SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

The role and regulation of myosin phosphatase in the physiological and pathological functions of the uterus

Ilka Keller M.D.

Supervisor: Dr. Beáta Lontay

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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

14:00, 14th of April 2025

Introduction

Protein phosphatases and the PP1 enzyme family

Most of the proteins in eukaryotic cells can be regulated by post-translational modifications, of which protein phosphorylation is of crucial importance. Phosphorylation is catalyzed by protein kinases which are attaching phosphate groups to the Ser/Thr or Tyr residues of substrates. The reversibility of this process is ensured by protein phosphatases. Protein phosphorylation regulates the activity, interactions, and stability of target proteins and plays a critical role in the control of physiological processes. An imbalance in the activity of kinases and phosphatases can trigger a malfunction from which metabolic and oncogenic disorders may arise. The diverse functions of protein phosphatases are based on the large number of combinations of their catalytic and regulatory subunits. 90% of Ser/Thr-specific protein phosphatases are phosphoprotein phosphatases (PPPs), which can be further subdivided into subgroups with either PP1 or PP2A catalytic subunits. PP1s play a significant role in key cellular processes such as apoptosis, differentiation, cell cycle regulation, and glucose homeostasis. Protein phosphatases with the PP1 catalytic subunit typically occur in a heterodimeric structure, where a regulatory subunit is bound to the protein phosphatase 1 catalytic subunit (PP1c). These regulatory subunits determine the target protein and present a possibility to modulate the enzyme's activity.

Metal ion-dependent protein phosphatases (PPMs) and role of the Mg^{2+}/Mn^{2+}-dependent protein phosphatase 1B (PPM1B)

Metal ion-dependent protein phosphatases (PPMs, also known as PP2C enzymes) are Ser/Thr-specific protein phosphatases that stabilize their structure by chelating divalent metal ions, primarily Mg^{2+} and Mn^{2+}, but occasionally Fe^{2+}, as well. Members of the PPM superfamily lack regulatory subunits, as their catalytic domain is responsible for both enzymatic activity and substrate recognition. PPM enzymes are involved in the fine-tuning of cell cycle and cell differentiation, as well as in the coordination of immune responses. Change in their expression drives cells toward malignant transformation, as similar alterations have been already documented in triple-negative breast cancer, bladder cancer, and hepatocellular carcinoma. Elucidating the tumor suppressor roles of PPM1A and PPM1B is particularly important for understanding and treating tumors with poor prognosis.

PPM1B was first identified in 1992, and to date five isoforms have been described of the protein, expressed in various tissues of the human body (e.g., skeletal muscle, cardiac muscle, gastrointestinal tract, and testis). The regulation of PPM1B can occur via microRNAs (e.g., miR-181a-5p, miR-186) and phosphorylation (e.g., by PKA at the Ser195side chain). PPM1B is involved in several pathways (e.g., TNF-α, AMPKα and p53), thereby it plays a crucial role in regulating cellular stress responses and survival. PPM1B' activity can be potently inhibited by the alkaloid sanguinarine (SNG). Degradation of PPM1B the protein has been reported in hepatocellular carcinoma. PPM1B has already been described in cytoplasmatic and nuclear localization as well. Research conducted on HepG2 cells identified the protein in the nucleus, where it interacts with the myosin phosphatase targeting subunit (MYPT1) protein, which is the regulatory subunit of myosin phosphatase (MP).

Structure, Function, and Regulation of Myosin Phosphatase (MP)

Myosin phosphatase (MP) is a member of the PP1 superfamily, but it structurally varies from other members of the PP1s as the PP1c catalytic subunit is bound not only to the MYPT1 regulatory subunit, but to the 20 kDa molecular weigth M20 subunit as well, whose function remains unclear.However, the description of MP is linked to studies investigating smooth muscle contraction, where MP dephosphorylates the 20 kDa myosin light chain (MLC20), the function of MP is not solely limited to the regulation of contractile properties, as the MYPT1 regulatory subunit enables the interactions of MP with numerous substrates. The regulation of MP activity can occur through inhibitory phosphorylation of the MYPT1 subunit at Thr696 and Thr853 residues by RhoA-activated protein kinase (ROK), as well as through protein-protein interactions. MP can also be inhibited through its PP1c subunit by various inhibitors, such as the protein kinase C-activated inhibitor (CPI-17) and okadaic acid.

Among the cytoplasmic interaction partners of MYPT1, the ezrin, radixin, and moesin (ERM) proteins, as well as adducin, which are critical for regulating cell adhesion and migration have been characterized. Moreover, MP interacts with Tau and MAP2, which are microtubule-associated proteins responsible for cytoskeletal remodeling processes. MP can also regulate the cell cycle through the dephosphorylation of retinoblastoma protein (pRb). In addition, MP influences basal smooth muscle tone through the dephosphorylation and activation of endothelial nitric oxide synthase (eNOS) and it also plays a role in neurotransmitter release by interacting with synaptosome-associated protein (SNAP-25) and syntaxin proteins.

