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Author: Anita Boratkó Margit Péter Csilla Csortos

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Regulation of merlin by protein phosphatase 1-TIMAP and EBP50 in endothelial cells

Anita Boratkó[a], Margit Péter[a], Csilla Csortos*

Department of Medical Chemistry, Faculty of Medicine, University of Debrecen;

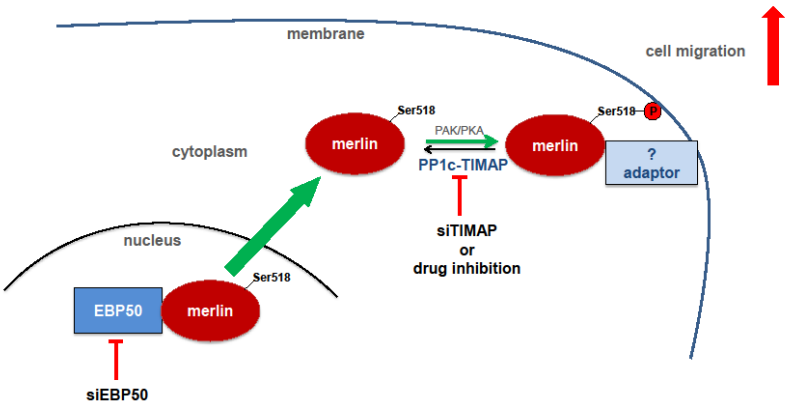
Egyetem tér 1., Debrecen, H-4032, Hungary

[a]Anita Boratkó and Margit Péter contributed equally to this work.

*Corresponding author: at Department of Medical Chemistry, Faculty of Medicine, University of Debrecen; Egyetem tér 1., Debrecen, H-4032, Hungary, tel.: +36 52412345 ext.61173; fax: +36 52412566

E-mail address: csortos@med.unideb.hu (C. Csortos)

Graphical abstract



Abstract

Merlin (*m*oesin-*e*zrin-*r*adixin *l*ike prote*in*), the product of neurofibromatosis type 2 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 by head to tail folding that is controlled by phosphorylation of 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. In this work interactions and their impact on the subcellular localization and phosphorylation state of the Ser518 side chain of merlin were investigated in endothelial cells. It is shown that merlin (dephospho-Ser518 form) interacts in the nucleus of endothelial cells with the scaffolding protein EBP50, a member of the Na^+/H^+exchanger regulatory factor family. Upon EBP50 depletion, merlin translocated from the nucleus, suggesting that binding of merlin to EBP50 is critical in the nuclear localization of merlin. Along with the translocation, the phosphorylation level of phospho-Ser518-merlin was increased in EBP50 depleted cells. TIMAP (TGFβ-inhibited membrane-associated protein), a type 1 protein phosphatase (PP1) regulatory subunit, was newly recognized as an interacting partner for merlin. Domain mapping using truncated mutant forms in GST pull down revealed that the N-terminal half of TIMAP (aa 1-290) and the FERM domain of merlin are the regions responsible for the interaction.The catalytic subunit of PP1 (PP1c) was present in all merlin-TIMAP pull down or immunoprecipitation samples demonstrating that merlin

actually interacts with the PP1c-TIMAP holoenzyme. On the other hand, from TIMAP depleted cells, without its targeting protein, PP1c could not bind to merlin. Also, when the phosphatase activity of PP1c-TIMAP was inhibited either with depletion of TIMAP or by treatment of the cells with specific PP1 inhibitor, there was an increase in the amount of phospho-Ser518 form of merlin in the membrane of the cells. These data strongly suggest that the PP1c-TIMAP- complex dephosphorylates phospho-Ser518-merlin. ECIS measurements indicate that phospho-merlin accelerates *in vitro* wound healing of the endothelial monolayer.

In conclusion, in endothelial cells, EBP50 is required for the nuclear localization of merlin and the PP1c-TIMAP holoenzyme plays an important role in the dephosphorylation of merlin on its Ser518 side chain, which influence cell migration and proliferation.

Abbreviations:

BPAEC: Bovine Pulmonary Artery Endothelial Cells; ERM: ezrin, radixin, moesin; FERM: band 4.1, ezrin, radixin, moesin; GST: glutathione S-transferase; ILK: integrin-linked kinase; IP: immunoprecipitation; MYPT: myosin phosphatase target subunit; NF2: neurofibromatosis type 2; NHERF: Na+/H+ exchanger regulatory factor; PAK: p21-activated kinase; PKA: cAMP-dependent protein kinase; PKC: protein kinase C; PP: protein phosphatase; PP1c: catalytic subunit of PP1; PP1c-MYPT1: myosin phosphatase;TIMAP: TGFβ-inhibited membrane-associated protein

Keywords: endothelial cells; merlin; protein phosphatase 1; TIMAP; EBP50

1. Introduction

Endothelial cell barrier integrity is critical to tissue and organ functions as the endothelium must meet the physiological requirements of the underlying tissue. EC integrity depends on the organization of the cytoskeletal elements and cell-cell junctions significantly. Protein-protein interactions, which may require assistance of further adaptors or linkers, are essential in the actual arrangement of the cytoskeletal and junction proteins.

ERM (*e*zrin, *r*adixin, *m*oesin) proteins serve as cross-linkers between cortical actin filaments and integral membrane proteins either through direct interaction or via adaptor molecules such as members of the Na^+/H^+ exchanger regulatory factor (NHERF) family. Yet, we observed nuclear localization of NHERF1/EBP50 during interphase in EC unlike the cytoplasm/membrane localization detected in epithelial cells (Boratko et al., 2012). Further, all three ERM proteins strongly interact with NHERF2 in EC, but NHERF1 is a poor protein partner of the ERM in this cell type (Boratko and Csortos, 2013), although both NHERF proteins have an ERM-binding domain (Murthy et al., 1998, Voltz et al., 2001, Reczek et al., 1997).

Biological activity of ERM is regulated by reversible phosphorylation. While ERM exist in an inactive, closed conformation in the cytoplasm, phosphorylation by Rho kinase or protein kinase C (PKC) on a conserved threonine residue triggers a conformational change and activation of ERM as cross-linkers (Matsui et al., 1998, Bretscher et al., 2002). Two protein phosphatase 1 (PP1) holoenzyme forms, myosin phosphatase (PP1c-MYPT1) (Fukata et al., 1998) and PP1c-TIMAP (Csortos et al., 2008), were identified to dephosphorylate ERM in different cell types. TIMAP (TGF-β inhibited membrane-associated protein) is highly abundant in EC and it is a regulatory subunit for δ isoform of the catalytic subunit of PP1 (PP1c). Our previous (Csortos et al., 2008, Czikora et al., 2011) and unpublished results have indicated that the PP1c-TIMAP complex is involved in maintaining the pulmonary EC barrier function via regulation of the phosphorylation level of ERM.

