

**SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)**

**IDENTIFICATION AND CHARACTERIZATION OF NEW  
INTERACTING PARTNERS OF PROTEIN PHOSPHATASE 1-  
TIMAP COMPLEX IN ENDOTHELIAL CELLS**

by Margit Péter

Supervisor: Csilla Csontos, PhD., D.Sc.



UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF MOLECULAR MEDICINE

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The Examination takes place at Department of Physiology, Faculty of Medicine, University of Debrecen at 11 AM, 10<sup>th</sup> of April, 2017

Head of the **Defense Committee**: Prof. László Csernoch, PhD, DSc

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, at 13 PM, 10<sup>th</sup> of April, 2017

## SCIENTIFIC BACKGROUND

### **Vascular endothelial function**

The vascular endothelium consists of closely connected cells lining the intima of the blood vessels. Endothelium has a central role in inflammation, hemostasis, vasoregulation, angiogenesis, and vascular growth. The main function of endothelium is to provide a semipermeable barrier between the blood and intestinal space and control the passage of macromolecules, fluid, proteins, nutrients and metabolic wastes. Appropriate functioning of the EC barrier, therefore, necessitates proper shape/cytoskeletal structure of individual cells and integrity of the EC monolayer. Integrity, however, requires uncompromised intercellular junctions and cell-matrix contacts. Vascular EC have gap, adherent, and tight junctions; the latter two play a role in paracellular permeability. These are communicating structures and contain proteins for several signaling pathways. The dynamic structures of the three major components of the EC cytoskeleton, F-actin, microtubules, and intermediate filaments, are critical importance in the balance of the competing contractile and tethering forces determining the actual shape of the cell. Multiple signal transduction pathways, regulating EC contraction and barrier function, involve the activity of several protein kinases and phosphatases. The activity of both the kinases and the phosphatases is strictly controlled, many of them by reversible phosphorylation/dephosphorylation.

### **Role of reversible phosphorylation in the endothelial barrier function**

#### ***Reversible protein phosphorylation, classification of protein phosphatases***

Reversible protein phosphorylation is one of the most important posttranslational modifications influencing the activity of proteins. The enzymes that play a key role in protein phosphorylation/dephosphorylation are protein kinases and phosphatases. Protein kinases responsible for the phosphorylation of proteins, and protein phosphatases catalyze the cleavage of phosphate groups. The presence or absence of a phosphate group on the Ser, Thr, Tyr or occasionally His amino acid side chain of a protein affects its conformation and may serve as an “on and off” signal in the regulation of its physiological activity. Protein phosphatases are classified into three families, according to their ability to dephosphorylate amino acid residues. Ser/Thr specific protein phosphatases dephosphorylate phospho-Ser or phospho-Thr residues, Tyr specific protein phosphatases dephosphorylate phospho-Tyr residues alone, and dual specificity protein phosphatases dephosphorylate phospho-Ser, phospho-Thr and phospho-Tyr residues. The Ser/Thr specific phosphatases are sorted into

three structurally distinct families, namely PPM family of  $Mg^{2+}$ -dependent protein phosphatases, like PP2C; and the PPP family which contains PP1, PP2A, PP2B (aka calcineurin), PP4, PP5, PP6 and PP7.

### ***Characterization of protein phosphatase 1***

Protein phosphatase 1 (PP1) is a highly conserved protein which is responsible for several cellular processes. PP1 is ubiquitously expressed in eukaryotic cells. The holoenzyme consists of a catalytic subunit (PP1c) and a regulatory subunit. The catalytic subunit of PP1 has three isoforms, namely PP1 $\alpha$ , PP1 $\delta$  (other name PP $\beta$ ) and PP1 $\gamma$ . So far more than 100 putative regulatory subunits of PP1 enzyme have been identified. The PP1 binding subunits have quite different features, except for the short conserved PP1c binding motif: R/KVxF/W. The interaction between the catalytic and regulatory subunits of PP1 plays a central role in the regulation of PP1 holoenzyme.

### ***Characterization of protein phosphatase 2A and protein phosphatase 2B***

PP2A is heterodimeric or heterotrimeric holoenzyme which shows a very diverse structure. PP2A is expressed in every eukaryotic cells and it is involved in many biological processes. The dimer PP2A consists of a structural subunit (A subunit or PR65) and a catalytic subunit (PP2A C). The heterodimeric form of PP2A can bind the regulatory subunit (B subunit) forming the heterotrimeric form of PP2A. PP2B or calcineurin is a  $Ca^{2+}$ /calmodulin-dependent protein phosphatase. PP2B plays a central role in many biological processes. PP2B consists of a catalytic A subunit (calcineurin A or CNA) and a conserved  $Ca^{2+}$ -binding B subunit (calcineurin B or CNB).

### ***Regulation of vascular endothelial cell-cell contacts by reversible protein phosphorylation***

Endothelial barrier function is regulated by the coordinated complex actions of protein kinases and phosphatases on cytoskeletal, cytoskeleton-associated and intracellular junction proteins. The phosphorylation level of these proteins is crucial in determining the actual physical state of cytoskeletal proteins, the interaction between regulatory, membrane associated and intercellular junction proteins.