MP can also influence gene expression through multiple pathways, by interacting with either the histone deacetylase 7 (HDAC7), or the protein arginine methyltransferase 5 (PRMT5). HDAC7 upon dephosphorylation and hence activation, translocates to the nucleus and modifies histone proteins, thus alters gene expression. PRMT5 forms an octameric methylosome complex with methylosome protein 50 (MEP50) and symmetrically dimethylates histone proteins (H2A and H4), thus the transcriptional balance of tumor suppressors and proto-oncogenes shifts in a way that promotes malignant transformation. By removing the activating Thr80 phosphorylation of PRMT5, MP acts as a tumor-suppressor. This function requires dephosphorylation of MYPT1 at the inhibitory Thr696 phosphorylation site in nuclear localization for which PPM1B emerges as the only potential candidate amongst the nuclear interaction partners of MYPT1. Defects in MP activity and expression have been observed in hepatocellular carcinoma-derived HepG2 cells and leukemic cell lines as well. Abnormal MP activity may contribute to the progression of cancer by driving cell proliferation and survival.

The structure and functions of SMTNL1 protein

SMTNL1 (smoothelin-like protein 1) was first isolated from rabbit ileum smooth muscle as a substrate of cGMP-dependent protein kinase (PKG), and the protein has been described in visceral and vascular smooth muscles, type IIa skeletal muscle fibers, and steroid hormone-sensitive tissues such as the endometrium and myometrium. SMTNL1 is regulated via phosphorylation by protein kinase A (PKA) or PKG at the Ser301 residue. This modification facilitates translocation to the nucleus where SMTNL1 acts as a co-activator of progesterone receptor B (PR-B) and regulates the expression of numerous genes, including MYPT1. Studies conducted on SMTNL1 KO mice revealed that SMTNL1 plays a role in regulating skeletal muscle plasticity, as muscle fiber composition of the *musculus plantaris* of KO mice underwent a isotype shift, with a decrease in the proportion of type IIb fibers and an increase in type IIa fibers. In a C2C12 *in vitro* hyperthyroidism model, SMTNL1 inhibited the fiber isotype switch towards type IIb fibers.

SMTNL1 KO mice also exhibited reduced reproductive fitness, characterized by higher embryonic lethality, smaller litter sizes, and longer intervals between pregnancies. During pregnancy, SMTNL1 regulates MYPT1 expression, inducing adaptations in the tone of vascular smooth muscle and the myometrium characteristic to gestation. Moreover, SMTNL1 KO mice showed an insulin-resistant phenotype in metabolic assays, and this phenomenon was

accompanied by a significant decrease in the expression of GLUT4 and IRS-1 at the molecular level, leading to disruptions of the insulin signaling cascade.

In an *in vitro* insulin-resistant model using C2C12 cells, SMTNL1, as a co-activator of the PR-B receptor, regulated in a progesterone-dependent manner the phosphorylation of key elements of insulin signaling, such as IRS-1 and PI3K. Additionally, SMTNL1 inhibited JNK and nPKCε activity, showcasing an insulin-sensitizing effect under insulin-resistant conditions.

Pathological alterations and tumors of the uterus

The endometrium is the mucosal lining of the uterine cavity, adapts to hormonal changes of the female reproductive cycle and plays a critical role in embryo implantation and the maintenance of gestation. Endometrial abnormalities are correlating with pathological conditions as well as malignant transformations. Such functional disorders are endometriosis, and polycystic ovary syndrome (PCOS), which causes severe hormonal imbalances and leads to insulin resistance, which increase the risk of developing endometrial hyperplasia and carcinoma and may result in infertility as well. Uterine tumors include endometrial carcinomas, uterine sarcomas and cervical cancer, which deserves special attention due to its significant prevalence. Infection with human papillomavirus (HPV) strains 16 and 18 significantly predisposes individuals to carcinoma development.

Regulatory elements of the cell cytoskeleton, particularly myosin phosphatase (MP), play a key role in the molecular mechanisms underlying pathological alterations and tumor formation in the uterus. MP expression is closely linked to hormonal effects, as SMTNL1, a co-activator of PR-B, decreases the expression of the MYPT1 regulatory subunit. Further exploration of the roles of MP and MYPT1 in endometrial epithelial cells could deepen our understanding of the pathogenesis of commonly occurring functional disorders such as PCOS and endometriosis.

Additionally, disruptions in the phosphatase-kinase balance, such as malfunctions in the ROK/MP axis, are directly associated with tumorigenesis. Studies have shown that decreased MYPT1 expression and its increased inhibitory phosphorylation contribute to tumor development. Research has also focused on the potential role of MP as an independent tumor suppressor. These findings shift the focus on the need to further investigate MP's role in uterine pathological changes and tumor development.

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Aims

The disruption of the physiological homeostasis of uterine tissues predisposes individuals to various pathological conditions and can serve as the foundation of the development of malignant cancer. During the female reproductive cycle, the SMTNL1 protein plays a crucial role in regulating endometrial differentiation as a PR-B co-activator, while also significantly influencing the success of the insulin signaling cascade. Additionally, the SMTNL1 protein exerts a critical regulatory function through its protein-protein interaction with MYPT1, whose activity is regulated by PPM1B, which exhibits reduced expression during the development of cervical carcinomas. Therefore, the objectives of our experimental work were as follows:

