



# Identification of Protein Phosphatase 4 Inhibitory Protein That Plays an Indispensable Role in DNA Damage Response

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**Protein phosphatase 4 (PP4) is a crucial protein complex that plays an important role in DNA damage response (DDR), including DNA repair, cell cycle arrest and apoptosis. Despite the significance of PP4, the mechanism by which PP4 is regulated remains to be elucidated. Here, we identified a novel PP4 inhibitor, protein phosphatase 4 inhibitory protein (PP4IP) and elucidated its cellular functions. PP4IP-knockout cells were generated using the CRISPR/Cas9 system, and the phosphorylation status of PP4 substrates (H2AX, KAP1, and RPA2) was analyzed. Then we investigated that how PP4IP affects the cellular functions of PP4 by immunoprecipitation, immunofluorescence, and DNA double-strand break (DSB) repair assays. PP4IP interacts with PP4 complex, which is affected by DNA damage and cell cycle progression and decreases the dephosphorylational activity of PP4. Both overexpression and depletion of PP4IP impairs DSB repairs and sensitizes cells to genotoxic stress, suggesting timely inhibition of PP4 to be indispensable for cells in responding to DNA damage. Our results identify a novel inhibitor of PP4 that inhibits PP4-mediated cellular functions and establish the physiological importance of this regulation. In addition, PP4IP might be developed as potential therapeutic reagents for targeting tumors particularly with high level of PP4C expression.**

**Keywords:** dephosphorylation, DNA damage response, DNA

double-strand break repair, protein phosphatase 4, protein phosphatase 4 inhibitory protein

## INTRODUCTION

DNA double-strand breaks (DSBs) induced by ionizing radiation (IR) and replication stress are the most deleterious lesions that lead to genomic instability and cellular transformation. To prevent these detrimental consequences, eukaryotic cells have evolved an elaborate and complex system, so called DNA damage response (DDR) comprising of DNA DSB repair, cell cycle arrest, apoptosis, and transcriptional regulation (Jackson and Bartek, 2009). The DDR in mammalian cells is generally initiated by the phosphatidylinositol-3 (PI3) kinase-like family of protein kinases that include DNA-dependent protein kinase catalytic subunit (DNA-PKcs), ataxia telangiectasia mutated (ATM), and ATM-and Rad3-related (ATR) (Matsuoka et al., 2007; Mu et al., 2007). In response to exogenous or endogenous DNA damage, these kinases phosphorylate proteins, such as H2A histone family member X (H2AX), replication protein A2 (RPA2), p53-binding protein 1 (53BP1), Krüppel-like associated box (KRAB)-associated protein 1 (KAP1) and deleted in breast cancer protein 1 (DBC1) that play a key role in DSB repairs, homologous recombination (HR) and non-homologous end joining (NHEJ)

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(Chowdhury et al., 2008; Lee et al., 2010; 2012; 2014; 2015; Nakada et al., 2008).

Previously, we and others demonstrated that the phosphorylation of key proteins including KAP1, H2AX, and RPA2, particularly in early response to DNA damage, is tightly controlled by protein phosphatase 4 (PP4), which is also essential to DSB repairs, cell cycle arrest, apoptosis in DDR (Lee et al., 2010; 2012; 2015; Nakada et al., 2008). PP4 not only plays a role as a counteractor of kinases, but also plays a critical role in initiating events in DDR (Chen et al., 2014; Lee and Chowdhury, 2011). Thus, the dephosphorylation of target proteins by PP4 is equally important in DDR as much as phosphorylation by kinases. In addition, by regulating the phosphorylation of target substrates, PP4 significantly contributes to various cellular processes, such as cell migration, immune response, stem cell development, glucose metabolism, and circadian system through the dephosphorylation of its target substrates (Chen et al., 2014; Li et al., 2015; Lyu et al., 2013; Martin-Granados et al., 2008; Toyo-oka et al., 2008; Xie et al., 2013; Zhao et al., 2015). Structurally, PP4 occurs as dimeric or trimeric complexes, and the catalytic subunit of PP4 complex, forms dimeric or trimeric complexes with regulatory subunits (PP4R1, PP4R2, PP4R3 $\alpha$ , PP4R3 $\beta$ ) conferring substrate specificity (Lee and Chowdhury, 2011; Pereira et al., 2011; Shi, 2009).

