



PPM1B suppresses cervical carcinoma invasion by dephosphorylating MYPT1 at Thr853 and inactivating the PRMT5 epigenetic axis

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ABSTRACT

The spatiotemporal regulation of protein phosphorylation regulates cellular homeostasis, yet the phosphatase networks that actively dismantle oncogenic kinase signaling remain elusive. Here, we identify Mg^{2+} -dependent protein phosphatase 1 (PPM1B) as a critical tumor suppressor in cervical carcinoma. Biochemically, PPM1B directly interacts with and dephosphorylates the myosin phosphatase (MP) regulatory subunit MYPT1 at its inhibitory Thr853 residue. We show that Rho-associated kinase (ROCK) activation by lysophosphatidic acid generates a high-affinity substrate profile, recruiting PPM1B to the Thr853-phosphorylated MYPT1. Crucially, this dephosphorylation drives the nuclear export of the MP holoenzyme, triggering a dual tumor-suppressive cascade. In the cytoplasm, reactivated MP dephosphorylates myosin light chain (MLC20) at Ser19 to prevent actomyosin-driven invasion. Concurrently, nuclear clearance of MYPT1 disrupts the PRMT5/histone H4 epigenetic axis, diminishing PRMT5 Thr80 phosphorylation and H4 symmetric dimethylation. Proteomic and phospho-kinase profiling indicates that PPM1B globally reprograms the malignant state, upregulating tumor suppressors (p53, Maspin) while attenuating epithelial-mesenchymal transition (EMT) drivers and survival networks (JNK, CREB). Finally, clinical biopsies of human squamous cell carcinoma demonstrate a strong correlation between reduced PPM1B expression and MYPT1 Thr853 hyperphosphorylation. Collectively, our findings establish PPM1B as a master catalytic switch that abrogates oncogenic signaling at both the cytoskeletal and epigenetic levels.

1. Introduction

Protein phosphorylation and dephosphorylation, as one of those fundamental post-translational modifications, that regulate every

cellular mechanism, such as cell proliferation, motility, apoptosis, and stress responses (Turdo et al., 2021). The balance between the activities of kinases and phosphatases provides appropriate cell signaling, while their alterations frequently enhance cells towards oncogenic

Abbreviations: AMPK, AMP activated protein kinase; BSA, Bovine serum albumin; BCA, Bicinchoninic acid; BCL-x, B-cell lymphoma extra-large; Chk-2, Checkpoint kinase 2; CREB, cAMP response element binding protein; DAPI, 4',6-diamidino-2-fenilindol; DMEM, Dulbecco's Modified Eagle's Medium; DTT, Dithiothreitol; ECL, Enhanced chemiluminescence; EDTA, Ethylenediaminetetraacetic acid; EGTA, Ethylene glycol-bisβ-aminoethyl ether-N,N,N',N'-tetraacetic acid; EMT, Epithelial-mesenchymal transition; ER, Estrogen receptor; ERB2, Erb-b2 receptor tyrosine kinase; ERB3, Erb-b3 receptor tyrosine kinase; FBS, Fetal bovine serum; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; GM-CSF, Granulocyte-macrophage colony-stimulating factor; GST, Glutathione S-transferase; H4, Histone H4; HCG, Human chorionic gonadotropin; HIF-1α, Hypoxia inducible factor 1-alpha; HNF-3β, Hepatocyte nuclear factor 3 beta; HGFR, Hepatocyte growth factor receptor; ILK, Integrin-linked kinase; JNK1/2, c-Jun N-terminal kinase 1/2; LPA, Lysophosphatidic acid; MLC20, Myosin light chain 20 kDa; MP, Myosin phosphatase; M20, 20 kDa small subunit of myosin phosphatase; MOPS, 3-N-morpholinopropanesulfonic acid; MSP/MST1, Macrophage-stimulating protein / Macrophage-stimulating 1; MYPT1, Myosin phosphatase target subunit 1; Na⁺/K⁺-ATPase, Sodium-potassium ATPase; PBS, Phosphate-buffered saline; PECAM-1, Platelet endothelial cell adhesion molecule 1; PI3K, Phosphatidylinositol 3-kinase; PIC, Protease inhibitor cocktail; PLA, Proximity ligation assay; PP1c, Catalytic subunit of protein phosphatase 1; PPM1B, Mg^{2+}/Mn^{2+} dependent protein phosphatase 1B; PRMT5, Protein arginine methyltransferase 5; RIPA, Radioimmunoprecipitation assay; ROK/ROCK, Rho-associated kinase; SDS, Sodium dodecyl sulfate; SDS-PAGE, SDS polyacrylamide gel electrophoresis; SNG, Sanguinarine; SPA, Sodium-potassium ATPase.; STAT2, Signal transducer and activator of transcription 2; TBS, Tris-buffered saline; TBST, TBS containing Tween-20; VEGF, Vascular endothelial growth factor; ZIPK, Zipper-interacting protein kinase.

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transformation (Turdo et al., 2021; Qi et al., 2018). Kinases have been extensively investigated in tumor-related signaling, however protein phosphatases are now increasingly recognized as critical tumor-suppressors whose deficiency or dysfunction can promote signaling pathways without control (Ahmed et al., 2017; Sipos et al., 2017).

In particular, metal-dependent serine-/threonine-specific phosphatases of the PP2C/PPM family play a pivotal role in maintaining cellular homeostasis (Sarkar et al., 2023). Among these phosphatases, PPM1B (Mg²⁺/Mn²⁺-dependent Protein Phosphatase 1B) has appeared as a multifunctional regulator involved in different signaling pathways related to the cell cycle, apoptosis, and stress response (Hanada et al., 1998; Zhu et al., 2025; Yu et al., 2024). PPM1B is known to dephosphorylate several substrates, including IκBα, TAK1, and AMPK, thus acting as a negative regulator of pro-survival and inflammatory signaling pathways (Hanada et al., 2001; Chida et al., 2013). Highlighted evidences also suggest that PPM1B may function as a tumor-suppressor, as its reduced expression or declined activity has been described in certain cancers (Wang et al., 2009). Nevertheless, its exact role in the regulation of cytoskeletal and motility-related processes, and how this contributes to the suppression of oncogenic phenotypes, remains poorly understood.

A crucial effector of cytoskeletal contractility and cell motility is the myosin phosphatase (MP) complex. MP is a heterotrimeric holoenzyme composed of a catalytic subunit (PP1cδ), a targeting subunit (MYPT1), and a small regulatory subunit (M20) (Lee et al., 2022). MP plays a central role in maintaining the dynamic balance between phosphorylation and dephosphorylation of the myosin light chain (MLC), thereby tightly controlling actomyosin-related cellular processes such as contraction, adhesion, migration, and cytokinesis (Kiss et al., 2019). Via dephosphorylation of MLC, MP enhances relaxation of actomyosin filaments and stabilizes cytoskeletal structures required for coordinated cell shape and motility regulation. Conversely, inhibition of MP activity results in permanent MLC phosphorylation, increased cortical tension, and the formation of stress fibers, which are frequently associated with oncogenic transformation and invasive cellular mechanisms (Wang et al., 2009). The MYPT1 subunit serves as a crucial structural and regulatory component of the phosphatase complex, responsible for coordinating the PP1cδ catalytic subunit toward specific substrates and subcellular organelles. MYPT1 also functions as a signaling integrator through interactions with various kinases and cytoskeletal elements. Its activity is precisely modulated by phosphorylation at multiple residues, Thr696 and Thr853, as the two major inhibitory sites (Grassie et al., 2011). However, those residues own different functions regarding to their phosphorylations. Phosphorylation of Thr696 by kinases such as Rho-associated kinase (ROK), zipper-interacting protein kinase (ZIPK), or integrin-linked kinase (ILK) acts as an autoinhibitory modification to eliminate the phosphatase activity of MP. ROK-specific phosphorylation of Thr853 rather hampers the association of MYPT1 with MLC, resulting in decreased MP activity and elevated myosin II-mediated contractility (Wang et al., 2009; Feng et al., 1999; Khasnis et al., 2014). This molecular shift plays an elementary role in cell motility and adhesion dynamics by coupling RhoA/ROK signaling to cytoskeletal tension (Ulke-Lemee and MacDonald, 2010). Importantly, dysregulation of this pathway has been observed in numerous pathological processes, including tumor progression, metastasis, and epithelial-mesenchymal transition (EMT) (Wei et al., 2016; Rodriguez-Hernandez et al., 2016). Elevated ROK activity or extended phosphorylation of MYPT1 have been observed in multiple cancer types, leading to increased invasive potential through cytoskeletal rearrangement and focal adhesion turnover (Kumper et al., 2016). Thus, the phosphorylation level of MYPT1 not only reflects the mechanical state of the cytoskeleton but also serves as a regulatory center linking oncogenic kinase signaling to cellular plasticity and migration capacity.

Previous work from our laboratory has identified a nuclear regulatory link between PPM1B, MYPT1, and protein arginine

methyltransferase 5 (PRMT5), demonstrating that PPM1B can influence chromatin-associated processes through modulation of the MYPT1/PRMT5/H4 signaling axis, particularly via dephosphorylation of MYPT1 at Thr696 (Sipos et al., 2017; Keller et al., 2025). Building on these findings, the present study aimed to investigate that PPM1B targets the functionally distinct Thr853 phosphorylation site of MYPT1 as well. Whether PPM1B also targets the Thr853 residue of MYPT1, and how this interaction affects MP activity and oncogenic signaling, remain to be elucidated. Understanding how this site is dynamically regulated, and by which phosphatase, could therefore provide insight into the mechanism of oncogenic transformation. Given the distinct cellular localization and regulatory role of Thr853, this site may represent a key node through which PPM1B modulates the tumor-suppressive functions of MP (Dedinszki et al., 2015).

Based on these considerations, we hypothesized that PPM1B directly dephosphorylates MYPT1 at Thr853, thereby reactivating myosin phosphatase and counteracting motility-associated oncogenic signaling. To test this, we examined the biochemical interaction between PPM1B and MYPT1, evaluated how PPM1B overexpression influences MYPT1 phosphorylation and downstream cytoskeletal targets, and assessed consequent changes in cellular invasion. In addition, proteome- and phospho-proteome analyses were performed to identify broader alterations in proto-oncogene and tumor-suppressor pathways triggered by PPM1B. Together, these findings elucidate a potential tumor-suppressive role for PPM1B through regulation of MP activity and cytoskeletal signaling.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma-Aldrich (Merck, Darmstadt, Germany) unless stated otherwise.

2.2. Antibodies

Antibodies used in this study are listed in [Supplementary Table S1](#).

2.3. Cell culture maintenance

HeLa (human epithelioid cervix carcinoma) and tsA201SV40-transformed human embryonic kidney cell lines (ECACC, Porton Down, Salisbury, United Kingdom, cat. #99040201 and #85120602, respectively) were cultured according to the manufacturer's guidelines. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Thermo Fisher, Waltham, MA, USA) with phenol red, supplemented with 10% foetal bovine serum (FBS) and 2 mM L-glutamine (hereafter referred to as complete growth medium), at 37 °C in a humidified atmosphere with 5% CO₂ content. Cells were subcultured at 80% confluency twice a week. All cell lines were authenticated by the supplier and routinely tested to confirm the absence of mycoplasma contamination.

2.4. Protein Extraction

Cells were rinsed with phosphate buffer solution (PBS), (pH 7.2; 2.7 mmol/L KCl, 1.5 mmol/L KH₂PO₄, 136.9 mmol/L NaCl, 8.9 mmol/L Na₂HPO₄·7 H₂O) and lysed in radioimmunoprecipitation assay buffer (RIPA) (25 mM Tris pH: 7.4, 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100) completed with protease and phosphatase inhibitor cocktails (PIC), 1 mM sodium fluoride (NaF), 1 mM tetrasodium pyrophosphate (Na₄P₂O₇), 1 mM β-glycerophosphate, and 1 mM sodium orthovanadate (Na₃VO₄). Lysates were sonicated and centrifuged (13,000 × g, 4 °C, 10 min). Supernatants were collected as total protein extracts. Protein concentrations were determined using the Pierce BCA kit (Thermo Fisher, Waltham, MA, USA).