1. To elucidate the role of the PPM1B protein in the MP/PRMT5/H4 proto-oncogene axis:
    - Confirmation of the physical and physiological interactions between PPM1B and MYPT1 proteins using *in vitro* phosphatase-kinase assays in HeLa and tsA201 cells.
    - To investigate the effect of PPM1B inhibition on the MP/PRMT5/H4 proto-oncogene axis in nuclear and cytoplasmic fractions of HeLa cells.
    - Examination of the effect of PPM1B protein transfection on the elements of the MP/PRMT5/H4 proto-oncogene pathway in HeLa cells.
    - To explore the association of PPM1B with the MP/PRMT5/H4 proto-oncogene axis by analyzing tissue lysates derived from human cervical carcinoma samples, and control counterparts.
2. To investigate the role of the SMTNL1 protein in the insulin signaling pathway and the differentiation of Ishikawa cells derived from human endometrial adenocarcinoma:
    - Examination of the effect of SMTNL1 transfection on Ishikawa cell differentiation in an *in vitro* pregnancy and gestational diabetes model.
    - Investigateion of the impact of SMTNL1 transfection on the migration of Ishikawa cells in an *in vitro* pregnancy and gestational diabetes model.
    - Assessment of the effect of SMTNL1 transfection on the insulin signaling pathway in Ishikawa cells under an *in vitro* pregnancy and gestational diabetes model.
    - To examine the impact of SMTNL1 transfection on glycogen storage and glucose uptake in Ishikawa cells in an *in vitro* pregnancy and gestational diabetes model.

Materials and Methods

Cell culture. Ishikawa, HeLa, and tsA201 cells were maintained according to the manufacturer's instructions and were cultured at 37 °C in a 5% CO_2 atmosphere. The composition of the medium for Ishikawa cells was as follows: Dulbecco's Modified Eagle's Medium (DMEM) containing phenol red and 1000 mg/L glucose, supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine, and 1% non-essential amino acids (NEAA). For HeLa and tsA201 cells, DMEM containing phenol red and 1000 mg/L glucose was supplemented with 2 mM L-glutamine and 10% FBS. The cells were passed three times a week

Transient transfection of Ishikawa, HeLa and tsA201 cells with FT-SMTNL1, PPM1B, or MYPT1 plasmids. Ishikawa, HeLa, and tsA201 cells were transfected using GeneJuice transfection reagent according to the instuctions of the distributor. Three groups were established: control, MOCK (transfected with an empty vector), and FT-SMTNL1/FT-MYPT1/FT-PPM1B groups. The concentration of the empty vector and SMTNL1 plasmid was uniformly 2.5 x 10^{-3} µg/µl, calculated for a final volume of 1500 µl. For PPM1B transfection, the concentration of the empty plasmid and FT-PPM1B vector was 1.7 x 10^{-3} µg/µl, while for MYPT1 transfection, it was 1.09 x 10^{-3} µg/µl. The treatments were maintained for 72 hours. The success of the transfection was verified using Western blot and immunofluorescent staining.

***In vitro* pregnancy (P4) and gestational diabetes (GDB) model of Ishikawa cells**. After transfection, cells were washed with 1×phosphate buffered saline (PBS), and the medium was replaced with phenol red-free complete growth medium adjusted to the treatment conditions. For the P4 model, the medium was supplemented by 5% FBS, 2 mM L-glutamine, 1% NEAA, 1 µM medroxyprogesterone acetate (MPA), 10 nM 17-β-estradiol, and 0.5 mM 8-Br-cAMP, with a final dilution of glucose concentration of 5.11 mM. In the GDB model, the P4 medium was supplemented with additional 100 nM insulin and the final diluted glucose concentration was 22.21 mM. Cells were treated for 72 hours.

Sanguinarine treatment and subcellular fractionation of HeLa cells. HeLa cells were grown in complete growth medium until 90% confluency, followed by a medium change to incomplete DMEM containing 0.1% FBS in order to initiate starvation for 24 hours. Cells were treated with 1 µM sanguinarine (SNG) and were processed to obtain whole-cell lysates or subcellular

fractions, producing nuclear and cytoplasmic fractions. Protein concentrations were measured by a BCA assay.

Cell viability assay of Ishikawa and HeLa cells. Cells were seeded at a density of 20,000 cells/well in a 96-well plate and transfected as described above. After 72 hours of treatment, cell viability was assessed using Alamar Blue assay on 530/590 nm wavelengths.

Wound healing assay of Ishikawa cells. Cells were fluorescently labeled using Vybrant™ DiD Cell-Labeling Solution, seeded onto collagen-coated plates, and cultured under control/P4/GDB conditions with or without FT-SMTNL1 transfection. After 48 hours of treatment, the confluent cell cultures were scratched, and migration potential was monitored in real-time for 24 hours by HCS microscope's 10 × air objective (NA: 1.15), capturing images hourly. The proportion of the free area was normalized to cell number.

Immunofluorescence staining of HeLa and Ishikawa cells. Cells were seeded onto 96-well plates, transfected, and maintained under control/P4/GDB conditions for 72 hours in case of Ishikawa cells, or went under 1 hour long 1µM SNG treatment in case of HeLa cells. Cells were fixed with 4% paraformaldehyde in 1×PBS (PFA/PBS) and were permeabilized with 0.2% Triton X-100 in 1 × PBS. Primary antibody incubation was performed overnight at 4 °C with a 1:100 dilution. Alexa488-conjugated secondary antibodies were incubated at room temperature for 1.5 hours at a dilution of 1:1000. Nuclei were stained with DAPI, and the actin cytoskeleton was labeled with Texas Red Phalloidin. Fluorescent signals were analyzed using the HCS microscope, and fluorescence intensity was normalized to cell counts obtained via DAPI staining.

Lysis of Ishikawa and HeLa cells for whole-cell lysates. Following transfection and/or control, P4, or GDB treatment, HeLa and Ishikawa cells were washed with 1×PBS and lysed in RIPA buffer. The lysates were centrifuged at 16,000 × g at 4 °C. Protein concentrations were measured using a BCA assay.