The functional importance of PP4 in cells is evident by the need of tight regulation of its activity required to maintain cell health. Previous studies have reported that PP4R2 and PP4R3 $\alpha$  are inactivated by phosphorylation during mitosis and PP4C is methylated on leucine residue at C-terminal to form active PP4 complex (Lee and Lee, 2014; Voss et al., 2013). However, further studies are required to elucidate the mechanism and novel regulators involved in the modulation of PP4 activity.

Recently, C19ORF43 has been characterized as a telomerase RNA component interacting RNase (TRIR). TRIR was detected in partial purified human telomere RNA (hTR) complex, and recombinant TRIR protein purified from prokaryotic system has RNase activity in *in vitro* assay (Xie et al., 2017). However, its role in human cells *in vivo* still remains unresolved. Previously, we and others proved that the critical role of the phospho-regulation of KAP1 by PP4 in mediating cellular functions, including efficient repair of DSBs and chromosomal relaxation induced by DNA damage (Goodarzi et al., 2011; Hu et al., 2012; Liu et al., 2013; Noon et al., 2010; Sripathy et al., 2006; Ziv et al., 2006). Interestingly, data from proteomic analysis suggested the presence of TRIR in the KAP1-associated complex (Kim et al., 2014). Taken together, we hypothesized that TRIR have a significant role in cells, especially in connection with PP4 as a new partner in complex. In this study, TRIR is referred to as protein phosphatase 4 inhibitory protein (PP4IP) because of its functional role related to PP4. PP4IP interacts with PP4 complex that is altered depending on the phase of the cell cycle and DNA damage. PP4-mediated dephosphorylation of target proteins is significantly inhibited by ectopic overexpression of PP4IP. Furthermore, premature activation of PP4 by depleting PP4IP in unperturbed cells impairs the formation of phospho-signals, which is critical for initiating efficient repair following DNA

damage. In the present study, we identified a novel inhibitory molecule of PP4 and elucidated the mechanism by which PP4 activity is regulated.

## MATERIALS AND METHODS

### Cell culture, antibodies and reagents

MDA-MB-231, MDA-MB-453, HeLa, HeLa S3, HCT116, SW620, 293T, HepG2, MCF-7, MRC-5, U2OS, 8988T, MIA-PaCa-2, PANC1, RPE1, A431, and SK-MEL2 cells were grown in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS). HT29, HL60, Huh7, A549, H1650, HCC827, AsPC-1, BxPC-3, Capan-1, and SNU-213 cells were grown in RPMI-1640 supplemented with 10% (v/v) FBS. For cell cycle synchronization, the cells were incubated with 2 mM thymidine diluted in 1 $\times$  phosphate-buffered saline (PBS) for 16 h and additional 8 h with fresh media. For secondary thymidine or nocodazole, the cells were incubated with 2 mM thymidine or 100 ng/ml nocodazole for 12 h. In case of double thymidine treatment, the cells were collected after the indicated times from release. For thymidine-nocodazole treatment, the cells were harvested immediately. Antibodies used were against  $\alpha$ -tubulin (Sigma-Aldrich, USA), HA (Sigma-Aldrich), PP4IP (Abnova, Taiwan), phospho-histone H2AX (Ser139) (Cell Signaling Technology, USA), RPA2 (Cell Signaling Technology), KAP1 (Cell Signaling Technology), phospho-KAP1 (Ser824) (Cell Signaling Technology), PP4C (Bethyl Laboratories, USA), PP4R1 (Bethyl Laboratories), PP4R2 (Bethyl Laboratories), PP4R3 $\alpha$  (Bethyl Laboratories), PP4R3 $\beta$  (Bethyl Laboratories), HDAC3 (EnoGene, USA), histone H3 (EnoGene). Camptothecin (CPT), etoposide, thymidine, and nocodazole were obtained from Sigma-Aldrich. Propidium iodide was purchased from Invitrogen (USA) and cell cycle analysis was performed with S3e cell sorter (Bio-Rad, USA).

