Roles of TIF1β Phosphorylation in Colorectal Cancer

大腸がんにおける TIF1β のリン酸化の役割

2017

筑波大学グローバル教育院

School of the Integrative and Global Majors in University of Tsukuba

Ph.D. Program in Human Biology

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PREFACE

The global cancer burden is increasing rapidly nowadays. Many people are diagnosed with cancer each year and more than half of the patients eventually die from it. Cancer is the second common cause of death following cardiovascular diseases. However, cancer has or will soon become the number one killer around the world with significant improvement in therapy and prevention of cardiovascular diseases. As population aging continues in many countries and elderly people are easier to be diagnosed with cancer, cancer will remain a major health problem around the world.

My scientific interest in cancer research had emerged since the first time I got involved in two projects when I was a master student in Taiwan, wherein I first studied the effects of Celastrol, a triterpene from traditional Chinese medicine, on modulating the anticancer effects of TRAIL in oral squamous cell carcinoma, and next I studied how cancer stem cells acquire and maintain their pluripotency in lung cancer. After I had joined Ph. D. Program in Human Biology which encouraged us to understand and solve the global issues, I decided to continuously work on cancer research to contribute and involve in this field.

In this dissertation, I focused on the biological functions of an epigenetic modulator, Transcription Intermediary Factor 1 beta (TIF1β), especially its phosphorylation status in cancer progression. Several studies have implicated that TIF1β is involved in tumor development, but its molecular mechanism has remained unclear. Interestingly, the Ser473 residue of TIF1β may serve as a molecular switch regulates the chromatin remodeling and expression of particular genes through its phosphorylation status. In addition, the dynamic state of phosphorylation has been postulated to involve in a wide

range of processes in cancer. The imbalance between kinases and phosphatases leads to change protein phosphorylation status which has also amply reported in cancer progression. Therefore, I investigated which phosphatase and kinases are responsible for regulating phosphorylation of TIF1β at Ser473 and its roles in cancer. This dissertation describes two projects aimed to contribute to understanding the biological functions underlying TIF1β phosphorylation in different perspectives. The first project (Chapter 1) entitled "Protein phosphatase 4 dephosphorylates TIF1β and increases drug resistance in poorly differentiated colorectal cancer", revealed protein phosphatase 4 involves in regulating of TIF1β dephosphorylation, slow proliferation but promote drug resistance in poorly differentiated colorectal cancer. The second project (Chapter 2) entitled "Oxidative stress-induced phosphorylation of ser473 in TIF1β by mitogen-activated protein kinases", revealed oxidative stress induces phosphorylation of TIF1β through p38 MAPKs in response to DNA damage. The diversities of TIF1β phosphorylation regulatory stimuli may suggest that TIF1β-Ser473 phosphorylation serves as a hub of the different stimuli to connect the common chromatin and centromere regulation.

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Chapter 1 Protein phosphatase 4 dephosphorylates TIF1β and increases drug resistance in poorly differentiated colorectal cancer

1.1 Abstract

Protein phosphatase 4 is a serine/threonine phosphatase that has critical functions in DNA double-strand break repair and cell cycle by the regulation of phosphorylation of its target proteins, such as Transcription Intermediary Factor 1 beta (TIF1β), which is an intermediary factor of transcription and epigenetic modulator of gene expression in several physiological processes including embryonic development and cellular differentiation as well as in cancer initiation and progression. However, gaps exist in our knowledge how PP4C functions in TIF1β phosphorylation and what the biological consequence is in cancer. Comparisons of immunohistochemistry staining on different differentiation status of human colorectal cancer (CRC) tissues showed that PP4C is upregulated in moderate and poorly differentiated CRC. Phosphorylated TIF1β is correspondingly decreased from well-differentiated to poorly differentiated tumors, suggesting a link among PP4C, TIF1β dephosphorylation, and tumor aggressiveness. We demonstrated that TIF1β phosphorylation is induced by Epidermal Growth Factor via RAS-MEK-ERK signaling pathway, which stimulates cell proliferation and it can be dephosphorylated by PP4C. In addition, ectopic expression of PP4C in human CRC cells promoted anticancer drug resistance. These results suggest that PP4C is involved in TIF1β dephosphorylation, slow proliferation but promotes drug resistance in poorly differentiated colorectal cancer.

1.2 Introduction

1.2.1 Post-Translational Modification: Phosphorylation

Protein phosphorylation on serine (S), threonine (T) and tyrosine (Y) residues is critical and well-reported, reversible, post-translational modification (PTM) regulates diverse intracellular processes such as the cell cycle, apoptosis, and differentiation [1]. Protein phosphorylation signaling is required to change protein structure, activity, and help modulate interaction with other protein. There is ample evidence within the literature that, in particular, dysregulation of the balance between kinases and protein phosphatases leads to change in protein phosphorylation status and subsequent cancer progression [2]. The balance between activation and deactivation of signaling pathways is regulated by kinases for phosphorylation and by a diverse range of phosphatases through dephosphorylation.

Phosphorylation is essential for cancer [3]. Although abundant evidence supports the role of the kinase in cancer [4], the mechanistic study of protein phosphatases [5] and their regulation has only recently expanded into research aimed at shedding light on the importance of these proteins in cancer. In addition, specific protein phosphatases may mediate cancer progression by acting as principal regulators of both kinase activation and the phosphorylation status of proteins involved in signal transduction.

1.2.2 Protein Phosphatase 4 (PP4) in Cancer

Protein phosphatases are divided into three major groups based on their substrate specificity: serine/threonine phosphatases, tyrosine phosphatases, and dual-specificity phosphatases [6]. Classification of protein serine/threonine phosphatases into five groups is based on their ability to specific substrates, biological functions and sensitivity to specific inhibitors [7].

Protein phosphatase 4 (PP4) is a protein phosphatase 2A (PP2A)-related, serine/threonine phosphatase that shares 65% amino acid sequence identity with PP2A [8]. It has been shown that PP4 is required for DNA double-strand breaks (DSBs). A PP4 phosphatase complex dephosphorylates replication protein A 32 kDa subunit (RPA2) to facilitate HDR-mediated repair of DSBs [9]. Another PP4C-PP4R2 containing phosphatase complex is involved in NHEJ-mediated repair of DSBs, partially through regulation the phosphorylation status of TIF1β [10]. Protein phosphatase 4 catalytic subunit (PP4C) plays a major role in microtubule growth/organization [11], apoptosis [12], tumor necrosis factor signaling [13], and pre-T-cell receptor signaling. Genetic deletion of the PP4 catalytic subunit PP4C in mice results in early embryonic lethality [14]. Several putative PP4C-containing complexes have been identified, but their biological functions remain unclear, and so far there are only a few substrates of PP4.

Of critical importance to the study of cancer progression, PP4 has been shown to be highly expressed in human breast and lung cancer while inhibition of PP4C expression sensitized such cells to cisplatin treatment [15]. High expression of PP4 is also correlated with poor prognosis in patients with stage II pancreatic ductal adenocarcinoma [16]. PP4 activated JNK-1 in prostate carcinoma cell lines, PC-3 and LNCaP, and increase the activities of c-Jun/activator protein (AP-1) and EGR-1 [17]. In addition, PP4 regulated the survival of both leukemic T cells and untransformed human peripheral blood T cells and had been demonstrated to play a significant role in the development and progression of leukemia [18]. These data suggested the PP4 may play a major role in human malignancies (Appendix 1). However, the expression and the functions of PP4 in the tumorigenesis and tumor progression remain unclear.

1.2.3 Transcription Intermediary Factor 1 beta (TIF1β)

Quantitative phosphoproteomics of PP4 depletion revealed that transcription intermediary factor 1 beta (TIF1β) is a putative substrate of PP4 [19]. TIF1β is also called tripartite motif-containing protein 28 (TRIM28) or KRAB-associated protein 1 (KAP1). It is a universal nuclear corepressor with conserved domains, *i.e.* RING fingers, B boxes, leucine zipper α helical coiled-coil region, plant homo domain finger, and bromo domain for Kruppel-associated box zinc finger proteins (KRAB-ZFPs) which bind DNA in a sequence-specific fashion [20] (Appendix 2). TIF1β is a critical regulator of normal development, differentiation (see TIF1β in the Transcription Factor Encyclopedia Database) and involved in maintaining pluripotency in mouse embryonic stem cells [21]. TIF1β is a scaffold for a multimolecular complex which can assemble epigenetic machinery to form heterochromatin structures resulting in gene silencing by recruiting histone methyltransferase SETDB1 [22], heterochromatin protein 1 (HP1) [23], or the NuRD-histone deacetylase complex [24].

In addition, several studies have implicated that TIF1β is involved in tumor development. TIF1β protein levels are increased in liver, gastric, lung, breast, and prostate cancer (see TIF1β in Human Protein Atlas websites, http://www.proteinatlas.org/, Appendix 3). Gastric cancer [25], non-small cell lung cancer [26], colorectal cancer [27] and cervical cancer patients [28] with high mRNA levels of TIF1β showing a significantly lower survival rate suggested that there were the biological functions and clinical significance of TIF1β in various cancers. Moreover, TIF1β can promote the invasion and metastasis of cancer cells by regulating the epithelial-mesenchymal transition (EMT) [29]. However, the molecular mechanism of TIF1β in tumor progression has not been identified.

1.2.4 Phosphorylated TIF1β at Serine 473

TIF1β has been identified many phosphorylation sites by nuclear phosphoprotein analysis [30]. Serine phosphorylation sites of TIF1β at serine 473 (Ser473) and serine 824 (Ser824) are most frequently studied. The Ser473 residue of TIF1β located near the upstream of the HP1 binding motif, PXVXL, which could compromise the binding of TIF1β and HP1 [31]. The phosphorylation status of TIF1β-Ser473 may act as a molecular switch through the interaction of HP1-TIF1β resulting the chromatin structures and the expression of particular genes (Appendix 4A). The human PP4 phosphatase complex, PP4C-PP4R2-PP4R3, was identified by mass spectrometry [32]. PP4C-PP4R2-PP4R3β directly dephosphorylates both of TIF1β phosphoresidues, serine 473 and serine 824 in DNA damage response [19]. Ataxia-telangiectasia-mutated (ATM) kinase induced TIF1β phosphorylation at Ser824 is essential for DNA damaged repair [33]. Although the phosphorylation of Ser473 has been reported, the functional implications of this modification have not been described. Our previous data demonstrated that the context sequence around Ser473 residue of TIF1β is highly conserved among mammals (Appendix 4B). A comparison of the immunohistochemistry staining data on basal layer of human normal oral epithelia and crypt region of mouse intestinal epithelial has shown that the phosphorylation of TIF1β at Ser473 is an event closely correlated with cell proliferation. In addition, phosphorylation of TIF1β at Ser473 is highly expressed in mouse intestine adenoma compared with adjacent normal tissue. Therefore, I would like to investigate the molecular mechanisms regulating phosphorylation of TIF1β at Ser473 through PP4 and its specific kinase, as well as its roles in tumor progression.

1.2.5 Colorectal Cancer (CRC)

The incidence of colorectal cancer (CRC) is increasing, and globally, this malignant disease has the third highest incidence (after breast and lung cancer) and the fourth highest mortality rate (after lung, liver and stomach cancer) [34]. In the first stages of its development, surgical intervention can treat CRC. In later stages, it has a high capacity to form secondary tumors, mainly in the liver and lungs. Nearly, one-fourth of the CRC patients have metastasis, and overall survival was not more than six months to one year. Although the mainstay treatment is surgical resection for patients with localized, non-metastatic disease, adjuvant chemotherapy can reduce the risk of recurrence by 40% to 50% in high-risk patients [35]. However, the current chemotherapy regiment has significant adverse effects. Therefore, an alternative therapeutic approach is needed for patients with colorectal cancer suffering from metastasis.

1.2.6 Hypothesis and Approach

A multistep process of the delicate homeostatic balance between cell proliferation, differentiation, and apoptosis is involved in the colorectal carcinogenesis. In addition, the balance of phosphorylation and de-phosphorylation is important in regulating cellular proliferation and division. In this research project, we would like to understand whether the homeostatic balance of phosphorylation is critical in the multistep process of colorectal carcinogenesis. Therefore, I would like to investigate the roles of PP4 in balancing phosphorylated TIF1β by using different differentiation status of patients with colorectal cancer and different ages of $APC^{min/+}$ mice (a popular animal model for studies of human colorectal cancer) and explore upstream signaling pathway and kinase of phosphorylated TIF1β in several human colon cancer cell lines.

1.3 Specific Aims

The expression and the prognostic significance of PP4 in patients in poorly differentiated colorectal cancer have not been studied yet. In addition, the mechanism of TIF1β, a putative substrate of PP4, involving in tumor progression is still unclear. PP4 directly dephosphorylates TIF1β phosphorylation at serine 473 and may switch its role in cell proliferation and differentiation during tumor progression.

Therefore, specific aims are listed in the following:

1. To examine whether PP4C expression is correlated with different pathology grades of human colorectal cancer (CRC) tissues
2. To compare PP4C expression and phospho-TIF1β level in CRC aggressiveness
3. To investigate the upstream signaling pathway of TIF1β phosphorylation
4. To confirm whether PP4C regulate dephosphorylation of TIF1β at Ser473
5. To evaluate the functions of ectopic expression of PP4C in CRC cells
6. To examine the potential of anticancer drug resistance in PP4C expressing cells

1.4 Materials and Methods

1.4.1 Antibodies

Mouse monoclonal antibody against TIF1β (clone 20A1) and anti-TIF1β-S473 phosphorylation antibodies were provided by Dr. Sheng-Chung Lee. Rabbit polyclonal antibody against p44/42 MAPK (Erk1/2, #9102), Phospho-p44/42 MAPK (Thr202/Tyr204) (#9101S) was from Cell Signaling, and Ki-67 was from Santa Cruz (sc-15402). Rabbit polyclonal antibody against PPP4C (A300-835A), PPP4R2 (A300-838A) and PPP4R3β (A300-842A) were from Bethyl Laboratories.

1.4.2 Colon Tumor Survey Tissue Array

Tissue microarray slides (CO20811), each containing 190 cases of adenocarcinoma, 1 each of papillary adenoma and signet ring cell carcinoma, 12 normal tissue, 4 adjacent normal tissue, single core per case, were obtained from US Biomax.

1.4.3 $APC^{min/+}$ Mouse Model

After receiving approval, all animal experiments were carried out by the Institutional Animal Experiment Committee of the University of Tsukuba, and in accordance with the regulation for animal experiments in the university and with the fundamental guidelines for the proper conduct of animal experiments.

$APC^{min/+}$ mice were examined at 8, 12, 16 and 20 weeks after birth. The intestinal tissues from each mouse were opened along the longitudinal axis and rinsed with PBS and then fixed in 12% buffered formalin for 24 hours at room temperature. After fixation, intestines were dehydrated and embedded in paraffin by embedded machine.

1.4.4 Immunohistochemistry Staining

Paraffin-embedded sections (3um) were deparaffinized in xylene and rehydrated in graded ethanol from 100%, 100%, 95%, 70% for each 5minutes and eventually in desterilized water for 5 minutes. Antigen retrieval was performed with an autoclave in 10mM citric acid buffer (pH 6.0) followed by cooled down 20 minutes on ice. Endogenous peroxidase activity was quenched with 5% H_2O_2 for 30 min. Non-specific binding was blocked with goat serum in PBS (Biogenex) for 30 min prior to being probed with primary antibodies for 2 hours. The Dako Real Envision detection system and HRP-rabbit/mouse antibodies (Dako) were used to detect primary antibodies with DAB chromogen. Slides were counters-stained in hematoxylin. Visualize staining of tissue under a microscope using a bright-field illumination.

1.4.5 Immunohistochemistry Multiple-staining

The sections were deparaffinized in xylene and rehydrated in graded ethanol from 100%, 100%, 95%, 70% for each 5 minutes and eventually in desterilized water for 5 minutes. Antigen retrieval was performed with an autoclave in 10mM citric acid buffer (pH 6.0) followed by cooled down 20 minutes on ice. Endogenous peroxidase activity was quenched with 5% H_2O_2 for 30 min. Non-specific binding was blocked with goat serum in PBS (Biogenex) for 30 min prior to being probed with primary antibodies for 2 hours. The Dako Real Envision detection system and HRP-rabbit/mouse antibodies (Dako) were used to detect primary antibodies with AEC (3-amino-9-ethylcarbazole) substrate chromogen (Dako). Slides were counters-stained in hematoxylin and were coverslipped using aqueous mounting media. Visualize and photograph staining of tissue under a microscope using a bright-field illumination.