## **Role of the myosin phosphatase enzyme and its regulatory subunits in the endothelial barrier function**

### ***The myosin phosphatase enzyme and the MYPT protein family***

Myosin phosphatase consists of protein phosphatase 1 catalytic subunit (PP1c) and two regulatory subunits namely a larger regulatory subunit myosin phosphatase target subunit 1 (MYPT1) and a smaller regulatory subunit (M20). The most typical features of MYPT1 structure are the PP1c binding motif (KVVF) close to the N-terminal part of the protein which is followed by the seven ankyrin repeats and a regulatory/inhibitory phosphorylation site (Thr696). The C-terminal part of the protein contains a leucine-zipper motif. Several MYPT1 related proteins, namely MYPT2, MYPT3, MBS58 (myosin binding subunit 85) and TIMAP (TGF $\beta$ -inhibited membrane associated protein) were identified. MYPT2 is expressed in skeletal muscle, heart and brain and it shares 61% homology with MYPT1. The MBS85 protein is expressed ubiquitously, the similarity with MYPT1 is 39%. The membrane-associated MYPT3 is expressed in heart, brain and kidney and it shows a very similar structure with TIMAP. The most common features of MYPT family members structures are – like MYPT1 – the PP1c binding motif and the ankyrin repeats near the NH<sub>2</sub>-terminus. MBS85 has a C-terminal leucine-zipper motif. MYPT3 and TIMAP shares same structural features, like PP1c binding motif, ankyrin repeats, and C-terminal prenylation motif (CAAX box) which allows the membrane localization of the proteins.

### ***TIMAP protein, a MYPT family member***

TIMAP is a 64 kDa protein expressed at high level in hematopoietic stem cells and endothelial cells, especially in vascular endothelial cells. TIMAP shares structural features of MYPT family members, i.e. PP1c binding motif (KVVF), five ankyrin repeats and an N-terminal nuclear localization signal. Unlike other members of the family – except for MYPT3 – there is a prenylation motif, a CAAX box, which allows the membrane localization of TIMAP. Based on its structure, TIMAP was expected to be a regulatory subunit of PP1c which was proved by our work group. Our research group also found that moesin, an ERM protein, is an interacting partner of TIMAP and showed that TIMAP is involved in the endothelial barrier maintenance through the regulation of ERM dephosphorylation. Another interacting partner, receptor for activated C kinase 1 (RACK1) was also identified by our workgroup. RACK1 ensures the prenylation and translocation of TIMAP to the plasmamembrane. The latest identified interacting partner is the endothelin converting enzyme 1 (ECE-1). TIMAP plays a role in the regulation endothelin 1 production of EC through the regulation of the

dephosphorylation of ECE-1. TIMAP was detected in the plasmamembrane and nucleus of endothelial cells, but the role of the nuclear localization of TIMAP is unclear yet.

### **eEF1A1 protein**

Eukaryotic elongation factor 1 A (eEF1A) is a highly conserved protein. The eEF1A has two isoforms, eEF1A1 and eEF1A2. Their primary sequences are 92% identical. Protein eEF1A1 was described to be expressed in brain, placenta, lung, liver, kidney and pancreas. The other isoform, eEF1A2, is expressed in brain, heart and skeletal muscle. The canonical function of eEF1A is to bind to amino-acid-tRNA (aa-tRNA) in a GTP-dependent manner and deliver it to the ribosome but the protein has many non-canonical functions as well, like turnover of missfolded proteins, actin bundling, apoptosis, and function in the viral life cycle. The elongation factor is also involved in the nuclear export of proteins. It was reported that eEF1A1 aids nuclear export of polyadenylate-binding protein 1 (PABP1) and von Hippel-Lindau tumor suppressor (VHL) proteins. A so called transcription-dependent nuclear export motif (TD-NEM) present in these proteins was shown to interact specifically with eEF1A1. Proteins which lack the motif that encodes TD-NEM fail to bind to eEF1A1.

### **Merlin protein**

Moesin-ezrin-radixinlike protein, merlin the product of neurofibromatosis type 2 (*NF2*) gene was first identified as a tumorsuppressor protein, but it also functions as a membrane-cytoskeletal linker and regulator protein of multiple signaling pathways. There are 10 known isoforms of merlin protein, but the most intensively studied are isoforms 1 and 2. The difference between the two isoforms is the absence or presence of exon 16 or 17, respectively. Merlin is the member of the Band 4.1 protein family like ERM proteins. Merlin has an N-terminal FERM (Four point one, ezrin, radixin and moesin) domain, which is followed by an  $\alpha$ -helical and a C-terminal domain. Merlin lacks the actin binding site, but it can bind to actin via the ERM proteins. The activity and localization of merlin is regulated by head to tail folding that is controlled by phosphorylation of merlin at Ser518 residue. Merlin localizes in the nucleus when its Ser518 residue is not phosphorylated and suppresses oncogenic expression in the nucleus by activating the Hippo tumor suppressor pathway and inhibits the CRL4<sup>DCAF1</sup> E3 ubiquitin ligase. Phosphorylated form of merlin is present in the cytoplasm and the plasmamembrane, and evokes a growth-permissive form. Ser518 side chain of merlin was reported to be phosphorylated by cAMP-dependent protein kinase (PKA) or p21-activated kinase (PAK). Less is known about the protein phosphatases specific for the phospho-sites of merlin in endothelial cells.