### siRNAs and plasmids

siRNAs were transfected with Lipofectamine 3000 (Invitrogen). The siRNAs were as follows: PP4C sense 5'-GGCCA-GAGAGAUCUUGGUAUU-3', antisense 5'-UACCAAGAUCUCUCUGGCCUU-3'; PP4R2 sense 5'-UAUACUGAGAGGUCUAAU AUU-3', antisense 5'-UAUUAGACCUCUCAGUAUAAU-3'; PP4IP sense 5'-CAAACUA GCCUCAAGACGGGAAUA-3', antisense 5'-UAUCCCGUCUUGAGGGC-UAGUU UG-3'; BRCA1 sense 5'-GCAGGAGCCA AAUCUAAU-3', antisense 5'-UUUAUAGAU UUGGCUCUCC-3'. PP4IP cDNA was synthesized with total RNA from HeLa cells, amplified and cloned into pOZ-FH-N, pLVX-DsRed-Monomer-C1 and pcDNA3-myc3 plasmids. Plasmids were transfected with Genjet (SignaGen Laboratories, USA) or Lipofectamine 3000.

### Immunofluorescence

Cells plated were rinsed twice with 1 $\times$  PBS, fixed with 4% (v/v) paraformaldehyde diluted in 1 $\times$  PBS for 15 min and washed with 1 $\times$  PBS for 5 min. Fixed cells were permeabilized with 0.4% (v/v) Triton X-100 diluted in 1 $\times$  PBS for 30 min and blocked with buffer containing 2% (v/v) FBS diluted in 1 $\times$  PBS for 1 h. After incubation with primary antibody diluted in blocking buffer for 1 h, cell were washed three times with

the blocking buffer, incubated with secondary antibody diluted in 2% FBS for 30 min and washed twice with blocking buffer and once with 1× PBS. The secondary antibody used was conjugated with Alexa Fluor 488 (Invitrogen). Slides were mounted using Dapi Fluoromount-G (SouthernBiotech, USA). Images were acquired using Laser Scanning Confocal Microscope System (Leica TCS SP5/AOBS/Tandem; Leica Microsystems, Germany) at the Korea Basic Science Institute. HeLa cells stably expressing DsRed-Monomer-PP4IP were mounted after permeabilization. Images were acquired using Evos FL Auto 2 (Thermo Fisher Scientific, USA).

### Co-immunoprecipitation

Cells were lysed in buffer containing 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% (v/v) NP-40, 5% (v/v) glycerol and protease inhibitor cocktail (Quartett, Germany). Lysates were incubated on ice for 20 min and centrifuged at 13,000g at 4°C for 20 min. The supernatants were transferred into new 1.5 ml tube and incubated with ANTI-FLAG M2 agarose beads (Sigma-Aldrich) at 4°C for 16 h. The immunoprecipitates were washed three times with buffer containing 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% (v/v) NP-40 and 5% (v/v) glycerol, resolved by SDS-PAGE and analyzed by immunoblot. Phosphorylation of PP4IP was assessed using the pIMAGO phosphoprotein detection kit (Tymora Analytical, USA) according to the manufacturer's instructions.

### RNA isolation and real-time quantitative polymerase chain reaction (PCR)

Total RNA was extracted from cells using TRIzol (Mentos, Korea) according to manufacturer's instructions. Complementary DNA was synthesized from 1 µg of the extracted RNA with TOPscript RT DryMIX (Enzygnomics, Korea) and amplified with TOPreal qPCR 2X PreMIX (Enzygnomics) in AriaMX real-time PCR system (Agilent, USA). Gene-specific primers used are as follows: PP4IP-F, 5'-AGCTTCTGGAGCT GTTCAA-3'; PP4IP-R, 5'-CCGTCTTGAGGGCTAGTTTG-3'; GAPDH-F, 5'-TGCACCACCAA CTGCTTAGC-3'; GAPDH-R, 5'-GGCATG-GACTGTGGTCATGAG-3'.

### Protein phosphorylation detection assay

We performed the protein phosphorylation assay using pIMAGO-based kit (Tymora Analytical) according to manufacturer's instructions. Cells stably expressing FH-PP4IP were lysed and FH-PP4IP immunoprecipitated by Flag antibody was resolved on SDS-PAGE and subsequently blotted onto protein membrane. After incubation of membrane with pIMAGO-Biotin solution for conjugation with phosphorylated FH-PP4IP, it was probed with avidin-HRP for detection.