Merlin (*m*oesin-*e*zrin-*r*adixin *li*ke protei*n*), the product of NF2 (neurofibromatosis type 2) tumor suppressor gene, shares its domain organization with ERM proteins, namely, its N-terminal FERM domain (band 4.1 (F), ezrin (E), radixin (R), moesin (M)) is followed by an α-helical and a C-terminal regulatory domain (Bretscher et al., 2002). Merlin was primarly recognized as a tumor suppressor since it regulates cell proliferation (Morrison et al., 2001). Lately, merlin seems to have a dual function in different regions of the cell. It is involved in regulating formation of membrane domains and in organizing cell junctions; also, it propagates antimitogenic signaling at the cell cortex. Merlin suppresses oncogenic gene expression by activating the Hippo tumor suppressor pathway and inhibits the $CRL4^{DCAF1}$ E3 ubiquitin ligase in the nucleus (McClatchey and Fehon, 2009, Gladden et al., 2010, Li et al., 2010, Cooper and Giancotti, 2014).

Similarly to ERM, Ser518 phosphorylation of merlin modulates its intra- and intermolecular associations and activity (Rong et al., 2004). Based on the sequence homology with the ERM, phospho-Ser518-merlin was thought to have an open form, while the dephosphorylated protein is presumably in a closed conformation. More recent results, however, suggest that phosphorylation of merlin at Ser518 enhances the FERM-C-ERMAD association and a more closed form, while dephosphorylation of this site results in a more open state of merlin (Yogesha et al., 2011, Sher et al., 2012). Nevertheless, dephospho-Ser518 form of merlin has nuclear localization, and it is referred as the active form inhibiting cell proliferation. On the other hand, phosphorylation of Ser518 evokes a growth-permissive form of the protein present in the cytoplasm and cell membrane (Li et al., 2012, Surace et al., 2004). Ser518 side chain of merlin was reported to be phosphorylated by cAMP-dependent protein kinase (PKA) or p21-activated kinase (PAK) (Xiao et al., 2002, Alfthan et al., 2004). Further, merlin was shown to be phosphorylated by several kinases at different other Ser/Thr side chains as well. While active or inactive conformation depends on Ser518 phosphorylation, Ser10, Thr230 and Ser315 are considered to be responsible for the stability of

merlin (Ye, 2007, Scoles, 2008, Li et al., 2012).Much less is known about the protein phosphatases specific for the phospho-sites of merlin. Based on the interaction detected between MYPT1 (myosin phosphatase target subunit 1) and merlin, myosin phosphatase (PP1c-MYPT1) was shown to control dephosphorylation of phospho-Ser518 of merlin in human tumor cell lines (Jin et al., 2006).

In the present work we investigated protein-protein interactions of merlin in relation with the subcellular localization and phosphorylation state of its Ser518 side chain in vascular endothelial cells.

2. Materials and Methods

2.1 Reagents

Materials were obtained from the following vendors: anti-c-myc antibody: Zymed Laboratories (South San Francisco, CA); anti-SLC9A3R1 antibody (NHERF1/EBP50), anti-PPP1R16B antibody (TIMAP): Abgent Inc. (San Diego, CA); anti-CD31 antibody, anti-merlin (D1D8) antibody, anti-phospho-merlin (Ser518), anti-rabbit IgG HRP-linked and anti-mouse IgG HRP-linked secondary antibodies: Cell Signaling Technology, Inc. (Beverly, MA); anti-PP1 delta antibody: Upstate Biotechnology (Lake Placid, NY), anti-lamin A/C antibody Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Substances for cell culturing were from PAA (Austria). Anti-MYPT1 anibody was a kind gift from Dr. Ferenc Erdődi's lab (Lontay et al., 2004). Anti-actin antibody and all other chemicals were obtained from Sigma (St Louis, MO).

2.2 Cell cultures and cell fractionation

Bovine Pulmonary Artery Endothelial Cells (BPAEC) (American Type Tissue Culture Collection, Rockville, MD, culture line-CCL 209) were used and maintained as described before (Boratko et al., 2013). Subcellular fractionation was done as described in (Boratko et al., 2015). The efficiency of fractionation was analyzed by immunoblotting using CD31 antibody as a membrane marker, lamin A/C antibody as a nuclear marker and actin antibody as a cytoplasmic marker.

2.3 Preparation of constructs

The wild-type TIMAP (NM_015568) and truncated forms were made as described earlier (Csortos et al., 2008, Boratko et al., 2013). pCMV-myc ezrin, radixin and moesin constructs were also made earlier (Boratko and Csortos, 2013). Human cDNA prepared from HPAEC cells was used for merlin (NM_000268.3) amplification with the following primers and cloned into pCMV-myc or pGEX-4T-2 vector: 5'-TTG AAT TCC CAT GGC CGG GGC CAT CG-3', 5'-TTC AGT

CGA CCT AGA GCT CTT-3'. Additional merlin constructs were derived from the full length using the following primer pairs: FERM domain: 5'-TTGAATTCCCATGGCCGGGGCCATCG-3', 5'-TTAGTCGACTCACCTTCTCCTCATAAATAGATCAT-3'; α-helical domain: 5'-TCAAGGATCCATGAAAGCCGATTCTTTGGAAGTTCA-3', 5'-TAAGTCGACTCAAGACAGGCTGTCA-3'; C-terminal domain: 5'-ATTGGATCCATGTTCGACTTCAAAGATACTGACA-3', 5'-TTCAGTCGACCTAGAGCTCTT-3'. All primers were synthesized by Integrated DNA Technologies (Coralville, IA). The DNA sequences of the constructs were confirmed by sequencing (Biomi Kft., Szeged, Hungary).

2.4 Bacterial recombinant protein expression and GST pull down

Escherichia coli BL21 (DE3) transformed with pGEX-4T-3 containing glutathione S-transferase (GST) and pGEX-4T-3 containing TIMAP wild type or mutants were induced and purify as described (Boratko et al., 2015). pGEX-4T-2 containing merlin full length and truncated forms were induced with 0.1 mM IPTG and grown at room temperature with shaking for 3 h.