## **AIMS OF THE STUDY**

Endothelium has a central role in inflammation, hemostasis, vasoregulation, angiogenesis, and vascular growth. TIMAP is highly abundant in endothelial cells. Our research group identified, that TIMAP is a PP1c regulatory subunit, and it is involved in the endothelial barrier maintenance through the regulation of ERM dephosphorylation. In addition RACK1 and ECE-1 was described as interacting partners of TIMAP, and it was shown that TIMAP is participated in the regulation of several processes in endothelial cells.

Specific aims of the presented work were the following:

### **I.**

- Identification a new interacting partner of TIMAP in endothelial cells.
- Structural domain mapping of the interaction.
- Investigation of the physiological role of the interaction.

### **II.**

- Detection of the interaction between merlin and TIMAP protein in endothelial cells.
- Structural domain mapping of the interaction.
- Investigation of the role of PP1c-TIMAP complex in the dephosphorylation of phospho-Ser518-merlin.

# MATERIALS AND METHODS

## Reagents and chemicals

All reagents and chemicals were purchased from Sigma (St Louis, MO, USA), unless stated otherwise.

## Methods

### *Reverse transcription (RT) and polymerase chain reaction (PCR)*

Total RNA was isolated from endothelial cells with ZR RNA MicroPrep™ kit (Zymo Research Corporation, Irvine, CA, USA). Phire® or Phusion® High-Fidelity DNA Polymerases (Thermo Scientific, Inc. Vantaa, Finland) were used to PCR. Coding regions of the proteins were amplified using specific primers.

### *Plasmids, cloning*

For cloning pGEX-4T-2, pGEX-4T-3 bacterial, or pCMV-Myc suitable for mammalian expression vectors (GE Healthcare, Piscataway, NJ, USA) were used.

### *Agarose gelelectrophoresis, DNA isolation from agarose gel*

To separate DNA fragments 1-1.2% agarose gels were used. DNA fragments were cut out from the gel on UV illuminator table (Hoefer Inc., Holliston, MA, USA). DNA was extracted from the agarose gel using GeneJet Gel Extraction Kit (Thermo Fisher Scientific, Inc., Waltham, USA) according to the manufacturer's protocol. Elution was done at 55°C in 30 µl nuclease free water. The purity and concentration of DNA was evaluated by NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, USA).

### *Restriction digestion and ligation*

FastDigest restriction enzymes (Thermo Fisher Scientific, Inc., Waltham, USA) were employed. The amounts of restriction enzymes used were kept lower than 10% of the total restriction volume. Recognition sites of the enzymes are as follow: BamHI: G˘GATCC, EcoRI: G˘AATTC, NotI: GC˘GGCCGC, SalI: G˘TCGAC, XhoI: C˘TCGAG.

During ligation the vector:insert molar ratio was 1:3. 5 units of T4 DNA Ligase (Thermo Fisher Scientific, Inc., Waltham, USA) were applied in each reaction. Ligation was performed at room temperature for 1 hour.

### ***Competent cell preparation and transformation***

A single colony of JM109 or BL31 (DE3) competent cells (Invitrogen Corporation, Carlsbad, CA, USA) was inoculated into 3 ml LB medium and was grown at 37°C for 16 hours. The overnight culture was diluted 1:100 ratio in LB and was grown at 37°C with shaking 180 rpm until OD<sub>600</sub> reached 0.3-0.6, after the culture was put on ice for 10 min. This was followed by centrifugation at 4°C with 2500 g for 5 min. Cells were resuspended in 30 ml of ice cold CaCl<sub>2</sub> solution (100 mM, pH 7.4) and centrifuged again (4°C, 2500 g, 5 min). Then cells were resuspended in 3 ml of ice cold CaCl<sub>2</sub> solution (100 mM, pH 7.4) containing 15% glycerol. Finally, 400 µl aliquots were made and stored at -70°C. Plasmids were added to 100 µl competent cells thawed on ice. After 20 min incubation on ice, 45 seconds heat shock (42°C) was applied, then cells were replaced on ice for 2 min. 900 µl SOC medium was added to the samples which were then cultured at 180 rpm and 37°C for 45 min. After that bacteria were spread onto LB agar containing 100 µg/ml ampicillin. Controls were spread onto LB agar with or without the antibiotic. Plates were incubated 16 hours at 37°C.

### ***Plasmid preparation***

A single colony of plasmid containing bacteria was inoculated into LB medium containing 100 µg/ml ampicillin antibiotic and was grown at 37°C, 180 rpm, 16 hours. GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to purify plasmid DNA. DNA sequencing was made in the Division of Laboratory Clinical Research, Department of Laboratory Medicine, Faculty of Medicine, University of Debrecen.

