TIMAP^{WT} does not alter MYPT1 phosphorylation on the ROCK-sensitive Thr 696 and Thr 853 sites in the MYPT 1 C-terminus.

Phosphorylation of CPI-17 on Thr38 enhances its inhibitory potency toward MYPT1 by ~1000 fold (Eto, Ohmori et al. 1995, Senba, Eto et al. 1999). However, we found no changes in total and pThr38 CPI-17 in cells overexpressing GFP-TIMAP^{WT}, compared to control GFP and GFP-TIMAP$^{V64A/F66A}$ (Figure 5.6 D). Therefore, we conclude that the enhanced MLC2 phosphorylation by overexpressed GFP-TIMAP^{WT} is not due to changes in the abundance or activating phosphorylation of CPI-17.

Overexpression of protein 14-3-3 has been shown to increase myosin II phosphorylation by an inhibitory binding to MYPT1 (Koga and Ikebe 2008). Since overexpression of GFP-TIMAP^{WT} induces massive MLC2 phosphorylation, we considered the possibility that GFP-TIMAP^{WT} interacts with MYPT1, inhibiting its activity and consequently enhancing MLC2 phosphorylation. To properly investigate the possibility of interaction between TIMAP and MYPT1, we would need to perform an *in vitro* binding assay. However, if GFP-TIMAP^{WT} interacts with MYPT1 in endothelial cells under the conditions of enhanced MLC2 phosphorylation, we should be able to co-immunoprecipitate the two protein together. However, we observed that MYPT1 does not co-immunoprecipitate with TIMAP (Figure 5.7 A), indicating that the two proteins are not in the same complex. Furthermore, confocal immunofluorescence analysis showed that GFP-TIMAP^{WT} co-localization with MYPT1 was restricted to some areas near the plasma membrane (Figure 5.7 B). Thus, these findings indicate that GFP-TIMAP^{WT} does not inhibit MYPT1 activity directly.

Altogether, our data suggest that GFP-TIMAP^{WT} does not act as a myosin phosphatase in endothelial cells. Nonetheless, a limitation of this study is that we do not have data on the role of endogenous TIMAP in regulating MLC2 phosphorylation. Having said that, the reason we chose not to silence endogenous TIMAP to study MLC2 phosphorylation is that we found that the changes in MLC2 phosphorylation in the infected cells are only observable during the proliferation stage of the cells, which is within the first 48 hours of infection. On the other hand, to efficiently silence TIMAP, we need to wait at least 72 hours after siRNA transfection, and since TIMAP depletion inhibits endothelial cell proliferation (Figure 3.1 and 3.3), changes in MLC2 phosphorylation become undetectable. The other reason is that inhibition of endogenous TIMAP expression by siRNA approach does not help in understanding the role of TIMAP/PP1c interaction or TIMAP phosphorylation in the regulation of MLC2 phosphorylation and myosin II dynamics, whereas the overexpression system enables us to use different mutants of TIMAP and map their particular function.

CHAPTER 6

General Discussion and Future Directions

6.1 Main Findings (Figure 6.1)

The main findings presented in this thesis are:

1. TIMAP maintains endothelial cell survival, proliferation and sprouting angiogenesis *in vitro* via a positive regulation of Akt Phosphorylation.
2. TIMAP/PP1c holoenzyme dephosphorylates pMLC2 *in vitro*.
3. PP1c- unbound TIMAP inhibits kinase-mediated phosphorylation of the activating Thr18/Ser19 sites of MLC2 *in vitro* and in endothelial cells.
4. Overexpressed TIMAP that is able to bind to PP1c dramatically increases global MLC2 phosphorylation via inhibition of the myosin phosphatase in endothelial cells.