For pull down assay BPAEC lysate was incubated with GST or GST-fused proteins coupled to glutathione Sepharose for O/N at 4°C. The beads were washed three times with 1X TBS then the GST fusion proteins were eluted by boiling the samples.

2.5 Immunoprecipitation and immunofluorescent staining

Immunoprecipitation and immunostaining of desired proteins utilizing appropriate antibodies were done as described before (Boratko et al., 2013). Confocal images were acquired with a Leica TCS SP8 confocal microscope using HC PL APO CS2 63x 1.40 NA oil immersion objective on a DMI6000 CS microscope at 25°C. Images were processed using LAS AF v3.1.3 software. Nonspecific binding of the secondary antibodies was checked in control experiments (not shown).

2.6 Transfection and gene silencing

BPAEC cells were transfected with pCMV-myc ezrin, radixin, moesin or merlin plasmids using Lipofectamine 2000 transfection reagents (Invitrogen Corporation, Carlsbad, CA), according to the manufacturer's instructions.

TIMAP was silenced using 50nM ON-TARGETplus SMARTpool siRNA (L-004065-00-0 HumanPP1R16B, Dharmacon) for 48 hours, EBP50 was silenced using 50nM siNHERF-1 (sc-63330, Santa Cruz Biotechnology, Inc.) for 72 hours in complex with Lipofectamine RNAiMAX.

ON-TARGETplus siControl nontargeting pool (D-001810-10-01-05; Dharmacon) was used as an irrelevant control.

2.7 Western blotting

Protein samples were separated by SDS-PAGE and transferred to 0.45 μm pore sized Hybond ECL Nitrocellulose Membrane (GE Healthcare, Picataway, NJ). Western blots were imaged using an alpha Innotech FluorChem FC2 Imager.

2.8 ECIS measurements

ECIS (Electric cell-substrate impedance sensing) model Zθ, Applied BioPhysics Inc. (Troy, NY) was used to monitor spreading and attachment of control or transfected cells seeded on type 8W10E arrays. Wound healing experiments were performed as described before (Boratko et al., 2016).

2.9 Statistical analysis

Statistical evaluations were performed by ANOVA using SigmaStat Software. Values were reported as mean ± SD. Differences were considered significant for $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). Densitometry of immunoblots was done by Image J software.

3. Results

3.1 EBP50 supports nuclear localization of merlin in endothelial cells

Although both EBP50 and NHERF2 proteins are expressed in endothelial cells, their subcellular localization is different, nuclear and cytoplasmic, respectively (Boratko et al., 2012). Unlike other cell types, we found that in endothelial cells ERM proteins prefer to bind NHERF2 (Boratko and Csortos, 2013). To test the possible interaction of merlin with EBP50 in endothelial cells, mammalian expression construct of merlin was created by subcloning the RT-PCR product into pCMV-myc vector as described in Materials and methods. Sequencing of the plasmid confirmed that the most abundant isoform 1 of merlin (Cooper and Giancotti, 2014) was cloned from human cDNA. BPAEC cells were transfected with expression constructs of ezrin, radixin, moesin or merlin, each cloned into pCMV-myc vector, and then EBP50 was immunoprecipitated from the lysates of the overexpressing cells. Total cell lysates and the IP complexes were tested in Western blots with anti-EBP50 and monoclonal anti-c-myc antibodies. EBP50 interacted only with

the c-myc tagged merlin, (Fig.1A). Truncated GST-tagged fragments of merlin were also created according to its domain structure (FERM domain aa1-311, α-helical region aa312-506 and C-terminal aa507-595), to identify the interacting region of merlin. BPAEC cells overexpressing EBP50 cloned into pCMV-myc vector (Boratko et al., 2012) were utilized in GST-pull down assay (Fig.1B). We identified that EBP50 binds to merlin's FERM domain in endothelial cells. Since Western blot detection of the 55 kDa EBP50 in immunoprecipitates is problematic, the recombinant c-myc tagged EBP50 was also used for immunoprecipitation (IP). Control or EBP50 overexpressing cells were subjected to IP with c-myc antibody. As expected, the recombinant EBP50 carrying the c-myc tag was able to interact with merlin but interestingly could not bind the Ser518 phosphorylated form (Fig.1C).

Localization and active or dormant state of merlin depend on the phosphorylation state of its Ser518 side chain.The unphosphorylated form of merlin is present in the nucleus and it translocates into the cytoplasmic and membrane region upon phosphorylation (Li et al., 2012, Cooper and Giancotti, 2014). Previously we also showed that EBP50 is present in the nucleus of endothelial cells (Boratko et al., 2012) suggesting that the EBP50-merlin interaction ensures nuclear localization of merlin. To test whether binding of merlin to EBP50 affects its localization, EBP50 was silenced using specific siRNA and the presence of merlin in different cellular fractions was tested. Purity of cytoplasmic, nuclear and membrane fractions was analyzed using antibodies specific for actin, lamin A/C and CD31, and efficiency of EBP50 silencing was verified with anti-EBP50 antibody. In nonsiRNA treated control cells merlin was present mainly in the nucleus and also in the cytoplasm, while in EBP50 silenced cells it disappeared from the nuclear fraction and translocated into the cytoplasm and the membrane fraction (Fig.1D). Western blot analysis of the total cell lysates showed that upon the EBP50 silencing the phosphorylation level of merlin on Ser518 also increased greatly (Fig.1E).

3.2 Merlin interacts with PP1c-TIMAP

Previously it was reported that myosin phosphatase (PP1c-MYPT1) is responsible for the dephosphorylation of phospho-Ser518-merlin in tumor cells (Jin et al., 2006). 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 (Csortos et al., 2008). 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 (Fig. 2A), also, endogenous PP1c-TIMAP was present in the pull down sample of purified GST-tagged merlin (Fig. 2B). Truncated TIMAP mutants (N-

terminal aa1-290 and C-terminal aa291-567) 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 ANK repeats – interacted with merlin, but the C-terminal fragment of TIMAP was not able to bind the endogenous merlin (Fig. 2C). The interaction between c-myc tagged recombinant merlin and GFP-tagged TIMAP (Boratko et al., 2013) was also shown by affinity chromatography (SupFig. 1). The recombinant N-terminal FERM-, central α-helical-, and C-terminal regulatory domains were used in pull down assay. PP1c -TIMAP was present only in the full length merlin and FERM-domain samples (Fig. 2D).