### ***Endothelial cell culturing***

Bovine Pulmonary Artery Endothelial Cells (BPAEC) (culture line: CCL 209) were obtained frozen at passage 8 (American Type Culture Collection, Rockville, MD, USA), and were utilized at passages 15-22. Cells were maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air in MEM supplemented with 10% (v/v) heat inactivated fetal bovine serum, 1% sodium pyruvate, 0.1 mM MEM non-essential amino acids solution (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

### ***Immunoprecipitation***

Cells were rinsed three times with 1x PBS and then collected and lysed with 600 µl immunoprecipitation (IP) buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 2 mM sodium vanadate, 1% NP-40) containing protease inhibitor cocktail. The lysate was centrifuged with 10 000 g for 15 min at 4°C. To avoid nonspecific binding, the supernatants

were precleared with 50  $\mu$ l of protein G Sepharose (GE Healthcare, Piscataway, NJ, USA) at 4°C for 3 hours with end-over-end rotation. Protein G Sepharose was removed by centrifugation at 4°C, and the supernatant was incubated with the appropriate volume of antibody at 4°C for 1 hour and then with 50  $\mu$ l of fresh protein G Sepharose at 4°C overnight with gentle rotation. The resin was washed three times with 300  $\mu$ l of IP buffer, boiled, and microcentrifuged for 5 min. The supernatant was analyzed by Western blotting.

### ***Transfection of endothelial cells***

TIMAP was transfected with pCMV-Myc TIMAP 1-257 wt and pCMV-Myc 1-257 TIMAP G252A mutant plasmids using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA). TIMAP and eEF1A1 were silenced using 50 nM ON-TARGET plus SMARTpool siRNA (L-004065-00-0, Dharmacon, Lafayette, CO, USA) and 10 nM EF-1  $\alpha$ 1 siRNA (sc-77232, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) respectively, using Lipofectamine RNAiMAX transfection reagent (Invitrogen Corporation, Carlsbad, CA, USA) in serum-free medium. ON-TARGET plus siCONTROL nontargeting pool (D-001810-10-01-05; Dharmacon, Lafayette, CO, USA) was used as an irrelevant control. After 6 hours medium was changed to complete medium. Cells were further incubated for 48 hours.

### ***Subcellular fractionation***

For subcellular fractionation Mem-PER<sup>TM</sup> Plus Membrane Protein Extraction Kit and ProteoJET<sup>TM</sup> Cytoplasmatic and Nuclear Protein Extraction Kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) were used according to the manufacturer's protocol. The efficiency of fractionation was analyzed by Western blotting using CD31 antibody as membrane marker,  $\beta$ -tubulin as a cytoplasmatic marker and lamin A/C antibody as a nuclear marker.

### ***Immunofluorescent staining and confocal microscopy***

Endothelial cells were plated onto 0.2% gelatin coated glass cover slip and grown. After washing, cells were fixed with 3.7% paraformaldehyde soluted in 1XTBS for 15 min, room temperature. The cells were permeabilized with 0.5% Triton X-100-TBS solution for 15 min, blocked with 2% BSA in 1XTBS for 30 min, and incubated primary and secondary antibodies respectively diluted in blocking solution for 1h, room temperature in dark. DAPI (4',6-diamidino-2-phenylindole, Molecular Probes, Eugene, OR, USA) or TO-PRO-3 Iodide (Thermo Fischer Scientific, Waltham, MA, USA) were used for staining nuclei. Between each following steps, the cells were rinsed three times with 1XTBS. Cover slips were rinsed and then mounted in ProLong Gold Antifade medium (Thermo Fisher Scientific, Inc., Waltham,

MA, USA). Images were acquired with Leica TCS SP8 confocal microscopy (Leica Microsystems, Wetzlar, Germany), using HC PL APO CS2 63 x 1.40 NA oil immersion objective on a DMI6000 microscopy at 25°C. Images were processed using LAS AF v3.1.3 software. Non-specific binding of the secondary antibodies was checked in control experiments.

### ***SDS-PAGE and Western blotting***

Protein samples were separated by 10-12% SDS-PAGE and stained with Coomassie Blue or Blue Silver solution. In Western blotting experiments proteins were separated by SDS-PAGE and transferred to 0.45 µm pore sized Hybond ECL Nitrocellulose Membrane (GE Healthcare, Piscataway, NJ, USA). Membranes were blocked with 5% low-fat dry milk powder in TBST and then they were incubated with primary antibody diluted in TBST containing 1% BSA for overnight at 4°C. After the washing steps the membranes were incubated with the HRP-conjugated secondary antibody diluted in TBST containing 1% BSA for 1 hour, room temperature. After washing the membrane was incubated with WesternBright ECL-HRP substrate (Advansta Inc., Menlo Park CA, USA). Images were acquired using Alpha Innotech FluorChem<sup>®</sup> FC2 Imager (Lab Merchant, London, UK).