Before next antibody staining, coverslips can be removed by dipping into the desterilized water. Slides with AEC product and hematoxylin will be washed off with ethanol/alcohol solution and 0.1M Glycine-HCl (0.1 M, pH 2.2), respectively. Slides were followed the same procedures of general immunohistochemistry staining: antigen retrieval, blocking and then, against the indicated antibodies with HRP-rabbit/mouse antibodies, the final step is detecting primary antibodies with AEC substrate chromogen.

1.4.6 Immunohistochemistry Evaluation

We performed quantitative analysis of the tissue specimen without knowledge of specimen identification. Scoring was based on intensity and percentage of positively stained cells for PP4C, TIF1β and phospho-TIF1β (Ser473) by immunohistochemistry as follows: low; 1–30%, medium; 30–70%, high; ≥70% (Supplementary Fig. S5). All discrepancies were resolved by a second examination using a multiheaded microscope.

1.4.7 Cell Lines and Cell Culture

HaCaT cells (spontaneously immortalized human keratinocyte cell line) were obtained from N.E. Fusenig and maintained in Dulbecco's modified Eagle's medium (SIGMA) supplemented with 1% of penicillin–streptomycin solution (Gibco) and 10% dialyzed fetal bovine serum (FBS). Epithelial human colorectal adenocarcinoma Caco-2 cells and human embryonic kidney (HEK) 293T cells were maintained in Dulbecco's modified Eagle medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% of penicillin–streptomycin solution (Gibco) at 37℃ in a 5% CO_2 humidified atmosphere. Human colorectal adenocarcinoma cell lines, DLD-1 and COLO 320 cells, were cultured in RPMI 1640 medium (Invitrogen), which is supplemented with 10% fetal bovine serum

(FBS) and 1% of penicillin–streptomycin (PS) solution, at 37℃ in a 5% CO_2 humidified incubator. After growth to subconfluent state, cells were washed once, made quiescent by incubation in serum- and supplement-free medium for 18 hours, and then used for experiments.

1.4.8 EGF Stimulation

For EGF stimulation experiment, after starvation for 18 hours, cells were treated with EGF for the indicated time periods, and western blot was performed. For kinase inhibitor experiment, after starvation for 18 hours, cells were incubated with EGF receptor kinase inhibitor AG1478 (1uM, SIGMA) and MEK Inhibitor U0126 (10uM, SIGMA) for 10 minutes respectively before challenged with EGF.

1.4.9 Western Blot Analysis

Cells were solubilized in NP-40 lysis buffer (20 mM Tris-HCl, ph7.5, 150 mM NaCl, 1% Nonidet P-40, 2000 KIU/ml aprotinin, 1 ug/ml leupeptin). After clearing by centrifugation, total cell lysates or immunoprecipitates obtained using the indicated antibodies were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electrotransferred to a mixed ester nitrocellulose membrane (Hybond-C Extra, GE Healthcare) and subjected to immunoblot analysis. Antibody-protein complexes were detected by using enhanced chemiluminescence (ECL) system (GE Healthcare, Buckinghamshire, UK) and were visualized by exposing membranes to LAS-4000 luminescent image analyzer (Fujifilm). Band signals were quantified by the software Multi-gauge V3.0 (Fujifilm).

1.4.10 Transfection and Plasmids

For plasmid transfection, HEK293T cells were reached about 85% confluence and transfected with plasmids using Lipofectamine 2000 reagent (Invitrogen) in Opti-MEM medium (Invitrogen) for 6 hours, after which the medium was replaced with fresh complete medium. After 24-48 hours, the transfected cells performed assays. We used Wild-type of Flag-mTIF1β plasmid was kindly provided by Dr. Sheng-Chung Lee (Institute of Molecular Medicine and Clinical Medicine, College of Medicine, National Taiwan University). Plasmids of the HRas wild-type, HRas G12V mutant form, ERK2 wild type, ERK2-dominant negative (DN) form and ERK-2 (L73P/S15D) constitutive active form (CA) expression constructs were kindly provided by Dr. Hiroyuki Suzuki (Department of Experimental Pathology, University of Tsukuba). The plasmid of pcDNA3.1+-DYK-PPP4C was purchased from GenScript (Clone ID: OHu16668).

1.4.11 Immunoprecipitation

Transfected-HEK293T cells were harvested and rinsed with normal saline. Whole cell extracts were prepared by treating the cells with lysis buffer which containing 20mM Tris/HCl, pH 7.4, 0.15M NaCl, 1mM DTT, 1mM EDTA, 1mM EGTA, 5% glycerol, 0.1% Triton X-100, 1X protease inhibitor (Roche) and 1X phosphatase inhibitor (Roche). Immunoprecipitation was performed by incubating the 500ug of protein from the cell lysates with anti-Flag M2 magnetic beads (Sigma) for 2 hours at 4℃. The M2 beads bound with precipitated proteins were washed 3 times with PBS+0.1% Tween20. The washed beads were added 2X SDS sample buffer and boiled at 95℃ for 5 minutes to dissociate the protein complex before western blot analysis.

1.4.12 Cell proliferation assay

Cells were seeded in the 24-well plate, cultured for the indicated time periods and counted with a hemocytometer.

1.4.13 Colony formation assay

Cells were trypsinized, and total 100 cells were reseeded in the 6-well plate for 2-weeks until each colony consisting 50 or more cells. Colonies were fixed with 10% neutral buffer formalin solution for 15 minutes and stained with 0.01% (w/v) crystal violet for 30 minutes. Visualize and photograph staining of colonies under a microscope using a bright-field illumination.

1.4.14 Cytotoxicity and cell growth

Cell survival was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Invitrogen) assay. Cells were seeded at a density of 5,000 cells per well in the 96-well plate and incubated at 37°C in humidified 5% CO_2 for 24 h. Serially diluted (RPMI or DMEM used as diluent) compound was added to give the intended final concentrations. Solvent tolerance testing up to 0.5% under identical conditions confirmed growth all cell lines was unaffected. Cells were then incubated an additional 72 h, and the MTT assay was performed according to the manufacturer's instructions (Molecular Probes, Eugene, OR). Absorbance values were determined at 570 nm on a Spectra Max 250 spectrophotometer (Molecular Devices, Sunnyvale, CA). All MTT assays were performed in triplicate. The 50% inhibitory concentration (IC50) values were defined as the drug concentrations required to reduce cellular proliferation to 50% of the untreated control well.

1.4.15 Sphere formation assay

Spheres were cultured in DMEM/F12 serum-free medium (Invitrogen) supplemented with B27 (Invitrogen), 20 ng/ml EGF (Sigma) and 20 ng/ml bFGF (R&D Systems) in an ultra-low attachment culture dish (Corning, NY, USA).

1.4.16 *In vivo* tumor formation assay

For cancer cell implantation, Five-week-old male ICR-nu/nu mice were purchased from Charles River. DLD-1 cells (1 x 10^7 cells/mouse) transfected with vector or PP4C in 200 μl of sterile PBS were subcutaneously injected into the flank region of the mice. After 1.5 months, the mice were sacrificed, and the tumors were weighed and measured. Tumor volume (V) was calculated using the following formula: ($width^2$ x length)/2. All animal experiments were approved by the animal experiment committee of the University of Tsukuba, performed in accordance with the university's animal experiment guidelines and the provisions of the Declaration of Helsinki in 1995.

1.4.17 *In vitro* phosphatase assay

HEK293T cells were transfected with Flag-PP4C or Flag-TIF1β to express particular proteins. Flag-PP4C and Flag-TIF1β proteins were purified by immunoprecipitation with anti-Flag antibody, respectively. Purified Flag-PP4C were then eluted with 3× Flag peptide. Different concentrations of eluted Flag-PP4C solution and purified Flag-TIF1β protein G-Sepharose beads were incubated together in *in vitro* phosphatase reaction buffer at 37 °C for 30 min and analyzed by Western blot.

1.4.18 Statistical analysis

Statistical analyses were carried out using a statics function in Microsoft Office (Microsoft, Redmond, WA, USA) and the t-test. Probability values of <0.05 were considered significant. *P<0.05, **P<0.01, ***P<0.001.

1.4.19 3-Dimensional Cell Culture System

Human dermal fibroblasts were mixed with a neutralized type I collagen gel (Cellmatrix type I-A, Nitta Gelatin Inc.) following the manufacturer's recommendations. The mixed solution was placed into 6 well plates (3 mL/well) and was hardened for 30 min in a CO_2 incubator at 37 °C in humidified air. HaCaT cells were then dispensed onto each gel at a density of 2×10^6 cells in 3 mL of complete 3D culture medium and were incubated overnight. The complete 3D culture medium consisted of a 1: 1 mixture of MCDB153 and DMEM, supplemented with 10% FBS, 100 µg/mL bovine pituitary extract (KOHJINBIO), 10 ng/mL EGF, 0.1 mM ethanolamine, 60 µM putrescine, 100 U/mL penicillin and 100 µg/mL streptomycin. The following day, the hardened gels were detached from the plates and incubation was continued for one week until the size of the contracted gel-stabilized. Cell strainers (Becton Dickinson) were placed upside-down into a fresh 6 well plate after removal of the strainer handles using sterilized scissors, and the 3D culture medium was poured into the wells until the nylon mesh of the strainers was covered. The contracted-gel discs were then placed on the mesh so that the HaCaT cells lay on the top of gel discs and the fluid level was adjusted to just below the upper edge of the gel. It was important that the gel was steeped sufficiently in the fluid, while at the same time its surface was exposed to air. Throughout the experiment, half of culture fluid was renewed every other day. After one week of an air-liquid interface culture, the gel

discs were fixed in a phosphate-buffered formalin solution, embedded in paraffin and vertical sections were stained with hematoxylin and eosin.

1.4.20 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RNA was isolated using TRIzol Reagent (Invitrogen) from cells following the manufacturer's instructions, and SuperScript^{TM} III First-strand synthesis system (Invitrogen) was performed in a final reaction volume of 20ul containing 600ng of total RNA in 1X RT buffer, 1X RT Random Hexamers, 1X dNTP Mix, 40U of RNase Out and 200U of SuperScript^{TM} III Reverse Transcriptase. The reaction mixture was incubated at 25℃ for 10min, 50℃ for 50min, and the reaction was terminated by heating at 85℃ for 5 min. Two microliters of the reaction mixture were amplified by polymerase chain reaction (PCR) with the pairs of primers. The PCR amplification was carried out in a 1X reaction buffer, 1mM dNTPs, 2U of Taq DNA polymerase, and 0.2uM each primer. The reactions were performed in a (BioRad) using the following protocol: Initial denaturing, 3min at 95℃; denaturing, 30 second at 95℃; annealing , 30 second at specific annealing temperature; and elongation, 30 second at 72℃ for a total of specific cycles, with a final extension of 72℃ for 4 minutes. Equal volumes of PCR samples were subjected to electrophoresis on a 1.5% agarose gel. The agarose gel was soaked in TBE buffer contained with 0.1% ethidium bromide (EtBr) and photographed under ultraviolet illumination.

1.5 Results

1.5.1 PP4C is upregulated in poorly differentiated CRC tissues

To investigate the roles of PP4C in colorectal cancer (CRC), we examined the tissue distribution of PP4C in a cohort of 208 CRC patients with adjacent non-tumorous tissues and normal colon tissues by immunohistochemistry. In cytopathology, the level of cellular differentiation is used as a measure of tumor aggressiveness. "Grade" is a marker of how differentiated a cell in a tumor is. The pathology diagnosis of grades in 1, 2 and 3 are equal to well-differentiated, moderately differentiated or poorly differentiated intestinal epithelium status. The data revealed that the expression of the PP4C protein was significantly upregulated in tumor tissues with moderately and poorly differentiated status compared with non-tumorous tissues (Figure 1A and 1B). As shown in Table 1, the potential correlation of PP4C expression with some clinicopathological parameters were examined in the patients which demonstrated that the PP4C protein expression is upregulated with grade (Grade 1, 29%; Grade 2, 53%; Grade 3, 56%) rather than with stage (Stage I, 49%; Stage II, 39%; Stage III, 17%; Stage IV, 20%). We further performed a meta-analysis of PP4C expression in overall survival of colorectal cancer from all available studies on Prognoscan (http://www.prognoscan.org/), the overall hazard ratio of 1.63 suggested that high expression of PP4C is associated with poor overall survival (Appendix 5). Taken together, these data provide the initial evidence that PP4C is upregulated in poorly differentiated CRC tissues, indicating that PP4C may be associated with increased tumor aggressiveness in CRC.

1.5.2 Phosphorylation level of TIF1β has a negative correlation with PP4C expression in CRC

Phosphoproteomic analysis revealed PP4C dephosphorylates TIF1β at Ser473 in the DNA damage response [19]. However, the regulation of PP4C on TIF1β in cancer associated with tumor aggressiveness is still unclear. To detect whether PP4C could dephosphorylate TIF1β in cancer, we first examined the phosphorylation level of TIF1β at Ser473 compared with PP4C expression on CRC patients by immunohistochemistry. The data revealed that the phosphorylation level of TIF1β was decreased while PP4C expression was increased in poorly differentiated CRC tissues (Figure 2A and 2B). As shown in Table 2, the potential correlation of TIF1β-Ser473 phosphorylation, with some clinicopathological parameters were examined in the patients, is decreased with grade (Grade 1, 54%; Grade 2, 45%; Grade 3, 34%) rather than with stage (Stage I, 45%; Stage II, 36%; Stage III, 67%; Stage IV, 0%). It has shown a negative correlation between PP4C expression and phosphorylation level of TIF1β in a tumor differentiation-dependent manner (Table 3). These data suggest the expression of PP4C may indicate the differentiation status and malignancy grades of the tumor, and PP4C may regulate TIF1β phosphorylation to promote tumor aggressiveness in CRC.

1.5.3 EGF induces TIF1β phosphorylation through RAS-MAPK-ERK signaling pathway

Next, we focused on possible signaling pathways inducing TIF1β phosphorylation. Epidermal Growth Factor (EGF) is a well-known growth factor that regulates cellular proliferation, differentiation, and survival [36]. Increased activity of the receptor for EGF has been observed in certain types of cancer, often correlated with mutations or amplification in the receptor and abnormal function such as constitutive receptor signaling independent of the levels of EGF or of binding of EGF [37]. Overexpressing EGF receptor (EGFR) is also seen in 22% to 75% of CRC. To test whether EGF induces TIF1β phosphorylation, we treated Caco-2 and DLD-1 cells with EGF and detected the phosphorylation level of TIF1β at Ser473 by western blots. TIF1β was phosphorylated within 1h, and the phosphorylation was decreased after 2h in Caco-2 cells and after 1h in DLD-1 cells of EGF treatment (Figure 3A). It suggested that TIF1β-Ser473 phosphorylation is transiently induced by EGF.

To identify which EGF signaling pathway is involved in TIFβ-Ser473 phosphorylation, Caco-2 and DLD-1 cells were treated with kinase inhibitors, including AG1478 (EGFR inhibitor), U0126 (MEK inhibitor), U-73122 (PLCγ inhibitor) or Rottlerin (PKCδ inhibitor) before EGF stimulation. As shown in Figure 3B, AG1478 (EGFR inhibitor) and U0126 (MEK inhibitor) prevented EGF-induced TIF1β phosphorylation in Caco-2 and DLD-1 cells compared with control (DMSO treatment). U-73122 (PLCγ inhibitor) or Rottlerin (PKCδ inhibitor) treatment has no effect. PKCδ has been reported that could meditate TIFβ-Ser473 phosphorylation in S phase of the cell cycle, but not in EGF signaling pathway.

To further confirm TIF1β-Ser473 phosphorylation is induced by EGF through

EGFR-MEK signaling pathway, we transfected wild-type of HA-HRas protein and constitutively active form of HA-HRas-G12V protein in HEK-293T cells and analyzed the endogenous TIF1β phosphorylation level by western blots. The data indicated that HRas-G12V protein increased TIF1β-Ser473 phosphorylation compared with wild type (Figure 3C). We also found that TIF1β-Ser473 phosphorylation is increased while transfected with a constitutively active form of ERK-2 protein (L73P/S15D) in HEK-293T cells compared with the wild-type of ERK-2 protein (Figure 3D). These data suggested that ERK protein has high possibility to be the specific kinase of TIF1β in EGF signaling pathway, and EGF could induce TIF1β-Ser473 phosphorylation through RAS-MEK-ERK signaling pathway on human colon cancer cell lines.