2.5. PPM1B protein overexpression

HeLa cells were transfected with two pRec-M11-based expression plasmids encoding either full-length, N-terminally Flag-tagged (FT) wild-type human PPM1B or its phosphatase-inactive R179G mutant, referred as FT-PPM1B and FT-PPM1B R179G, respectively (GeneCopoeia, Rockville, Maryland, USA), using GeneJuice Transfection Reagent (Novagen, Merck Millipore, Darmstadt, Germany) in serum-free DMEM, following the manufacturer's protocol. A mock control was transfected with an equal amount of empty vector and transfection reagent. Untreated groups served as negative control. All biochemical, imaging or functional assays were performed 72 h after transient transfection of HeLa cells, unless otherwise indicated.

2.6. Subcellular fractionation following Sanguinarine treatment or FT-PPM1B overexpression

HeLa cells were subjected to either PPM1B inhibition or transient overexpression of the phosphatase prior to subcellular fractionation. For phosphatase inhibition experiments, cells were serum-starved for 24 h (0.1% FBS) and treated with 1 μ M Sanguinarine (SNG) for 1 h, while untreated cells served as controls, as previously described (Keller et al., 2025). For overexpression experiments, HeLa cells were transiently transfected with either the empty vector or FT-PPM1B plasmid as described above. Total protein was extracted as described above. For subcellular fractionation, pelleted cells were resuspended in Buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 10 $\times$ PIC, 1 μ M Microcystin-LR) and centrifuged (16,000 $\times$ g, 1 min) to isolate the cytoplasmic supernatant. Remaining pellets were extracted in Buffer B (20 mM HEPES pH 7.9, 420 mM NaCl, 0.5 mM EDTA, 0.5 mM EGTA, 10 $\times$ PIC, 1 μ M Microcystin-LR) on ice for 25 mins and centrifuged (12,000 $\times$ g, 10 mins) to collect the nuclear fraction. Protein concentrations were quantified by BCA assay (Thermo Fisher).

2.7. Lysophosphatidic acid treatment and Duolink proximity ligation assay

The Duolink PLA assay (DUO92002, DUO92004, DUO92014; Sigma-Aldrich) was performed as it was described before (Keller et al., 2025). Shortly, for one of the experiments involved PLA assay, mock and FT-PPM1B-transfected cells plated on CellCarrier Ultra plates (PerkinElmer) were treated with 1 μ M LPA for 30 mins. Meanwhile, during the other PLA-based experiment only mock and FT-PPM1B transfected cells were used without any further treatment. Cells were fixed in 4% PFA/TBS, and permeabilized with 0.2% Triton X-100/TBS. Samples were incubated overnight at 4 $^{\circ}$ C with primary antibodies (1:100) against MYPT1, MYPT1^{pT853}, MYPT1^{pT696}, MLC20, MLC20^{pS19}, PRMT5, PRMT5^{pT80}, Rb and PPM1B. Positive controls included either anti-MYPT1 and anti-Rb or anti-MYPT1 and anti-PPM1B antibodies; primary antibodies were omitted for negative controls. Following standard PLA probe ligation and amplification, cells were counterstained with Texas Red-Phalloidin and DAPI. Images were acquired using an Opera Phenix High-Content Screening system (40 $\times$ water-immersion objective). Cytoplasmic and nuclear PLA spots were automatically quantified using Harmony software (v4.8) and normalized to DAPI-stained nuclei across ≥ 3 replicate wells (≥ 9 fields/well) per condition.

2.8. Human cervix tissue samples

Normal and tumor tissue lysates were purchased from Protein Biotechnologies (San Diego, California, USA), solubilized and stored in radioimmunoprecipitation assay buffer. Following centrifugation of lysates at 16,000 $\times$ g, at 4 $^{\circ}$ C for 4 mins, SDS sample buffer (Bio-Rad Laboratories, Hercules, California, USA) was added to supernatants and the samples were incubated for 5 mins at 100 $^{\circ}$ C and the samples were

used for Western blot analysis.

2.9. Database search

We gained histological images from an open-source database (ProteinAtlas, www.proteinatlas.org). Searches were run for PPM1B and MYPT1 proteins. Slides of diaminobenzidine (DAB) staining of those phosphatases were available from patients with normal and tumorous cervix. Each inserted picture was taken from certain parts of the slides. Descriptions of the patients and links to the slides are written in the legend of the figures.

2.10. Western Blot analysis

Protein samples (20–30 μ g) were separated on 10% polyacrylamide gels or 4–20% Criterion precast gels at 200 V for 45 mins and transferred to nitrocellulose membranes using common or Criterion blotter at 400 mA for 60 mins or 100 V for 75 min, respectively. Membranes were blocked with 3% (m/v) BSA/1 $\times$ TBST, at room temperature for 1 h. Primary and secondary antibodies were applied according to the dilutions stated in [Supplementary Table S1](#). Detection was performed using WesternBright ECL reagents (Advansta Inc., San Jose, California, USA) and visualized with a ChemiDoc Touch Imaging System (Bio-Rad Laboratories, Hercules, California, USA).

2.11. Confocal fluorescence microscopy and high content screening analysis

HeLa cells were cultured in 96-well CellCarrier Ultra glass bottom plates (PerkinElmer, Shelton, Connecticut, USA) for 24 h, serum-starved also for 24 h, and transfected with empty vector (mock) or FT-PPM1B as described in “PPM1B Protein Overexpression” section. The procedure was conducted following the protocol found in the work of Keller et al (Keller et al., 2025). Primary antibodies against PPM1B, MYPT1, MYPT1^{pT853}, PRMT5, PRMT5^{pT80}, H4 and H4 dimethylation were applied at 1:100 dilution in 1% BSA/TBST overnight at 4 $^{\circ}$ C. Compartment specific quantification of Alexa FluorTM 488 fluorescent signals was performed based on DAPI and Texas Red Phalloidin based F-actin staining for nuclear and cytoplasmic regions respectively, and sum of Alexa FluorTM 488 fluorescent signal per at least 9 pixels of 1 well was collected from nuclear, cytoplasmic and total cell territories. Results were corrected for autofluorescence and secondary antibody background and normalized to the number of DAPI-stained nuclei. Final data were converted to relative numbers compared to the control samples which were taken as 100%.

Qualitative experiment using PLA assay was conducted on HeLa cells under control versus FT-PPM1B-transfected conditions. LPA treatment was applied in certain groups. Experimental procedures were executed as they are described before in the previous sections. Images were acquired using a Leica TCS SP8 confocal laser scanning system (Leica Microsystems, Wetzlar, Germany) mounted on a Leica DM6000 microscope and equipped with a Leica HC PL APO 63 $\times$ /1.4 NA Oil CS2 objective. Fluorescence emission was collected using photomultiplier tube (PMT) detectors, with laser power, detector gain, and offset kept constant across all samples. Bidirectional scanning was performed at 100 Hz with PhaseX correction enabled. Images were acquired with the pinhole set to 2 Airy units at a resolution of 2048 $\times$ 2048 pixels with a zoom factor of 0.75 $\times$. Fluorescence was acquired sequentially in three channels: DAPI (λ_{ex} = 405 nm, λ_{em} = 410–500 nm), Duolink[®] green fluorescent detection probes (λ_{ex} = 495 nm, λ_{em} = 527 nm), and Alexa FluorTM 633-conjugated phalloidin (λ_{ex} = 638 nm, λ_{em} = 645–700 nm). Final images represent single optical sections acquired at the cellular mid-plane.

2.12. RhoA Kinase and PPM1B phosphatase assays

Assays were executed in the same manner as detailed before (Keller et al., 2025). GST and GST-MYPT1 purified from *E. coli* were conjugated to Glutathione Sepharose 4B beads (P1743–1VL, Merck, Darmstadt, Germany). GST-MYPT1 was phosphorylated using recombinant ROK enzyme. After washing the phosphorylated samples with TBS, dephosphorylation was initiated via completing the reaction buffer with recombinant PPM1B enzyme for 45 mins, at 30°C in both cases. To test the specificity of PPM1B, the experiment was repeated, first setting the reaction buffer without MgCl₂ and MnCl₂ and then in the presence of 1 µM SNG. Reactions were analyzed by Western blot using GST and phospho-specific MYPT1^{PT853} antibodies.

2.13. Invasion assay

Cell invasion was evaluated using Corning® BioCoat™ Matrigel® Invasion Chambers. HeLa cells were transfected with FT-PPM1B, FT-PPM1B R179G, or an empty vector as previously described. Twenty-four hours post-transfection, cells were serum-starved for 24 h, trypsinized, and seeded into the upper chambers. Media supplemented with 20% FBS was added to the lower wells as a chemoattractant. After a 24-h incubation at 37 °C, non-invading cells on the upper surface of the membrane were removed with a cotton swab. The inserts were rinsed in PBS, and invaded cells were detached and stained using a lysis buffer containing CyQuant GR fluorescent dye (Invitrogen). Fluorescence was measured at 485/535 nm using a Tecan Spark Multimode microplate reader (Tecan). Invaded cell numbers were calculated using a standard curve generated from serial dilutions of known cell concentrations.

2.14. Proteome profiler

Under the condition of control and overexpression of PPM1B in HeLa cells, expression and phosphorylation levels of numerous oncogenesis-related proteins were detected with R&D Systems Proteome Profiler Human XL Oncology Array kit and R&D Systems Proteome Profiler Human Phospho-Kinase Array kit (Minneapolis, MN, USA). HeLa cells were either transfected by mock or FT-PPM1B and lysed, as they are described in “PPM1B Protein Overexpression” and “Protein Extraction” sections. 200 µg of protein was applied, following the instructions of the manufacturer. Visualization was performed by ChemiDoc Touch Imaging System. Dot intensities were quantified with ImageJ software. Raw values were corrected via subtraction of the background intensities. Final data were plotted as heatmaps. Some significant hits were validated by semi-quantitative Western blot analysis or semi-quantitative RT-PCR.

2.15. RNA isolation and semi-quantitative RT-PCR analysis

The protocol of the experiment was used as it is described precisely before (Keller et al., 2024). Briefly, total RNA was isolated from FT-PPM1B transfected HeLa cells. RNA concentration was determined using a NanoDrop Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription was performed using cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). The resulting cDNA was used for PCR amplification with a 2 × Xceed qPCR SYBR Green Master Mix (Institute of Applied Biotechnologies, Prague–Strašnice, Czech Republic). Primer sequences are provided in Supplementary Table S2. qPCR reactions were performed using a LightCycler 480 system (Roche Applied Science, Penzberg, Germany). Cp values were normalized to the geometric mean of housekeeping genes, GAPDH and β-actin. Final data were normalized to the values obtained from mock vector-treated samples.

2.16. Statistical analysis

Western blots, protein arrays and immunofluorescence signals were quantified using ImageJ software. Data were visualized as bar graphs and analyzed using unpaired two-tailed *t*-tests for two-group comparisons or one-/two-way ANOVA with Tukey’s *post hoc* test for multiple comparisons in GraphPad Prism 8 software (<https://www.graphpad.com/updates/prism-10-1-1-release-notes>). Phosphorylated proteins were normalized to total protein expression, and expressed as relative numbers, while total protein expression was normalized to the loading control, and all data were represented as relative numbers compared to control or mock samples which were taken as 100%, except for the experiments of ROK kinase/PPM1B phosphatase assays and cell fractionation, where raw data are presented for total proteins and their modifications. Data are presented as mean ± SD (n = independent replicates, at least 3). The difference between groups was considered significant in case of *p* < 0.05. Grubbs’ test was used to identify outliers, and normality was confirmed prior to statistical analysis.