SDS-PAGE electrophoresis and Western Blot analysis. Protein samples were incubated in 6×SDS sample buffer at 100 °C. A total of 30–40 µg of protein was separated using SDS-PAGE on 4–20% Criterion gels. After transfer, membranes were blocked with 5% (m/V) BSA/TBST for 1.5 hours at room temperature. Primary antibody incubations were performed overnight at 4 °C, then on the following day HRP-conjugated secondary antibody incubation happened for 1,5 hours

on room temperature. Chemiluminescent signals were visualized using WesternBright ECL reagents and captured with the ChemiDoc Touch Imaging System.

Glycogen quantification in Ishikawa cells. At the end of the treatment, transfected Ishikawa cells cultured under control, P4, or GDB conditions were detached with trypsin, centrifuged, and lysed in 0.1 M KH_2PO_4 solution. Following the addition of 25% TCA, concentrated H_2SO_4, and 6% phenol solution, glycogen content was measured at 490 nm. Concentrations were calculated using a standard curve and normalized to protein concentrations determined by BCA assay.

2-NDBG-glucose uptake assay in Ishikawa cells. For the 2-NBDG (2-N-7-Nitrobenz-2-oxa-1,3-diazol-4-yl-Amino-2-Deoxyglucose) uptake assay, transfected Ishikawa cells in control, P4, or GDB conditions were cultured for 72 hours. Then, cells were incubated in glucose-free medium for 1 hour and then were supplemented with 2-NBDG containing media in 100 μM/well concentration for an additional hour. Cells were then washed with 1×PBS and lysed in 0.1 M KH_2PO_4 (pH 11.0) supplemented with 1% Triton X-100. Fluorescence intensity was measured with excitation at 485 nm and emission at 535 nm using a Tecan Spark Multimode microplate reader. Results were normalized to protein concentrations measured using BCA assay.

RNA isolation and semi-quantitative RT-PCR analysis of Ishikawa cells. Transfected Ishikawa cells were treated under control, P4, or GDB conditions for 72 hours and total RNA was extracted from culture using TRIzol reagent and chloroform, followed by isopropanol and 75% ethanol. RNA concentration was measured with NanoDrop fluorospectrometer, and purity was assessed by the 260/280 nm absorbance ratio with accepted range in between 1.8–2.0. Reverse transcription was performed using 0.6 μg of RNA per sample with a cDNA Synthesis. cDNA was amplified using RT-PCR with the 2× Xceed qPCR SyberGreen Master Mix, with human primes for GAPDH, Cyclophillin A and MUC-1. The PCR program included an initial step at 95 °C for 3 minutes, followed by 50 cycles of 95 °C for 3 seconds, 60 °C for 30 seconds, and 72 °C for 90 seconds. Amplifications were carried out by a LightCycler 480 instrument using triplicates for each gene and sample.

Processing of human cervical carcinoma lysates. 1 mg/ml control and tumor tissue lysates in RIPA buffer were purchased from Protein Biotechnologies. Samples were incubated in SDS sample buffer at 100°C for 5 minutes. Western blot analysis was used to examine the protein content of the samples.

Immunoprecipitation. tsA201 cells were transfected with FT-PPM1B or FT-MYPT1 plasmids and were lysed in TBS/EDTA buffer. Protein A Sepharose (PAS) resin was blocked, antibodies were conjugated, and the transfected lysates were immunoprecipitated. The success of immunoprecipitation was verified by Western blot analysis.

Pull-down assay of MYPT1 and PPM1B proteins. *E. coli* lysates expressing glutathione-S-transferase (GST) or GST-MYPT1 were incubated with Glutathione Sepharose 4B beads in binding buffer. The GST or GST-MYPT1 complexes immobilized on the beads were incubated with lysates from MOCK-transfected or FT-PPM1B-transfected tsA201 cells. The success of the pull-down assay was validated by Western blot analysis.

Duolink PLA Protein-Protein Interaction Assay in HeLa Cells. HeLa cells were fixed in 4% PFA/TBS solution, permeabilized, and incubated with primary antibodies establishing interaction groups as follows: negative control (without primary antibody incubation), MYPT1-PP1cβ, MYPT1-pRb, MYPT1-PPM1B, and MYPT1-PPM1A. After primary antibody incubation, proximity ligation assay (PLA) probes were added, followed by ligation and amplification by fluorescently labeled nucleotides. Fluorescent signals were registered by Opera Phoenix™ microscope and normalized to cell count.

Myosin phosphatase activity assay. HeLa cells, upon reaching 90% confluence, were serum-starved for 24 hours in medium containing 0.1% FBS. Cells were treated for 1 hour by 1 µM SNG or 50 nM okadaic acid (OA), or with a both of that. After 1 hour, cells were lysed in a buffer containing 50 mM Tris/HCl pH 7.4, 150 mM NaCl, 0.15% (w/v) Triton X-100, supplemented with 1x PIC, 1 mM PMSF, and 50 mM 2-mercaptoethanol. Cell lysates were sonicated and centrifuged. Supernatants were preincubated for 1 minute at 30°C and then incubated for another minute with 1 µM 32P-labeled turkey gizzard myosin (^{32}P-myosin). The reaction was stopped using 10% (v/v) trichloroacetic acid (TCA) and 6 mg/ml BSA. Following centrifugation, the precipitated proteins were collected, and the released radioactively labeled phosphate (^{32}Pi) was measured using a scintillation counter (Perkin Elmer). The measured phosphatase activity values were normalized to the amount of MYPT1 in the samples.