3.3 PP1c-TIMAP controls dephosphorylation of merlin

Immunoprecipitation experiments were utilized to verify the interaction of the endogenous proteins in EC. PP1c -TIMAP was present in the merlin IP samples and *vica versa*, TIMAP IP samples contained PP1c and merlin verifying the interaction. Interestingly, phospho-Ser518-merlin was also detectable in both types of IP samples (Fig. 3A). To test whether a PP1 type phosphatase does dephosphorylate phospho-Ser518-merlin, specific protein phosphatase inhibitors were applied. Cells were treated with 1μM tautomycetin to inhibit PP1, 5 nM okadaic acid or 2μM cyclosporine A to block PP2A and PP2B activity, respectively. The phosphorylation level of Ser518-merlin in the cell lysates increased after the treatment with the specific inhibitor against PP1. Densitometry of Western blots showed a 2-fold increase compared to control, okadaic acid or cyclosporine A treated samples (Fig. 3B). These results confirmed that the phosphorylation level of the Ser518 site is regulated via the activity of a type 1 protein phosphatase and strongly suggest that TIMAP directs the enzyme to merlin.In the pull down (Fig. 2A-B) and IP (Fig. 3A) samples PP1c was always present along TIMAP. To further prove that TIMAP directs PP1c to merlin, merlin was immunoprecipitated from nonsiRNA treated and TIMAP depleted cells (Fig. 3C). 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 EC, no interaction was detected with merlin regardless the presence or absence of TIMAP (Fig. 3C) further strengthening the critical role of TIMAP in dephosphorylation of phospho-Ser518-merlin.

Next, effect of TIMAP silencing on merlin phosphorylation and localization was studied. Phospho-Ser518-merlin blots and their statistical analysis showed that the phosphorylation level of merlin was significantly enhanced in TIMAP depleted cell (Fig 4A). Membrane, cytoplasmic and nuclear fractions of nonsiRNA and siTIMAP RNA treated EC were obtained. Purity of the fractions

was analyzed using marker antibodies, CD31 for membrane-, actin for cytoplasmic- and lamin A/C for nuclear fractions. In control cells, PP1c-TIMAP was present in all subcellular fractions. In TIMAP silenced cells membrane localization of PP1c was not detectable, probably due to the lost targeting of the missing regulatory TIMAP subunit (Fig. 4B top panel). Merlin was detectable mainly in the cytoplasm and nucleus of nonsiRNA treated cells, but showed a great enrichment in the membrane in TIMAP depleted cells and the amount of the nuclear merlin was decreased. Statistical analysis of the Western blots proved that these changes indicating the translocation of merlin from the nucleus to the membrane were significant (Fig. 4B bottom panel) confirming that the increased phosphorylation level and the enrichment of phospho-Ser518 merlin in the cell membrane is due to inactivation of PP1 by TIMAP silencing.

3.4 EBP50- and TIMAP governed subcellular localization of merlin

Subcellular localization of merlin was studied by immunoflurescent staining of control, non-siRNA, EBP50 specific siRNA and TIMAP specific siRNA transfected endothelial cells (Fig. 5). As expected from the results of cell fractionations, while majority of merlin localizes to the nuclei and perinuclear region of control cells (b,e), it shows definite cytoplasmic and membrane appearance in the depleted cells (h,k). Membrane localization of merlin is even more pronounced in TIMAP-depleted cells.

Proliferation and cell migration are important in wound healing (Keese et al., 2004, Sharma et al., 2003). Therefore, to assess cell proliferation and migration when amounts of available TIMAP and merlin proteins are altered, wound healing of TIMAP-depleted and merlin overexpressing endothelial cells were investigated by ECIS measurements (Fig. 6). Compared to control cells, wound healing was slower when the cells overexpressed merlin, and it was faster after TIMAP depletion. This effect was even larger, when merlin was overexpressed in TIMAP depleted cells. These results suggest that non-phosphorylated form of merlin inhibits cell migration and proliferation, but the phosphorylated merlin form aids these processes of the endothelial cells.

4. Discussion

Merlin is a tumor suppressor and is involved in regulation of cell proliferation. However, the mechanism(s) affected by merlin in tumor and normal cells are not completely understood yet. Although there is still some uncertainty about conformation-localization-activity relations of merlin, it is well accepted that the phosphorylation state of Ser518 side chain of merlin influences the proteins biological activity through the modulation of its conformation and intra- and/or intermolecular interactions. Based on the observation that the N- and C-terminal domains are

constitutively in close proximity to each other (Hennigan et al., 2010), it was recently proposed that merlin may be present in the cell in varying states between fully open or closed forms depending on its phosphorylation state at Ser518 and available protein partners (for review see (Scoles, 2008, Cooper and Giancotti, 2014)). Therefore, like ERM, reversible phosphorylation of Ser518 in merlin is critical to its biological activity.

Earlier poor binding of EBP50 to ERM in endothelial cells was revealed (Boratko and Csortos, 2013), which raised the question: what proteins do interact with this scaffolding protein in this cell type? Here we demonstrated that EBP50 binds merlin and the interaction affects the localization of merlin in endothelial cells. Earlier it was shown with recombinant proteins that merlin FERM domain binds EBP50, but merlin C-terminal does not (Nguyen et al., 2001, Murthy et al., 1998). Despite the different behavior of EBP50 in endothelial and epithelial cells, our results also showed that in endothelial cells merlin interacts with the FERM domain of EBP50. Also, colocalization of the proteins was reported in membrane ruffles, microvilli and filopodia of HeLa and COS-7 tumor cell lines (Murthy et al., 1998). However, in interphase endothelial cells, EBP50 localizes to the nucleus (Boratko et al., 2012) and according to previous findings of others, dephospho-Ser518-merlin also localizes to the nucleus (Li et al., 2012). Therefore, one can expect that the dephospho-Ser518-merlin form interacts with EBP50. Indeed, we could not detect phospho-Ser518-merlin binding to EBP50. Furthermore, merlin translocated from the nuclear fraction to the cytoplasm in EBP50-depleted cells. This translocation seems to disturb the kinase-phosphatase equilibrium which is responsible for the actual phosphorylation state of merlin as well, because an increase in the amount of phospho-Ser518-merlin has been detected in the absence of EBP50. Nevertheless, these results suggest that EBP50 is the nuclear scaffolding partner of dephospho-Ser518-merlin in EC. Recent work revealed important data on the mechanism by which merlin activates the Hippo pathway, inhibits the $CRL4^{DCAF1}$ E3 ubiquitin ligase in the nucleus and suppresses tumorigenesis (Li et al., 2010, Li et al., 2014). According to the current proposed model, dephospho-Ser518-merlin translocates to the nucleus and inhibits the E3 ligase which ultimately leads to phosphorylation and inactivation of the YAP/TAZ transcriptional coactivators (Li et al., 2014). TAZ has a PDZ-domain binding motif, which can be a link between the components of the Hippo pathway signaling complex and EBP50 that contains two PDZ domains, but this possibility has not been studied yet. Also, it is interesting to note that a significant fraction of confluent merlin-depleted HUVEC entered into S phase (Li et al., 2010) proving the regulatory effect of merlin on endothelial cell proliferation.