### ***Preparation of GST-fusion proteins and in vitro pull down assay***

*Escherichia coli* BL21 transformed with pGEX-4T-3 containing glutathione S-transferase (GST) or pGEX-4T-2 containing merlin, or merlin FERM domain, merlin  $\alpha$ -helical domain, merlin C-terminal domain coding DNA sequence (from *NF2* gene, accession number: NM\_000268.3), pGEX-4T-3 containing TIMAP 1-257 amino acids coding, TIMAP 1-257 G252A amino acids coding (from PPP1R16B gene, accession number: NM\_015568.3), eEF1A1 (from EEF1A1 gene, accession number: NM\_001402.5) fused with GST, or pCMV-Myc containing TIMAP 1-257 amino acids coding, TIMAP 1-257 G252A amino acids coding were induced with 0.1-1 mM isopropyl  $\beta$ -D-thiogalactoside (IPTG) and grown at 25°C or 37°C with shaking for 3 hours. Cells were harvested by centrifugation, sonicated using Branson Sonifer (AMS Materials, LLC, Jacksonville, FL, USA) homogenizer in lysis buffer (50 mM Tris-HCl (pH 7.5), 0.1% Tween 20, 0.2% 2-mercaptoethanol, Protease Inhibitor Coctail Set III (Calbiochem, Darmstadt, Germany), and proteins were isolated by affinity chromatography on glutathione Sepharose 4B (GE Healthcare, Piscataway, NJ)) according to the manufacturer's protocol. For pGEX-4T-3 TIMAP 1-257 and pGEX-4T-3 TIMAP 1-257 G252A mutant constructs *E. coli* BL21 (DE3) cells were induced with 0.5 mM IPTG and

grown at 25°C for 3 h. Harvested cells were sonicated in 0.2% sarcosyl containing lysis buffer. After centrifugation the supernatant of the samples were diluted in lysis buffer to contain 0.008% sarcosyl and proteins were isolated by affinity chromatography on glutathione Sepharose 4B.

BPAEC grown in 100-mm culture flasks were washed twice with 1x ice-cold PBS, scraped, and lysed in 600 µl lysis buffer. The lysates were incubated with GST or GST-fused proteins coupled to glutathione Sepharose for 3 hours at 4°C. The beads were washed three times with 1x TBS then the GST fusion proteins were eluted by boiling the samples.

#### ***Endothelial cell treatment with specific phosphatase inhibitors***

BPAE cells were treated with 1 µM tautomycetim as a potent PP1 inhibitor for 30 min, 5 µM okadaic acid as a potent PP2A inhibitor for 30 min, and 2 mM cyclosporin A as a potent PP2B inhibitor for 30 min. Control cells were treated with DMSO (vehicle). After that cells were scraped in lysis buffer and boiled in 1x SDS sample buffer.

#### ***LC-MS/MS analysis***

Proteins were resolved by SDS-PAGE and stained with Blue Silver solution. Liquid Chromatography with Tandem Mass Spectrometry Detection was performed by Dr. Tamás Janáky at the Department of Medical Chemistry, Faculty of Medicine, University of Szeged. Eluted peptides were analyzed by Data Dependent Acquisition and the three most abundant precursor ions were selected for MS/MS. Data were evaluated with ProteinLynx Global Server 2.4 software and Mascot 2.04 data browser.

#### ***Statistical analysis***

Statistical evaluations were performed by ANOVA using SigmaStat Software (Systat Software Inc., London, United Kingdom). Values were reported as mean ± SD. Differences were considered significant for  $p < 0.001$  (\*\*\*). Densitometry of immunoblots was done by Image J software (National Institutes of Health, Bethesda, MA, USA).

## RESULTS AND CONCLUSIONS

### Detection of a new interacting partner of TIMAP

#### *eEF1A1 is a new interacting partner of TIMAP*

Using pull down followed by LC-MS/MS analysis, a new protein partner of TIMAP, namely, the eukaryotic elongation factor 1 A 1 (eEF1A1, accession number: P68104) was identified. The interaction was verified by Western blotting of the GST-TIMAP pull down samples. Furthermore, eEF1A1 has been cloned from human pulmonary artery EC (HPAEC) by RT-PCR and subcloned into pGEX-4T-2 for bacterial expression. In this set of the experiments the recombinant elongation factor was employed in GST pull down as bait, and endogenous TIMAP from EC lysate was fished out. Then, the interaction of endogenous proteins, TIMAP, PP1c and eEF1A1, was proved by immunoprecipitations from EC lysates utilizing specific anti-TIMAP and anti-eEF1A1 antibodies. TIMAP co-immunoprecipitated with eEF1A1, and *vice versa*. In addition, PP1c was also present in both precipitates.

#### *Mapping the TIMAP-eEF1A1 interaction domains*

Further GST pull down assays, with several truncated TIMAP forms, showed that the TIMAP sequence responsible for the interaction is located in the middle region of the protein. Beside the full length (amino acids 1–567) TIMAP, eEF1A1 bound to those TIMAP mutants which contain ankiryin 4 and ankiryin 5 repeats in the 165–290 amino acid region.