1.5.4 PP4C dephosphorylates EGF-induced TIF1β phosphorylation

In order to investigate whether PP4C regulates the phosphorylation level of TIF1β upon EGF stimulation, the status of TIF1β phosphorylation was measured in the HEK-293T cells transfected with pcDNA3.1-FLAG-PP4C. Western blot analysis of proteins extracted from HEK-293T PP4C-overexpressing cells revealed that PP4C caused a corresponding decrease in the EGF-induced phosphorylation state of TIF1β on Ser473 (Figure 4A). To determine whether PP4C can directly dephosphorylate EGF-induced TIF1β-Ser473 phosphorylation, we immunopurified Flag-PP4C and Flag-TIF1β respectively and performed in vitro dephosphorylation assay. PP4C dephosphorylates pTIF1β-Ser473 in a dose-dependent manner (Figure 4B). In response to EGF in control DLD-1 cells, there is a rapid increase in pTIF1β-Ser473, which peaks within 1h and significantly drops by 4h. In the presence of PP4C, there is a significantly lower amount of pTIF1β-Ser473 in cells at 2h after EGF stimulation (Figure 4C). In the absence of

PP4C, the intensity of EGF-induced TIF1β-Ser473 phosphorylation was stronger and longer compared with control and PP4C-overexpressing DLD-1 cells (Supplementary Fig. S1). Taken together, those data provide further evidence to support the involvement of PP4C in regulating the phosphorylation status of TIF1β at Ser473.

1.5.5 PP4C suppresses proliferation and tumorigenesis

It has been reported that PP4C had several roles in promoting and inhibiting of cell proliferation, we, therefore, tested the effect in PP4C-overexpressing DLD-1 cells. First, PP4C-overexpressing DLD-1 cells were established and confirmed by western blot with Flag antibody as shown in Figure 5A. We used MTT assay to measure cell proliferation for four days. All clones of PP4C-overexpressing DLD-1 cells had lower proliferation rate compared with control DLD-1 cells (Figure 5B). By colony formation assay, PP4C-overexpressing DLD-1 cells showed a significant decrease in colony numbers as compared to control (Figure 5C). We further investigated the functional significance of PP4C in sphere-forming activities. The sphere-forming activities of PP4C-overexpressing cells significantly reduced the sphere number compared with control DLD-1 cells (Figure 5D). In addition, we carried out xenograft assays in ICR-nu/nu mice. Control and PP4C-overexpressing DLD-1 cells were s.c. injected into ICR-nu/nu mice for one and half months, the tumors were collected and measured. As shown in Figure 5E, the tumors of the PP4C-overexpressing cells were significantly smaller than control cells in ICR-nu/nu mice. Taken together, those data suggested that ectopic expression of PP4C on DLD-1 cells suppress cell proliferation and tumorigenesis ability.

1.5.6 PP4C increases anticancer drug resistance in DLD-1 cells

Since most conventional chemotherapy agents preferentially target high proliferating cells, we hypothesized that low proliferative PP4C-overexpressing DLD-1 cells would be relatively drug resistant. To test this hypothesis, we monitored the viability of PP4C-overexpressing DLD-1 cells and control DLD-1 cells upon treatment with different chemotherapeutic drugs including cisplatin, doxorubicin, and etoposide. Cisplatin, doxorubicin, and etoposide are potent chemotherapeutic agents currently used in the treatment of many cancers. We found that all clones of PP4C-overexpressing DLD-1 cells were more drug resistant than control. (Figure 6A-C). Furthermore, PP4C-overexpressing cells were preferentially drug resistant to doxorubicin and etoposide compared to cisplatin. Altogether, our results highlighted the importance of regulation of TIF1β-Ser473 phosphorylation, through a homeostatic balance between PP4C and Ras/MEK/ERK signaling pathway in cellular proliferation as well as tumor aggressiveness on human colorectal cancer.

1.6 Discussion

The mammalian cell continuously receives signals from its surroundings to which it must respond appropriately, for instance, growth factor signals are integrated with internal state information and lead to decisions on cell growth, proliferation, or differentiation [38-40]. Many human diseases, including cancer, arise through deregulation of this information processing capability, such as protein phosphorylation. Several studies have demonstrated that TIF1β is overexpressed in several types of cancer and the overexpression of TIF1β is associated with poor prognosis [25-29]. The activity of TIF1β is regulated by phosphorylation in cancer development. In this study, we found phosphorylation of TIF1β at Ser473 was highly expressed in crypt region of mouse intestinal epithelium and adenoma of APCmin/+ mice confirmed that phosphorylated TIF1β-Ser473 is an event closely correlated with cellular proliferation, differentiation and benign tumor growth (Supplementary Fig. S2). It is interesting to speculate that signal from TIF1β-Ser473 phosphorylation may stimulate cell proliferation, which has been hypothesized its role during cell cycle progression. Furthermore, our current finding is the first report demonstrated that TIF1β phosphorylation at Ser473 is transiently activated by EGF through Ras-MAPK-ERK signaling pathway and its phosphorylation level can be suppressed in the presence of protein phosphatase 4. EGF is one of the high-affinity ligands of EGFR, and EGF/EGFR system is well-known to induce growth, differentiation, migration, adhesion and cell survival through various interacting signaling pathways [36, 37]. TIF1β-Ser473 phosphorylation is transiently induced by EGF not only in human CRC cells but also in other types of cell (Supplementary Fig. S3). Ras/Raf phosphorylates MEKs, and it activates the extracellular signal-regulated kinase (ERK), which then activates many of transcriptional regulators to induce cell growth and proliferation [41].

Therefore, we considered that phosphorylation site of TIF1β at Ser473 might serve as a regulator of cell growth and proliferation through EGF signaling pathway in cancer.

Specific protein phosphatases may play a vital role as kinases in cancer by acting as important regulators of protein phosphorylation involved in signal transduction, cellular proliferation, differentiation and tumor growth. Therefore, protein phosphatases can consider as one of the molecular targets of anticancer agents. In this study, we found that PP4C dephosphorylates EGF-induced TIF1β-Ser473 phosphorylation on CRC cells. PP4C has shown to be overexpressed in many types of cancer and has implications for tumor prognosis. This is the first report demonstrated that PP4C protein expression is upregulated with pathology grading system in colorectal cancer patients. In pathology, grading is a measure of the cell appearance in tumors. The grade score (Grade 1 to Grade 3) increases with the lack of cellular differentiation - it reflects how much the tumor cells differ from the cells of the normal tissue they have originated from. Based on the upregulation of PP4C in the poorly differentiated CRC tissues, we hypothesized that PP4C might promote certain phenotypes during CRC aggressiveness. We also found that PP4C expression is correlated with the low level of TIF1β phosphorylation. A negative correlation between PP4C expression and TIF1β phosphorylation in CRC differentiation-dependent manner (grading) suggested that PP4 may involve in regulation of TIF1β phosphorylation in CRC tumor aggressiveness.

In this study, we confirmed that PP4C suppressed DLD-1 cells proliferation, which has been demonstrated that overexpression of PP4C can suppress HEK293T cells proliferation by inducing apoptosis and promoting cell cycle arrest in G1 [42]. PP4C also suppressed cell migration compared with control (Supplementary Fig. S4). Furthermore, we found that PP4C also suppressed tumor growth and potentially increase anticancer drug resistance in DLD-1 cells. To our surprise, Dr. Sanjun Cai’s team had already

reported that PP4C, promotes cell growth and migration, has a potential role in tumor progression in SW480 and HT29 human colorectal cancer cell lines [43]. Moreover, Huang et al. reported that PP4 played dual roles during cell proliferation when both up-regulation and inhibition of PP4 inhibited cell proliferation [44]. The discrepancies among the various reports may be associated with the use of different cell types and techniques.

TIF1β-Ser473 has been suggested to be phosphorylated by PKCδ in early S-phase under normal culture conditions [31]; has been listed its temporal dynamics upon growth-factor stimulation and as one of potential ionizing radiation-induced phosphorylation sites by quantitative mass spectrometry experiment-based proteomics [45, 46]. TIF1β-Ser473 phosphorylation is required in DNA damage response and has been identified to be regulated by mitogen-activated protein kinase-activated protein (MAPKAP) kinase 2 (MK2) under Kaposi's sarcoma-associated herpesvirus (KSHV) infection [47]. Dr. David K. Ann's team recently reported that TIF1β-Ser473 phosphorylation is induced by nutrient depletion in breast cancer [48]. The discrepancies among the various reports may be associated with the use of different cell types and different stimulations.

This is the first study demonstrating that TIF1β can be post-translationally modified upon growth signal in cancer. Ideally, we would like to observe the functions of pTIF1β-Ser473 signals which is direct regulated by PP4C in colorectal cancer. However, due to unstable of cell line generations, our repeated efforts failed to get DLD-1 cell lines stably expressing mutant TIF1β-S473E or wild type TIF1β in the present or absent of PP4C. Moreover, whether PP4C directly regulate TIF1β-Ser473 phosphorylation status to suppress cellular proliferation and tumor growth deserves more attention. In the current study, we demonstrated that pTIF1β-Ser473 is transient induced upon EGF induction. Although the specific kinases of TIF1β in EGF signaling are unclear, it is possible that

ERK1/2 directly or indirectly regulates TIF1β-Ser473 phosphorylation upon EGF stimulation.

In conclusion, we show that there is a negative correlation between PP4C expression and TIF1β-Ser473 phosphorylation in CRC patients with pathology grading system (Figure 7A). TIF1β-Ser473 phosphorylation is transiently induced by EGF through Ras-MEK-ERK signaling pathway and is suppressed in the presence of PP4C on CRC cells (Figure 7B). In addition, ectopic expression of PP4C in CRC cell suppresses cell proliferation, tumor growth and promotes anticancer drug resistance. Our finding provides evidence supporting the potential value of PP4C in the regulation of TIF1β phosphorylation on CRC aggressiveness and suggesting that inhibition of PP4C may be an option to enhance the chemotherapeutic effect of conventional anticancer drugs toward CRC.

Chapter 2 Oxidative stress-induced phosphorylation of ser473 in TIF1β by mitogen-activated protein kinases

2.1 Abstract

Reactive oxygen species (ROS) cause significant damage to macromolecules including DNA and can be easily produced by large amounts of bacteria and dietary metabolites in the intracolonic cavity. One of ROS, hydrogen peroxide, is thought to be a signal molecular which plays a major role in the growth of tumor cells. TIF1β is a pleiotropic regulator of a diver range of the cellular process. Notably, phosphorylation of TIF1β at Serine 473 is required for an efficient DNA repair and cell survival in response to DNA damage. However, the effect of oxidative stress on TIF1β on the biological behaviors of colorectal cancer cells have not been determined. We report here that brief exposure of DLD-1 cells and HCT116 cells to hydrogen peroxide induces phosphorylation of TIF1β-Ser473. Hydrogen peroxide stimulation for short periods also induced phosphorylation of p42/44 MAPKs and p38 MAPKs. H_2O_2-induced phosphorylation of TIF1β was reduced when U0126 inhibited p42/44 MAPKs activity and when SB203580 inhibited p38 activity. It suggested that p42/44 MAPKs and p38 MAPKs are the mediators of oxidative stress-induced phosphorylation of ser473 in TIF1β. These data demonstrate the regulation of TIF1β phosphorylation by oxidative stress in colorectal cancer cells.

2.2 Introduction

Reactive oxygen species (ROS) include oxygen radicals, hydroxyl radicals, and hydrogen peroxide, are formed as a natural byproduct of the cellular metabolism of oxygen and play critical roles in the determination of cell fate by a wide variety of cell response, such as proliferation, differentiation, and cell death [49]. Under normal conditions, cells control ROS levels by balancing the generation of ROS with their scavenging system. Under oxidative stress conditions, the excessive amount of ROS results in significant damage to DNA, lipids, proteins and other macromolecules.

Large amounts of bacteria and dietary metabolites can easily produce ROS in the colon and rectum [50]. However, exposure of an organism to oxidative stress can result in imbalances of homeostasis and tumor formation. Studies had suggested that ROS were involved in the pathogenesis of colorectal cancer [51-53]. A special intermediate in a redox reaction, H_2O_2, can cross cell membranes in a free manner and modify protein and DNA into radicals, and it is thought to be a signal molecular regulating the growth of tumor cells [54]. Low levels of ROS may enhance cell survival and proliferation, whereas high levels of ROS may cause cell death. Also, microsatellite instability is caused by H_2O_2-induced oxidative DNA damage [55], which is associated with colorectal cancer. Therefore, not only ROS are implicated in the etiology of disease states [56, 57], but also the resulting DNA damage may also be a direct contributor to harmful biological consequence. Considering the particular environment in colon and rectum, I think it is necessary to evaluate the effects of ROS on the progression of human colorectal cancer.

Several studies have demonstrated that Transcription Intermediary Factor 1 beta (TIF1β) is highly expressed in several types of cancer [26, 28, 29] and high expression of TIF1β is associated with poor prognosis in colorectal cancer patients [27]. TIF1β is an

epigenetic modulator that can regulate chromatin remodeling and gene expression through interacting with heterochromatin protein 1 (HP1) and other chromatin factors. TIF1β has been suggested to influence DNA repair in heterochromatin through regulation of HP1 proteins and its interaction [58]. TIF1β also mediated cell survival after DNA damage in MCF-7 and HCT116 cells [59]. Interestingly, phosphorylation of TIF1β at serine 473 is required for efficient DNA repair and cell survival in response to DNA damage [60].

Several factors which can change the microenvironment or cellular responses were reported to induce phosphorylation in serine 473 of TIF1β. For instance, Mitogen-activated protein kinase-activated protein (MAPKAP) kinase 2 (MK2) under Kaposi's sarcoma-associated herpesvirus (KSHV) infection regulates phosphorylation of TIF1β-Ser473 to activate the proto-oncogene STAT3 [47]. Protein kinase Cδ (PKCδ) has been reported to regulate TIF1β phosphorylation at Ser473 in early S-phase under normal culture conditions for cell cycle progression and the proliferation of cells [31]. Phosphorylation of TIF1β-Ser473 is temporal dynamics upon growth-factor stimulation [45]. In addition, TIF1β-Ser473 phosphorylation is induced by nutrient depletion in breast cancer [48]. The diversities of TIF1β-Ser473 phosphorylation regulatory stimuli among the various reports may suggest that TIF1β-Ser473 phosphorylation serves as a hub of the different stimuli to connect the common chromatin and centromere regulation as well as response to DNA damage. However, little is known about the effect of oxidative stress on TIF1β-Ser473 in the biological functions of colorectal cancer cells.

In this study, we reported that H_2O_2 induces phosphorylation of TIF1β-Ser473 in a concentration- and time- dependent manner. In addition, H_2O_2 activated p42/44 MAPKs, and p38 MAPKs provide us a hint that those kinases may regulate phosphorylation of TIF1β under oxidative stress. Our data showed that H_2O_2-induced phosphorylation of

TIF1β-Ser473 could be suppressed by kinases inhibitors (U0126 and SB203580). Together, we suggest the oxidative stress induced TIF1β-Ser473 phosphorylation through the mediators, p42/44 MAPKs and p38 MAPKs, in colorectal cancer cells.

2.3 Specific Aims

ROS, especially hydrogen peroxide (H_2O_2), could be one of the important factors cause microsatellite instability involved in colorectal cancer progression. High expression of TIF1β is associated with poor prognosis in colorectal cancer patients. In addition, TIF1β may mediate cell survival after DNA damage through its Ser473 phosphorylation. Therefore, specific aims are listed in the following:

1. To examine whether H_2O_2 stimulate TIF1β phosphorylation at Ser473
2. To explore whether H_2O_2-induced phosphorylation of TIF1β-Ser473 effect on growth of cancer cells
3. To investigate the kinases responsible for H_2O_2-induced phosphorylation of TIF1β at Ser473

2.4 Materials and Methods

2.4.1 Cell lines and cell culture

Human colorectal adenocarcinoma HCT116 cells and human embryonic kidney (HEK) 293T cells were maintained in Dulbecco's modified Eagle medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% of a penicillin-streptomycin solution (Gibco) at 37°C in 5% CO_2 humidified atmosphere. Human colorectal adenocarcinoma DLD-1 cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% of a penicillin-streptomycin solution at 37℃ in a 5% CO_2 humidified atmosphere. After growth to subconfluent state, cells were washed once, made quiescent by incubation in serum- and supplement-free medium for 18 hours, and then used for experiments.

2.4.2 H_2O_2 treatment

For H_2O_2 stimulation experiment, after starvation for 18 hours, cells were treated with 1mM of H_2O_2 for the indicated time periods or different concentration of H_2O_2 for 15 mins, and western blot was performed. For kinase inhibitor experiment, after starvation for 18 hours, cells were incubated with MEK Inhibitor U0126 (10uM, SIGMA) and p38 MAPK SB203580 (10uM, Cayman Chemical) for 1 hour, respectively, before challenged with H_2O_2.

2.4.3 Antibodies

Mouse monoclonal antibody against TIF1β (20A1) and rabbit polyclonal antibody against TIF1β-S473 phosphorylation (Poly6446) were from Biolegend. Rabbit polyclonal antibody against p44/42 MAPK (Erk1/2, #9102), phospho-p44/42 MAPK

(Thr202/Tyr204) (#9101S), p38 MAPK (#9212), and phospho-p38 MAPK (Thr180/Tyr182) (#9211S) were purchased from Cell Signaling.