We identified TIMAP as an interacting protein partner of merlin in EC. Domain mapping of the interaction revealed that the N-terminal half (aa1-290) of TIMAP binds merlin, similarly to its other interactions (Boratko et al., 2013, Boratko et al., 2015). The less ordered C-terminal half

(aa291-567) of TIMAP could not bind merlin from EC lysate. As many other interacting partners of merlin (Scoles, 2008), TIMAP binds to the FERM domain. TIMAP is a regulatory subunit of the δ isoform of PP1c, as expected, because it is a component of the PP1c-TIMAP phosphatase complex, PP1c was present in the immune complexes made by specific antibodies against merlin, phospho-Ser518-merlin or TIMAP proteins.

With the application of inhibitors specific for major phospho-Ser/Thr-protein phosphatases (PP1, PP2A, and PP2B), we could show that a type 1 phosphatase dephosphorylates phospho-Ser518-merlin in EC. So far, myosin phosphatase (PP1c-MYPT1) was reported to be responsible for dephosphorylation of phospho-Ser518-merlin in human tumour cell lines (Jin et al., 2006, Serrano et al., 2013). It was shown by GST pull-down that the N-terminal part of the α-helical domain of merlin interacts with MYPT1. Also, with the aid of CPI17, a specific inhibitor of PP1-MYPT1, results were presented which signify the role of this phosphatase holoenzyme form in dephosphorylation of phospho-Ser518 side chain of merlin in the studied cell lines (Jin et al., 2006). In addition, a more recent work demonstrated an integrin-linked kinase (ILK) mediated inactivation of merlin via inhibition of PP1c-MYPT. Intriguingly, silencing of MYPT1, the regulatory subunit of the phosphatase did not alter the Ser518 phosphorylation level in PC3 prostate cancer cells (Serrano et al., 2013).

Our finding that merlin interacts with the PP1c-TIMAP complex suggested that phospho-Ser518-merlin is dephosphorylated at least in part by PP1c-TIMAP in the endothelial cells. In fact, we detected TIMAP and PP1c in the immunoprecipitate of merlin prepared from endothelial cell lysate, but MYPT1 was undetectable. Furthermore, when TIMAP was depleted, neither PP1c nor MYPT1 were immunoprecipitated with merlin indicating that PP1c-MYPT1, at least directly, is not responsible for dephosphorylation of merlin in EC. Furthermore, silencing of TIMAP in our experiments, not only initiated translocation of merlin to the plasma membrane, but resulted in a significant increase as well in the phosphorylation level of Ser518 side chain of merlin. These results strongly indicate that TIMAP, which is a highly expressed protein in endothelial cells (Cao et al., 2002), is involved in the regulation of the phosphorylation level of merlin and PP1c-TIMAP dephosphorylates phospho-Ser518-merlin in endothelial cells.

In proliferating cells, activation of the Rac/PAK and PKA pathways leads to phosphorylation of the Ser518 side chain in merlin evoking an inactive conformation. In accordance with the decreased amount of merlin in the nucleus and the increased phospho-Ser518-merlin level in TIMAP silenced cells, one can expect that the proliferation of TIMAP depleted cells is enhanced due to the decreased inhibition of proliferation. Recently, in agreement with the hypothesis, we have shown a faster *in vitro* wound healing of TIMAP depleted endothelial cells compared to the non-siRNA control transfected cells (Boratko et al., 2016). Overexpression of merlin alone slowed

the wound healing process compared to the control. When TIMAP was depleted, we observed an increase in the phospho-Ser518-merlin level and the wound healing was faster. However, overexpression of merlin did not diminish the faster wound healing of TIMAP depleted cells, but made the process even faster. These seemingly conflicting results emphasize the importance of the actual phosphorylation state of merlin in the regulation of cell migration and proliferation. The inhibitory effect of the recombinant merlin is probably due to moderate excess of the overexpressed non-phosphorylated form of merlin, even though specific kinases and protein phosphatase 1 may mutually work on its phosphorylation level. However, the phosphatase is inhibited in the TIMAP depleted cells, therefore the overexpressed merlin which becomes phosphorylated will remain in the phosphorylated state and this larger pool of phospho-merlin may accelerate cell migration and proliferation.

5. Conclusions

Merlin - EBP50 interaction is vital in the nuclear localization of merlin in endothelial cells. We found that the scaffolding protein, EBP50 keeps merlin (nonphospho-Ser518-merlin form) in the nucleus, since merlin translocated from the nuclei to the cytoplasm and membrane of EBP50 depleted cells. Characterization of the newly identified interaction of merlin with the PP1 regulatory subunit, TIMAP, revealed that PP1c-TIMAP is also involved in the regulation of localization and reversible phosphorylation of merlin and eventually migration and proliferation of this cell type. Drug inhibition of PP1 or silencing of TIMAP increases phosphorylation level of merlin-Ser518, phospho-merlin translocates to the membrane and supports cell migration.

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7. Conflict of Interest

None declared.