6.2 TIMAP and its Role in Glomerular Endothelial Cell Survival, Proliferation and Angiogenesis

Our first study of TIMAP demonstrated that it is predominantly expressed in developing vessels and cultured endothelial cells, and is downregulated by TGF□1 (Cao, Mattagajasingh et al. 2002). Previous work from the lab showed that TGF□1 promotes glomerular endothelial capillary lumen formation by stimulating a sub-group of

endothelial cells to undergo apoptosis (Choi and Ballermann 1995, Fierlbeck, Liu et al. 2003). The fact that TIMAP expression is repressed by TGF1 (Cao, Mattagajasingh et al. 2002) suggests that TIMAP might antagonize the anti-survival and anti-proliferative effects of TGF1 in endothelial cells. In this thesis, we present a novel finding that TIMAP protein expression is important in maintaining glomerular endothelial cell survival, proliferation and sprouting angiogenesis *in vitro*.

Taken together the data that TGF1 is crucial for glomerular capillary lumen formation (Choi and Ballermann 1995, Fierlbeck, Liu et al. 2003), that TIMAP expression is inhibited by TGF1 in glomerular endothelial cells (Cao, Mattagajasingh et al. 2002), and that TIMAP depletion stimulates glomerular endothelial cell apoptosis, but inhibits their proliferation (Obeidat, Li et al. 2014), we can postulate that TIMAP might play a role in glomerular endothelial capillary formation, and that its expression may be differentially regulated during this process. Accordingly, TIMAP might be required during the proliferation stage of capillary formation, but its expression may be repressed by TGF1 during the lumen formation stage, which requires cell apoptosis. Hence, it would be plausible to investigate whether TIMAP plays a role in glomerular endothelial capillary development *in vivo*. This can be examined in TIMAP conditional knockout and TIMAP transgenic mice. Also, it would be important to determine whether TIMAP expression is differentially regulated during glomerular endothelial capillary development, which can be examined by studying TIMAP mRNA and protein expression levels in the developing mouse kidney at different stages of development.

As discussed in chapter 1 (Figure 1.7), the TGF1 pathway in endothelial cells is complex due to the existence of two signaling pathways, ALK1 and ALK2, and its mechanism of action is very much dependent on the cell context, development stage and experimental conditions. Thus, to understand the role of TIMAP in TGF1-mediated regulation of endothelial cell processes we need to determine which of the two TGF1 pathways, ALK1 or ALK2, regulates TIMAP expression. This can be accomplished by virtue of ALK-specific inhibitors that are commercially available. Furthermore, to examine whether TIMAP antagonizes TGF1 signaling in glomerular endothelial cells, we can evaluate TGF1-stimulated apoptosis, capillary lumen formation and target genes expression in cells overexpressing TIMAP.

Given that TIMAP is a PP1c regulatory subunit, we can speculate that the mechanism of action of TIMAP in the TGF1-mediated regulation in endothelial cells is predicted to be via its serine/threonine phosphatase property. Indeed, this would be plausible given the fact that the TGF1 pathway is a cascade of activating serine/threonine phosphorylation events beginning with the TGF1 receptors phosphorylation and proceeding through the various Smads phosphorylation.

Moreover, studying the role of TIMAP in angiogenesis can be expanded to involve tumor angiogenesis, hypoxia- induced angiogenesis and retinal angiopathies. It would be interesting to find that TIMAP protein expression is augmented in these processes, and that theses processes are attenuated in TIMAP knockout mice.

6.3. TIMAP and its regulation of MLC2 phosphorylation

TIMAP is PP1c regulatory subunit that, according to amino acid sequence analysis, belongs to the family of myosin phosphatase regulatory subunits (Cao, Mattagajasingh et al. 2002, Grassie, Moffat et al. 2011). TIMAP structure and the mechanism of regulation of its associated phosphatase activity are very similar to MYPT3 (Skinner and Saltiel 2001, Cao, Mattagajasingh et al. 2002, Yong, Tan et al. 2006, Li, Kozlowski et al. 2007, Grassie, Moffat et al. 2011). MYPT1, MYPT2 (Mizutani, Okamoto et al. 2010), MYPT3 (Yong, Tan et al. 2006) and MBS85 (Tan, Ng et al. 2001) all have been shown to exhibit myosin phosphatase function. However, as also noted by others (Grassie, Moffat et al. 2011), it was not known whether TIMAP, similar to other MYPTs, acts as a myosin phosphatase. In this thesis, we present novel findings on how TIMAP might regulate myosin II phosphorylation *in vitro* and in endothelial cells depending on its binding to PP1c. These findings are further discussed below.