### In vitro dephosphorylation assay

The *in vitro* dephosphorylation assay was performed as described (Lee et al., 2010; 2014). PP4IP-expressed or -knockout HCT116 cells were transfected with Flag-empty or Flag-PP4C plasmids and collected after 48 h. 293T cells were transfected with FH-KAP1 for 48 h, incubated with 10 µM CPT for 1 h and followed by further incubation for 1 h after release. HCT116 and 293T cells were immunoprecipitated

using Flag antibody-agarose beads. Immunoprecipitates were incubated with buffer containing 50 mM HEPES, 100 mM NaCl, 2 mM DTT, 0.01% Brij 35 for 1 h at 30°C in ThermoMixer C (Eppendorf, Germany). Lambda (λ) phosphatase (New England Biolabs, USA) serves as a control. After incubation, samples were resolved on SDS-PAGE, and the relative phosphatase activity was determined using antibody against anti-KAP1 pSer824 as the loss of FH-KAP1 phosphorylation immunoreactivity.

### Clonogenic assay

HeLa cells transiently transfected with PP4IP siRNAs or FH-PP4IP plasmids were irradiated with the indicated doses, seeded with 1,000 cells on 6-well plate in triplicate and incubated for 2 weeks for colony formation. After incubation, cells were rinsed with 1× PBS, fixed with buffer (acetic acid:methanol = 1:7) for 5 min, and stained for 2 h with 0.5% (w/v) crystal violet resolved in 25% (v/v) methanol and 75% (v/v) distilled water. Colonies were then rinsed with tap water and dried in the air. Surviving colonies of > 1 mm diameter were counted.

### Cell fractionation

Cell fractionation was performed as described previously (Chowdhury et al., 2008). HeLa cells harvested from 35 mm dishes were suspended with 100 µl of hypotonic buffer containing 10 mM Tris-HCl (pH 7.3), 10 mM KCl, 1.5 mM MgCl<sub>2</sub> and 10 mM β-mercaptoethanol followed by centrifugation at 800g at 4°C for 3 min. Supernatants were aspirated and the pellet were resuspended with 100 µl of hypotonic buffer followed by centrifugation at 1,000g at 4°C for 4 min. Supernatants (cytosolic fractions) were transferred to new 1.5 ml tube and the pellets were resuspended with 100 µl of hypotonic buffer and centrifuged at 1,000g at 4°C for 10 min. Isolated pellets were lysed with 100 µl of buffer containing 30 mM NaCl, 10% (v/v) glycerol, 50 mM Tris-HCl (pH 7.5), 0.2% (v/v) NP-40, 1 mM EDTA and protease inhibitor cocktail (Quartett) and incubated on ice for 30 min, and lysates were centrifuged at 14,000g at 4°C for 10 min. Supernatants (nuclear fraction) were transferred to new 1.5 ml tube and the pellets were used as chromatin fraction. Cytosol and nuclear soluble fractions were mixed with 100 µl of 2× SDS sample buffer, and chromatin fractions were mixed with 200 µl of 2× SDS sample buffer. The mixtures were subjected to immunoblot analysis.

### CRISPR/Cas9 Genome editing

A PP4IP-knockout cell line was constructed by transfecting HCT116 cells with PP4IP CRISPR/Cas9 KO plasmid (Santa Cruz Biotechnology, USA). After 48 h of transfection, GFP-positive clones were sorted by fluorescence-activated cell sorting (BD FACSAria III; BD Biosciences, USA), seeded in 96-well plates and cultured further for expansion. Each clone was verified for PP4IP-knockout by immunoblot analysis.

### DNA DSBs repair assay

HeLa cells, containing a single, stably integrated copy of the artificial recombination substrate DR-GFP with an I-SceI site (HR) or EJ5-GFP with two I-SceI sites (NHEJ), were transfected

ed with rare-cutting I-SceI endonuclease. HeLa-DRGFP or -EJ5GFP cells were transfected with siRNAs against BRCA1, PP4C or PP4IP. After 16 h, cells were transfected with I-SceI endonuclease plasmid, and GFP-positive cells were assayed after 48 h by FACS (BD FACSCalibur [BD Biosciences] and S3e Cell Sorter [Bio-Rad]). For transfection of expression vectors, FH-PP4C and FH-PP4IP plasmids were co-transfected with I-SceI expression plasmid.