3. Results

3.1. PPM1B binds to and dephosphorylates MYPT1 at Thr853

Our previous work established the interaction between PPM1B and MYPT1, demonstrating that PPM1B dephosphorylates the regulatory subunit at its Thr696 residue in the nucleus (Keller et al., 2025). In the current study we aimed to determine whether this catalytic process also targets the phospho-Thr853 residue of MYPT1. We first performed *in vitro* kinase and phosphatase assays on GST-MYPT1 (Fig. 1A.). The assays utilized purified, recombinant ROK and PPM1B enzymes to phosphorylate and dephosphorylate GST-MYPT1, respectively. To evaluate the metal ion dependency of PPM1B and to validate its specific activity, reactions were conducted with or without Mg²⁺/Mn²⁺ ions and sanguinarine (SNG), a validated and widely used benzo(c)phenanthridine PPM1B inhibitor. Western blot analysis using anti-GST and anti-MYPT1^{PT853} antibodies (Fig. 1A) revealed that ROK treatment significantly increased Thr853 phosphorylation on GST-MYPT1 by 5.5-fold (*p* < 0.0001) compared to GST-MYPT1 negative control. Subsequent addition of PPM1B significantly reduced the Thr853 phosphorylation by 1.9-fold (*p* < 0.0001) relative to the ROK-phosphorylated GST-MYPT1. Conversely, the presence of SNG or the absence of metal ions prevented this reduction, maintaining the phosphorylation level established by ROK. Samples with SNG or without metal ions indicated a significant elevation by 2-fold (*p* = 0.0003) and 1.8-fold (*p* = 0.0007) compared to PPM1B-treated samples, respectively (Fig. 1A). To assess the catalytic activity of PPM1B on the MYPT1^{PT853} residue in a cellular context, HeLa cells were treated with SNG and subjected to subcellular fractionation to allow the measurement of the phosphorylation level in the nucleus and cytoplasm, separately (Fig. S1A). Results of the fractionation upon SNG treatment are detailed in the Supplementary materials. Finally, we investigated the conditions required for PPM1B-MYPT1 interaction to determine whether recruitment of PPM1B to MYPT1 depends on Thr853 phosphorylation or occurs independently of this modification (Fig. 1B). To induce MYPT1 Thr853 phosphorylation, cells were treated with lysophosphatidic acid (LPA), a potent Rho-associated kinase (ROK) activator known to enhance phosphorylation of ROK substrates, including MYPT1. Validation of LPA effect on the phosphorylation level of MYPT1 Thr853 residue was conducted first using immunofluorescent staining (Fig. S1, B and C). In selected samples, cells were transfected with FT-PPM1B plasmids prior to LPA treatment to evaluate the effect of increased PPM1B levels. Following treatment, protein–protein proximity between PPM1B and Thr853-phosphorylated MYPT1 was assessed by Proximity Ligation Assay (PLA) (Fig. 1B). Negative and positive controls are shown and described in Supplementary materials (Fig. S1, D and E). LPA treatment significantly increased PPM1B-MYPT1^{PT853} interactions both in control and FT-PPM1B-overexpressing cells compared

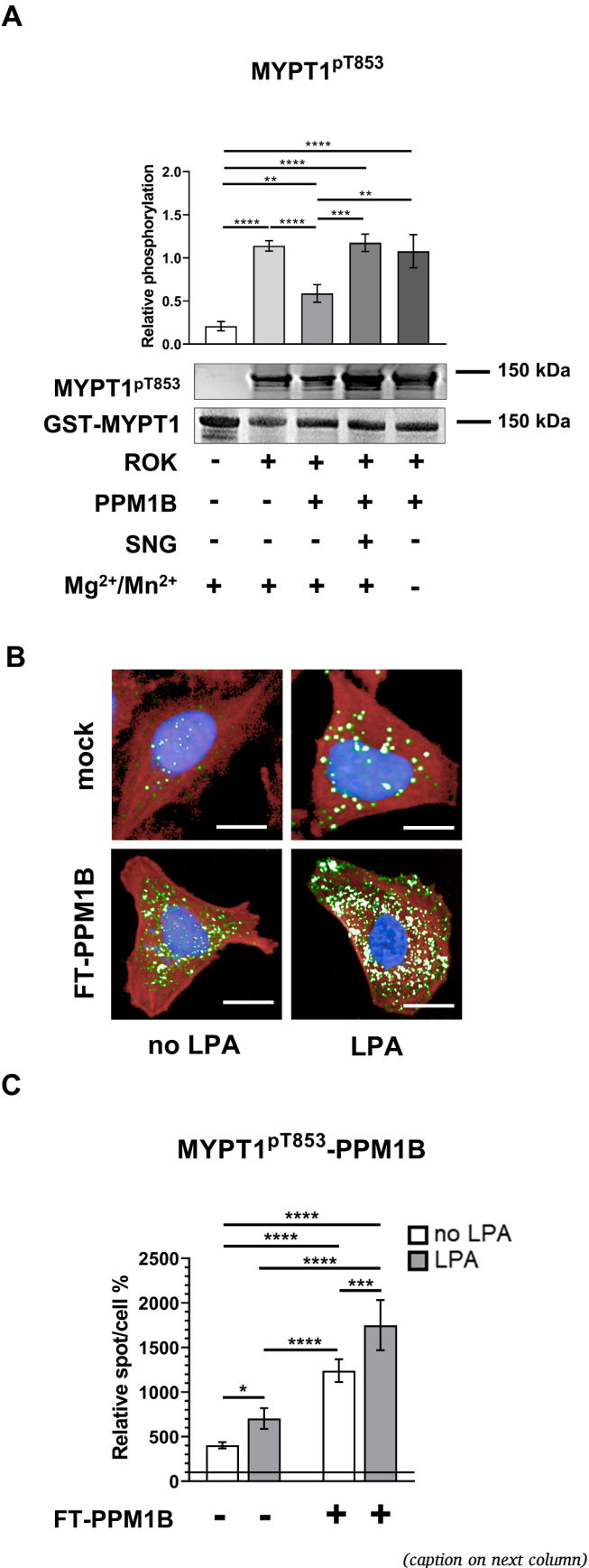


Fig. 1. PPM1B directly interacts with and dephosphorylates MYPT1 at Thr853. The enzyme–substrate interaction between PPM1B and MYPT1 was assessed by GST-MYPT1 pull-down assay. Recombinant proteins were immobilized on Glutathione-Sepharose 4B beads, phosphorylated with purified ROK, then incubated with purified PPM1B in the presence of Mg²⁺ and Mn²⁺ to induce dephosphorylation of GST-MYPT1. Where indicated, 1 μM SNG was added and/or metal ions were chelated. Western blot analysis was performed using anti-GST and anti-MYPT1^{pT853} antibodies. Data represent relative phosphorylation levels (mean ± SD, n = 5–11). Statistical analysis was performed using one-way ANOVA or unpaired two-tailed t-test depending on the number of groups, with p < 0.01 (**), p < 0.001 (***) and p < 0.0001 (****) (A). PPM1B–MYPT1 interactions were further analyzed by Duolink PLA. Nuclei and F-actin were labeled with DAPI and Texas Red–phalloidin, respectively (scale bar: 10 μm). Anti-PPM1B antibody was co-incubated with anti-MYPT1^{pT853} (B) under control or FT-PPM1B-transfected conditions, in the presence or absence of LPA. PLA signals were quantified as relative number of spots per cell (%). Data are shown as mean ± SD (n = 3–15). Statistical analysis was performed using two-way ANOVA with p < 0.05 (*), p < 0.001 (***) and p < 0.0001 (****) (C).

with their respective untreated counterparts in total cell area by 300.2% (p = 0.0457) and 510% (p = 0.0007). Overexpression of recombinant PPM1B significantly increased the number of PLA interaction spots irrespective of the presence or absence of LPA compared with the corresponding control samples by 837% (p < 0.0001) and 1046.8% (p < 0.0001) (Fig. 1C). Compartment-specific analysis revealed that FT-PPM1B overexpression significantly increased nuclear PPM1B–MYPT1^{pT853} interactions compared to the mock samples either with or without LPA-treatment by 684% (p < 0.0001) and by 644.4% (p < 0.0001), respectively. Following addition of LPA a significant reduction was observed both in mock and FT-PPM1B groups independently of the presence or absence of the recombinant protein by 216.4% (p = 0.0224) and 256% (p = 0.0444), respectively (Fig. S1F). In FT-PPM1B-overexpressing group a significant increase was experienced with respect to the cytoplasmic interactions both in non-treated and LPA-treated groups compared to their mock counterparts by 511% (p < 0.0001) and 548.7% (p < 0.0001), respectively. Unexpectedly, LPA treatment significantly increased cytoplasmic PPM1B–MYPT1^{pT853} interactions in the mock and FT-PPM1B samples compared to their non-treated pairs by 231.5% (p = 0.0083) and 269.2% (p = 0.0009), respectively (Fig. S1G). To determine whether the observed compartment-specific pattern was unique to phosphorylated MYPT1, PLA was repeated using antibodies recognizing total MYPT1 (Fig. S1H). Compartment-specific analysis of PPM1B–MYPT1 interaction in the nuclear area revealed a significant elevation under FT-PPM1B-transfected condition compared to the mock samples both in untreated and LPA-treated groups by 713.8% (p < 0.0001) and 239.7% (p < 0.0001), respectively. Both in mock and FT-PPM1B-overexpressing cells, the application of LPA reduced the interaction by 680.1% (p = 0.0001) and 1154.2% (p = 0.0031), respectively (Fig. S1I). In the cytoplasmic area FT-PPM1B overexpression indicated a significant increase in PPM1B–MYPT1 interaction only upon LPA treatment by 470.3% (p = 0.0006). LPA treatment significantly reduced the frequency of PPM1B–MYPT1 interactions both in mock and FT-PPM1B groups by 920.3% (p < 0.0001) and 338.5% (p = 0.028), respectively (Fig. S1J).

3.2. PPM1B overexpression decreases Thr853 inhibitory phosphorylation and alters the nuclear-cytoplasmic distribution of MYPT1 in HeLa cells

Representative PLA experiments demonstrated negative and positive controls to validate the assay and detect the number of interactions between PPM1B and MYPT1 under control and FT-PPM1B overexpressed conditions. (Fig. S2A) Furthermore, the proximity of PPM1B to Thr696-phosphorylated MYPT1 was depicted as well to show the co-existing interactions of those regulatory sites with PPM1B (Fig. S2B). As Thr853 is the predominant inhibitory phosphorylation site regulating myosin phosphatase activity, subsequent analyses focused on changes in