#### *Role of TD-NEM in the interaction between TIMAP-eEF1A1*

Moreover, careful analysis of the TIMAP sequence revealed that within this region, the sequence of 250–260 amino acids (250DHGVRVDVKDW<sub>260</sub>) may correspond to the TD-NEM motif (DxGxxDxxL). It was reported in a publication that eEF1A1 assists nuclear export of proteins with the TD-NEM motif, and the conserved amino acid residues in the motif are crucial both in binding of eEF1A1 to TD-NEM and the nuclear export. Among the studied side chains, the mutation of the first conserved amino acids (underlined in the TD-NEM motif above) caused the biggest effect in eEF1A1 binding and in the nuclear export. Therefore, we created two additional TIMAP recombinants, both spanning amino acids 1–257. One of these truncated forms has the wild type sequence, but in the other one we inserted a G252A mutation in the TD-NEM-like motif. After successful purification, pull down experiments were carried out to test the effect of mutation of the potential TD-NEM motif. TIMAP 1–257 G252A mutant showed dramatically decreased binding capacity of

endogenous eEF1A1 from EC lysate compared to the wild type truncated TIMAP (amino acids 1–257). Similarly, wild type truncated TIMAP (amino acids 1–257) was able to bind GST-eEF1A1, while the G252A mutant of TIMAP 1–257 could not bind the recombinant elongation factor. All these results demonstrated the importance of TD-NEM in the interaction of TIMAP with eEF1A1.

***TD-NEM like motif of TIMAP affects its subcellular localization***

Since eEF1A1 is involved in the nuclear export of proteins having TD-NEM motif, we hypothesized, that the TD-NEM motif and interaction of TIMAP with eEF1A1 may have a significance in the nuclear export of TIMAP. Next, therefore, coding sequences for the 1-257 and 1-257 G252A mutants of TIMAP were subcloned into pCMV-Myc vector suitable for mammalian protein expression. BPAEC cells were transfected and localization of the overexpressed proteins was analyzed by immunofluorescent staining using anti-c-myc-tag antibody. Both recombinant forms were found in the nucleus and cytoplasm of EC. Membrane, nuclear and cytoplasmic fractions of transfected EC were isolated, and analyzed by Western blotting. As expected, no membrane localized recombinant TIMAP was found in the cells due to lack of the C-terminal prenylation motif in the truncated forms. The distribution of recombinant TIMAP forms (amino acids 1-257 wild type and mutant) were compared in the nuclear and cytoplasmic fractions. In agreement with our hypothesis, the TD-NEM mutant form showed predominant nuclear localization, suggesting decreases nuclear export, still, considerable amount of the protein was present in the cytoplasmic fraction.

To further clarify the possible role of eEF1A1 in the nuclear export of TIMAP, the effect of eEF1A1 depletion on localization of the endogenous TIMAP was studied. Mixture of siRNAs targeted to eEF1A1 was purchased and the efficiency of depletion was verified by Western blotting. Cytoplasmic, nuclear and membrane fractions of EC transfected with siRNAs specific for eEF1A1 or with nonsiRNA were obtained and tested by Western blotting. Interestingly, eEF1A1 was present not only in the cytoplasmic, but also in membrane fraction of nonsiRNA transfected EC in considerable amount. However, no change in the localization of TIMAP was found in the eEF1A1 depleted samples, suggesting that eEF1A1 has a replaceable role, if any, in the nuclear export of TIMAP.

## **Investigation of the interaction between merlin and PP1c-TIMAP complex**

### ***Examination of merlin's isoforms in endothelial cells***

*NF2* maps to the long arm of chromosome 22 and encodes 10 merlin isoforms, but isoforms 1 and 2 are the most intensively studied forms. Merlin isoform 2 contains the alternatively spliced exon 16, but merlin isoform 1 not. The longer, dominant merlin isoform 1 has an extended carboxy-terminal tail that is encoded by exon 17. Merlin isoform 2, on the other hand, contains the alternatively spliced exon 16 which ends in a stop codon, encoding 11 unique residues following amino acid 579 as compared to merlin isoform 1. Notably, merlin isoform 2 lacks carboxy-terminal residues required for intramolecular binding between the amino-terminal FERM domain and the carboxy-terminal tail, possibly leading to a constitutively open conformation. We investigated which isoform(s) of merlin express(es) in EC. Primer pair was designed to detect these two isoforms. The presence or absence of exon 16 results in a 45 bp difference between the two isoforms (isoform 1: 493 bp, isoform 2: 538 bp). PCR was performed with the employment of HPAEC (Human Pulmonary Artery Endothelial Cells) cDNA. Our results indicated that endothelial cells express both isoform 1 and 2. For further experiments we used merlin isoform 1 for cloning and overexpressing the protein.