### Statistical analysis

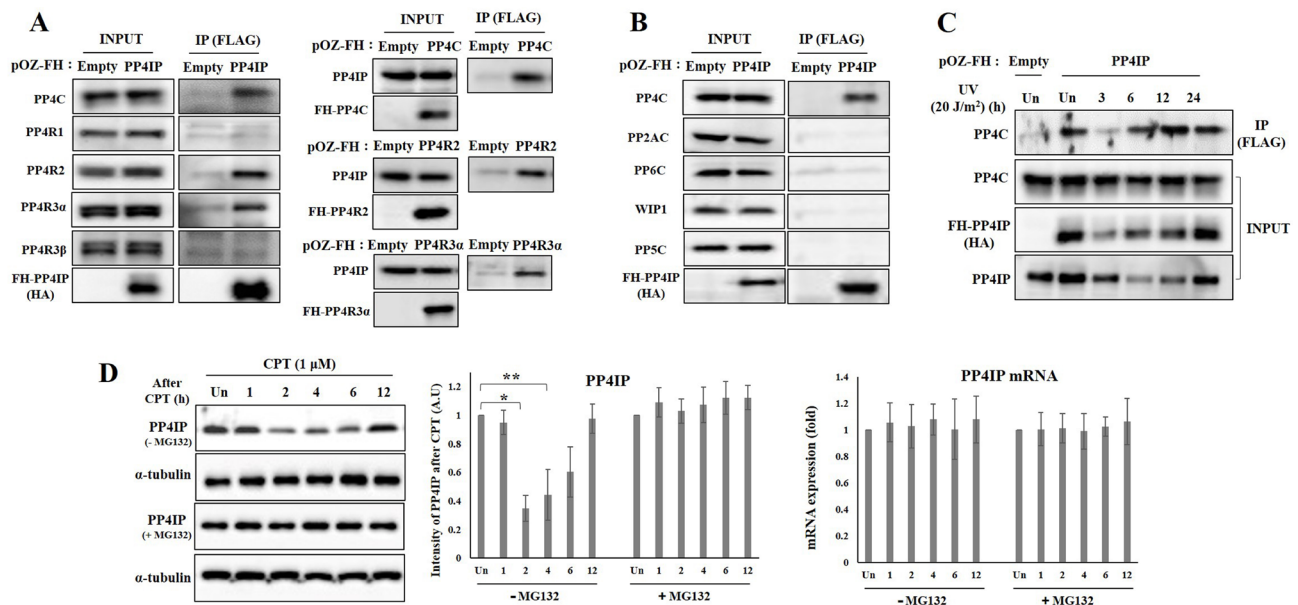
All statistical calculations were performed using Microsoft Excel 2016 (Microsoft, USA). Unless mentioned otherwise, all statistics were evaluated by two-tailed Student's *t*-test. Experiments were performed in triplicate and bars display mean  $\pm$  SD. A *P* value of 5% or lower is considered to be statistically significant.