Thr853 phosphorylation and MYPT1 subcellular expressions. Western blot analysis following subcellular fractionation was performed on mock and FT-PPM1B-overexpressing cells to evaluate the compartment-specific distribution of components of the MP/PRMT5/H4 signaling axis. To validate the purity of the fractions, we visualized tubulin and lamin A/C as cytoplasm- and nucleus-specific markers, respectively. Successful FT-PPM1B overexpression was first verified in an independent experiment by Western blot analysis using both anti-Flag and anti-PPM1B antibodies (Fig. S3, A and B, respectively). Compared with mock-transfected cells, normalized Flag density increased to 1555% ($p = 0,0012$), while total PPM1B expression increased to 252% ($p = 0,0004$). Quantitative immunofluorescence further confirmed efficient overexpression (Fig. S3C), demonstrating a significant elevation in cellular PPM1B fluorescence intensity to 337% of the untransfected control ($p = 0,0062$) (Fig. S3D). First, MYPT1 expression was investigated. Total MYPT1 level showed a significant increase in the cytoplasmic fraction by 1,15-fold ($p = 0,0484$) and a 1,41-fold ($p = 0,001$) reduction in the nuclear fraction upon FT-PPM1B overexpression compared to their paired mock samples. In total lysates significant changes were not detectable (Fig. 2A). To visualize subcellular distribution of MYPT1, immunofluorescence staining was conducted under mock or FT-PPM1B transfection conditions (Fig. 2B). Fluorescence intensities were quantified in the total cell area, in the cytoplasm and the nucleus, delineated by DAPI and Texas Red-phalloidin staining, respectively. Total cellular MYPT1 expression remained stable despite FT-PPM1B overexpression (Fig. 2C). Cytoplasmic MYPT1 expression showed a significant accumulation by 8% ($p = 0,0319$) and 7.3% ($p = 0,045$) compared to control and mock values, respectively (Fig. 2D). Conversely, nuclear MYPT1 intensity decreased significantly by 19.25% ($p = 0,0022$) and 11.65% ($p = 0,0344$) (Fig. 2E). These findings indicate the translocation of MYPT1 from the nucleus to the cytoplasm following by PPM1B overexpression (Fig. 2B). Immunoblotting highlighted significant reduction of MYPT1 pThr853 phosphorylation in total fraction by a 1,37-fold decrease ($p = 0,0353$), in the cytoplasmic fraction by 1,62-fold decrease ($p = 0,0247$) and nuclear fraction by a 3,37-fold decrease ($p = 0,0207$) in the presence of the recombinant PPM1B relative to their mock pairs (Fig. 2F). The spatial distribution of Thr853 phosphorylated MYPT1 exhibited a consistent decrease across the cellular compartments (Fig. 2G). When intensities were assessed across the total cell area, overall phosphorylation exhibited a significant reduction of 11.26% ($p = 0,0294$) and 12.83% ($p = 0,0185$), consistent with the observed shift in MYPT1 localization (Fig. 2H). Cytoplasmic MYPT1^{pThr853} phosphorylation decreased by 12.33% ($p = 0,0391$) and 11.62% ($p = 0,0342$) compared to control and mock samples, respectively (Fig. 2I). Similarly, nuclear phosphorylation was significantly reduced by 22.67% ($p = 0,0207$) and 21.17% ($p = 0,0345$) (Fig. 2J). Together, the fractionation and immunofluorescence analyses consistently demonstrate that PPM1B overexpression promotes redistribution of MYPT1 from the nucleus to the cytoplasm while concomitantly reducing inhibitory Thr853 phosphorylation in both compartments.

3.3. MP reduces PRMT5 Thr80 phosphorylation without altering its expression or nuclear localization in HeLa cells

Given the previously described functional association between nuclear MYPT1 and PRMT5 and the observed redistribution of MYPT1 from the nucleus to the cytoplasm, we next examined the expression and post-translational modification of PRMT5 upon FT-PPM1B overexpression. Representative PLA analysis did not reveal a definitive proximity signal between PPM1B and PRMT5 or its phosphorylated form at the Thr80 residue under the experimental conditions examined (Fig. S2C). Western blot analysis revealed no significant changes in total PRMT5 expression in either total lysates, cytoplasmic fractions, or nuclear fractions following FT-PPM1B overexpression (Fig. 3A). Using quantitative immunofluorescence, we evaluated PRMT5 expression and

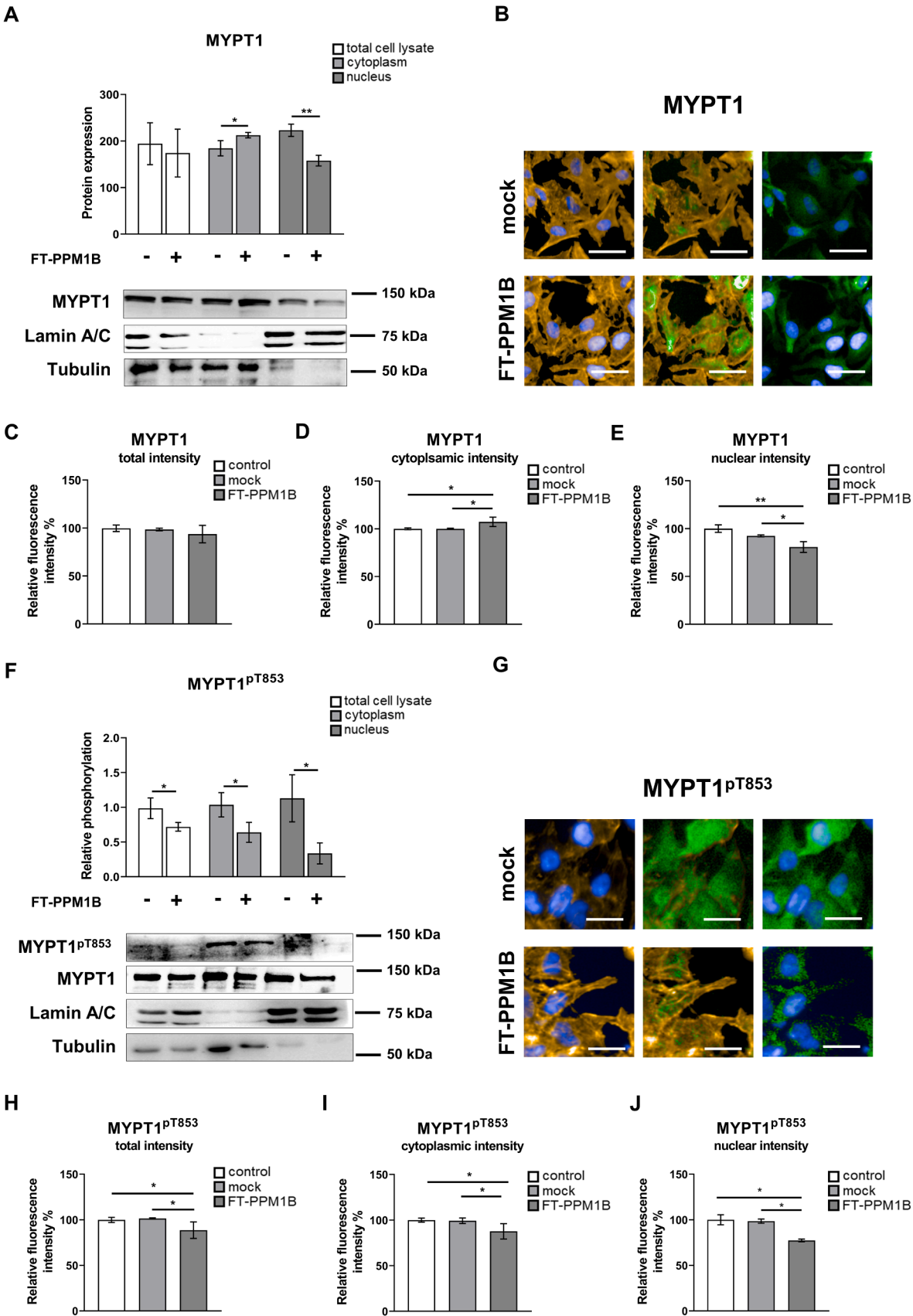
its Thr80 phosphorylation levels in the total cell area, in the cytoplasmic and finally the nuclear compartments (Fig. 3, B and G). Following FT-PPM1B transfection, expression levels of PRMT5 (Fig. 3B) remained unchanged in total cell area as well as in both compartments compared to controls (Fig. 3, C-E). Analysis of PRMT5 phosphorylation at its Thr80 residue following subcellular fractionation upon FT-PPM1B transfection demonstrated a significant, 1,602-fold decrease ($p = 0,0479$) in the total lysate compared to the mock counterpart. Similar changes were observed in the cytoplasmic fraction by a 1,36-fold reduction ($p = 0,0065$) and in the nuclear samples by a 2,03-fold decrease ($p = 0,0107$) relative to their mock pairs (Fig. 3F). These findings were further supported by quantitative immunofluorescence. The activating phosphorylation of PRMT5 at its Thr80 residue was uniformly reduced in all investigated compartments (Fig. 3G). After FT-PPM1B overexpression, values in total cell area revealed a significant overall reduction (Fig. 3H) in PRMT5 phosphorylation by 17.5% ($p = 0,0014$) and 11.1% ($p = 0,0353$) relative to control and mock samples, respectively (Fig. 3H). Likewise, cytoplasmic PRMT5 Thr80 phosphorylation decreased by 17.6% ($p = 0,0003$) and 15.6% ($p = 0,0019$) relative to control and mock cells, respectively (Fig. 3I). Nuclear PRMT5 Thr80 phosphorylation was similarly reduced by 15.3% ($p = 0,0008$) and 11.8% ($p = 0,0159$), respectively (Fig. 3J). Collectively, these results indicate that FT-PPM1B overexpression markedly reduces PRMT5 Thr80 phosphorylation without affecting the overall expression level or subcellular distribution of PRMT5.

3.4. PPM1B overexpression diminishes symmetrical dimethylation of H4 while maintaining baseline H4 expression and subcellular distribution

Finally, fractionation was used to examine the expression level and the symmetric dimethylation of H4 as one of the nuclear substrates of PRMT5 (Fig. 4, A and F, respectively). Total H4 protein expression remained unchanged in total lysates, cytoplasmic fractions, and nuclear fractions following FT-PPM1B overexpression (Fig. 4A). Quantitative immunofluorescence yielded comparable results (Fig. 4B). Expression level of H4 remained stable in all cellular compartments (Fig. 4, C-E). In agreement with the reduction in PRMT5 Thr80 phosphorylation observed above, immunoblot analysis revealed a significant decrease in H4 symmetric dimethylation following FT-PPM1B overexpression. FT-PPM1B-transfected total lysate showed a 1,49-fold decrease ($p = 0,0171$) compared to the mock sample. In the cytoplasmic fraction, H4 symmetric dimethylation showed a 2.02-fold decrease ($p = 0,0173$), whereas in the nuclear fraction it decreased 1.46-fold ($p = 0,0152$) relative to the corresponding mock samples (Fig. 4F). Consistent with the immunoblot analysis, symmetrical dimethylation of H4 was markedly reduced upon immunofluorescent labeling (Fig. 4G). Total cellular H4 symmetric dimethylation was reduced by 28.6% ($p = 0,0049$) and 27.8% ($p = 0,0044$) (Fig. 4H) compared to the control and mock values, respectively. At the subcellular level, cytoplasmic intensity of the modification did not show significant alterations (Fig. 4I), whereas nuclear H4 symmetric dimethylation was diminished by 21.7% ($p = 0,0026$) and 21.2% ($p = 0,0029$) (Fig. 4J) compared to the control and mock values, respectively. Overall, these observations suggest that FT-PPM1B overexpression selectively reduces H4 symmetric dimethylation without affecting total H4 expression or its subcellular distribution.

3.5. Catalytically active PPM1B suppresses HeLa cell invasion by downregulating the MYPT1/MLC20 axis

Based on our previously established model for investigating the role of PPM1B in oncogenesis, (Keller et al., 2025) we utilized a point mutation within the phosphatase gene (R179G) that results in a complete loss of catalytic activity. In this study, we aimed to assess the impact of wild-type (WT) versus mutant PPM1B R179G overexpression on the invasive potential of HeLa cells at both physiological (Fig. 5A) and



(caption on next page)

Fig. 2. Distribution of MYPT1 and MYPT1^{pT853} in HeLa cells upon PPM1B overexpression. Western blot analysis was conducted following subcellular fractionation using anti-MYPT1, anti-MYPT1^{pT853}, anti-lamin A/C and anti-tubulin antibodies. Total lysates, cytoplasmic and nuclear fractions were examined under mock and FT-PPM1B treatments. Columns show raw expression and phosphorylation levels. Values are shown as mean $\pm$ SD, $n = 3-5$. Data were compared using an unpaired two-tailed t -test where $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****) (C,D,E and H,I,J). Immunofluorescence staining was performed in control, mock and PPM1B-overexpressing HeLa cells using fluorescent Alexa FluorTM 488-conjugated anti-mouse/rabbit secondary antibody (as green) following incubation with anti-MYPT1 (B) and anti-MYPT1^{pT853} (G) antibodies. Nuclei and F-actin were labeled with DAPI (as blue) and Texas Red-phalloidin (as yellow), respectively (scale bar: 10 μ m). Relative intensities in total cellular, cytoplasmic and nuclear compartments were quantified for MYPT1 expression and Thr853 phosphorylation. Data are presented as mean $\pm$ SD ($n = 3-9$). Statistical analysis was performed using one-way ANOVA, where $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****) (C,D,E and H,I,J).