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FIGURE LEGENDS

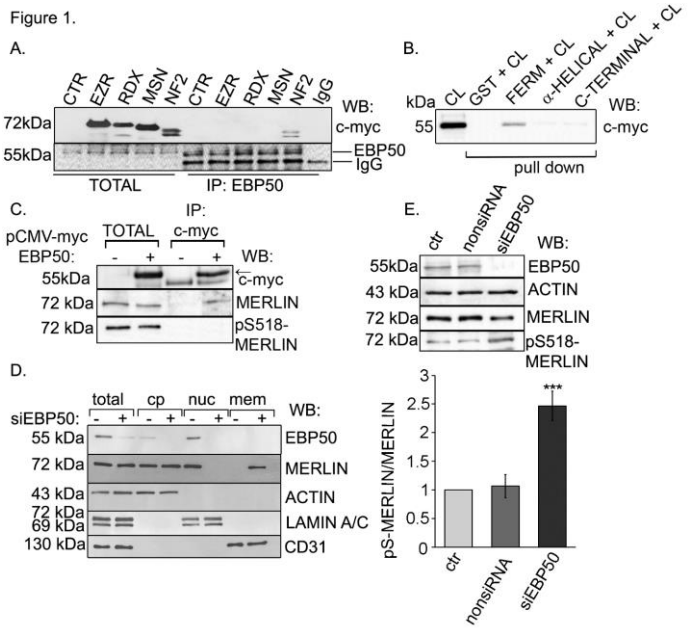

Figure 1. EBP50 affects localization of merlin in endothelial cells.

A) pCMV-myc ezrin (EZR), -radixin (RAD), -moesin (MSN) or -merlin (NF2) was overexpressed in BPAEC cells, and then EBP50 was immunoprecipitated. Total lysates and IP complexes were probed for EBP50 and c-myc antibodies.

B) GST and GST-merlin fragments (FERM, α-helical and C-terminal) were loaded onto glutathione-Sepharose. After the washing steps, the resin samples were incubated with pCMV-myc EBP50 transfected BPAEC lysate (CL) (pull down). Non-binding proteins were washed out and the samples were boiled with 1x SDS sample buffer. Western blot of the transfected endothelial cell lysate and the eluted fractions after the pull down probed with c-myc antibody is shown.

C) c-myc was immunoprecipitated from control (-) and pCMV-myc EBP50 (+) transfected cells. Total lysates and IP complexes were probed with c-myc, merlin and phospho-Ser518 merlin antibodies. Extra bands on the c-myc blot correspond to IgG, arrow points out c-myc tagged EBP50.

D) Subcellular fractionation of nonsiRNA (-) or EBP50 specific siRNA (+) treated cells was made. Total lysates, cytoplasmic (cp), nuclear (nuc) and membrane (mem) fractions were analyzed with anti-EBP50 and anti-merlin antibodies. Purity of the fractions was tested with anti-actin as cytoplasmic, anti-lamin A/C as nuclear and CD31 as membrane marker antibody.

E) Cell lysates of control, nonsiRNA or EBP50 specific siRNA treated cells were analyzed by Western blot, using EBP50, merlin, phospho-Ser518 merlin and actin antibodies (upper panel). Phospho-merlin to merlin ratios determined by densitometry of the Western blots are shown (lower panel). The error bars correspond to SE from 3 independent experiments. Statistical analysis was done with ANOVA. ($p<0.001$)

Figure 2.

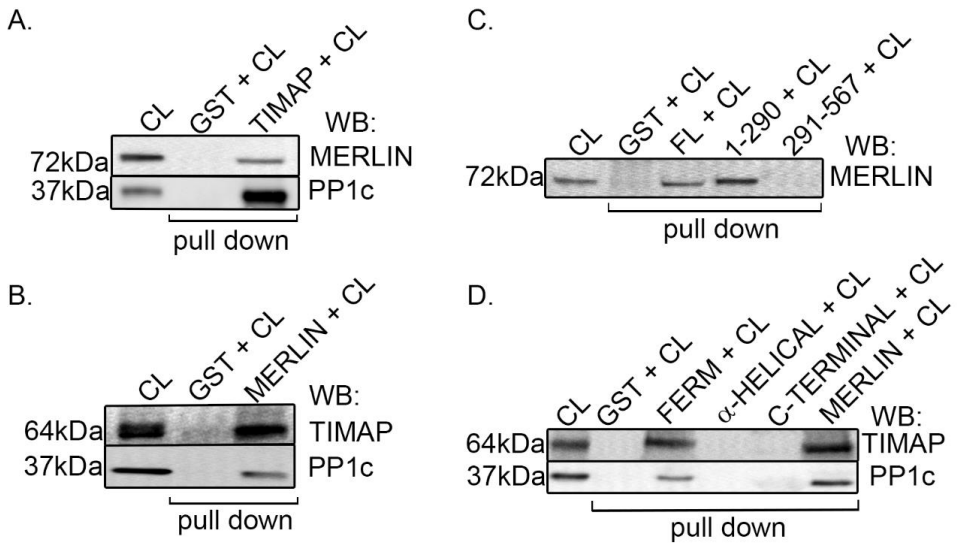

Figure 2. Merlin interacts with TIMAP-PP1c in endothelial cells.

Bacterially expressed glutathione S-transferase (GST) and GST-tagged wild type TIMAP (A), GST and GST-tagged wild type merlin (B), GST, GST-TIMAP (full length, FL) and additional GST-TIMAP fragments (C) or GST, GST-merlin and GST-merlin fragments (D) were loaded onto glutathione-Sepharose. After the washing steps, the resin samples were incubated with BPAEC lysate (CL) (pull down). Non-binding proteins were washed out and the samples were boiled with 1x SDS sample buffer. Western blot of the endothelial cell lysate and the eluted fractions after the pull down probed with antibodies specific for merlin, PP1c or TIMAP are shown.