## RESULTS

### Interaction of PP4IP with PP4 complex

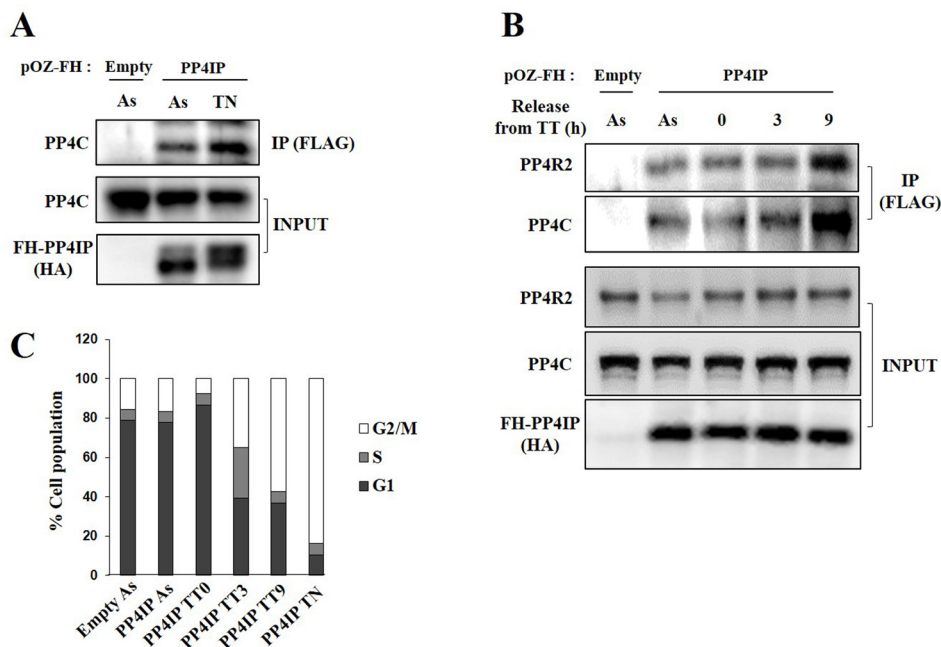
To identify the interaction between PP4IP and PP4, we used HeLa S3 cells stably expressing Flag-HA (FH)-PP4IP, FH-PP4C, FH-PP4R2, or FH-PP4R3 $\alpha$  for immunoprecipitation. We found that PP4IP associates with PP4C, PP4R2 and PP4R3 $\alpha$ , but not

with PP4R1 and PP4R3 $\beta$  through the reciprocal immunoprecipitation (Fig. 1A). Furthermore, there is no detectable interaction with other Serine/Threonine phosphatases, implying that PP4IP interacts with PP4 in a phosphatase-specific manner (Fig. 1B). In response to UV radiation the interaction between PP4IP and PP4 significantly decreases after 3 h, which is subsequently recovered back by 12 h, but this is because the consequent decrease in the expression of PP4IP (Fig. 1C). Comparable result was observed in cells treated with CPT at various time points (Fig. 1D). We observed that decreased expression of PP4IP detected at 2 h following CPT treatment, which went up back to normal at later time point, is due to proteasome-induced protein degradation after DNA damage, independent of the downregulation of mRNA expression (Fig. 1D). As shown in Figure 1D, the amount of PP4IP mRNA detected by quantitative real-time PCR is not altered to considerable level in cells treated with CPT. Interestingly, there is strikingly enhanced interaction between PP4IP and PP4 in cells arrested at mitotic phase by either thymidine-nocodazole or double-thymidine treatment (Fig. 2). According to a previous study showing that PP4 was inactivated by phosphorylation during mitosis in order to secure microtubule nucleation (Voss et al., 2013) and data in this study showing that robust interaction of PP4IP with PP4C at mitotic phase, compared