molecular levels (Fig. 5, B-D), while mock transfected cells served as control. The success of mutant PPM1B overexpression was validated via Western-blot analysis using anti-Flag and anti-PPM1B antibodies (Fig. S3, A and B). Flag signal was significantly increased by 959% ($p = 0.0171$) compared to the mock control (Fig. S3A). Total PPM1B expression was elevated as well by 233% ($p = 0.0008$). The expression levels upon wild-type FT-PPM1B and mutant FT-PPM1B R179G overexpression were comparable (Fig. S3B). Cell invasion was evaluated using a Boyden chamber assay, with quantification based on fluorescence labelling of invaded cells (Fig. 5A). Cells overexpressing FT-PPM1B exhibited a significantly diminished invasion capacity, showing a reduction of 37.7% ($p = 0.0372$) and 54.7% ($p = 0.0071$) relative to mock-transfected and FT-PPM1B R179G-expressing cells, respectively. In contrast, overexpression of the catalytically inactive R179G mutant yielded no significant change in the number of invading cells compared to the mock control (Fig. 5A). To elucidate the underlying molecular mechanisms driving this phenotype, cell lysates were generated under the same transfection conditions. Subsequently, samples were analyzed by Western blot analysis (Fig. 5, B-D). In the cells expressing wild-type FT-PPM1B, the inhibitory phosphorylation of MYPT1 at Thr853 was markedly reduced by 42.3% ($p = 0.0076$) and 28% ($p = 0.0476$) compared to the mock and FT-PPM1B R179G-mutant samples, respectively (Fig. 5B). Furthermore, while total MLC20 expression did not change significantly across all experimental groups (Fig. 5C), its activating phosphorylation at Ser19 was correspondingly diminished by 53% ($p = 0.0402$) and 62.3% ($p = 0.0205$) relative to mock and mutant conditions, respectively (Fig. 5D). Together, these data indicate that PPM1B impairs cell invasion in a catalytic activity-dependent manner by modulating the MYPT1/MLC20 signaling axis. Representative PLA analysis did not reveal a definitive proximity signal between PPM1B and MLC20 or phospho-MLC20 under the experimental conditions examined (Fig. S2C).

3.6. Human tumor samples and tissue slides show reduced expression of MYPT1 and PPM1B, along with elevated level of Thr853 phosphorylation of MYPT1

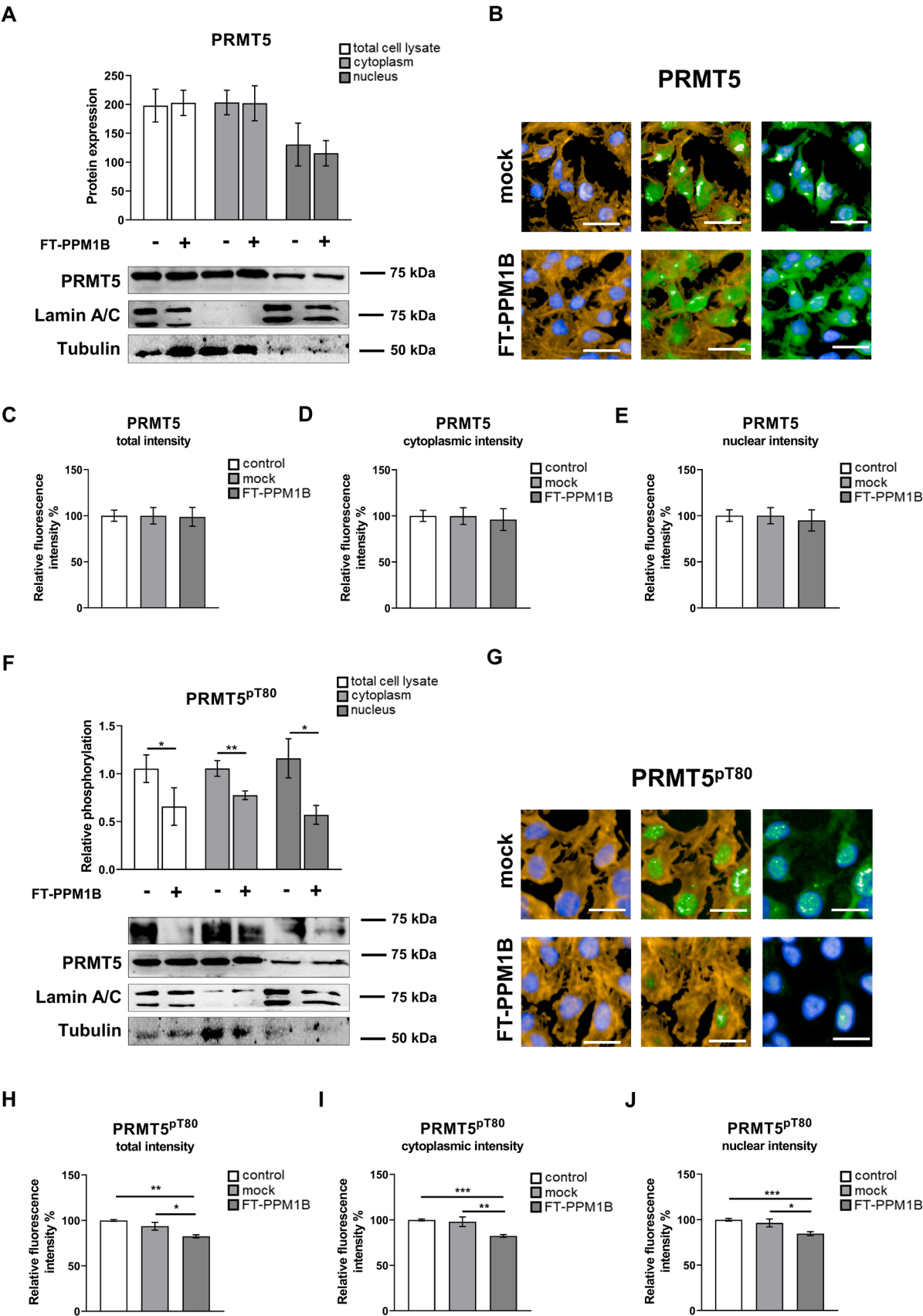
We planned to evaluate expression intensity of MYPT1, PPM1B and also the phosphorylation level of MYPT1 in human tumor samples. For those purposes human normal and cervical carcinoma biopsy lysates were purchased and analyzed using Western blot analysis (Fig. 6A), moreover tissue slide images were obtained from ProteinAtlas database (Fig. 6B). Western blot data indicates a significant elevation at the inhibitory Thr853 phosphorylation of MYPT1–166.2% ($p = 0.0023$) (Fig. 6A) compared to healthy biopsy lysates. According to the shared information and data about the patients to whom the tissue samples belong we used normal cervix tissue samples of a 65-year-old female patient (patient ID:2434) to detect PPM1B expression and a 34-year-old female patient (patient ID: 4030) to detect MYPT1 expression (Fig. 6B). To observe changes in the expressions upon cervical cancer, tissue samples were taken from a 55-year-old female patient with squamous cell carcinoma (patient ID: 470) for PPM1B detection and a 70-year-old female patient with squamous cell carcinoma (patient ID:3382) for MYPT1 detection (Fig. 6B). Slides showed harsher staining for both PPM1B and MYPT1 in normal cervical tissues in the case of expressions, while we experienced less intense chromogenic signals in cancerous samples (Fig. 6B).

3.7. PPM1B overexpression alters the expression profile of proto-oncogenes and tumor-suppressors in HeLa cells

Since we described a significant change in the inhibitory phosphorylation of nuclear MP, we sought to determine the broader impact of PPM1B overexpression on key oncogenic networks. To achieve this, a Human Oncology XL Protein Array was performed using control and PPM1B-overexpressing samples. Differentially expressed proteins were grouped according to their biological functions and relevance to cancer progression (Fig. 7, A and C). Overall, PPM1B overexpression was associated with a broad suppression of pro-tumorigenic signaling pathways accompanied by the induction of several tumor suppressors. Among growth factor- and receptor-related proteins, numerous proto-oncogenic factors, including DLL1, Angiopoietin-1/Tie-2, VEGF, HGFR, and members of the ErbB receptor family, were significantly downregulated (Fig. 7A). In parallel, proteins involved in cell adhesion, epithelial-mesenchymal transition (EMT), extracellular matrix remodeling, angiogenesis, proteolysis, inflammation, and stress responses also exhibited predominantly decreased expression (Fig. 7, A and C). These coordinated changes suggest an overall reduction in signaling pathways that promote proliferation, invasion, metastasis, and tumor progression. In contrast, several tumor suppressors were significantly upregulated following PPM1B overexpression. Notably, p53 and p27/Kip protein levels increased significantly, whereas the anti-apoptotic protein BCL-x was reduced (Fig. 7A). The increase in p53 was independently confirmed by Western blot analysis, demonstrating an 1146% increase relative to control cells ($p = 0.002$) (Fig. 7B). Similarly, the tumor suppressors thrombospondin-1 and Maspin were significantly elevated (Fig. 7C), and RT-qPCR further confirmed a 335.7% increase in Maspin mRNA expression ($p = 0.0446$) (Fig. 7D). P-values for all statistical comparisons are provided in Supplementary Table S3. Collectively, these findings indicate that PPM1B overexpression induces a coordinated shift toward a less oncogenic molecular profile, characterized by widespread suppression of proto-oncogenic pathways together with activation of multiple tumor-suppressive mechanisms.

3.8. PPM1B upregulation modulates the phosphorylation profile of proto-oncogene and tumor-suppressor kinases in HeLa cells

To further explore the broad intracellular effects of PPM1B, we analyzed HeLa cell lysates using a Human Phospho-Kinase Array. This screening revealed significant alterations in the phosphorylation states of several crucial signaling molecules, reflecting both the direct and indirect consequences of elevated PPM1B phosphatase activity (Fig. 8A). Interestingly, despite the increased protein phosphatase activity in the FT-PPM1B-transfected cells, several kinases exhibited significantly altered phosphorylation levels. For instance, a notable elevation in the phosphorylation of the tumor suppressor p53 at Ser46 was observed by 350% ($p = 0.0089$) (Fig. 8A). In contrast, activating phosphorylation of the pro-survival Ser/Thr kinase Akt1/2/3^{pS473} showed a significant reduction upon PPM1B overexpression by 29% ($p = 0.016$) (Fig. 8A). Similarly, several other key targets demonstrated significantly reduced phosphorylation, aligning with the expected suppressive effects of PPM1B overexpression. Phosphorylation of the stress-activated protein kinase JNK1/2^{pT183/pY185}, pT221/pY223 exhibited a significant reduction by 42% ($p = 0.0041$) (Fig. 8A). Likewise, the phosphorylation of the transcription factor CREB^{pS133} showed a marked decrease of 40.75%



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Fig. 3. Immunofluorescence analysis of PRMT5 expression and Thr80 phosphorylation in PPM1B-expressing HeLa cells. Western blot analysis was conducted following subcellular fractionation using anti-PRMT5, anti-PRMT5^{PT80}, anti-lamin A/C and anti-tubulin antibodies. Total lysates, cytoplasmic and nuclear fractions were examined under mock and FT-PPM1B treatments. Columns show raw expression and phosphorylation levels. Values are shown as mean $\pm$ SD, $n = 3$ –5. Data were compared using an unpaired two-tailed t -test where $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) (C,D,E and H,I,J). Immunofluorescence staining was performed on control, mock and FT-PPM1B-transfected HeLa cells using fluorescent Alexa FluorTM 488-conjugated anti-rabbit secondary antibody (as green) following incubation with anti-PRMT5(B) and anti-PRMT5^{PT80}(G) antibodies. Nuclei and F-actin were labelled with DAPI (as blue) and Texas Red-phalloidin (as yellow), respectively. Scale bar: 10 μ m. Relative intensity values of total cellular, cytoplasmic and nuclear compartments were quantified and depicted as columns for PRMT5 expression and its Thr80 phosphorylation. Values indicate mean $\pm$ SD, $n = 3$ –9. Statistical comparison was performed using one-way ANOVA where $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) (C,D,E and H,I,J).