***In vitro* RhoA kinase and PPM1 phosphatase assay.** *E. coli* lysates expressing GST or GST-MYPT1 were incubated with Glutathione Sepharose 4B beads in binding buffer, then the beads were washed with 1xTBS and were incubated with ROK enzyme (20 ng/µl) and ATP (1 mM) C

in ROK assay buffer supplemented with 1 μM microcystin-LR and 1x PIC. Control samples without ROK were included. After phosphorylation, the beads were washed with 1xTBS and incubated with or without PPM1B in PPM1B assay buffer. To verify the ion-dependency of PPM1B, an environment with chelated Mg^{2+}/Mn^{2+} ions was also established. PPM1B was inhibited by SNG in a separate group. Effects on MYPT1 Thr696 phosphorylation was validated by Western blot.

Statistical analysis. Western blot images were analyzed by Image J software. The values of posttranslationally modified proteins were normalized to their unmodified total proteins. The unmodified protein signals were normalized to loading controls. In experiments on Ishikawa cells or measurements of MP activity, the mean value of the control group was set as 100%. In case of HeLa cells, results were presented as relative values. Bar charts were generated using GraphPad Prism 8 software, and statistical analyses were conducted with the same software. Differences between two groups were analyzed using an unpaired two-tailed t-test. One-way or two-way ANOVA with Tukey's *post hoc* test was used for comparisons of multiple groups. Each bar in the charts represents the mean ± SD of a group, with "n" indicating the number of independent experiments. Outliers were removed using Grubbs's test in GraphPad Prism 8. Statistical significance was considered at $p < 0.05$ (*).

Results and conclusions

The role of PPM1B in regulating the MP/PRMT5/H4 signaling pathway

Protein-protein interaction between between MYPT1 and PPM1B

The interaction between MYPT1 and PPM1B was validated using immunoprecipitation, pull-down assay, and Duolink PLA proximity ligation assay, which results proved the physical interaction of the two proteins. The PPM1B-MYPT1 substrate-enzyme interaction was also assessed by ROK kinase combined with PPM1B phosphatase assay. In the *in vitro* phosphatase assay, ROK phosphorylated GST-MYPT1 was used, and the effect of PPM1B MYPT1 Thr696 phosphorylation was assessed by Western blot. The results confirmed that PPM1B targeted and dephosphorylated the Thr696 inhibitory phosphorylation site of MYPT1. Sanguinarine (SNG) was used to inhibit PPM1B activity, leading to a significant increase of MYPT1 Thr696 phosphorylation. Additionally, removing Mg^{2+}/Mn^{2+} ions from the environment demonstrated the metal ion dependence of PPM1B, as MYPT1 phosphorylation on the Thr696 side chain significantly increased under these conditions. The regulatory effect of the PPM1B-MYPT1 interaction on myosin phosphatase (MP) activity was examined in HeLa cells utilizing MP activity assay. Inhibition of PPM1B with SNG caused a notable decrease in MP enzymatic activity, while okadaic acid (OA) treatment also reduced MP activity. The combination of OA and SNG exhibited an additive effect, further reinforcing that PPM1B inhibition reduces MP activity. Collectively, these findings indicate that PPM1B plays a direct regulatory role in MP activation by dephosphorylating the regulatory subunit MYPT1.

Our findings confirm that PPM1B interacts with the MYPT1 regulatory subunit of MP. *In vitro* kinase assay demonstrated that the interaction between PPM1B and MYPT1 modulates the inhibitory phosphorylation site Thr696 of MYPT1. Previous studies have described PPM1B as a nuclear interacting partner of MYPT1 in HepG2 cells. While nuclear protein-protein interactions between these enzymes were previously confirmed, our Duolink Proximity Ligation assay data suggests that their interaction may also occur outside the nucleus, potentially influencing cytoplasmic signaling pathways.

PPM1B inhibition increases the inhibitory phosphorylation of MYPT1 in the nuclear fraction of HeLa cells

Our preliminary experiments demonstrated that PPM1B inhibition by SNG increases the inhibitory phosphorylation of MYPT1 *in vitro* and decreases MP holoenzyme activity in HeLa cells. We examined the effects of PPM1B inhibition in HeLa cell fractions, including whole cell lysates, cytoplasmic, and nuclear fractions. SNG treatment did not alter PPM1B protein expression or localization. However, the amount of nuclear MYPT1 increased 1.58-fold, indicating that PPM1B inhibition induces MYPT1 translocation to the nucleus. MYPT1 phosphorylation on the Thr696 residue significantly increased upon SNG treatment, with a 2.048-fold increase in whole-cell lysates and a 2.137-fold increase in the nuclear fraction compared to untreated counterparts. These results suggest that PPM1B inhibition facilitates MYPT1 translocation from the cytoplasm to the nucleus through increased Thr696 phosphorylation.