6.3.1 TIMAP/PP1c holoenzyme Dephosphorylates pMLC2 *in vitro*

We showed that PP1c effectively dephosphorylates pMLC2 in the absence and presence of TIMAP (Figure 4.1 A), and that PP1c-bound TIMAP does not inhibit pMLC2 dephosphorylation (Figure 4.1 B). However, under the same experimental conditions, TIMAP inhibited PP1c-mediated dephosphorylation of pLAMR1 (Figure 4.1 B), previously identified substrate of TIMAP/PP1c (Kim, Li et al. 2005). The phosphatase

activity of MYPTs is regulated by phosphorylation on their C-termini (Grassie, Moffat et al. 2011). For instance, phosphorylation of MYPT1 on the inhibitory sites Thr696 and Thr853 by ROCK inhibits its associated myosin phosphatase activity (Feng, Ito et al. 1999). Whereas, phosphorylation of MYPT3 on the activating sites Ser340/341/353 by PKA enhances its associated phosphatase activity toward pMLC2 *in vitro* (Yong, Tan et al. 2006), and phosphorylation of TIMAP on the activating sites Ser333/337 by PKA and GSK3 enhances its associated phosphatase activity toward the artificial substrate (DiFMUP) and phosphorylase (a) *in vitro* and LAMR1 *in vivo* (Li, Kozlowski et al. 2007, Shopik, Li et al. 2013). In this thesis, we did not attempt to investigate the role of TIMAP phosphorylation on its associated phosphatase activity toward pMLC2. Therefore, it would be reasonable to investigate whether phosphorylation of TIMAP plays a role in regulating TIMAP/PP1c- mediated dephosphorylation of pMLC2. This will further our understanding of the phosphatase function of TIMAP/PP1c holoenzyme toward pMLC2.

The fact that we do not observe differences in the phosphorylation state of MLC2 in endothelial cells overexpressing the PKA/GSK3 phosphorylation site mutants of TIMAP, TIMAP$^{S333A/S337A}$ and TIMAP$^{S333E/S337E}$ (Figure 5.3 A and B) and those overexpressing the wild type TIMAP (Figure 5.1) suggests that phosphorylation at these particular sites might not be involved in regulating the TIMAP/PP1c phosphatase activity toward pMLC2. Nonetheless, other undiscovered phosphorylation sites on TIMAP could play a role in regulating this activity. For example, one possible phosphorylation site that might be predicted to regulate TIMAP/PP1c phosphatase activity toward pMLC2 would be the serine residue in the KVSF PP1c binding motif of TIMAP, which does not exist in

other MYPT family members. We can potentially produce phosphorylation mutants of TIMAP at this site by mutating it to alanine for the phosphorylation deficient mutant and to aspartic acid or glutamic acid for the phosphorylation mimetic mutant.

6.3.2 TIMAP Inhibits Kinase-mediated Phosphorylation of MLC2

We have previously reported that TIMAP can interact with LAMR1 and inhibit its phosphorylation by PKA and PKC independently of its associated phosphatase activity (Shopik, Li et al. 2013). Similarly, TIMAP also inhibits its-bound PP1c phosphorylation by cyclinA/cdk2. Therefore, we entertained the idea that TIMAP might similarly interact with MLC2 and inhibit its phosphorylation in the absence of PP1c. Indeed, we found that the two proteins interact directly (Figure 4.2 A and B), and that TIMAP-bound MLC2 cannot be phosphorylated by ROCK and MLCK on the activating phosphorylation sites Thr18/Ser19 (Figure 4.2 C-E). We could recapitulate this function of TIMAP of regulating MLC2 phosphorylation in endothelial cells by overexpressing the PP1c-deficient TIMAP, which also reduces MLC2 phosphorylation (Figure 4.5). These findings suggest that TIMAP has the property of regulating protein phosphorylation independently of PP1c. Also, since TIMAP and PP1c exist as a complex in cells, it would imply that under physiological conditions TIMAP might dissociate from PP1c to interact with MLC2/other proteins and mask their phosphorylation. As discussed in chapter 4, to further investigate this kinase inhibition function of TIMAP toward MLC2 and its physiological relevance, we would need to determine the ability of TIMAP to bind to and inhibit kinase-mediated MLC2 phosphorylation when TIMAP is bound to PP1c.