($p = 0.0004$) (Fig. 8A). These array-based observations were subsequently validated by Western blot analysis. The increased phosphorylation of p53 at Ser46 was also validated, showing an elevation of 51.6% ($p = 0.0013$) (Fig. 8B). The modulation of Akt1/2/3^{pS473} was confirmed by a similar decrease of 51.6% ($p = 0.0013$) (Fig. 8, C and D). Similarly, Western blot analysis confirmed the reduction in JNK1/2 phosphorylation, demonstrating a decrease of 47% ($p = 0.0334$) (Fig. 8, E and F). In agreement with the array results, CREB^{pS133} phosphorylation was also reduced, with Western blot revealing a 60.33% decrease ($p = 0.0429$) (Fig. 8, G and H). Beyond the described changes, proteins exhibiting nonsignificant alterations and their corresponding p -values are presented in the Supplementary material (Fig. S4A, and Table S4, respectively).

4. Discussion

The spatiotemporal regulation of protein phosphorylation networks is a fundamental determinant of cellular homeostasis (Day et al., 2016), and the uncoupling of these networks—often via the hyperactivation of kinases or the functional loss of phosphatases—is a primary driver of oncogenic transformation (Burton et al., 2024). While the role of ROK in driving tumor progression through actomyosin hypercontractility is well established (Kumper et al., 2016; Northcott et al., 2018), the compensatory tumor-suppressive phosphatase networks remain uncharacterized. In this study, we elucidate a pleiotropic, tumor-suppressive signaling cascade governed by PPM1B. We establish that PPM1B directly dephosphorylates the MYPT1 regulatory subunit of MP at its strictly conserved inhibitory Thr853 residue. This catalytic event not only reactivates the holoenzyme but dynamically shifts its subcellular localization, triggering a cascade that dismantles the epigenetic PRMT5/H4 oncogenic axis, reverses actomyosin-driven invasive phenotypes, and globally reprograms the proteomic landscape of cervical carcinoma cells.

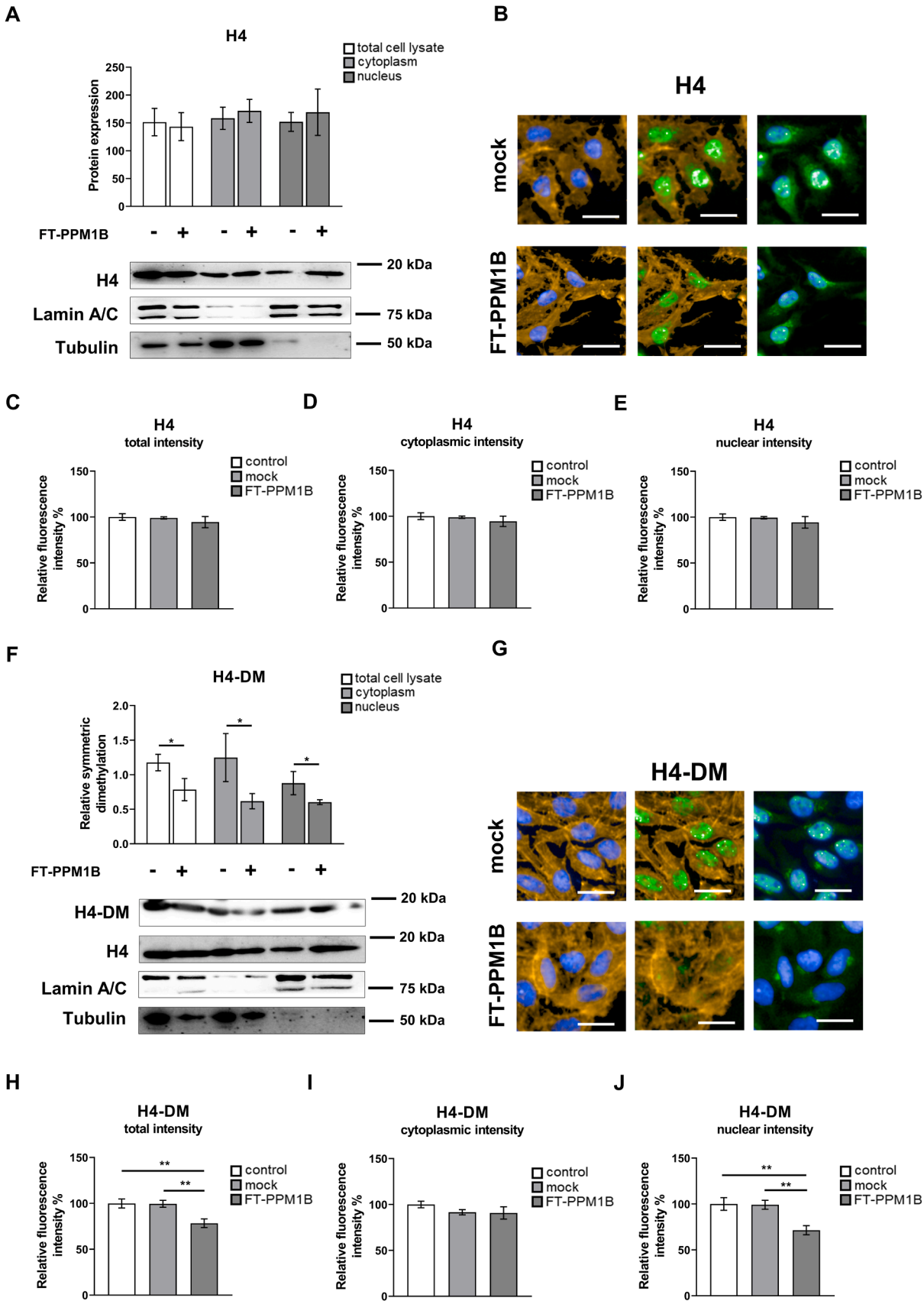
The MYPT1 subunit acts as the critical signaling regulator of the MP holoenzyme, directing the PP1c δ catalytic subunit to its specific substrates (Hartshorne et al., 2004). Our findings add a new layer of complexity to the regulation of MYPT1, particularly regarding its two well-characterized inhibitory sites, Thr696 and Thr853. While ROK has historically been viewed as the master kinase for both residues, an expanding network of enzymes—including ZIPK, ILK, Raf-1, and p21-activated kinases—specifically targets Thr696, suggesting a highly redundant or context-dependent upstream signaling hierarchy. The structural mechanism driving this inhibition involves conformational rearrangements that direct the phosphorylated region into the substrate-binding groove of the PP1c catalytic center, thereby attenuating MP activity. However, the precise *in vivo* contribution of Thr853 remains controversial; our data weigh into the long-standing debate between studies showing no functional inhibition in smooth muscle and those identifying Thr853 as an equivalent, primary ROK target. Given the potential cooperative priming suggested by Khasnis et al., as well as cell-type-specific variations in kinase accessibility, our results support a model where these two sites operate independently yet synergistically to fine-tune cellular phosphatase activity across diverse physiological environments. The inhibitory phosphorylation of MYPT1 at Thr696 and Thr853 by ROK sterically hinders the interaction between the phosphatase and its primary substrates (Ito et al., 2004). Previous work from our laboratory demonstrated that PPM1B acts as an upstream activator

of MP by targeting the phospho-Thr696 residue (Keller et al., 2025). Here, our *in vitro* protein phosphatase and *in situ* proximity ligation assays unequivocally establish that PPM1B also directly targets the phospho-Thr853 residue. The clinical relevance of this specific interaction is underscored by our analysis of primary human cervical carcinoma biopsies, which revealed a significant elevation of phosphorylation of MYPT1 on the Thr853 residue compared to healthy adjacent tissue. This suggests that the functional attenuation of PPM1B, and the subsequent hyperphosphorylation of MYPT1, are prevalent mechanisms utilized by cervical malignancies to sustain oncogenic signaling.

Our *in vitro* and subcellular fractionation data collectively demonstrate a metal ion-dependent, sanguinarine-sensitive dephosphorylation of Thr853 by PPM1B that is highly restricted to the nucleus, underscoring a tightly controlled spatial regulation of this network. Most notably, our proximity ligation assays reveal a dynamic, phosphorylation-directed interaction between these proteins (Figure S2B). While baseline assays confirm a steady-state association between PPM1B and the myosin phosphatase complex (Fig. 1), further targeted analysis reveals a preferential, high-affinity interaction between PPM1B and the Thr853-phosphorylated subpopulation of MYPT1 (Figure S2). This targeted association strongly points toward a substrate-driven feedback loop. Under conditions where upstream kinases (such as ROK) phosphorylate these residues to modulate complex activity, this specific modification simultaneously generates a structural motif that promotes the recruitment of PPM1B. Consequently, PPM1B does not merely bind the complex statically, but is actively recruited to the phosphorylated signaling pool to facilitate localized dephosphorylation and fine-tune downstream PRMT5 signaling.

Crucially, PPM1B-mediated dephosphorylation of MYPT1 at Thr853 drives a profound spatial redistribution of MP holoenzyme from the nucleus to the cytoplasm. Since the PP1c catalytic subunit lacks intrinsic targeting sequences, MYPT1 dictates the subcellular localization of the functional MP complex (Sarkar et al., 2023). This trafficking is strictly governed by conformational changes that expose or obscure intrinsic nuclear localization and export (NES) signals, alongside phosphorylation-dependent interactions with transport machinery such as importins and 14–3–3 proteins (Hanada et al., 1998, 2001; Zhu et al., 2025). We propose that dephosphorylation at Thr853 by PPM1B triggers a conformational shift that favors nuclear export, likely by masking retention signals or unmasking the C-terminal leucine-rich NES. This spatial dynamic is highly consistent with previous reports demonstrating that MYPT1^{pT853} accumulates predominantly in the perinuclear and nuclear compartments, in contrast to the cytoskeletal localization of pThr696-MYPT1 (Chida et al., 2013; Lontay et al., 2005). Functionally, kinase-driven nuclear accumulation of the MYPT1 phosphorylated on Thr853 residue has been shown to mediate the Crm1-dependent export and transcriptional inhibition of targets like Nkx2.5 (Yu et al., 2024). Therefore, our findings establish PPM1B as a critical regulatory switch that not only modulates MP activity but also enforces its cytoplasmic retention, thereby directly antagonizing its nuclear functions.

Upon establishing its cytoplasmic presence, the reactivated MP complex achieves its canonical function by dephosphorylating MLC20 at Ser19. Because elevated Ser19 phosphorylation is necessary for stress fiber assembly, focal adhesion maturation, and the traction forces driving mesenchymal and amoeboid invasion, its targeted reduction is



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Fig. 4. Redistribution and modification of H4 upon PPM1B overexpression in HeLa cells. Western blot analysis was conducted following subcellular fractionation using anti-H4, anti-H4-symmetrical DM, anti-lamin A/C and anti-tubulin antibodies. Total lysates, cytoplasmic and nuclear fractions were examined under mock and FT-PPM1B treatments. Columns show raw expression and symmetric dimethylation levels. Values are shown as mean $\pm$ SD, $n = 3-5$. Data were compared using an unpaired two-tailed t -test where $p < 0.05$ (*) (A and F). Immunofluorescence staining was performed on control, mock and FT-PPM1B-transfected HeLa cells using fluorescent Alexa Fluor™ 488-conjugated anti-rabbit secondary antibody (as green) following incubation with anti-H4(B) and anti-H4-symmetrical DM (G) antibodies. Nuclei and F-actin were stained with DAPI (as blue) and Texas Red-phalloidin (as yellow), respectively. Scale bar: 10 μ m. Relative intensity values of total cellular, cytoplasmic and nuclear compartments were quantified and shown in form of columns for H4 expression and H4-symmetrical DM modification. Values indicate mean $\pm$ SD, $n = 3-9$. Statistical comparison was performed using one-way ANOVA where $p < 0.01$ (**), $p < 0.001$ (***) (C,D,E and H,I,J).