2.4.4 Transfection and plasmids

For plasmid transfection, HEK293T cells were reached about 85% confluence and transfected with plasmids using Lipofectamine 2000 reagent (Invitrogen) in Opti-MEM medium (Invitrogen) for 6 hours, after which the medium was replaced with fresh complete medium. After 24-48 hours, the transfected cells performed assays. We used Wild-type of Flag-mTIF1β, S473A of Flag-mTIF1β and S473E of Flag-mTIF1β plasmids were kindly provided by Dr. Han-Yi Chou (Graduate Institute of Oral Biology, School of Dentistry, National Taiwan University). We used Wild-type of p38α-HA plasmid was kindly provided by Dr. Hidenori Ichijo (Laboratory of Cell Signaling, Graduate School of Pharmaceutical Sciences, The University of Tokyo). All plasmids were sequenced before use.

2.4.5 Western Blot Analysis

Cells were solubilized in NP-40 lysis buffer (20 mM Tris-HCl, ph7.5, 150 mM NaCl, 1% Nonidet P-40, 2000 KIU/ml aprotinin, 1 ug/ml leupeptin). After clearing by centrifugation, total cell lysates or immunoprecipitates obtained using the indicated antibodies were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electrotransferred to a mixed ester nitrocellulose membrane (Hybond-C Extra, GE Healthcare) and subjected to immunoblot analysis.

2.4.6 Immunoprecipitation

The cell lysates were precleared with protein G-Sepharose beads (GE Healthcare) for 30 minutes at 4℃ with end-over-end rotation and then precipitated with Flag antibody (M2, Sigma) for 2 hours at 4℃. The immune complexes were precipitated by incubation with protein G-Sepharose beads for 30 minutes at 4℃, followed by three times wash with NP-40 lysis buffer. The immunoprecipitated proteins and aliquots of total cell lysates were subjected to western blotting.

2.4.7 Lambda Protein Phosphatase Assay

Cell lysates were collected from HEK293T cells and HCT116 cells in NP-40 lysis buffer without phosphatase inhibitor after treatment with hydrogen peroxide and were then incubated with λ protein phosphatase for 30 minutes at 37°C. Phosphatase was then inactivated by incubating the lysate at 65°C for 10 minutes.

2.5 Results

2.5.1 The phosphorylation of TIF1β on Ser473 is induced by hydrogen peroxide treatment in the colorectal cancer cell lines.

To explore the effect of oxidative stress on TIF1β-Ser473 phosphorylation, DLD-1 cells and HCT116 cells were treated for 15 minutes with different concentrations of H_2O_2 (Figure 8A and 8B) or treated with 1mM of H_2O_2 for various periods of time (Figure 8C and 8D). TIF1β-Ser473 phosphorylation was induced by H_2O_2 in a concentration- and time- dependent manner. Increased TIF1β phosphorylation was easily observed after an hour treatment of H_2O_2 in DLD-1 cells and a 15min treatment of H_2O_2 in HCT116 cells. We used the 1mM of H_2O_2 and shortest time (15min) for eliciting cellular signaling changes. This increase in phosphorylation was constant through 2hr of treatment with H_2O_2 in both DLD-1 and HCT116 cells.

To verify the specificity of this antibody, ectopically expressed FLAG-tagged TIF1β or mutants were immunoprecipitated from HEK293T cells with FLAG M2 beads and subjected to Western blotting. Unlike WT TIF1β, TIF1β Ser-473 mutants were not detected by the anti-phospho-TIF1β-Ser473 antibody (Figure 9A). To further ensure the band detected by the phospho-specific antibody under oxidative stress is indeed due to phosphorylation, cell lysates from HEK293T cells and HCT116 cells were incubated with lambda protein phosphatase, which is a Mn^{2+}-dependent protein phosphatase with activity towards phosphorylated serine, threonine and tyrosine residues, after H_2O_2 treatment to remove any phosphorylation. As shown in Figure 9B and 9C, the band corresponding to TIF1β phosphorylation is reduced with lambda phosphatase treatment, confirming that the TIF1β phospho-specific antibody was indeed detecting only the phosphorylated protein under oxidative stress.

2.5.2 Phosphorylation of TIF1β-Ser473 induced by hydrogen peroxide is mediated through MAPKs

Mitogen-activated protein kinases (MAPKs) have three subgroups: the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinase (JNKs), and the p38 MAPKs. They are involved in both cell growth and cell death [61]. ROS have been reported to activate ERKs, JNKs and p38 MAPKs, however the mechanism by which ROS can activate these kinases are still unclear [62]. To determine which kinase might be responsible for phosphorylation of TIF1β under oxidative stress, we examined the phosphorylation of p42/44 MAPKs and p38 MAPKs during different periods and different concentrations of H_2O_2 treatment. p42/44 MAPKs were activated in response to H_2O_2 treatment in DLD-1 cells (Supplementary Fig. S6). However, in HCT116 cells, only p38 MAPK were significantly activated in response to H_2O_2 treatment in concentration- and time- dependent manner (Figure 10). To further investigate the involvement of p42/44 MAPKs or p38 MAPKs in inducing TIF1β phosphorylation under oxidative stress, we firstly used U0126, a specific inhibitor of MEK, to block its activity. U0126 blocked p42/44 MAPKs phosphorylation and activation; however, it failed to inhibit TIF1β phosphorylation in DLD-1 cells under oxidative stress (Supplementary Fig. S7) but successfully prevented H_2O_2-induced phosphorylation of TIF1β in HCT116 cells (the middle panel of Figure 11A). This effect could be due to some differences between the cell line and basal levels of phosphorylation on p42/44 MAPKs. Since p38 MAPKs were also shown to be phosphorylated and activated by H_2O_2 in HCT116 cells, the involvement of p38 MAPKs in TIF1β phosphorylation was also explored. Cells were treated with a p38 inhibitor, SB203580 before treated with H_2O_2 (the right panel of Figure 11A). SB203580 prevented H_2O_2 induced phosphorylation of TIF1β, suggesting that p38

MAPKs is involved in regulating TIF1β phosphorylation during H_2O_2 treatment (Figure 11B). Taken all together, p42/44 MAPKs and p38 MAPKs which can be activated by H_2O_2 are the mediators of TIF1β phosphorylation under oxidative stress.

2.6 Discussion

ROS naturally and ubiquitously occur in all aerobic organisms. In the colon, there are large amounts of bacteria, and dietary metabolites to produce ROS. Diet rich in fat also increased the ROS production in feces, which may damage the stem cells in the colon [63]. Therefore, ROS could be one of the important factors involved in colorectal cancer progression. Hydrogen peroxide (H_2O_2) is well investigated in the inflammatory response and oxidant-induced stress. Recently, more evidence suggested that H_2O_2 can act as a signaling molecule involved in many cellular functions such as apoptosis and proliferation. In addition, the regulation of series of genes involved in carcinogenesis and progression is associated with the role of H_2O_2. Several reports suggested that high expression of TIF1β is related to poor prognosis in colorectal cancer patients and phosphorylation of TIF1β is related to DNA repair in response to DNA damage. Here, we showed that TIF1β phosphorylation is induced by hydrogen peroxide at Ser473 in concentration- and time- dependent manner. Its phosphorylation is mediated by p42/44 MAPKs and p38 MAPK which are also activated by oxidative stress.

Whether TIF1β is a direct substrate for both p42/44 MAPKs and p38 MAPKs or p42/44 MAPKs and p38 MAPKs cause the phosphorylation of TIF1B through other signaling intermediates is unknown at this time. It will be interesting to determine in the future how phosphorylation at Serine 473 of TIF1β, under conditions of increased oxidative stress, results in activation of different MAPKs signaling pathways, its interaction with other proteins and determine the cell fate to survive or die, which could make further diverse cellular changes, especially in cancer. Future studies will also characterize kinases/phosphatases which are responsible for the regulation of TIF1β phosphorylation under oxidative stress as well as its role in colorectal cancer progression.

Chapter 3 Summary, Significance, and Perspective

TIF1β is a multi-functional protein that might contribute to a tumor in different ways. This dissertation describes the consequences of S473 phosphorylation upon growth factor induction and under oxidative stress.

According to my observation, I could generalize the following conclusions:

I. Protein phosphatase 4 Catalytic subunit (PP4C) is upregulated in poorly differentiated CRC tissues, and there is a negative correlation between PP4 expression and phosphorylated TIF1β status in colorectal cancer tissues.

The immunohistochemistry data revealed that the expression of the PP4C protein was significantly upregulated with grade, rather than with stage, which is associated with tumor aggressiveness in CRC. The data also revealed that the phosphorylation level of TIF1β was decreased while expression of PP4C was increased in moderately and poorly differentiated CRC tissues suggested that PP4 may regulate TIF1β phosphorylation to promote tumor aggressiveness in CRC.

II. Epidermal growth factor (EGF) induced TIF1β phosphorylation at Ser473 via RAS-MEK-ERK signaling pathway, and it can be dephosphorylated by PP4C.

EGF, a well-known growth factor regulates cellular proliferation, induced TIF1β-Ser473 phosphorylation through Ras-MEK-ERK signaling pathway in colorectal cancer cells. In the presence of PP4C, by transient or stable expressed, significantly lower levels of TIF1β-Ser473 phosphorylation were observed throughout the time course after EGF stimulation supported the involvement of PP4C in the regulation of TIF1β-Ser473 phosphorylation.

III. PP4C suppressed cell growth and increase drug resistance in poorly differentiated colorectal cancer cells.

PP4C-expressing DLD-1 cells have shown a significant decrease the proliferation rates, colony numbers, sphere numbers, migration ranges, and tumor sizes compared with control DLD-1 cells. In addition, PP4C-exrpessing DLD-1 cells were preferentially drug resistant to anticancer drugs than control DLD-1 cells.

IV. Oxidative stress, hydrogen peroxide, induced TIF1β phosphorylation through activation of p42/44 MAPKs and p38 MAPKs.

H_2O_2 induced phosphorylation of TIF1β-Ser473 in a concentration- and time-dependent manner in colorectal cancer cell lines, DLD-1 and HCT116 cells. In addition, H_2O_2 activated p42/44 MAPKs and p38 MAPKs to regulate phosphorylation of TIF1β in response to DNA damage.

Several factors which can change the microenvironment or cellular responses were reported to induce phosphorylation in serine 473 of TIF1β for cell cycle progression and the proliferation of cells or for cells in response to DNA damage. To our knowledge, this is the first study directly demonstrating that TIF1β can be post-translationally modified upon growth signal in cancer cells. Ideally, we would like to observe the functions of pTIF1β-Ser473 signals which is direct regulated by PP4C in colorectal cancer. However, due to unstable of cell line generations, our repeated efforts failed to get DLD-1 cell lines stably expressing mutant TIF1β-S473E or wild type TIF1β in the present or absent of PP4C. Moreover, whether PP4C directly regulate TIF1β-Ser473 phosphorylation status to suppress cellular proliferation and tumor growth deserves more attention.

Interestingly, PP4C may also regulate TIF1β-Ser473 phosphorylation status under

oxidative stress in response to DNA damage. In this dissertation, we demonstrated that pTIF1β-Ser473 are transiently induced upon EGF induction and under oxidative stress in different perspectives. According to our results, TIF1β-Ser473 phosphorylation is likely mediated by the cooperation of ERK1/2 or p38 MAPKs and other kinases not identified in response to growth factor or oxidative stress in cancer.

Our results highlighted the importance of regulation of TIF1β-Ser473 phosphorylation, through a homeostatic balance between PP4C and Ras/MEK/ERK signaling pathway in cellular proliferation as well as tumor aggressiveness on human colorectal cancer. Our findings suggest not only the specific phosphatase of TIF1β for cancer cells response to growth factors has revealed, but also another response for cancer cells to adapt the oxidative stress during tumor progression and cancer therapy.

FIGURES

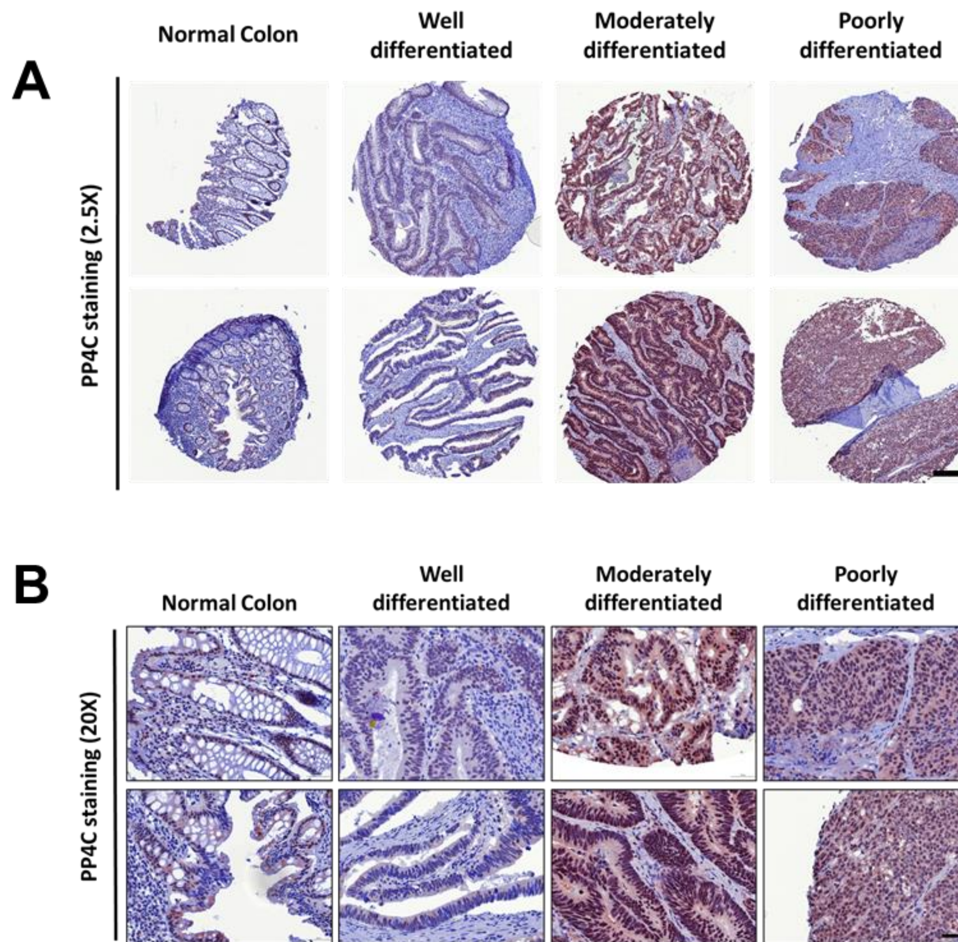


Figure 1 The expression of PP4C protein in different differentiation status of human colorectal cancer patients.

Human colon epithelium specimens from patients suffering from colon adenocarcinomas were analysis by immunohistochemistry staining with the PP4C antibody. The grade of low, middle and high in pathology diagnosis is equivalent to well-differentiated, moderately differentiated or poorly differentiated, respectively, under a microscope.