PPM1B modulates gene expression indirectly by regulating PRMT5 activity and therefore changing symmetric dimethylation of histone H4

Previous studies reported that nuclear MP inhibits PRMT5 activity by dephosphorylating its Thr80 activating phosphorylation site. We investigated how PPM1B inhibition by SNG affects PRMT5 activity and H4 symmetric demethylation in several fractions of HeLa cells. Our results showed that SNG treatment did not change PRMT5 expression but significantly increased PRMT5 phosphorylation on the Thr80 residue in all examined fractions, including whole-cell lysate, cytoplasm, and nuclear fraction. H4 symmetric dimethylation increased 1.54-fold in whole-cell lysate and 3.25-fold in the nuclear fraction following SNG treatment, while H4 protein expression remained unchanged. Fluorescent imaging indicated no change in PRMT5 and H4 localization or expression following treatment. However, increased PRMT5 Thr80 phosphorylation and H4 symmetric dimethylation detected by Western blot were also observed in fluorescent images. SNG application confirmed that reduced PPM1B activity leads to increased phosphorylation of MYPT1 at Thr696 phosphorylation site, which is associated with elevated PRMT5 Thr80 phosphorylation. Activation of PRMT5 results in increased symmetric dimethylation of the H4 protein, clearly contributing to oncogenesis.

FT-PPM1B overexpression reduces PRMT5 activity through MP activation in HeLa cells

To identify isoform specific effects associated with PPM1B, HeLa cells were transfected by FT-PPM1B plasmid. Overexpression was validated using Western blot and immunofluorescent staining, and the effects of transfection on cell viability were assessed by Alamar Blue assay. Cell viability decreased by 25.25% in the MOCK transfected group compared to the untreated control, but FT-PPM1B transfection did not affect cell viability. PPM1B overexpression did not influence MYPT1 expression but increased MP activity and reduced PRMT5 activity by enhancing MYPT1 Thr696 phosphorylation and reducing PRMT5 Thr80 phosphorylation, which resulted in decreased H4 symmetric demethylation. Our findings indicate that PPM1B regulated MP activity is directly linked to PRMT5 activity, which supports that PPM1B plays a key role in MP regulation within the nucleus, and reduced PPM1B activity contributes to tumorigenesis.

Findings of the modified PPM1B-MYPT1-PRMT5-H4 axis in human cervical carcinoma tissue lysates correlates with our *in vitro* results

Based on the semi quantitative Western blot analysis of human cervical carcinoma tissues, PPM1B and MYPT1 expression was decreased in tumor samples compared to their control counterparts, while PRMT5 phosphorylation increased in tumor samples. Symmetric dimethylation of H4 was also elevated in tumor tissues, and the expression of the tumor suppressor pRb decreased in tumor samples. These findings confirm the important role of the PPM1B/MP/PRMT5/H4 axis in cervical cancer. In summary, our research confirms that PPM1B is a critical upstream regulatory element of the MP/PRMT5/H4 signaling pathway and exhibits tumor-suppressive functions. Pharmacological activation of PPM1B and MP offers a potential therapeutic approach, particularly for the treatment of cervical cancer.

The role of SMTNL1 protein in *in vitro* insulin resistant model of Ishikawa cells contractile properties and insulin signaling pathway

The role of SMTNL1 protein in the contractile properties of insulin resistant Ishikawa endometrial epithelial cells

The SMTNL1 plays a crucial role in the steroid hormone-dependent regulation of various physiological processes of endometrial epithelial cells. To further explore the role of SMTNL1, the Ishikawa cell line, which represented the columnar epithelium of the endometrium, were applied in our experiments. Ishikawa cells were transfected with FT-SMTNL1 plasmid, and the

overexpression was validated using Western blot and immunofluorescent staining. The effect of transfection on cell viability was assessed by Alamar Blue assay, which showed no significant changes in between the transfected groups compared to control. Different hormonal treatments were applied to mimic pregnancy (P4) as well as insulin resistant conditions combined with P4 to simulate gestational diabetes (GDB). GDB treatment decreased endogenous SMTNL1 expression in Ishikawa cells, validated by Western blot analysis. To confirm the insulin resistant state, the expression and phosphorylation of Akt-1 protein was examined. Acute insulin treatment increased Akt-1 Ser473 phosphorylation in the control group, but changes in phosphorylation were not detectable under GDB conditions, confirming the successful establishment of insulin resistance in Ishikawa cells.

Morphological changes in Ishikawa cells during differentiation induced by steroid hormones were observed by HCS microscopy. P4 treatment induced morphological changes compared to untreated control, shifting cells to a columnar shape, which change in appearance was hindered in GDB group. FT-SMTNL1 transfection facilitated these morphological changes synergistically with progesterone even in the GDB-treated group, where differentiation was blocked previously due to the insulin resistant conditions. The expression of the differentiation marker MUC-1 was analyzed using both immunofluorescence and RT-PCR. Results showed that MUC-1 mRNA expression significantly increased in response to both P4 and GDB treatment upon FT-SMTNL1 overexpression. In the GDB model, where MUC-1 expression was reduced, FT-SMTNL1 restored its levels. The synergistic effect of FT-SMTNL1 transfection and progesterone was particularly evident under P4 treatment conditions.

SMTNL1 overexpression reduces MYPT1 expression and inhibits MP activity. Changes in the expression of cytoskeletal proteins and their upstream regulators contribute to the physiological processes of cell shape and differentiation. A key regulatory element responsible for cytoskeletal changes in endometrial epithelial cells is myosin phosphatase (MP), whose regulatory subunit, MYPT1 interacts with SMTNL1. As co-regulator of PR-B, SMTNL1 reduces MYPT1 expression, as verified by previous studies.