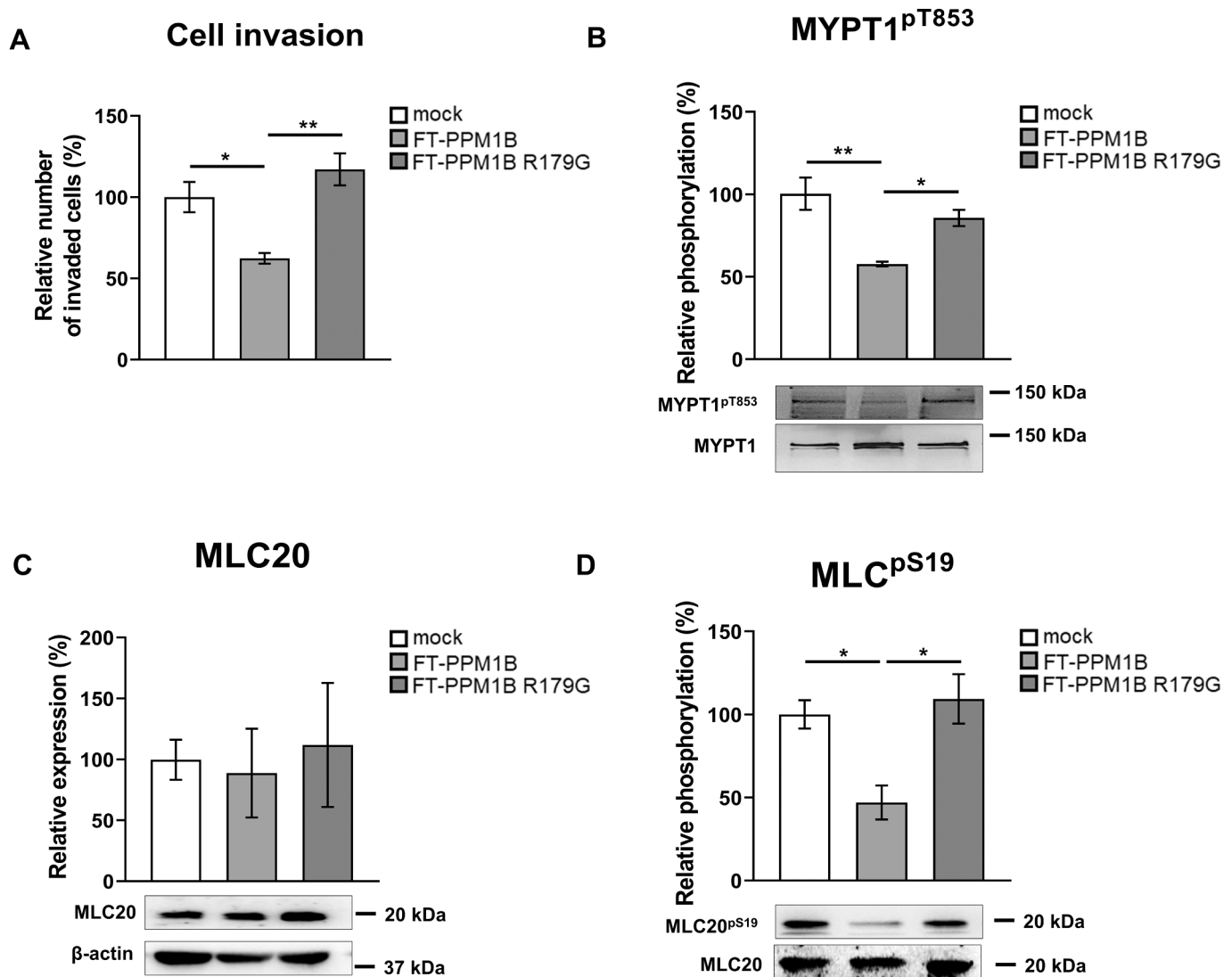


Fig. 5. Invasion capacity of wild-type and inactivated PPM1B expressing HeLa cells. Following transfection with empty vector, wild-type FT-PPM1B or R179G FT-PPM1B plasmids, HeLa cell invasion was analyzed using Boyden chambers (A). Invaded and CyQuant GR-labelled cells were lysed, and fluorescence signals were measured and analyzed. Columns represent the relative number of invaded cells in %. Values represent mean $\pm$ SD, $n = 3$. Comparison among multiple groups was performed using one-way ANOVA, where $p < 0.01$ (**). Mock, wild-type and mutant PPM1B-transfected cell lysates were analyzed by Western blot using anti-MYPT1, anti-MYPT1^{pT853} (B), anti-MLC20 (C), anti-MLC20^{pS19} (D) and anti- β -actin antibodies. Columns show relative expressions and phosphorylations in %. Values are shown as mean $\pm$ SD, $n = 3$. Data were compared using one-way ANOVA where $p < 0.05$ (*).

highly consequential (Terry et al., 2012; Vicente-Manzanares et al., 2009). We demonstrated that wild-type PPM1B overexpression profoundly decreases MLC20 Ser19 phosphorylation—without affecting total MLC20 levels—thereby biochemically relaxing the actomyosin cytoskeleton and severely suppressing invasive capacity. Crucially, the catalytically inactive PPM1B-R179G mutant failed to alter MLC20 phosphorylation or inhibit invasion, confirming that this tumor-suppressive phenotype relies strictly on PPM1B phosphatase activity rather than secondary scaffolding effects. This mechanism closely

aligns with the established biphasic requirement for MLC20 phosphorylation in cancer cell motility [46]. Optimal phosphorylation dictates non-muscle myosin II activation to coordinate the dynamic contraction-relaxation cycles necessary for leading-edge protrusion and trailing-edge detachment. However, just as hyper-phosphorylation induces a rigid, overly contractile state, the profound hypo-phosphorylation driven by excessive PPM1B activity abolishes the mechanical force required for migration (Totsukawa et al., 2000). Ultimately, both extremes severely disrupt dynamic actomyosin

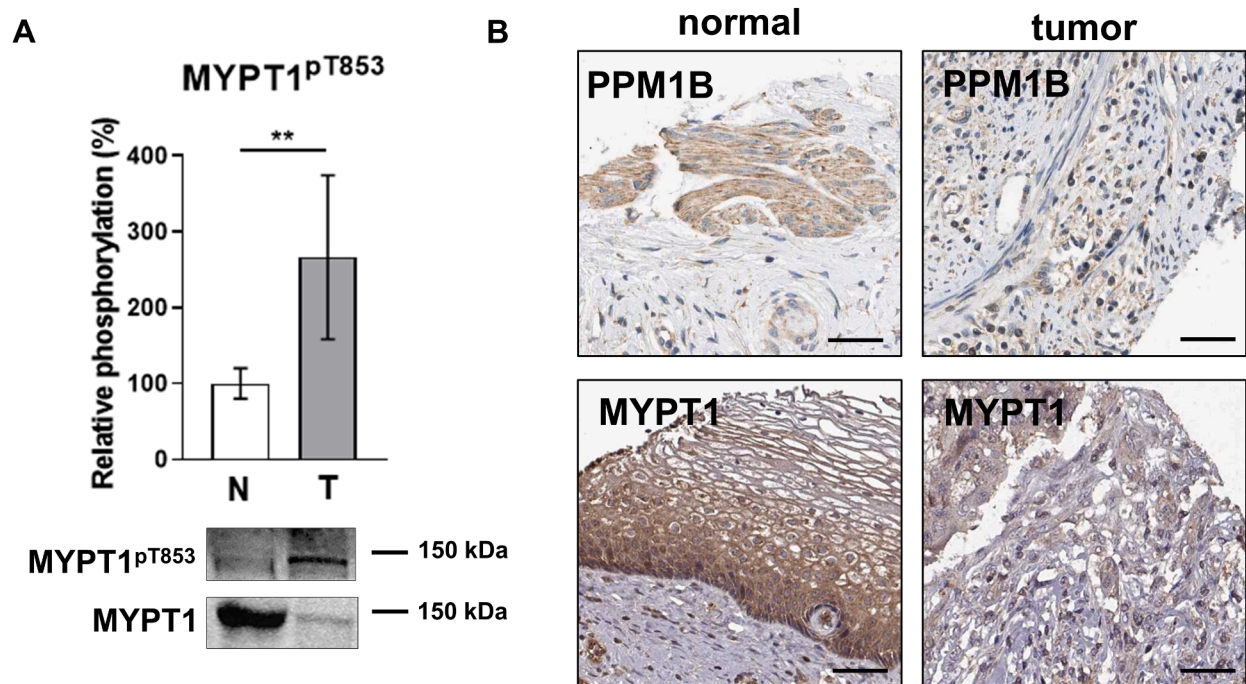


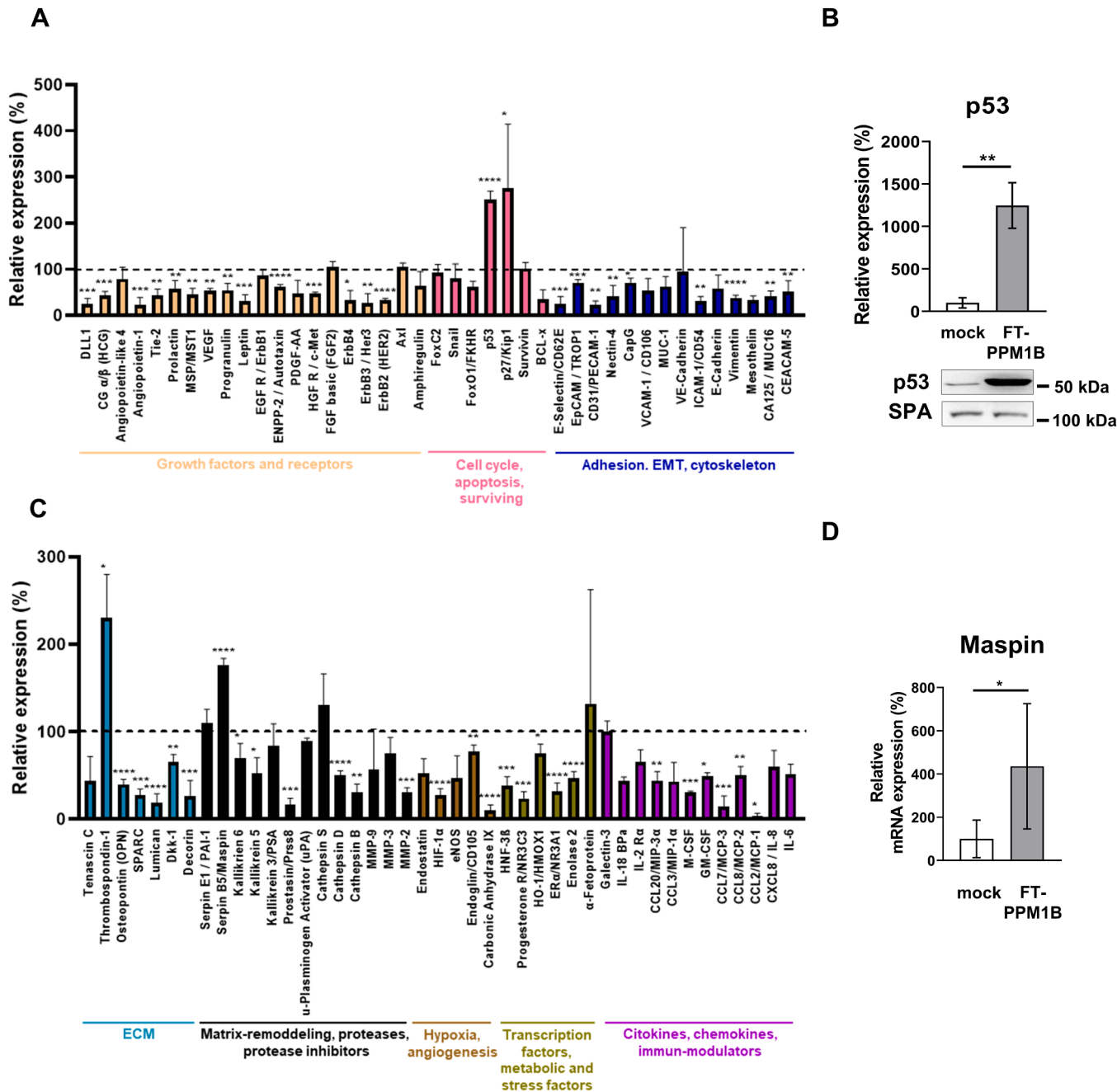
Fig. 6. MYPT1/PPM1B Expression and MYPT1 Thr853 Phosphorylation in Human Squamous Cell Carcinoma Cervical carcinoma cell lysates were purchased and analyzed by Western blot using anti-MYPT1, anti-MYPT1^{pT853} (A). Values mean relative phosphorylation in % and are shown as mean $\pm$ SD, $n = 6-7$. Comparison between two groups was performed using an unpaired two-tailed t-test where $p < 0.05$ (*). For presenting tissue slides (B) we used shared data from ProteinAtlas database under the following links: <https://www.proteinatlas.org/ENSG00000138032-PPM1B/tissue/cervix#img> (normal tissue, PPM1B), <https://www.proteinatlas.org/ENSG00000138032-PPM1B/cancer/cervix+cervix#img> (cancer tissue, PPM1B), <https://www.proteinatlas.org/ENSG00000138032-MYPT1/tissue/cervix#img> (normal tissue, MYPT1), <https://www.proteinatlas.org/ENSG00000138032-MYPT1/cancer/cervix+cervix#img> (cancer tissue, MYPT1).

remodeling, extinguishing migratory speed and directional efficiency [47–49].