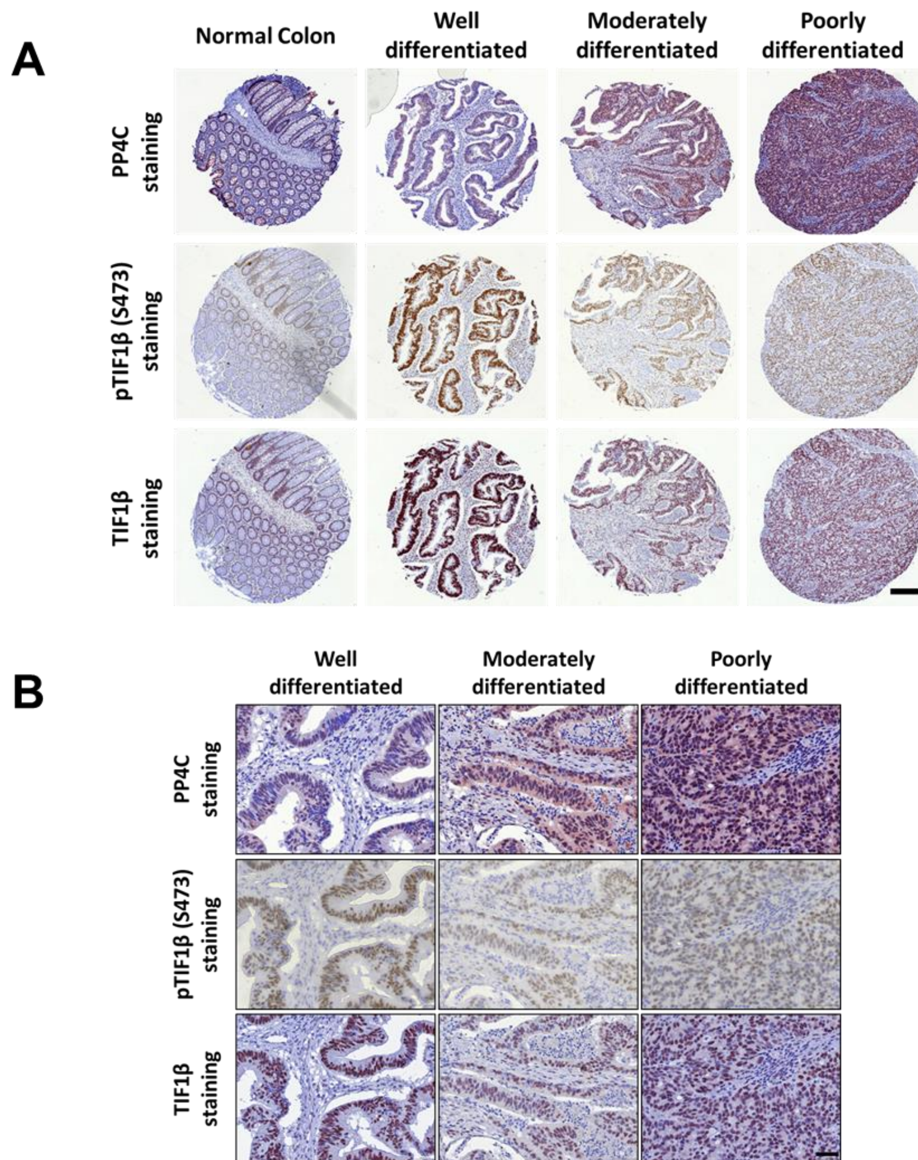


Figure 2 A negative correlation between TIF1β phosphorylation and PP4C expression in colorectal cancer patients.

Human colon epithelium specimens from patients suffering from colon adenocarcinomas were analysis by immunohistochemistry staining with the indicated antibodies: PP4C, TIF1β, and phosphorylated-TIF1β (Ser473).

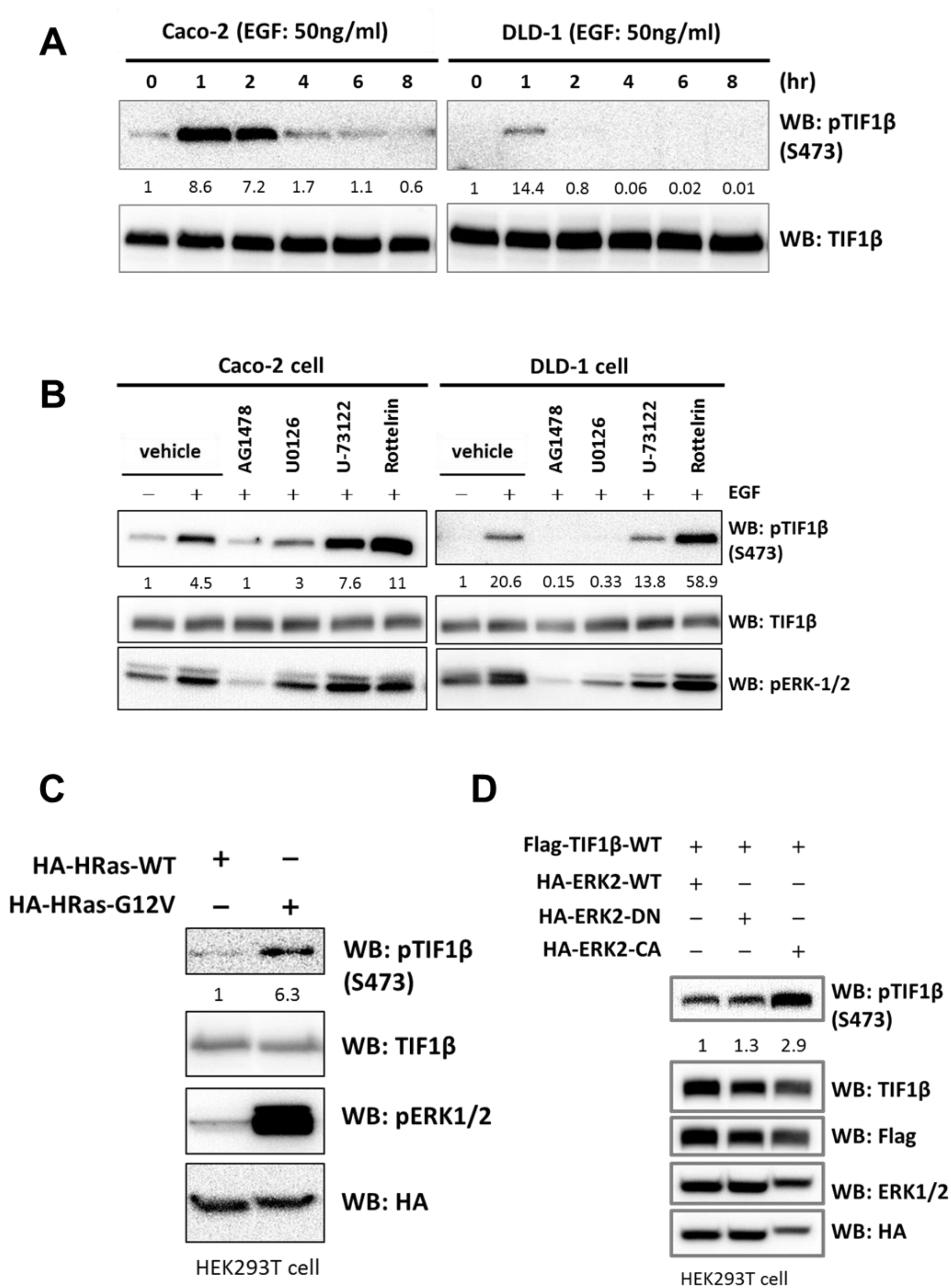


Figure 3 EGF stimulates TIF1β phosphorylation through Ras-MEK-ERK signaling pathway.

(A) Caco-2 and DLD-1 cells were treated with EGF (50 ng/ml) for the indicated time periods, and western blots were performed. Western blots of phospho-TIF1β (Ser473) and total TIF1β expression were detected. (B) Caco-2 and DLD-1 cells were incubated with

inhibitors as indicated (AG1478: 1 μM, U0126: 10 μM, U73122: 10 μM, and Rottlerin: 5 μM) for 10 minutes before EGF induction for 30 minutes, analyzed by western blot and detected as in (A). Phospho-ERK 1/2 expression level was used as a positive control of EGF stimulation and effect of inhibitors. HEK-293T cells were transfected with (C) HA-HRas-WT and HA-HRas-G12V; or with (D) TIF1β-WT, ERK2-WT, ERK2-donminant negative (DN) form and ERK-2 (L73P/S15D) constitutively active (CA) form and analyzed TIF1β phosphorylation level by western blot. The relative level of TIF1β phosphorylation (pTIF1β /TIF1β) were measured by NIH Image and are shown below the panel.

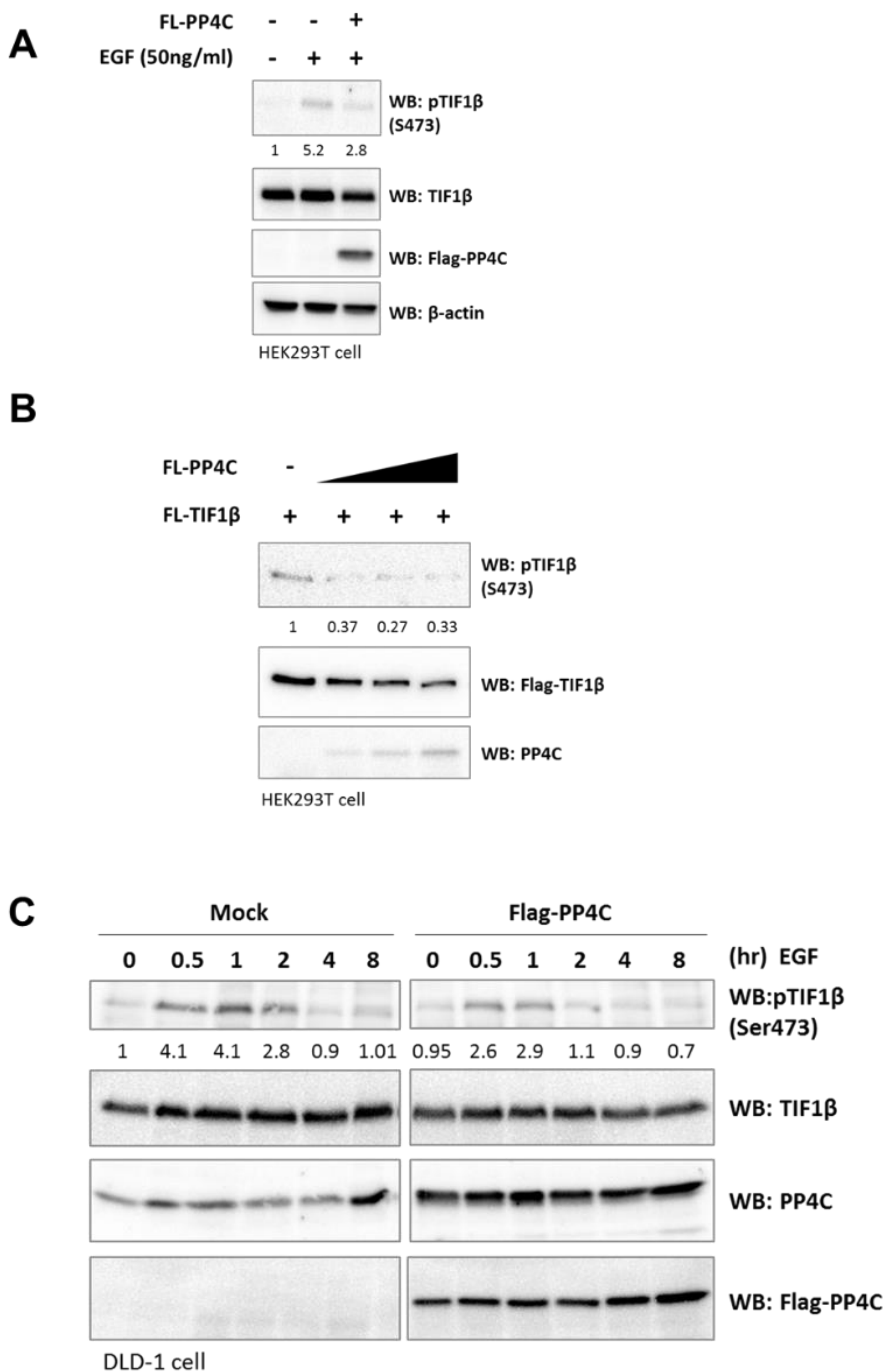


Figure 4 PP4C dephosphorylates EGF-induced TIF1β phosphorylation.

(A) HEK-293T cells were treated with EGF (50 ng/ml) for 2 hours after transfected with PP4C and analyzed the endogenous TIF1β-Ser473 phosphorylation level by western blot.

(B) In vitro dephosphorylation assay: HEK293T cells were transfected with Flag-PP4C or Flag-TIF1β and purified by IP with anti-Flag antibody, respectively. Purified Flag-PP4C were then eluted with 3× Flag peptide. Different concentrations of eluted Flag-PP4C and purified Flag-TIF1β were incubated at 37 °C for 30 min and analyzed by western blot. (C) DLD-1 cells stably expressed Flag-PP4C were treated with EGF (50 ng/ml) for the indicated time periods compared with DLD-1 mock cells. Western blots were performed, and relative level of TIF1β phosphorylation (pTIF1β /TIF1β) were measured by NIH Image and are shown below the panel.

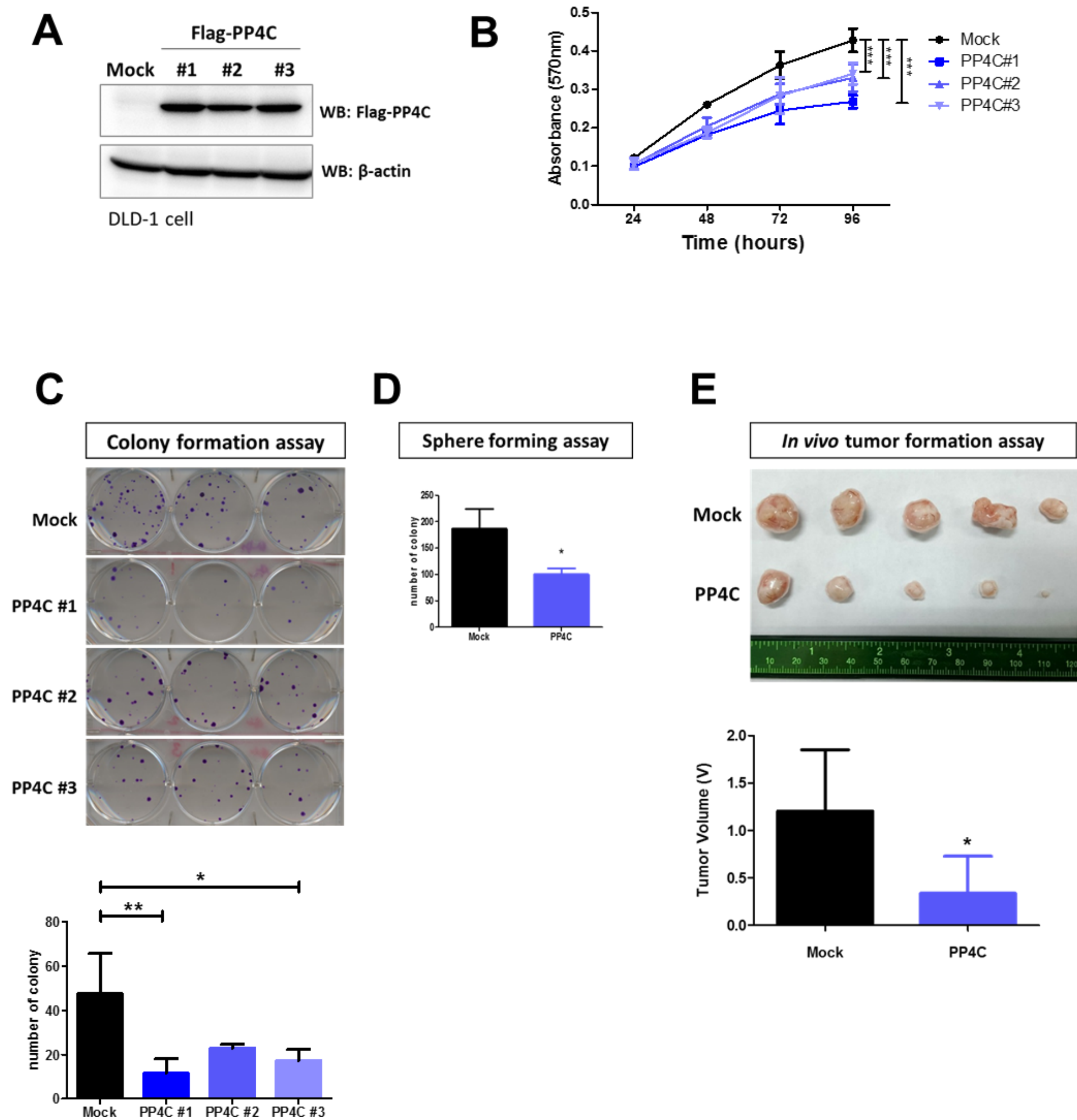


Figure 5 Ectopic expression of PP4C in DLD-1 cell suppresses cellular proliferation and tumor growth *in vitro* and *in vivo*.

(A) Ectopic expression of PP4C in DLD-1 cell compared with mock and western blots were performed with antibodies against Flag and β-actin (loading control). (B) Growth curve of DLD-1 mock cell and PP4C-overexpressing cell over 96 hours. Cell density was determined using MTT assay. Data represent means±SD, n=3. ***P<0.001. (C) Colony formation assay: Morphology of colony growth of DLD-1 mock cell and PP4C-overexpressing cell. Down panel shows the quantitative analysis of the colony forming

assay. (D) DLD-1 mock cell and PP4C-overexpressing cell were cultured in sphere formation medium for 12 days. Sphere size larger than 100 μm in diameter were counted. The means ± SDs are shown. (E) DLD-1 mock cell and PP4C-overexpressing cell were injected s.c. into ICR-nu/nu mice. After 1.5 months, the tumors were harvested and photographed. Tumor volume was calculated. The means ± SDs are shown.