Furthermore, since we previously showed that phosphorylation of TIMAP alters its binding affinity to PP1c (Li, Kozlowski et al. 2007), it would be interesting to examine whether the kinase inhibition function of TIMAP is regulated by its phosphorylation state.

Given that inhibition of kinase- mediated MLC2 phosphorylation by TIMAP requires a direct interaction between the two proteins (Figure 4.2), it would be logical as well to map the protein sites/domains required for this interaction.

6.3.3 Overexpression of TIMAP that is able to bind PP1c Strongly Enhances MLC2 Phosphorylation in Endothelial Cells.

Although we could demonstrate that TIMAP/PP1c acts as a myosin phosphatase *in vitro*, we could not provide evidence that this is also true in endothelial cells. In contrast, we present considerable evidence that overexpressed TIMAP that can form a complex with endogenous PP1c (Figure 5.3 C) strongly stimulates MLC2 phosphorylation in otherwise un-stimulated endothelial cells. Contrary to the kinase inhibition effect of TIMAP on MLC2 phosphorylation, which occurs in the absence of PP1c binding, the enhanced MLC2 phosphorylation by the overexpressed TIMAP is strictly dependent on the ability of TIMAP to bind to PP1c.

The evidence provided by the ROCK and MLCK inhibition experiments (Figure 5.4) implies that the endogenous myosin phosphatase activity is attenuated by the overexpression of wild type TIMAP, but not the PP1c-deficient TIMAP. Despite the fact that MYPT1 is the dominant myosin phosphatase in most non-muscle cells, we did not

find any mechanistic evidence on how TIMAP would inhibit MYPT1 activity in endothelial cells, at least under the experimental conditions presented in this thesis. Altogether, we conclude that TIMAP, although belongs to the MYPT family, does not act as a typical MYPT in endothelial cells. Nevertheless, our study sheds light on a novel un-conventional way of regulating myosin phosphorylation by a MYPT.

6.4 Future Directions on Possible coupling of Akt and Myosin II Pathways by TIMAP in Endothelial Cells.

TIMAP is actively expressed in developing vessels (Cao, Mattagajasingh et al. 2002), but its expression is downregulated in differentiated vessels (unpublished data). We showed that TIMAP depletion attenuates endothelial cell proliferation and sprouting angiogenesis (Figure 3.1 and 3.2). These effects are correlated with decreased activating and inhibitory phosphorylation of Akt and PTEN, respectively (Figure 3.3 A and 3.4 C). Given that overexpression of wild type TIMAP strongly augments Akt and MLC2 phosphorylation (Figure 3.3 B and 5.1), that PI3K/Akt segregate from PTEN during chemotaxis and cytokinesis to ensure continuous Akt activation (Funamoto, Meili et al. 2002, Janetopoulos, Borleis et al. 2005), that myosin II and PTEN co-localize under conditions of chemotaxis and cytokinesis (Pramanik, Iijima et al. 2009), and that TIMAP co-localizes with PTEN and MLC2 in endothelial cell projections (Figure 3.4 A and 4.4), we can speculate that a crosstalk might exist between the PI3K/Akt axis and myosin II downstream of TIMAP under conditions of endothelial cell proliferation and/or migration.