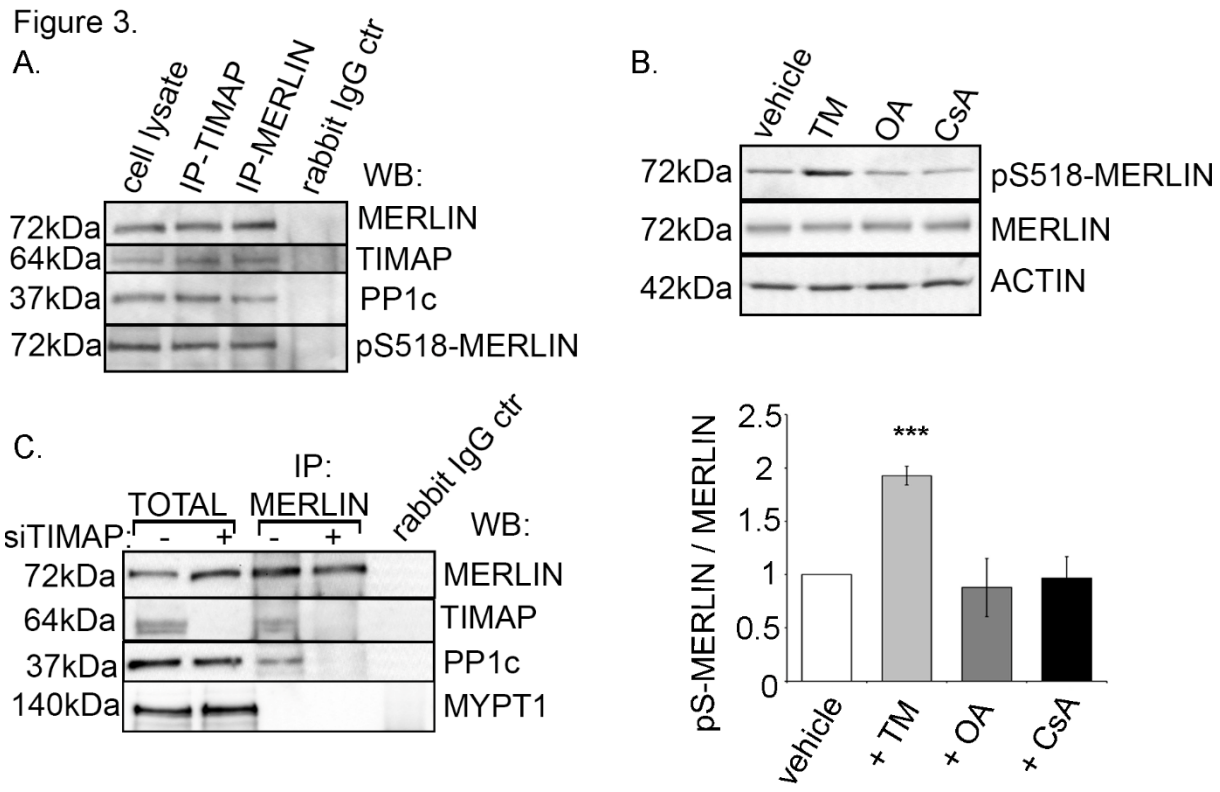

Figure 3. **PP1c is involved in dephosphorylation of merlin.**

A) TIMAPor merlin was immunoprecipitated from lysates of BPAEC . Total lysates and IP complexes were probed for merlin, TIMAP, PP1c and phospho-Ser518-merlin. Representative data of three independent experiments are shown.

B) BPAEC were treated with vehicle (DMSO), 1 µM tautomycetin (TM), 5 nM okadaic acid (OA), or 2 µM cyclosporine A (CsA) for 30 min and then phosphorylation level of merlin was studied by Western blot (upper panel). Phospho-merlin to merlin ratios determined by densitometry of the Western blots are shown compared to the ratio of the corresponding vehicle control (lower panel). The error bars correspond to SE (n=3). Statistical analysis was done with ANOVA. ($p<0.001$)

C) Merlin was immunoprecipitated from lysates of non-siRNA or TIMAP specific siRNA treated BPAEC. Total lysates and IP complexes were probed for merlin, TIMAP, PP1c and MYPT1. Representative data of three independent experiments are shown.

Figure 4.

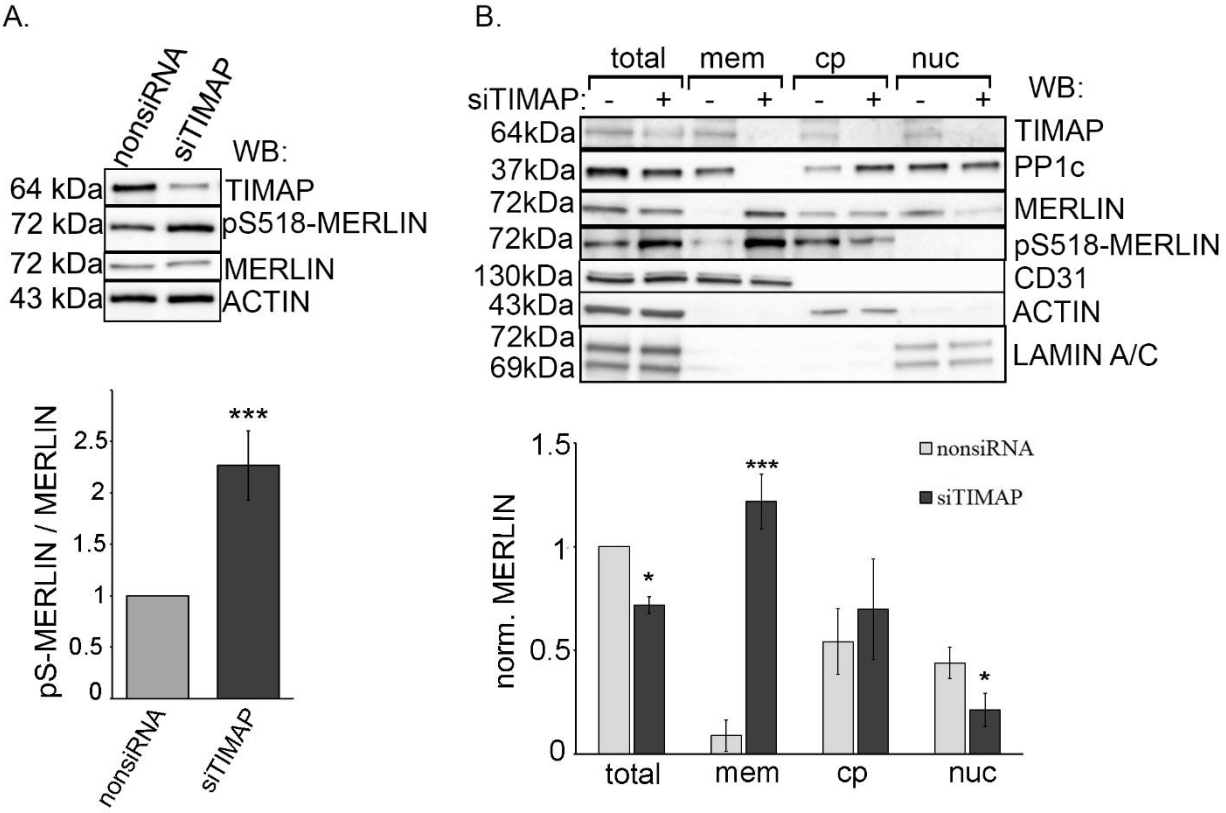

Figure 4. TIMAP silencing augments membrane localization of merlin.