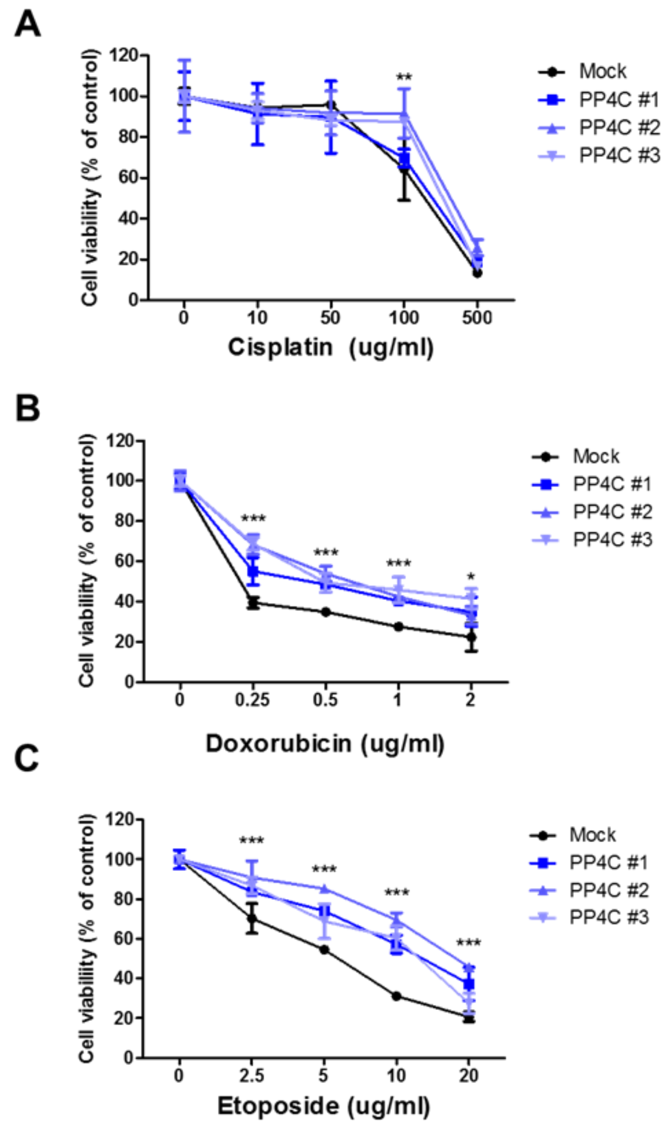


Figure 6 Ectopic expression of PP4C increases anticancer drug resistance in colorectal adenocarcinoma DLD-1 cell line.

DLD-1 stably expressed PP4C were exposed to different anticancer drugs in the indicated concentration. Cell density was determined over the following 72hours using MTT assay. Cell density in the presence of (A) cisplatin, (B) doxorubicin and (C) etoposide. Data represent means±SD, n=3. *P<0.05, **P<0.01, ***P<0.001.

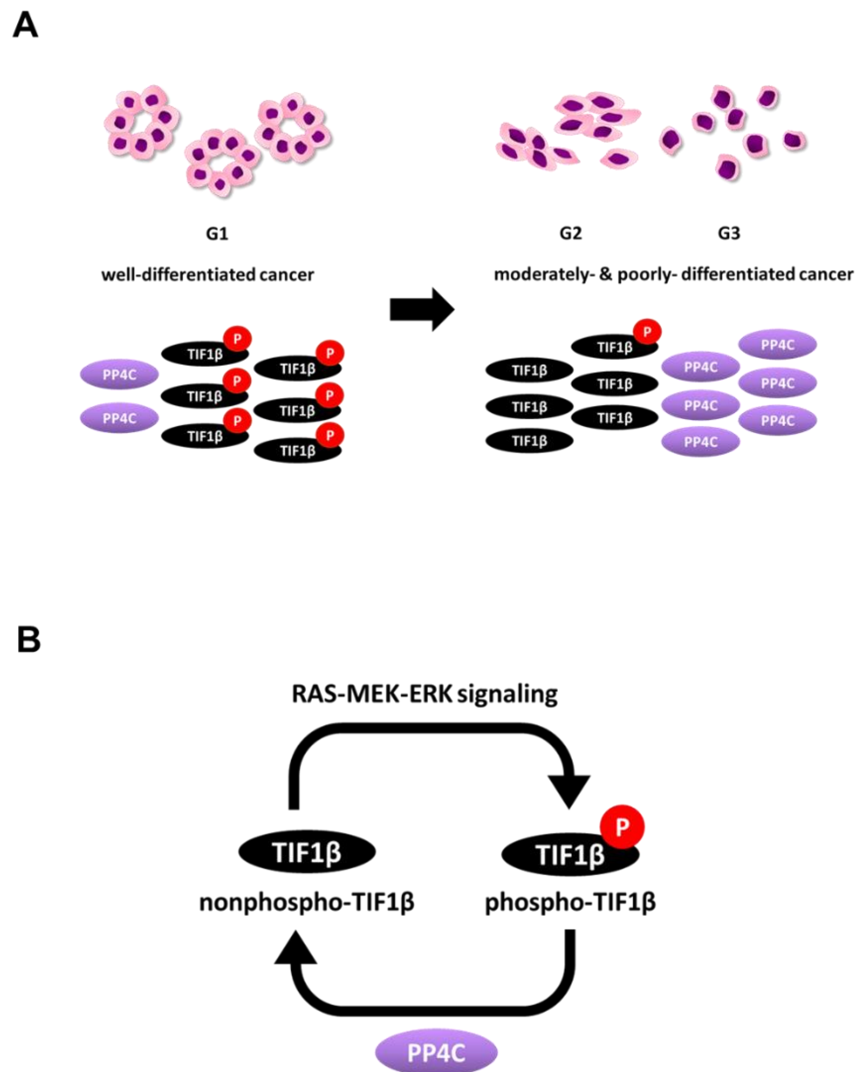


Figure 7 Expression of PP4C and regulation of TIF1β phosphorylation in colorectal cancer aggressiveness.

(A) Overview of PP4C protein expression and TIF1β-Ser473 phosphorylation between well-differentiated tumor (Grade 1, G1) and moderately- (Grade 2, G2), poorly differentiated (Grade 3, G3) tumor of CRC. (B) Schematic representation of the regulation of TIF1β phosphorylation and dephosphorylation through PP4C and Ras-MEK-ERK signaling pathway.

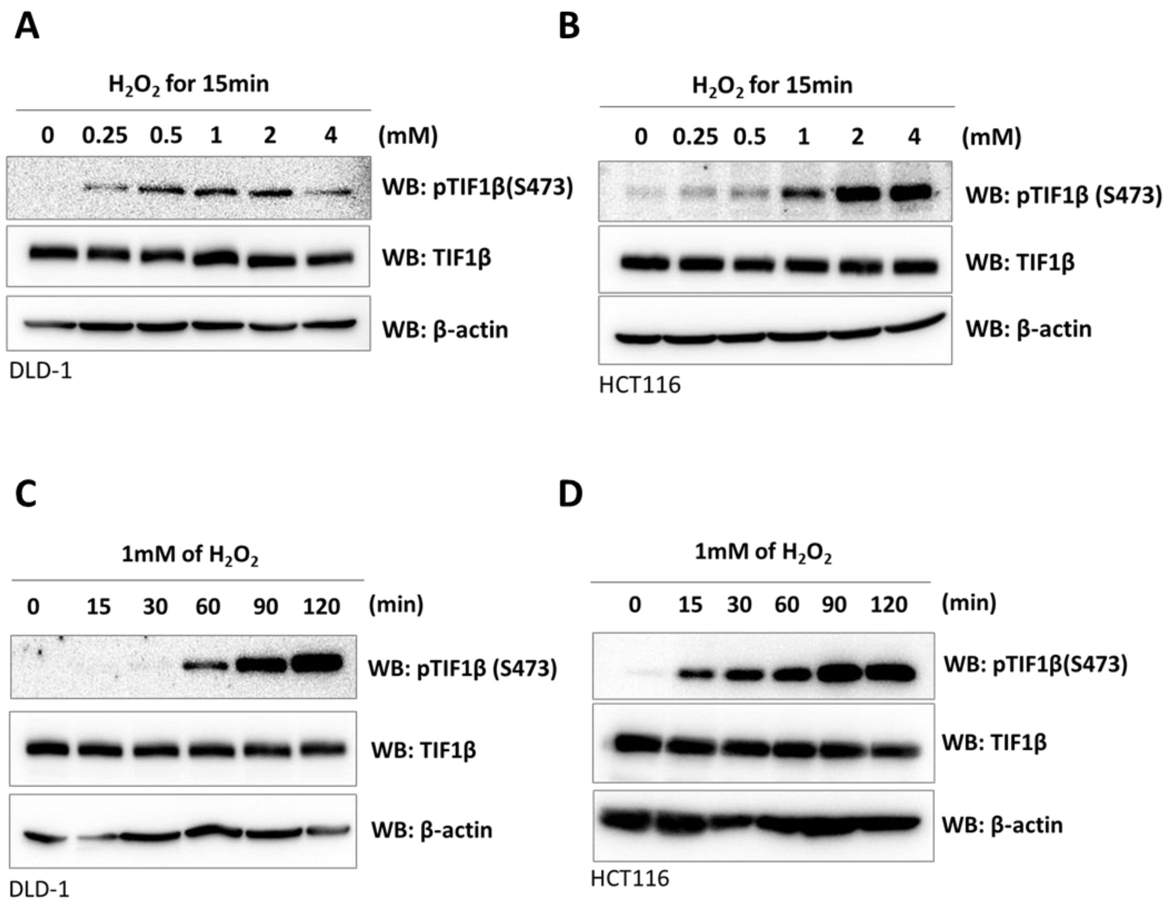


Figure 8 Effect of H_2O_2 on TIF1b –Ser473 phosphorylation in colorectal cancer cells, DLD-1 and HCT116, in a concentration- and time-dependent manner.

DLD-1 and HCT116 were incubated with H_2O_2 at the indicated concentration for 15 min (A-B) or with 1mM of H_2O_2 for the shown time (C-D). Whole cell lysates were immunoblotted to detect endogenous expression of phosphorylated TIF1β, total TIF1β, and β-actin, respectively.

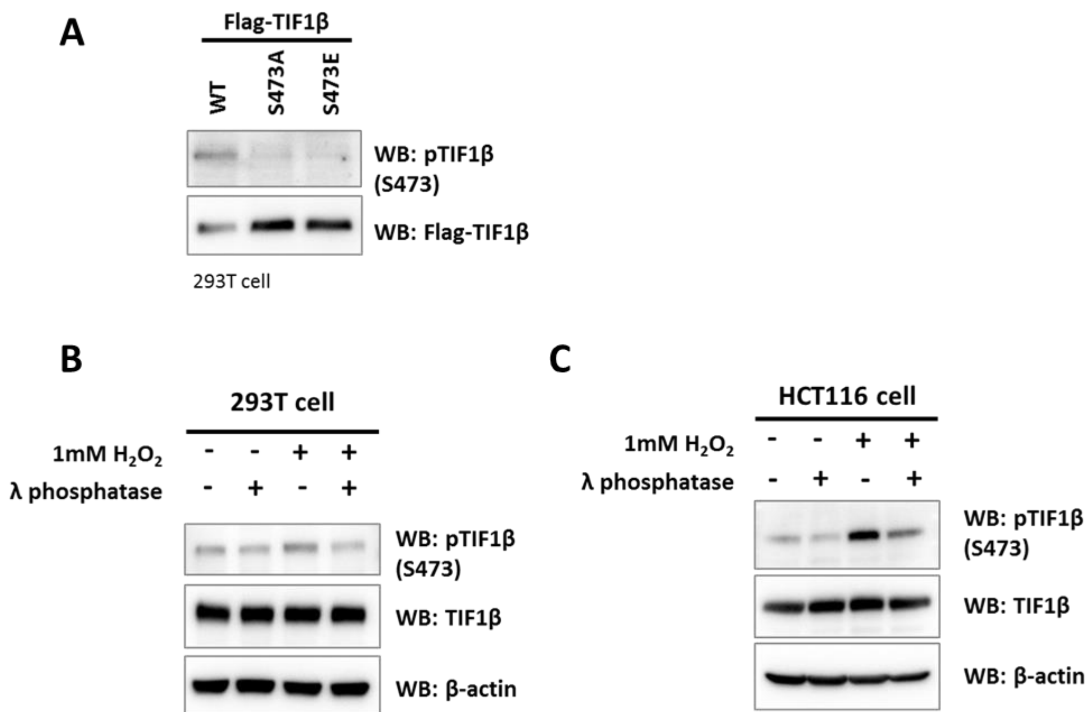


Figure 9 Characterization of anti-TIF1β-Ser473 phosphorylation antibody under oxidative stress.

HEK293T cells transfected with TIF1β-wild type, S473A, and S473E mutant, respectively (A). HEK293T cells (B) and HCT116 cells (C) were incubated with H_2O_2 for 15 min and treated with or without λ phosphatase for 30 min. Whole cell lysates were immunoblotted to detect endogenous expression of phosphorylated TIF1β, total TIF1β, and β-actin, respectively.

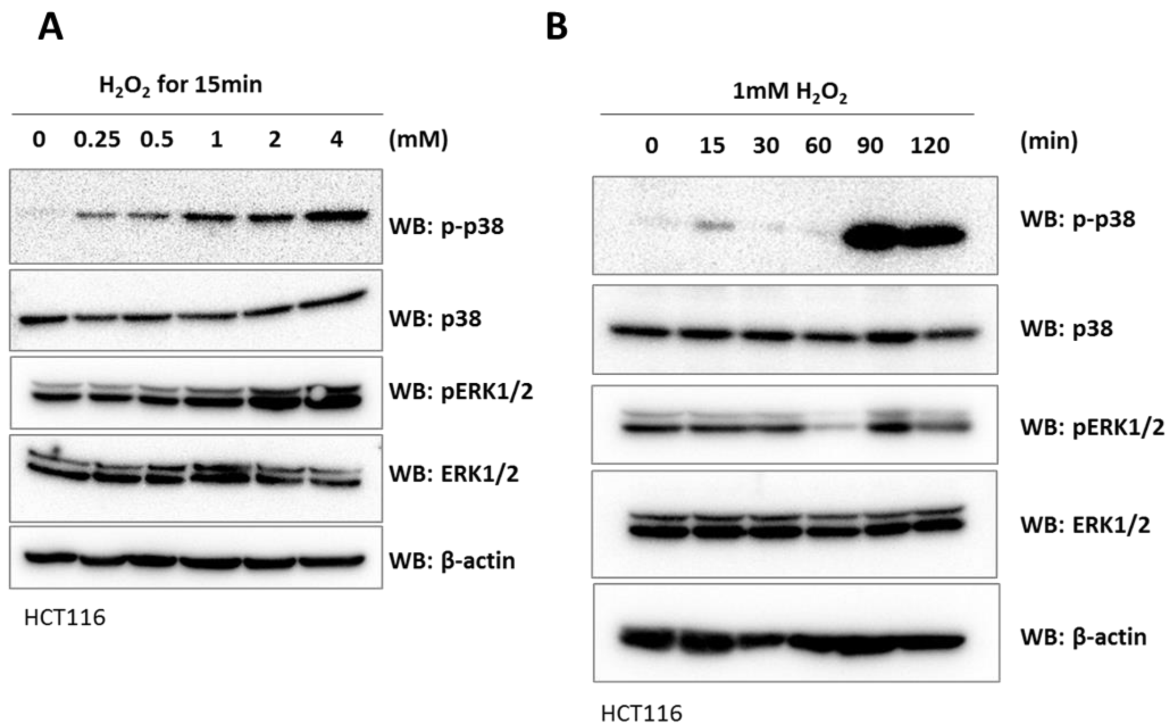


Figure 10 Effect of H_2O_2 on ERK1/2 and p38 MAPK phosphorylation.