In FT-SMTNL1 transfected Ishikawa cells cultured under control, P4, or GDB conditions, FT-SMTNL1 overexpression reduced MYPT1 expression in a progesterone-dependent manner in both P4 and GDB treatments. Immunofluorescence intensity analysis showed that MYPT1 expression decreased by 35.34% (P4) and 38.66% (GDB) compared to non-transfected controls. Western blot analysis further demonstrated that FT-SMTNL1 transfection reduced MYPT1

expression by 39.49% in P4 and by 50.91% in GDB conditions compared to non-transfected P4 and GDB treated controls. MP activity was analyzed by examining the inhibitory phosphorylation of the MYPT1 regulatory subunit at the Thr696 phosphorylation site, which phosphorylation increased upon FT-SMTNL1 transfection, particularly in the GDB model, with a 113.66% increase compared to the non-transfected counterpart.

FT-SMTNL1 overexpression indirectly regulates MLC20 phosphorylation via MP inhibition.

After describing the effect of FT-SMTNL1 transfection on the MYPT1 regulatory subunit, we aimed to investigate the expression and phosphorylationof the physiological substrate of MP, namely MLC20. FT-SMTNL1 transfection did not affect MLC20 expression but increased its Ser19 phosphorylation. Western blot and data obtained from immunofluorescence intensity showed that MLC20 Ser19 phosphorylation increased by 37.78% in P4 and by 40.91% in GDB model upon FT-SMTNL1 overexpression compared to non-transfected P4 and GDB groups. P4 treatment reduced MLC20 phosphorylation, while GDB treatment increased it compared to controls untreated by hormones.

FT-SMTNL1 overexpression inhibits the migratory capacity of Ishikawa cells.

FT-FT-SMTNL1 overexpression significantly reduced cell migration, particularly under P4 and GDB treatments, by 59.68% in P4 and by 32.14% in GDB model upon FT-SMTNL1 transfection. While P4 hormone treatment alone increased migration, FT-SMTNL1 overexpression counteracted this effect. GDB treatment independently reduced migration, which was further inhibited by FT-SMTNL1 transfection. Overall, we examined the impact of FT-SMTNL1 transfection on cytoskeletal elements. MP activity, which regulates the contractility and migration of endometrial cells, is influenced by the MYPT1 protein. In our experiments, FT-SMTNL1 transfection reduced MYPT1 expression in a progesterone-dependent manner and inhibited MP activity by increasing the inhibitory phosphorylation of MYPT1 at Thr696 residue. This phenomenon correlated with increased phosphorylation of MLC20 at the Ser19 site. These changes were associated with a decrease in cell migratory capacity. The effects induced by FT-SMTNL1 transfection confirmed that regulating migration-related mechanisms plays a key role in the development of endometrial dysfunctions.

SMTNL1 regulates IRS-1 and Akt-1 Ser phosphorylation and activity in Ishikawa cells.

Fertility disorders are often associated with insulin resistance. Previous studies using C2C12 cells and *smtnl KO* mice highlighted the role of SMTNL1 in insulin signaling. Our experiments aimed

to investigate the effects of FT-SMTNL1 transfection on IRS-1 and Akt-1 expression and phosphorylation in Ishikawa cells under P4 and GDB conditions. IRS-1 expression remained unchanged under all experimental conditions, but IRS-1^{pS612} phosphorylation increased by 34.87% in the GDB model, as a marker of insulin resistance. FT-SMTNL1 transfection reduced this phosphorylation under both P4 and GDB treatments. Similarly, Akt-1 expression was unaffected, but Akt-1^{pS473} phosphorylation was decreased, indicating insulin resistance in the P4 and GDB groups compared to untreated controls. FT-SMTNL1 transfection increased Akt-1^{pS473} phosphorylation in both models. These results suggest that FT-SMTNL1 overexpression alleviates molecular signs of insulin resistance in gestational diabetes.

FT-SMTNL1 transfection indirectly regulates MAPK activity in Ishikawa cells.

In further experiments, we aimed to examine upstream regulatory elements of insulin signaling, particularly MAPK kinase and its activity, which affects IRS-1 Ser612 phosphorylation in insulin resistance. T202/Y204 phosphorylation, indicative of ERK½ activity, increased in response to GDB treatment but was reduced by FT-SMTNL1 transfection. ERK½ activity is enhanced by nPKCε, whom expression was also decreased under FT-SMTNL1 transfection in the GDB treatment. Additionally, the expression and activity of the phosphatases PP2A and DUSP9 increased following FT-SMTNL1 transfection. PP2A expression was elevated under GDB conditions, while DUSP9 expression increased under both P4 and GDB treatments. FT-SMTNL1 transfection reduced ERK½ activity indirectly on a progesterone-dependent manner, by downregulating nPKCε expression and upregulating PP2A and DUSP9 phosphatase expression. These effects indirectly inhibited ERK½ activity, resulting in reduced IRS-1^{pS612} phosphorylation and highlighting the role of the MAPK pathway in insulin signaling regulation. The use of the U0126 ERK½ inhibitor clarified that MAPK ERK½ regulates glucose uptake and insulin signaling in Ishikawa cells. U0126 reduced ERK½ activity, which was accompanied by decreased IRS-1^{pS612} phosphorylation and increased glucose uptake under control, P4, and GDB conditions.