### ***Detection of the interaction between merlin and PP1c-TIMAP complex***

Previously it was reported that myosin phosphatase (PP1c-MYPT1) is responsible for the dephosphorylation of phospho-Ser518 merlin in tumor cells. In endothelial cells, another member of the MYPT family of PP1 regulatory subunits, namely TIMAP is highly expressed and regulates dephosphorylation of moesin, an ERM family member. Therefore, we hypothesized that the PP1c-TIMAP complex can be involved in merlin dephosphorylation as well in the endothelial cells. First, interaction between merlin and PP1c-TIMAP was tested by several methods. In pull down assay recombinant GST-tagged TIMAP interacted with endothelial merlin and PP1c, also, endogenous PP1c-TIMAP was present in the pull down sample of purified GST-tagged merlin. Immunoprecipitation (IP) experiments were utilized to verify the interaction of the endogenous proteins in EC. PP1c-TIMAP was present in the merlin IP samples and *vice versa*, TIMAP IP samples contained PP1c and merlin verifying the interaction. Interestingly, phospho-Ser518-merlin was also detectable in both precipitates.

### ***Mapping the PP1c-TIMAP and merlin interaction domains***

Truncated TIMAP mutants (N-terminal amino acids 1-290 and C-terminal amino acids 291-567) were created earlier and used for domain mapping of the interaction. The full length and the N-terminal fragment of TIMAP – containing the nuclear localization signal, the PP1c-binding motif and the five ankyrin repeats – interacted with merlin, but the C-terminal fragment of TIMAP was not able to bind the endogenous merlin. The recombinant N-terminal FERM-, central  $\alpha$ -helical-, and C-terminal regulatory domains were created and used in pull down assay. PP1c-TIMAP was present only in the full length merlin and FERM-domain samples. Domain mapping of the interaction revealed, that the N-terminal half (amino acids 1-290) of TIMAP binds merlin, similarly to its other interactions. The less ordered C-terminal half (amino acids 291-567) of TIMAP could not bind merlin from endothelial cell lysate. As many other interacting partners of merlin, PP1c-TIMAP complex binds to the FERM domain.

### ***Effects of silencing of TIMAP on the merlin-PP1c-TIMAP interaction and phosphorylation state of merlin***

To prove that TIMAP directs PP1c to merlin, merlin was immunoprecipitated from nonsiRNA treated and TIMAP depleted cells. PP1c was only present in the merlin IP complex prepared from the lysate of nonsiRNA treated cells, where TIMAP bound to merlin. Importantly, no interaction was found between merlin and PP1c in TIMAP depleted cells. This suggests that TIMAP is the sole regulatory protein directing PP1c to merlin. Still total cell lysates and IP complexes were tested for MYPT1 as well. Although MYPT1 is expressed in endothelial cells, no interaction was detected with merlin regardless the presence or absence of TIMAP further confirming the critical role of TIMAP in dephosphorylation of phospho-Ser518-merlin. Subcellular localization of phospho-Ser518-merlin was studied by immunofluorescent staining of nonsiRNA and TIMAP specific siRNA transfected endothelial cells using phospho-Ser518-merlin antibody, anti-CD31 antibody as membrane marker and DAPI as nuclei dye. We found that in TIMAP depleted cells phospho-Ser518-merlin was enhanced in the plasmamembrane compared to nonsiRNA treated cells. These results indicate that TIMAP play a role in the regulation of phospho-Ser518-merlin dephosphorylation.

### ***Inhibition of PP1 enzyme increases the phosphorylation level of merlin at Ser518 side chain***

To test which protein phosphatase dephosphorylates phospho-Ser518-merlin, specific protein phosphatase inhibitors were applied. Cells were treated with 1  $\mu$ M tautomycin to inhibit PP1, 5 nM okadaic acid or 2  $\mu$ M cyclosporine A to block PP2A and PP2B activity, respectively.

The phosphorylation level of merlin at Ser518 residue in the cell lysates increased after the treatment with the specific inhibitor against PP1. Densitometry of Western blotting showed a two-fold increase compared to control, okadaic acid or cyclosporine A treated samples. These results confirmed our hypothesis that the phosphorylation level of the Ser518 site is regulated via the activity of a type 1 protein phosphatase.