HCT116 cells were incubated with H_2O_2 at the indicated concentration for 15 min (A) or with 1mM of H2O2 for the shown time (B). Whole cell lysates were immunoblotted to detect endogenous expression of phosphorylated ERK1/2, total ERK1/2, phosphorylated p38 MAPK, total p38 MAPK, and β-actin, respectively

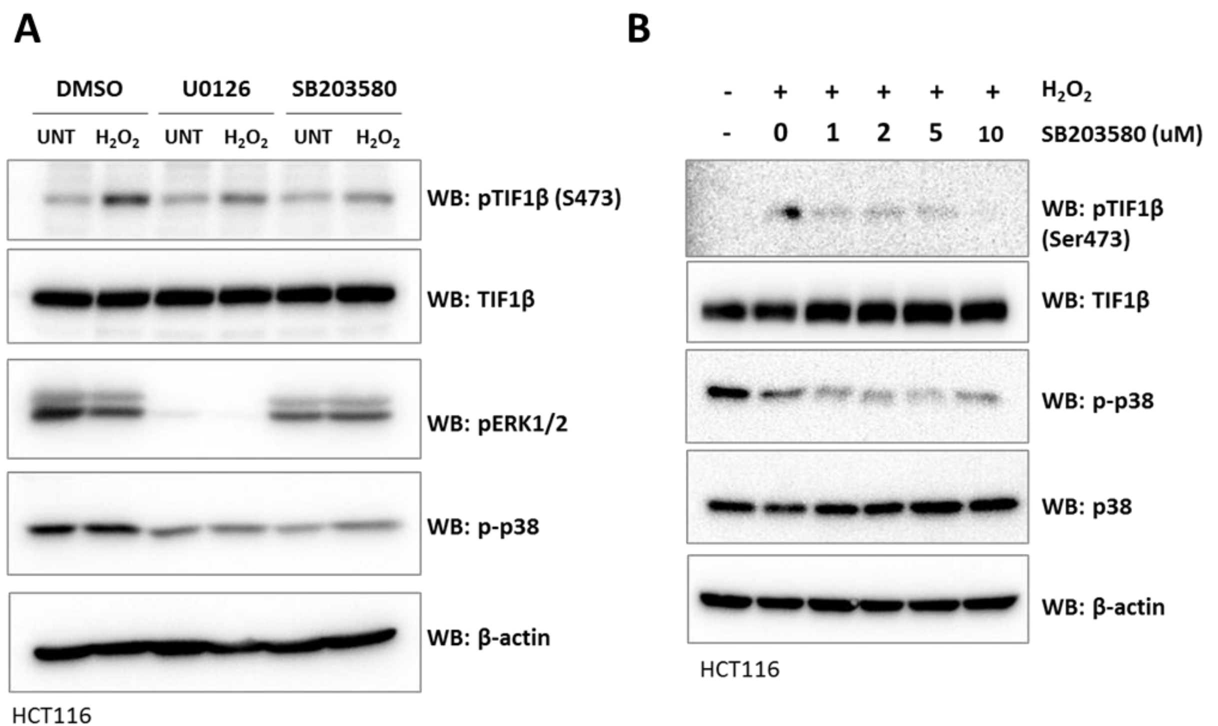
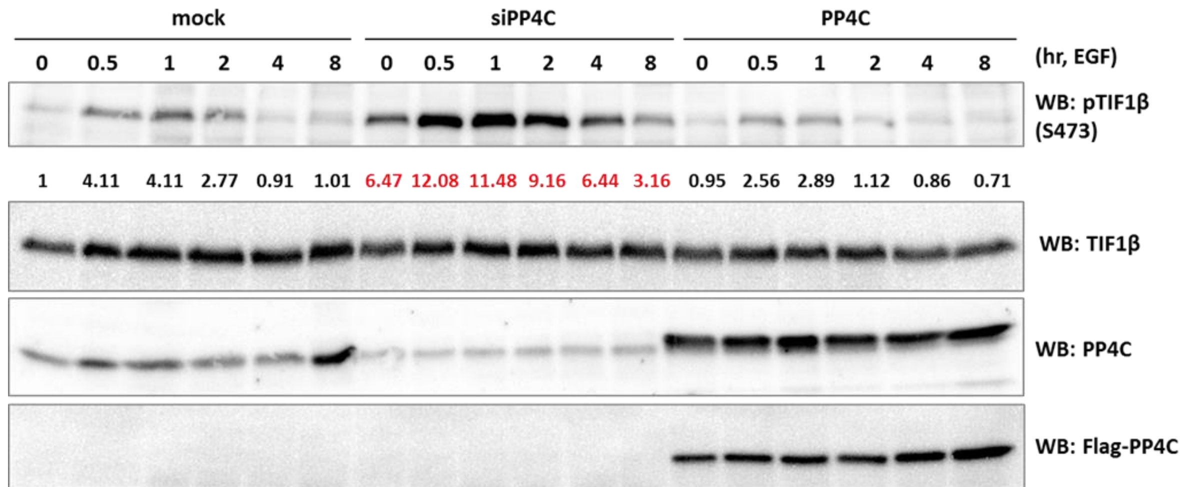


Figure 11 Effect of ERK1/2 inhibitor (U0126) or p38 MAPK inhibitor (SB203580) on H_2O_2-induced TIF1b –Ser473 phosphorylation in HCT116 cells.

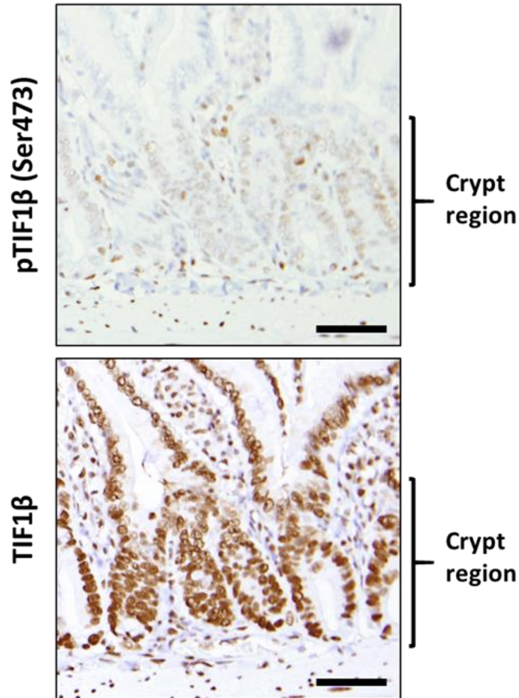
HCT116 cells were incubated with DMSO, U0126, and SB203580 for 1 hour before H_2O_2 stimulation (A). HCT116 cells were incubated with SB203580 at the indicated concentration for 1hour before H_2O_2 stimulation (B). Whole cell lysates were immunoblotted to detect endogenous expression of phosphorylated TIF1β, total TIF1β, phosphorylated ERK1/2, phosphorylated p38 MAPK and β-actin, respectively.

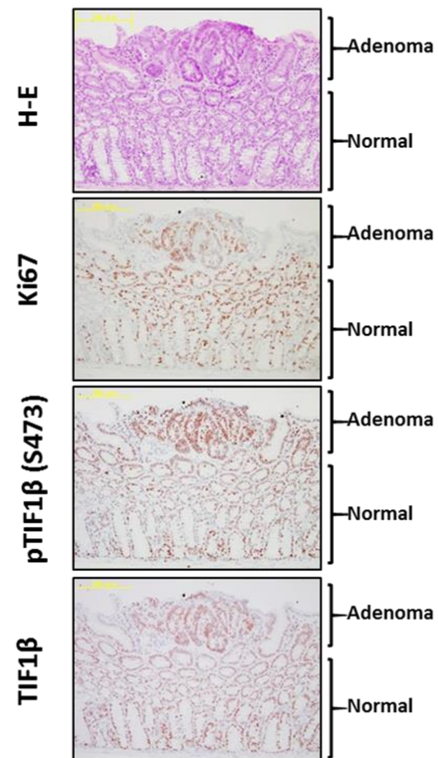
SUPPLEMENTARY DATA



Sup. 1 Comparison of TIF1β phosphorylation level induced by EGF in the absent or present of PP4C.

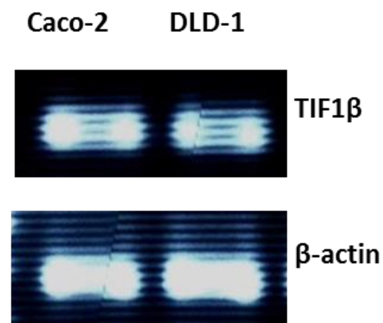
At 72 h posttransfection of PP4C specific siRNA, cells were treated with EGF (50ng/ml) for the indicated time as well as DLD-1 mock cell and PP4C-overexpressing DLD-1 cell. Cell pellets were collected, and western blots were performed. The relative level of TIF1β phosphorylation (pTIF1β /TIF1β) were measured by NIH Image and are shown below the panel.

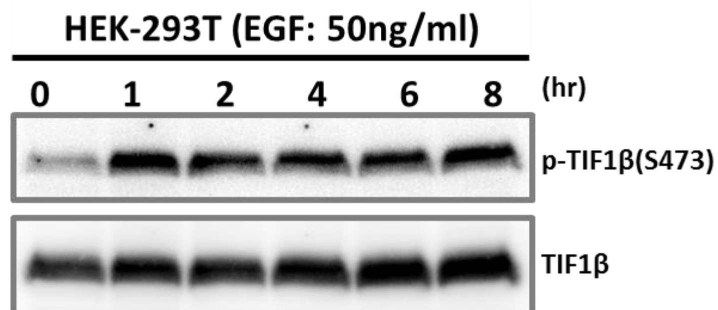




Sup. 2 TIF1β-Ser473 phosphorylation is an event associated with epithelial cells proliferation, differentiation, and tumor progression.

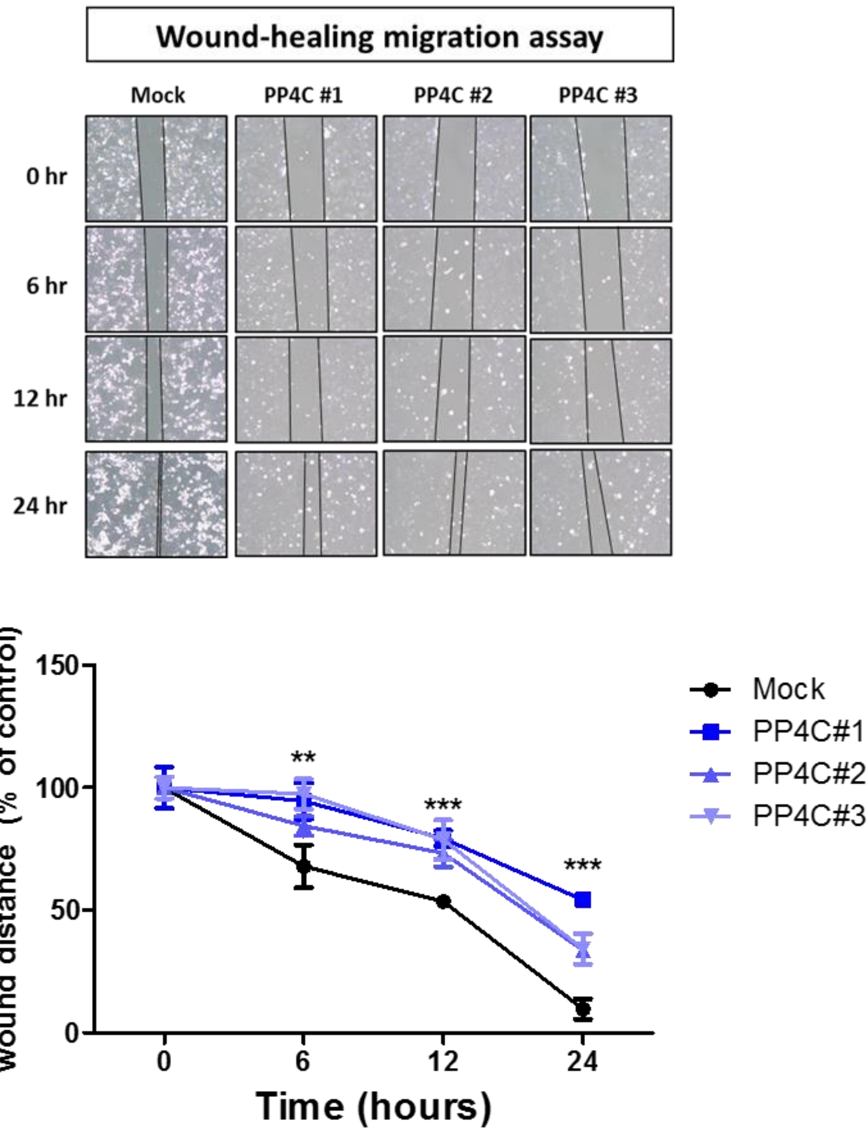
(A) Wild-type C57BL/6 mouse: normal intestinal epithelium and (B) $APC^{min/+}$ mice: adjacent normal tissue and adenoma of large intestine were analysis by immunohistochemistry staining with the indicated antibodies: ki-67 (a marker of proliferation), TIF1β, phosphorylated-TIF1β (S473) and hematoxylin-eosin staining.





Sup. 3 EGF-induced TIF1β-Ser473 phosphorylation in a different cell line in a time-dependent manner.

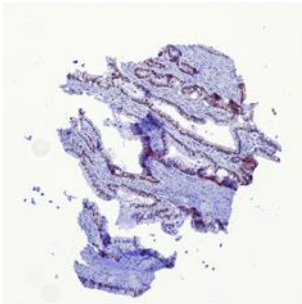
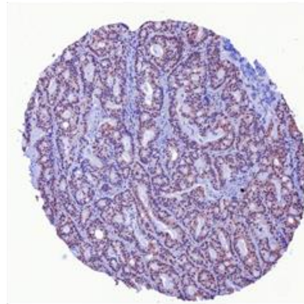
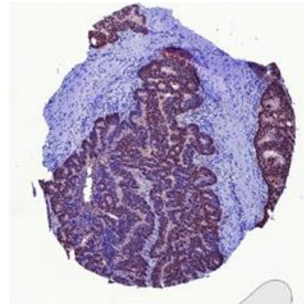
(A) RT-PCR analysis using TIF1β primer showed TIF1β expression in human colorectal cancer Caco-2 and DLD-1 cells. (B) HEK293T cells were treated with EGF (50ng/ml) for the indicated time periods, and western blot was performed. Western blots of TIF1β-Ser473 phosphorylation level and TIF1β expression level were detected.



Sup. 4 Ectopic expression of PP4C in DLD-1 cell suppresses cell proliferation and migration.

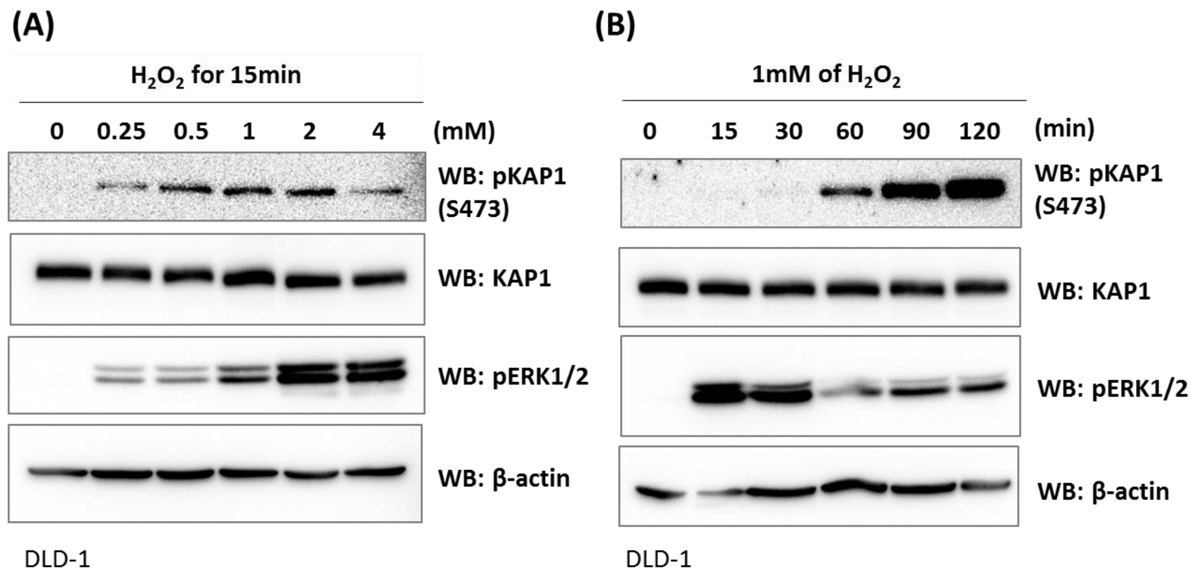
Wound-healing migration assay: DLD-1 mock cells and PP4C-overexpressing cells were seeded on 6-wells plate for 48h and were wounded with a sterile pipette tip to remove cells from two perpendicular linear scratches.