The nuclear export of MYPT1 bears severe consequences for the nuclear oncogenic machinery, particularly the MP/PRMT5/H4 axis (Sipos et al., 2017). PRMT5 is frequently overexpressed in solid tumors (Elurbide et al., 2024; Lattouf et al., 2019), where it orchestrates the symmetric dimethylation of histone H4 to epigenetically silence tumor-suppressor genes (Bonday et al., 2018; Zheng et al., 2019). PRMT5 activity is heavily dependent on the activating phosphorylation of its Thr80 residue (Sipos et al., 2017). Our data demonstrate that PPM1B overexpression uniformly abolishes PRMT5 Thr80 phosphorylation levels and concurrently eradicates H4 symmetric dimethylation. The structural and epigenetic shifts induced by PPM1B are reflected in a global, systemic rewiring of the cellular proteome. Our XL Oncology Array data revealed a coordinated downregulation of proto-oncogenes and drivers of epithelial-mesenchymal transition, alongside a robust upregulation of key tumor-suppressors. Although the lack of robust E-cadherin upregulation suggests that PPM1B overexpression does not induce a canonical, full-scale mesenchymal-to-epithelial transition, the marked downregulation of mesenchymal and cell-adhesion markers—specifically vimentin, EpCAM, ICAM-1, and CD31—strongly implies a suppression of partial EMT (pEMT) dynamics. Rather than acting as a definitive master regulator of a fixed epithelial state, PPM1B likely serves as a critical checkpoint that destabilizes the motile, invasive phenotype of these cancer cells. The expression of these EMT markers is heavily reliant on intact mechanotransduction signaling pathways (such as the YAP/TAZ axis), which require a rigid, highly contractile cytoskeleton to enter the nucleus and drive transcription. By relaxing cytoskeletal tension via the MYPT1/MLC20 axis, PPM1B disrupts this mechanosensory feedback loop, blocking the mechanotransduction pathways that trigger EMT genes. Simultaneously, PPM1B overexpression triggered the dramatic upregulation of classical tumor-suppressors, including p53 (Hernandez Borrero and El-Deiry,

2021), the cyclin-dependent kinase inhibitor p27/Kip (Kwon et al., 1996), and the metastasis suppressor Maspin (SERPINB5) (Hernandez Borrero and El-Deiry, 2021; da Silva et al., 2025). The robust transcriptional induction of Maspin—independently verified by RT-qPCR—further solidifies the role of PPM1B as a regulator of cellular plasticity, actively enforcing a non-motile, epithelial-like state.

The extensive phenotypic alterations driven by PPM1B are linked to the modulation of global kinase networks. Our phospho-kinase array demonstrated a targeted suppression of stress-activated survival kinases, notably JNK1/2 and the transcription factor CREB that was escorted with a decrease of the activating phosphorylation of specific kinase, Akt (pSer473). However, we also observed a paradoxical increase in the phosphorylation of other kinases, such as p38 α (pThr180/pTyr182) and Chk-2 (pThr68), despite the globally elevated phosphatase background. This unexpected kinase activation likely represents a highly coordinated, secondary cellular stress response. The profound structural collapse of the actin cytoskeleton, combined with the acute epigenetic change of the genome, likely induces substantial cellular stress, triggering the activation of the Chk-2 and p38 α stress-kinase cascades. This hypothesis is strongly supported by the hyperphosphorylation of p53 at Ser46, since p38 phosphorylates the p53 tumor-suppressor at Ser46 shifting its transcriptional focus toward activating pro-apoptotic target genes (Bulavin et al., 1999). Unlike other p53 phosphorylation sites that merely induce cell cycle arrest, pSer46 is a specific, high-threshold mark generated by stress kinases that commits the cell to irreversible apoptosis or senescence in response to severe oncogenic stress (Oda et al., 2000). Together, these data suggest that PPM1B acts as a dual-edged sword against malignancy: it directly inhibits the mechanical and epigenetic machinery required for invasion, while simultaneously inflicting structural stress that hyperactivates the p53 tumor-suppressive signaling network.



5. Conclusions

In summary, our findings position PPM1B as a crucial negative regulator of cervical carcinoma progression. By serving as the direct catalytic switch that dephosphorylates MYPT1 at Thr853, PPM1B uncouples oncogenic kinase signaling from the cytoskeleton and the epigenome. The invasive cancer phenotype is synergistically reversed by the subsequent clearance of nuclear MYPT1, the disruption of the PRMT5 axis, and the inhibition of actomyosin contractility. These data illuminate the sophisticated interactions between structural

mechanobiology and epigenetic regulation, and consequently, these data strongly support the investigation of PPM1B-activating compounds as an effective, multifaceted therapeutic intervention for metastatic solid tumors.

Declarations

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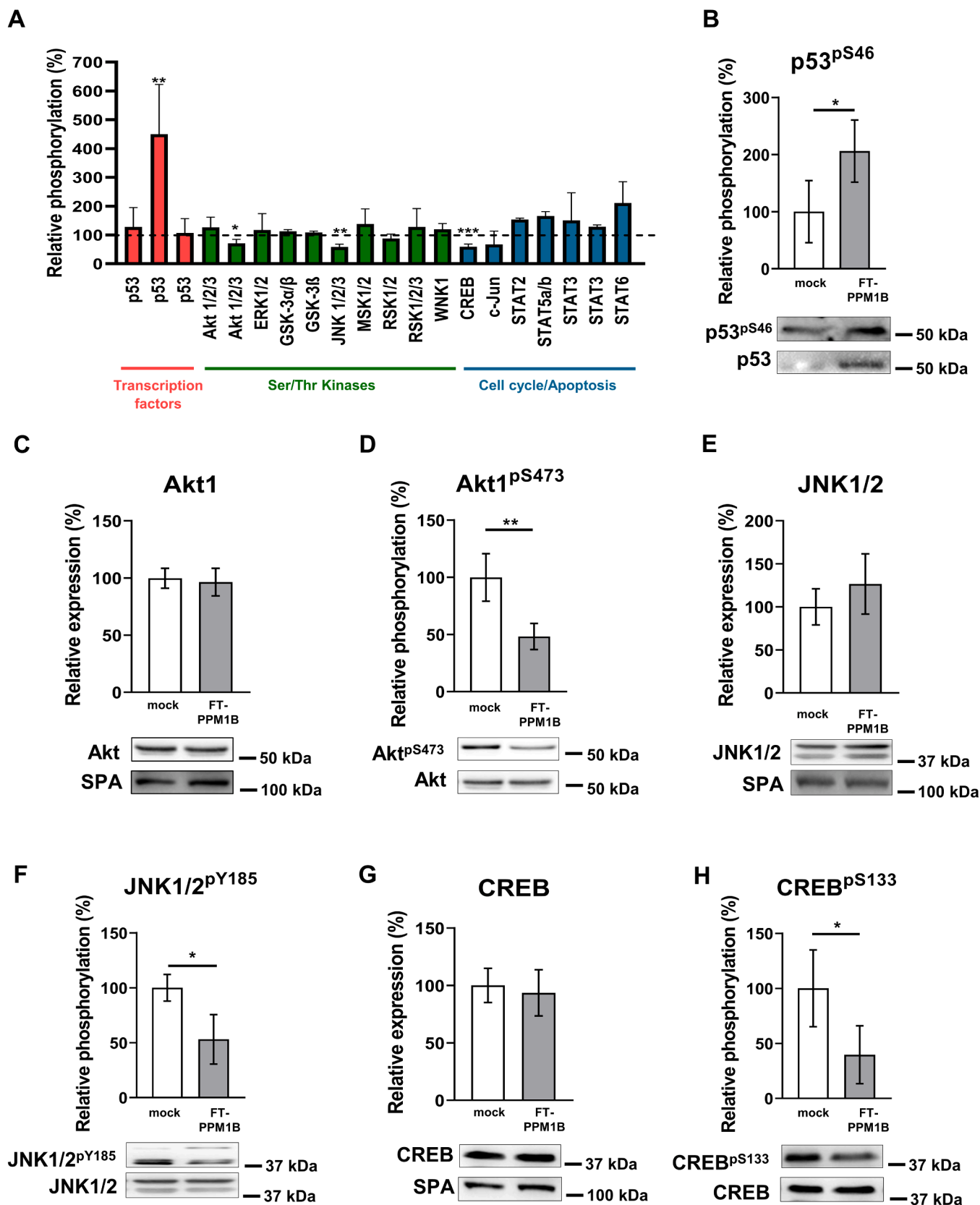


Fig. 8. PPM1B-dependent Phosphorylations of key oncogenic and tumor-suppressor kinases. Phosphorylation levels of several regulatory kinases were investigated using the R&D Systems Proteome Profiler Human Phospho-Kinase Array Kit in control and PPM1B-overexpressing samples. Each phosphorylation site was detected in duplicate. Relative phosphorylations in % are presented as bar charts and heat maps (mean $\pm$ SD, $n = 4$). Two groups were compared using unpaired two-tailed t -tests where $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) (A). Control and FT-PPM1B-transfected cell lysates were analysed by Western blot using anti-p53 and anti-p53^{pS46}(B), anti-Akt1 (C), anti-Akt1^{pS473}(D), anti-JNK1/2(E), anti-JNK1/2^{pY185}(F), anti-CREB(G), anti-CREB^{pS133}(H), and anti-Na⁺/K⁺-ATPase (SPA) antibodies. Columns of relative protein expression values are presented as mean, $n = 3-6$. Statistical analysis was performed using unpaired two-tailed t -tests where $p < 0.05$ (*), $p < 0.01$ (**).

CRediT authorship contribution statement

Ádám Ungvári: Writing – original draft, Visualization, Validation, Methodology, Formal analysis. **Ilka Keller:** Visualization, Methodology, Formal analysis. **Zoltán Kónya:** Methodology. **Richárd Kinter:** Methodology, Formal analysis. **Demény Máté Ágoston:** Methodology. **Zoltán Ödön Nagyenyedi:** Methodology. **Andrea Kiss:** Validation, Methodology. **Ferenc Erdódi:** Writing – review & editing. **Beata Lontay:** Supervision, Resources, Funding acquisition, Data curation, Conceptualization.

Declaration of Generative AI and AI-assisted technologies in the writing process

Generative AI and AI-assisted technologies were used in the writing process to check grammar and spelling.

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Declaration of Competing Interest

The authors have declared that no financial and non-financial competing interests exist.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.ejcb.2026.151559](https://doi.org/10.1016/j.ejcb.2026.151559).

Data availability

<https://doi.org/10.6084/m9.figshare.31742770.v1>

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