A great number of studies showed that regulation of MLC2 phosphorylation and myosin II activity is crucial for cell proliferation, particularly the cytokinesis stage (Matsumura 2005). In addition, the dynamic phosphorylation/dephosphorylation of myosin II is the limiting step of cell migration, as the constitutive phosphorylation of MLC2 inhibits focal adhesion turnover and rear retraction required for cell migration to proceed (Kolega 2003, Lontay, Kiss et al. 2005, Bavaria, Ray et al. 2011). In view of the fact that angiogenesis depends on the directional migration of tip endothelial cells (Hellstrom, Phng et al. 2007) and on the proliferation of stalk cells (Eilken and Adams 2010), that TIMAP overexpression augments MLC2 and Akt phosphorylation in a dose dependent manner, and that TIMAP expression is downregulated in non-dividing differentiated cells, we can postulate that changes in TIMAP protein expression alter MLC2 and Akt phosphorylation, and consequently regulate endothelial cell migration and proliferation during vessel development.

The fact that TIMAP stimulates Akt and MLC2 phosphorylation without affecting ERK1/2 and ERM phosphorylation makes it unlikely that TIMAP would have an effect on the activity of receptor tyrosine kinases and the ERM proteins. However, this raises the intriguing possibility that TIMAP might modify the function of a common regulator of Akt and MLC2 phosphorylation. One possible candidate is the integrin-linked kinase (ILK). ILK is a serine/threonine kinase that contains 4 ankyrine repeats (Hannigan, Leung-Hagesteijn et al. 1996) important for its localization to focal adhesions (Li, Zhang et al. 1999) and a phosphoinositide phospholipid-binding motif (Delcommenne, Tan et al. 1998). It interacts with the cytoplasmic domains of integrin β1 and β3 (Hannigan, Leung-Hagesteijn et al. 1996). Similar to TIMAP, overexpression of ILK induces S473 Akt

phosphorylation (Delcommenne, Tan et al. 1998). This effect is dependent on PI3K and the kinase domain of ILK, and is correlated with an inhibition of GSK3 activity, a known downstream effector of Akt. Furthermore, ILK and Akt are constitutively active in PTEN-null prostate cancer cells, and inhibition of ILK with a small molecule ILK inhibitor attenuates S473 Akt phosphorylation, resulting in cell cycle arrest and apoptosis (Persad, Attwell et al. 2000). Similarly, using the ILK inhibitor, we could inhibit the stimulated S473 Akt and S9 GSK3 phosphorylation in glomerular endothelial cells infected with AdGFP-TIMAP^{WT} (Obeidat M, unpublished data), suggesting that this enhanced phosphorylation by the overexpressed GFP-TIMAP^{WT} is dependent, at least in part, on ILK activity. Interestingly, ILK is also considered to be a MLC2 kinase, which induces MLC2 phosphorylation and vascular smooth muscle contraction independently of Ca^{2+} (Deng, Van Lierop et al. 2001, Wilson, Sutherland et al. 2005). Alternatively, it can also stimulate MLC2 phosphorylation via an inhibitory phosphorylation of MYPT1 (Kiss, Muranyi et al. 2002). Our preliminary data shows that inhibition of ILK with the small molecule ILK inhibitor can reduce MLC2 phosphorylation stimulated by the overexpressed GFP-TIMAP^{WT}. Taken together these data, and the fact that TIMAP, like ILK, localizes to focal adhesions (Obeidat M, unpublished data), stimulates Akt and MLC2 phosphorylation and is important for cell survival and proliferation, we can postulate that TIMAP might be regulating ILK-mediated Akt and MLC2 phosphorylation in endothelial cells. Obviously, these hypotheses need further investigation.