α-PP4C positively stained cells

Low (L) 1–30%	Medium (M) 30–70%	High (H) ≥70%
		

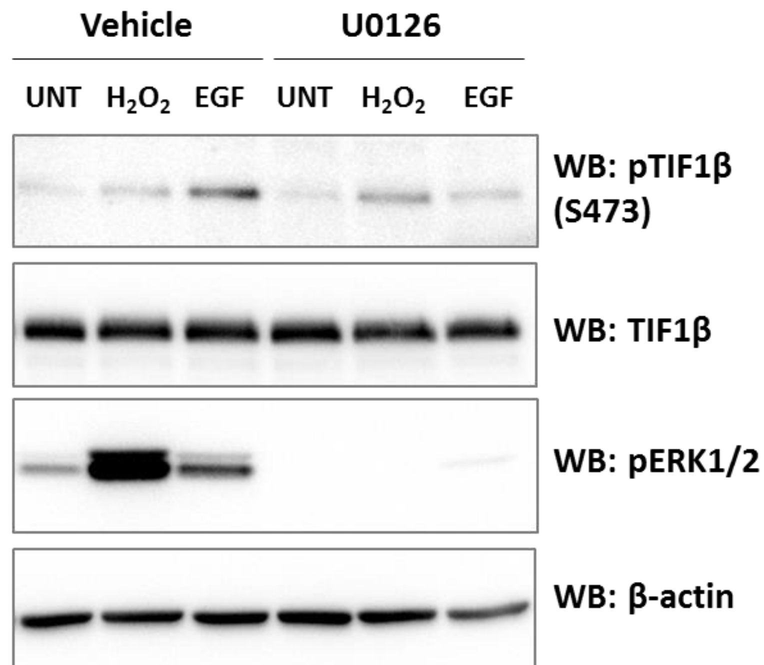
Sup. 5 Example of positively stained cells on immunohistochemistry staining.

The low percentage of positively stained cells: (L) 1–30%, medium percentage of positively stained cells: (M) 30–70%, and a high percentage of positively stained cells: (H) ≥70% with the anti-PP4C antibody on CRC patients' tissues.



Sup. 6 Effect of H_2O_2 on phosphorylation of TIF1b –Ser473 and pERK1/2 in human colorectal cancer DLD-1 cells in a dose- and time- dependent manner.

(A) DLD-1 cells were incubated with H_2O_2 at the indicated concentration for 15 minutes or (B) with 1mM of H_2O_2 for the indicated time. Whole cell lysates were immunoblotted to detect endogenous expression of phosphorylated TIF1β, total TIF1β, phosphorylated ERK1/2, and β-actin, respectively.



Sup. 7 H_2O_2 stimulation induced TIF1β-Ser473 phosphorylation but not through MEK-ERK signaling pathway.

DLD-1 cells were incubated with U0126(10 uM) for 10 min before challenged with EGF (50ng/ml) or H_2O_2 (1mM) for 30min and analyzed by western blot.

TABLES

Parameter	All cases	Negative	Positive			%
			Low (L)	Medium (M)	High (H)	(M+H)/All
Normal colon	16	6	9	1	0	6%
Colon cancer						
Grade						
1	35	3	22	6	4	29%
2	101	8	39	29	25	53%
3	32	3	11	13	5	56%
Stage						
I	92	7	40	24	21	49%
II	89	15	39	24	11	39%
III	6	2	3	0	1	17%
IV	5	2	2	0	1	20%

Table 1 Associations between PP4C expression levels and clinicopathological features of CRC patients in grade and stage.

Percentage of positively stained cells: Low(L); 1–30%, medium(M); 30–70%, high(H); ≥70%

Parameter	All cases	Negative	Positive			%
			Low (L)	Medium (M)	High (H)	(M+H)/All
Normal colon	16	0	11	5	0	31%
Colon cancer						
Grade						
1	35	0	16	12	7	54%
2	101	1	55	37	8	45%
3	32	1	20	11	0	34%
Stage						
I	92	0	51	35	6	45%
II	89	3	54	23	9	36%
III	6	1	1	4	0	67%
IV	5	2	3	0	0	0%

Table 2 Associations between phosphorylation level of TIF1β at Ser473 and clinicopathological features of CRC patients in grade and stage.

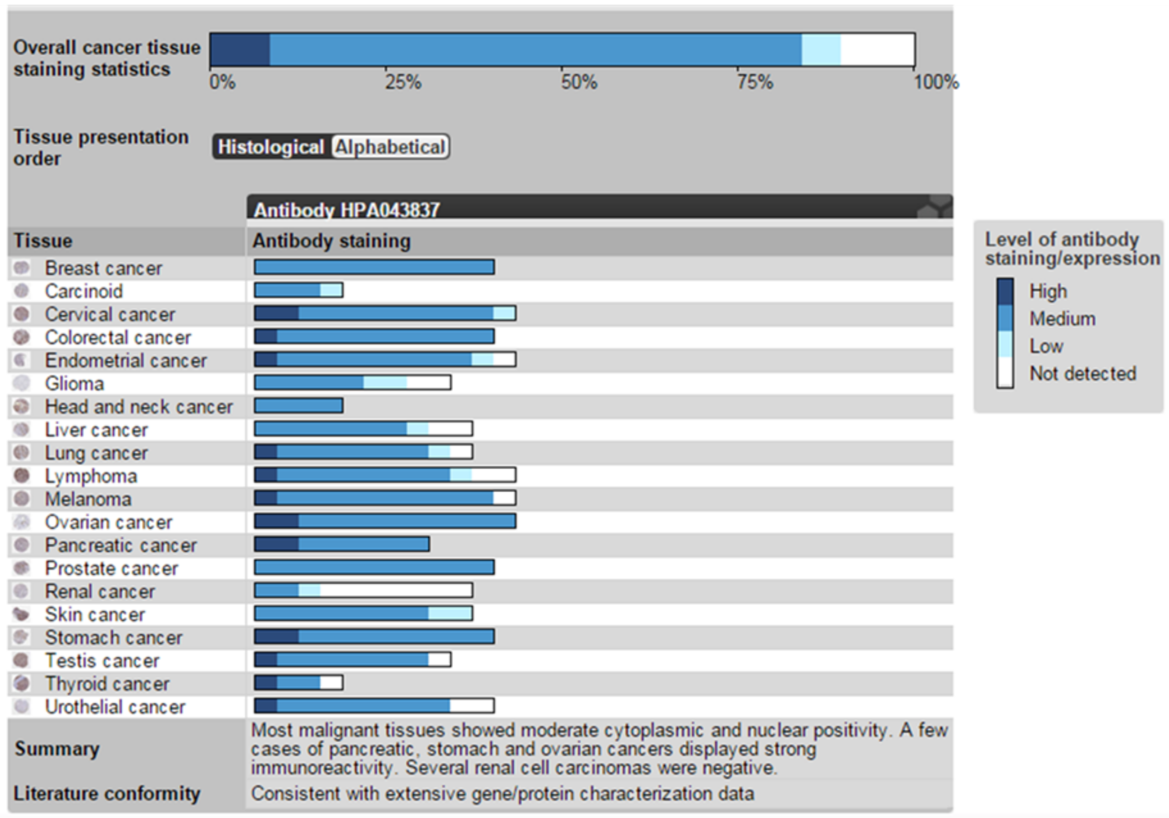
Percentage of positively stained cells: Low(L); 1–30%, medium(M); 30–70%, high(H); ≥70%

Grade	PP4C(+) phospho-TIF1β(+++)	PP4C (+++) phospho-TIF1β(+)
	Case patients, no. (%) (n = 20)	Case patients, no. (%) (n = 28)
1	11 (55)	2 (7)
2	7 (35)	17 (61)
3	2 (10)	9 (32)

Table 3 A negative correlation between PP4C expression and TIF1β-Ser473 phosphorylation in CRC poorly-differentiated tumor.

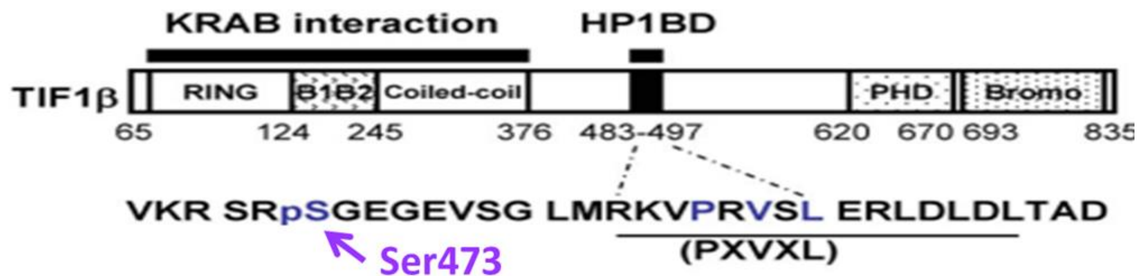
Comparison of PP4C (+) / phospho-TIF1β (+++) with PP4C (+++) / phospho-TIF1β (+) of CRC patients in grading.

APPENDIX



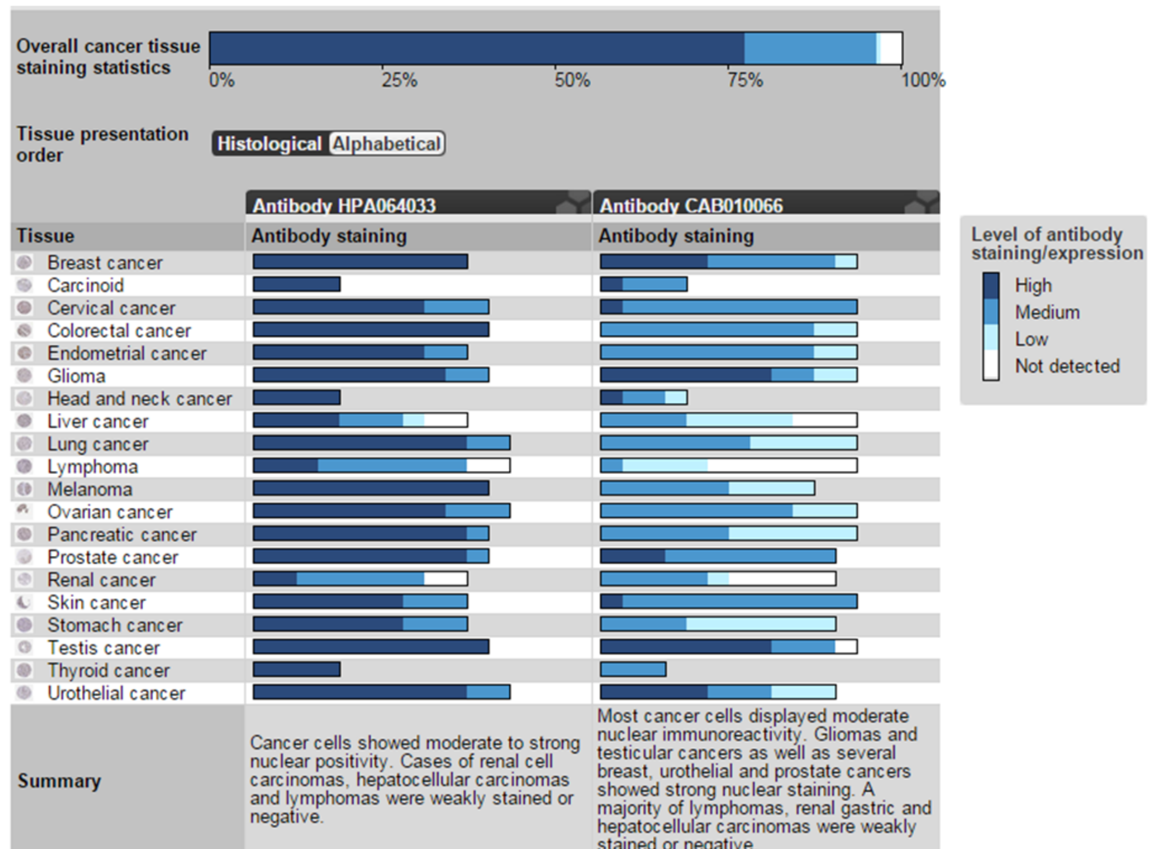
Appendix 1 Expression levels of PP4C in various cancer tissues of human.

The cancer tissue shows PP4C (Protein phosphatase 4, catalytic subunit) antibody staining in 20 different cancers from the cancer atlas of Human Protein Atlas. The overall cancer tissue staining statistics shows the fraction of patient samples with staining intensity, using all the available antibodies to the protein targets encoded by PP4C gene.



Appendix 2 A schematic representation of the conserved domains of TIF1β.

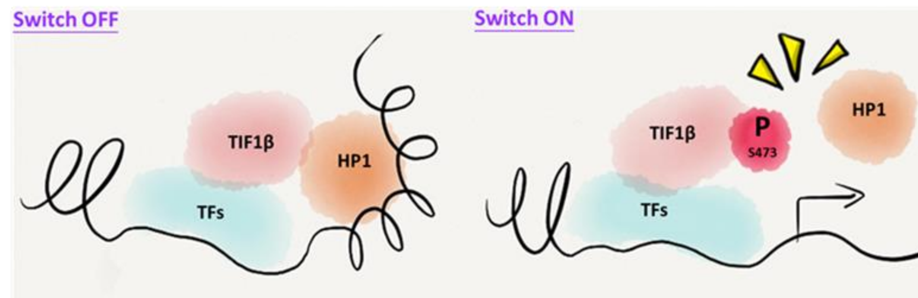
Numbers refer to amino acid positions. TIF1β includes several conserved domains (RING fingers, Type 1 and 2- B boxes, a leucine zipper α-helical coiled-coil region, a plant homeodomain finger, and bromodomain for Krüppel-associated box zinc finger proteins [KRAB-ZFPs]).



Appendix 3 Expression levels of TIF1β in various cancer tissues of a human.

The cancer tissue shows TIF1β antibody staining in 20 different cancers from the cancer atlas of Human Protein Atlas. The overall cancer tissue staining statistics shows the fraction of patient samples with staining intensity, using all the available antibodies to the protein targets encoded by TIF1β gene.

(A) **Molecular switch**



(B) **Highly conserved among mammals**

Serine 473

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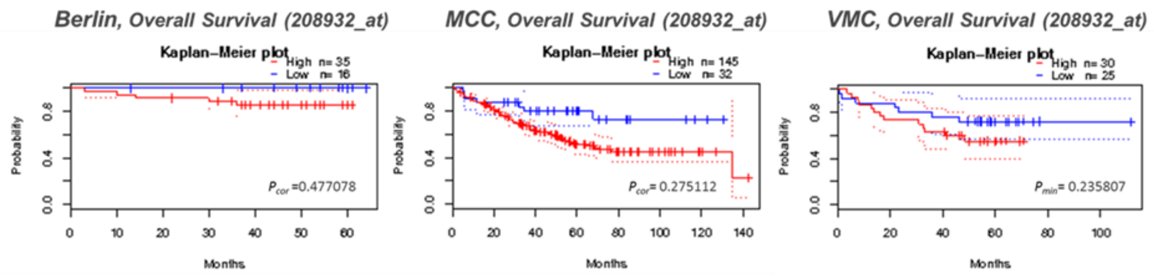
                                    *
Homo sapiensSSAEPHVSGVKRSRSGEGEVSGLMRKVPRVSLERLDLDLT    498
Macaca mulat...EPHVSGVKRSRSGEGEVSGLMRKVPRVSLERLDLDLT    628
Callithrix jSSAEPHVSGVKRSRSGEGEVSGLMRKVPRVSLERLDLDLT    498
Bos taurus  SSAEPHVSGVKRPRSGDGEVSGLMRKVPRVSLERLDLDLT    443
Canis familiSSAEPHVSGVKRSRSGEGEVSGLMRKVPRVSLERLDLDLT    491
Rattus norveSSAEPHVSGMKRSRSGEGEVSGLMRKVPRVSLERLDLDLT    499
Mus musculusSSAEPHVSGMKRSRSGEGEVSGLLRKVPRVSLERLDLDLT    498
Consensus      ephvsg kr rsg gevsgl rkvprvslerldldlt
```

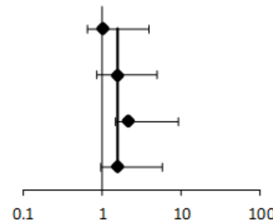
Appendix 4 The primary structure around Serine 473 phosphorylation site in TIF1β protein is highly conserved among vertebrates and may serve as a molecular switch on gene regulation.

(A) The phosphorylation/de-phosphorylation of TIF1β-Ser473 may serve as a "molecular switch" which regulates its interaction with HP1 protein and genes expression of proliferation or differentiation in tumor progression. (B) Alignment of Serine 473 phosphorylation site in TIF1β protein across several species is analyzed by using NCBI database and DNAMAN software.



Study, No.	Rate (95% CI)
Study 3	1.03 (0.38-2.83)
Study 2	1.6 (0.74-3.46)
Study 1	2.12 (0.63-7.08)
Effect Summary	1.603 (0.65-4.1)

Random effects model (i^2 = 0%)



Appendix 5 Two studies of PP4C gene expression in overall survival of colorectal cancer via Prognoscan database.

Kaplan-Meier plots and Microsoft Excel spreadsheet for meta-analyses and shown as forest plots for high and low PP4C-expressing groups in colorectal cancers.

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