## SUMMARY

TIMAP (TGF $\beta$ -inhibited membrane associated protein) is a highly abundant in endothelial cells (EC). It is a member of the MYPT (myosin phosphatase target subunit) protein family, and a regulatory subunit of PP1c. In this study, we identified eEF1A1 (eukaryotic translation elongation factor 1 A 1) as a new interacting partner of TIMAP. Besides its canonical role in translation, eEF1A1 may participate in the nuclear export of proteins with TD-NEM (transcription-dependent nuclear export motif), a newly recognized nuclear export signal (DxGxxDxxL) which is crucial in binding to eEF1A1 and in the nuclear export. TIMAP localizes in the nucleus and at the plasma membrane in EC. Since TIMAP contains a region (DHGVRVDV) highly homologous to the TD-NEM motif, we hypothesized that eEF1A1 may be involved in the nuclear export of TIMAP. We found that this TD-NEM-like motif of TIMAP has a critical role in its specific binding to eEF1A1. However, according to our results eEF1A1 is not or not exclusively responsible for the nuclear export of TIMAP. Merlin (moesin-ezrin-radixin like protein), the product of neurofibromatosis type 2 (*NF2*) gene, was primarily recognized as a tumor suppressor, but it also functions as a membrane-cytoskeletal linker and regulator of multiple signaling pathways. The activity and localization of merlin is regulated phosphorylation at the Ser518 side chain. Merlin localizes in the nucleus when the Ser518 side chain is not phosphorylated, while the phosphorylated form is present in the cytoplasm and the plasma membrane. We showed that EC express both merlin 1 and 2 isoforms. Furthermore, we identified a protein-protein interaction between PP1c-TIMAP and identified the N-terminal part of TIMAP and FERM domain of merlin as critical regions of the interaction. In addition, we detected that TIMAP is able to bind phospho-Ser518-merlin. In EC co-immunoprecipitation of merlin with PP1c-TIMAP was also shown, but there was no detectable interaction between merlin and PP1c in TIMAP depleted cells. In addition MYPT1 is expressed in EC, but no interaction was detected with merlin regardless the presence or absence of TIMAP. Results of immunofluorescent staining of TIMAP depleted and nonsiRNA treated EC suggest that the complex regulates dephosphorylation of phospho-Ser518-merlin. To test whether PP1c is alone responsible for dephosphorylation of merlin, EC lysates were treated with specific phosphatase inhibitors. In the presence of tautomycin, a potent PP1 inhibitor, the phosphorylation level of merlin was elevated implying that a type-1 phosphatase dephosphorylates phospho-Ser518-merlin. In summary, this work focused on two newly identified interacting partner of TIMAP in EC, which provide important informations about the role of TIMAP protein in EC.

## **NEW FINDINGS**

1. eEF1A1 protein is a new interacting partner of TIMAP in EC.
2. The region in TIMAP responsible for eEF1A1 binding is within the amino acids 165-290 (ankyrin repeats 4. and 5).
3. The TD-NEM like motif of TIMAP is essential in the interaction between TIMAP and eEF1A1.
4. Merlin isoforms 1 and 2 are expressed in EC.
5. Merlin is an interacting partner of PP1c-TIMAP complex in EC.
6. The N-terminal fragment of TIMAP interacted with merlin, and the FERM domain of merlin interacted with PP1c-TIMAP complex.
7. PP1c-TIMAP complex is involved in the regulation of dephosphorylation of phospho-Ser518-merlin in EC.

## **KEYWORDS**

endothelial cells, reversible protein phosphorylation, protein phosphatase 1, TIMAP, eEF1A1, merlin, phospho-Ser518-merlin, pull down, immunoprecipitation

# LIST OF PUBLICATIONS RELATED TO THE DISSERTATION



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## List of publications related to the dissertation

1. Boratkó, A.\*, **Péter, M.\***, Csortos, C.: Regulation of merlin by protein phosphatase 1-TIMAP and EBP50 in endothelial cells.  
*Int. J. Biochem. Cell Biol.* 82, 10-17, 2017.  
DOI: <http://dx.doi.org/10.1016/j.biocel.2016.11.010>  
\*Anita Boratkó and Margit Péter contributed equally to this work.  
IF: 3.905 (2015)
2. Boratkó, A., **Péter, M.**, Thalwieser, Z., Kovács, E., Csortos, C.: Elongation factor-1A1 is a novel substrate of the protein phosphatase 1-TIMAP complex.  
*Int. J. Biochem. Cell Biol.* 69, 105-113, 2015.  
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# CONFERENCE PRESENTATIONS

## Oral presentations

**Margit Péter**, Anita Boratkó, Csilla Csontos: Investigation of interaction between TIMAP and merlin in endothelial cells.

Cell Biology and Signaling Conference (a training course)  
Galyatető, 20-22 May, 2016

**Margit Péter**: Role of protein-protein interaction in endothelial cells

Annual Symposium of the Doctoral School of Molecular Medicine  
Debrecen, 1 September, 2015

**Margit Péter**, Anita Boratkó, Csilla Csontos: EBP50 protein and its interacting partners in vascular endothelial cells

Cell Biology and Signaling Conference  
Egerszalók, 29-31 May, 2015

**Margit Péter**: Protein-protein interactions in endothelial cells

Annual Symposium of the Doctoral School of Molecular Medicine  
Debrecen, 4 September, 2014

**Margit Péter**, Anita Boratkó, Csilla Csontos: Investigation of a new interacting partner of TIMAP

Signaling pathways in cancer biology (a training course)  
Mátraháza, 16-18 May, 2014

## Poster presentations

Anita Boratkó, **Margit Péter**, Csilla Csontos: Interaction of TIMAP-PP1c complex with merlin

Annual Conference of the Hungarian Biochemical Society  
Szeged, 28-31 August, 2016

**Margit Péter\***, Anita Boratkó\*, Csilla Csontos: A TIMAP-PP1c komplex szerepének vizsgálata a merlin<sup>Ser518</sup> szabályozásában

\*equal contribution

46<sup>th</sup> Membrane-Transport Conference  
Sümege, 17-20 May, 2016

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