Glycogen storage and glucose uptake in Ishikawa cells are promoted by FT-SMTNL1 transfection in a progesterone-dependent manner

Glycogen storage in the uterine endometrium is essential for maintaining its homeostasis. Our experiments examined the effect of FT-SMTNL1 transfection on glycogen storage and glucose uptake in Ishikawa cells. FT-SMTNL1 transfection increased glycogen storage, particularly upon P4 and GDB treatments, which was accompanied by enhanced glucose uptake under similar conditions. Reduced glycogen storage in the P4 and GDB groups was a consequence of hormonal

treatment as well as the established hyperglycemic-hyperinsulinemic condition. FT-SMTNL1 transfection exhibited strong insulin-sensitizing effects in P4 and GDB treated cell groups. No significant changes were observed in GLUT4 transporter expression; thus, alterations in glycogen storage and glucose uptake were attributed to changes in the transporter's localization.

In summary, our findings demonstrate that SMTNL1 plays a critical role in regulating insulin signaling and differentiation of endometrial cells, as well as their migratory capacity. Our research highlights that the SMTNL1 protein could have a prominent role in the function of the female reproductive system and in addressing metabolic disorders, particularly in understanding pregnancy maintenance and insulin resistance-related issues.

Summary

The PPM1B protein plays a crucial role in regulating the activity of MP within the nucleus, a function essential for balancing the MP/PRMT5/H4 proto-oncogene axis in a tumor-protective manner. Amongst the intranuclear interactors of MP, PPM1B is the only protein phosphatase that, due to its interaction with the MYPT1 subunit, is capable to dephosphorylate the Thr696 sidechain, thereby facilitating the tumorsuppressor function of MP. This process decreases the activation of the PRMT5 protein, hence reducing the symmetric dimethylation of the H4 protein, a post-translational modification whose upregulation unsettles the protooncogene/tumorsuppressor expression balance by reducing the expression of tumorsuppressors and increasing that of protooncogenes. Our experimental results confirmed the interaction between PPM1B and MYPT1 in the cytoplasm and nuclus of HeLa cells derived from cervix cancer. Based on our result arising from utilizing the PPM1B inhibitor SNG, we observed that the inhibition of PPM1B enhanced the activating phosphorylation of PRMT5 on the Thr80 sidechain, thereby elevating the symmetric dimethylation of H4 histones. These changes were also validated in tissue lysates derived from human cervical carcinoma biopsies. Correlatingly, PPM1B transfection prevented these alterations. The role of PPM1B is particularly crucial in human cervical cancer tissues, where its expression is lower compared to control tissues, which is consistent with previous research identifying PPM1B as a tumor suppressor factor. Based on these findings, PPM1B is a promising therapeutic target in cancer treatment.

The SMTNL1 protein was previously identified as a PR-B coactivator in steroid hormone-dependent tissues. In this study, we characterized its role during the differentiation of endometrial epithelial cells and its influence on insulin signaling in *in vitro* models of pregnancy and gestational diabetes. FT-SMTNL1 transfection regulated cytoskeletal elements, such as MYPT1 and MLC20, in a progesterone-dependent manner, providing a molecular basis for the regulation of cell contractility and migration. FT-SMTNL1 transfection inhibited cell migration in a progesterone-dependent manner and reversed molecular changes characteristic of insulin resistance by indirectly modulating IRS-1 phosphorylation that resulted in an enhanced glucose-uptake and glycogen content of Ishikawa cells. These findings suggest that SMTNL1 could be a therapeutic target not only for endometrial dysfunctions but also for insulin resistance and cancer.

List of publications

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List of publications related to the dissertation

1. **Keller, I.**, Ungvári, Á., Major, E., Horváth, D., Kónya, Z., Tóth, E., Erdődi, F., Kiss, A., Lontay, B.: Magnesium-dependent-protein phosphatase 1B regulates the protein arginine methyltransferase 5 through th modulation of myosin phosphatase.
   J. Biol. Chem. 301 (2), 1-16, 2025.
   DOI: http://dx.doi.org/10.1016/j.jbc.2024.108107
   IF: 4 (2023)
2. **Keller, I.**, Ungvári, Á., Kinter, R., Szalmás, A. F., Kókai, E., Lontay, B.: Smoothelin-like protein 1 promotes insulin sensitivity and modulates the contractile properties of endometrial epithelial cells with insulin resistance.
   Front Endocrinol (Lausanne). 15, 1-16, 2024.
   DOI: http://dx.doi.org/10.3389/fendo.2024.1375771
   IF: 3.9 (2023)

List of other publications

3. Tamás, I., Major, E., Horváth, D., **Keller, I.**, Ungvári, Á., Haystead, T. A. J., MacDonald, J. A., Lontay, B.: Mechanisms by which smoothelin-like protein 1 reverses insulin resistance in myotubules and mice.
   Mol. Cell. Endocrinol. 551, 1-12, 2022.
   DOI: http://dx.doi.org/10.1016/j.mce.2022.111663
   IF: 4.1
4. Major, E., Győry, F., Horváth, D., **Keller, I.**, Tamás, I., Uray, K., Fülöp, P., Lontay, B.: Smoothelin-like protein 1 regulates development and metabolic transformation of skeletal muscle in hyperthyroidism.
   Front Endocrinol (Lausanne). 12, 1-17, 2021.
   DOI: http://dx.doi.org/10.3389/fendo.2021.751488
   IF: 6.055

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5. Major, E., **Keller, I.**, Horváth, D., Tamás, I., Erdődi, F., Lontay, B.: Smoothelin-like Protein 1 Regulates the Thyroid Hormone-Induced Homeostasis and Remodeling of C2C12 Cells via the Modulation of Myosin Phosphatase.
Int. J. Mol. Sci. 22 (19), 1-18, 2021.
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