A) Western blot analysis of nonsiRNA and siTIMAP RNA treated cells with the indicated antibodies are shown. Phospho-merlin to merlin ratios determined by densitometry of the Western blots are shown (lower panel). The error bars correspond to SE from 3 independent experiments. Statistical analysis was done with ANOVA. ($p<0.001$)

B) Subcellular fractionation of nonsiRNA or siTIMAP RNA treated BPAEC cells. The fractions were analyzed with anti-TIMAP, anti-PP1c, anti-merlin, and anti-phosphoSer518-merlin antibodies. Purity of the fractions was analyzed with anti-CD31 as membrane, anti-lamin A/C as nuclear, and anti-actin as cytoplasmic marker antibody. Densitometry of Western blots is shown. Amount of merlin protein was normalized against actin (total and cp), CD31 (mem) or lamin A/C (nuc). Error bars correspond to SE (n=3). Statistical analysis was done with ANOVA ($p<0.05$ * ; $p<0.001$ ***). MEM: membrane fraction; CP: cytoplasmic fraction; NUC: nuclear fraction.

Figure 5.

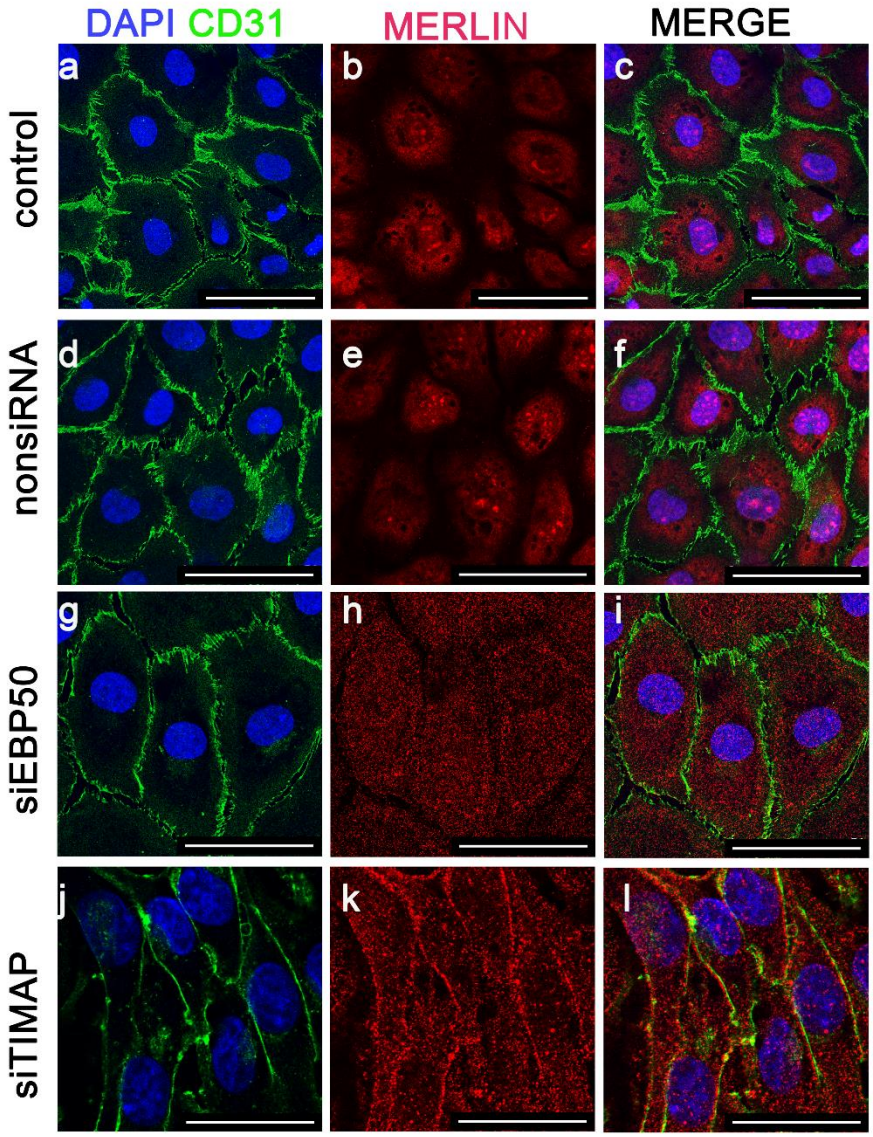

Figure 5. Translocation of merlin in EBP50- or TIMAP depleted cells.

Immunofluorescence staining of confluent control, non-siRNA, siEBP50 or siTIMAP RNA treated BPAEC using anti-merlin (red), anti-CD31 (green) primary antibodies is presented. Nuclei of cells were stained with DAPI (blue).

Figure 6.

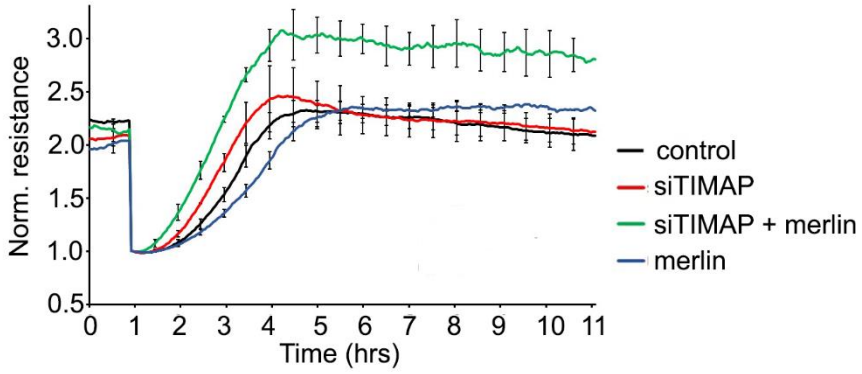

Figure 6. ***In vitro*** **wound healing of merlin/siTIMAP transfected cells.**

Control (black), siTIMAP RNA (red) or pCMV-myc merlin (blue) transfected, or siTIMAP RNA and pCMV-myc merlin cotransfected (green) BPAEC cells were plated onto two 8W10E arrays. After cells achieved monolayer density (about 1000 Ω impedance) an alternate current of 5 mA at 60 kHz frequency was applied for 30 sec duration to establish wounds in the cell layer (at 1 h); after that the impedance was measured for 10 h. Error bars represent SD from three parallel samples.