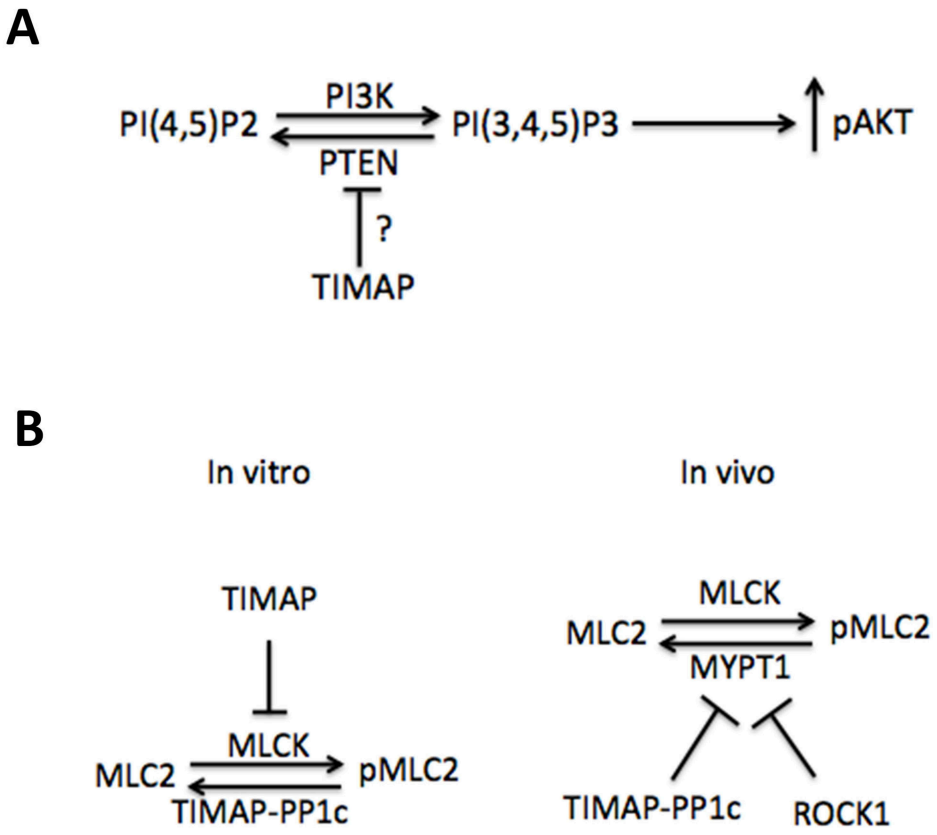

Figure 6.1: Final Model of the major findings. A. The role of TIMAP in the PI3K/Akt. B. The role of TIMAP in MLC2 phosphorylation.

6.5 Conclusion

It is known that TIMAP is a prenylated endothelial regulatory subunit of PP1c in the MYPT family whose expression is upregulated in developing vessels and downregulated by TGF1. However, the biological and physiological role of TIMAP in vessel development, as well as its function as a myosin phosphatase has not been investigated. Our study provides novel evidence that TIMAP, at least *in vitro*, is vital for endothelial cell survival, proliferation and angiogenesis. We also show that this role is associated with maintaining AKT phosphorylation and activation. Furthermore, we discovered that, unlike other MYPTs, TIMAP does not act as a conventional myosin phosphatase, and that it differentially regulates MLC2 phosphorylation on the activating sites Thr18/Ser19 depending on whether it interacts with PP1c.

The main challenges in this study have been 1). The lack of proper mouse model that could have allowed us to present physiological evidence of the role of TIMAP, and guided us to determine the underlying mechanism of TIMAP action in living cells. 2). While we were able to show that TIMAP/PP1c acts as a myosin phosphatase *in vitro*, we could not recapitulate this finding *in vivo*. 3). In our various experiments, we always approached TIMAP as a regulatory subunit of PP1c and, therefore, we attempted to identify phosphorylation targets that might be dephosphorylated by TIMAP/PP1c, however, we did not succeed in providing evidence that TIMAP acts as PP1c regulatory subunit in cells. Nevertheless, we presented robust evidence that TIMAP function is dependent on the level of TIMAP expression, its interaction with other proteins, and its interaction with PP1c. Taken together these findings with the fact that TIMAP itself is a

substrate of PP1c-mediated dephosphorylation (Li, Kozlowski et al. 2007), we can conclude that TIMAP might regulate various biological activities by virtue of its protein-protein interaction, and that this in turn is regulated by TIMAP interaction with PP1c. This thesis provides a platform for further studies investigating the role of TIMAP in endothelial cell development and in regulation of myosin II dynamics.

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