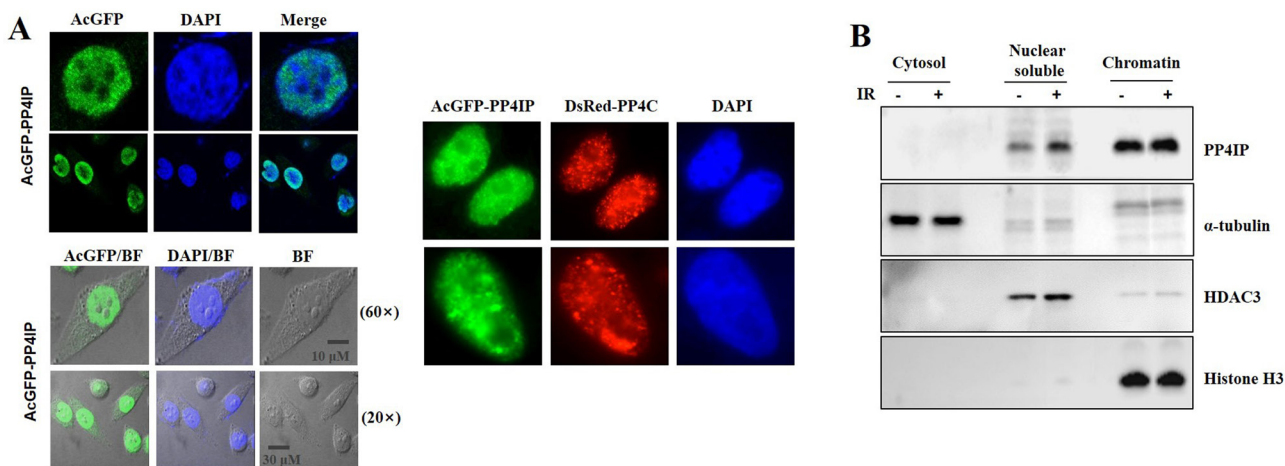


**Fig. 1. PP4IP interacts with PP4.** (A) HeLa S3 cells stably expressing Flag-HA-empty (FH-empty), FH-PP4IP, FH-PP4C, FH-PP4R2, or FH-PP4R3 $\alpha$  were subjected to immunoprecipitation using Flag antibody-agarose bead conjugates. The immunoprecipitates were probed with antibodies against PP4C, PP4R1, PP4R2, PP4R3 $\alpha$ , and PP4R3 $\beta$ . FH-PP4C, FH-PP4IP, FH-PP4C, FH-PP4R2, and FH-PP4R3 $\alpha$  were detected with antibody against HA. (B) HeLa S3 cells stably expressing FH-empty or FH-PP4IP were subjected to immunoprecipitation using Flag antibody-agarose bead conjugates. The immunoprecipitates were probed with antibodies indicated. (C) Interaction between PP4IP and PP4 is impaired upon UV damage. The cells were treated with 20 J/m<sup>2</sup> and harvested at the indicated time points. The immunoprecipitates were immunoblotted with antibody against PP4C. (D) The downregulation of PP4IP after DNA damage arises in the post-translational level, but not post-transcriptional level. HCT116 cells were treated with 1  $\mu$ M of CPT for 1.5 h and the collected at various time points. In the other set of experiment, cells were pretreated with 10  $\mu$ M of MG132 for 3 h before CPT treatment. The expression of PP4IP was assessed by immunoblotting (left panel) and quantitative PCR (right panel). The intensity of proteins was represented graphically using ImageJ. \**P* = 0.0064, \*\**P* = 0.0033. Data are expressed as mean  $\pm$  SD of three independent experiments. CPT is a topoisomerase inhibitor which induces DSBs. MG132 is a proteasome inhibitor. Un, untreated.





**Fig. 2. The interaction of PP4IP with PP4 is increased in cells arrested at mitotic phase.** (A and B) The cells stably expressing FH-empty or FH-PP4IP were incubated with thymidine-nocodazole (A) or double thymidine (B) and collected after the indicated times from release. The immunoprecipitates produced by Flag antibody were probed with antibody against PP4C (upper and lower panel), PP4R2, and HA. (C) For cell cycle analysis of synchronized cells, DNA content was quantified with propidium iodide (PI) staining. Results were depicted graphically. As, asynchronous; TN, thymidine-nocodazole; TT, double thymidine.



**Fig. 3. Cellular localization of PP4IP.** (A) PP4IP is localized in nucleus and colocalized with PP4C. HeLa cells stably expressing AcGFP-PP4IP and/or DsRed-PP4C were fixed and observed using Evos FL auto 2 microscopy. (B) PP4IP in nucleus is mainly associated with chromatin. HeLa cells were fractionated into three parts: cytosol, nuclear soluble, and chromatin. Each fraction was resolved on SDS-PAGE and immunoblotted with antibody against PP4IP. HDAC3,  $\alpha$ -tubulin, and histone H3 were served as loading controls for nuclear soluble, cytosol, and chromatin, respectively. BF, bright field; IR, ionizing radiation.

to that in asynchronous cells (Fig. 2), the inactivation of PP4 maybe correlates with the interaction of PP4 with PP4IP and therefore, it could be speculated that PP4IP might play an inhibitory role against the cellular functions of PP4 through the protein-protein interaction. In addition, we monitored that subcellular localization of PP4IP and found out that the expression of PP4IP is restricted to the nuclei and it colocalizes well with PP4C (Fig. 3A). From cell fractionation study, we observed that the majority of PP4IP in nucleus is included at chromatin and the DNA damage by irradiation did not causes any notable changes of PP4IP expression in different cellular

compartments, compared to the loading control (Fig. 3B). This result is in agreement with a previous study that PP4C, PP4R2 and PP4R3 in nuclei were mainly associated with chromatin (Chowdhury et al., 2008).

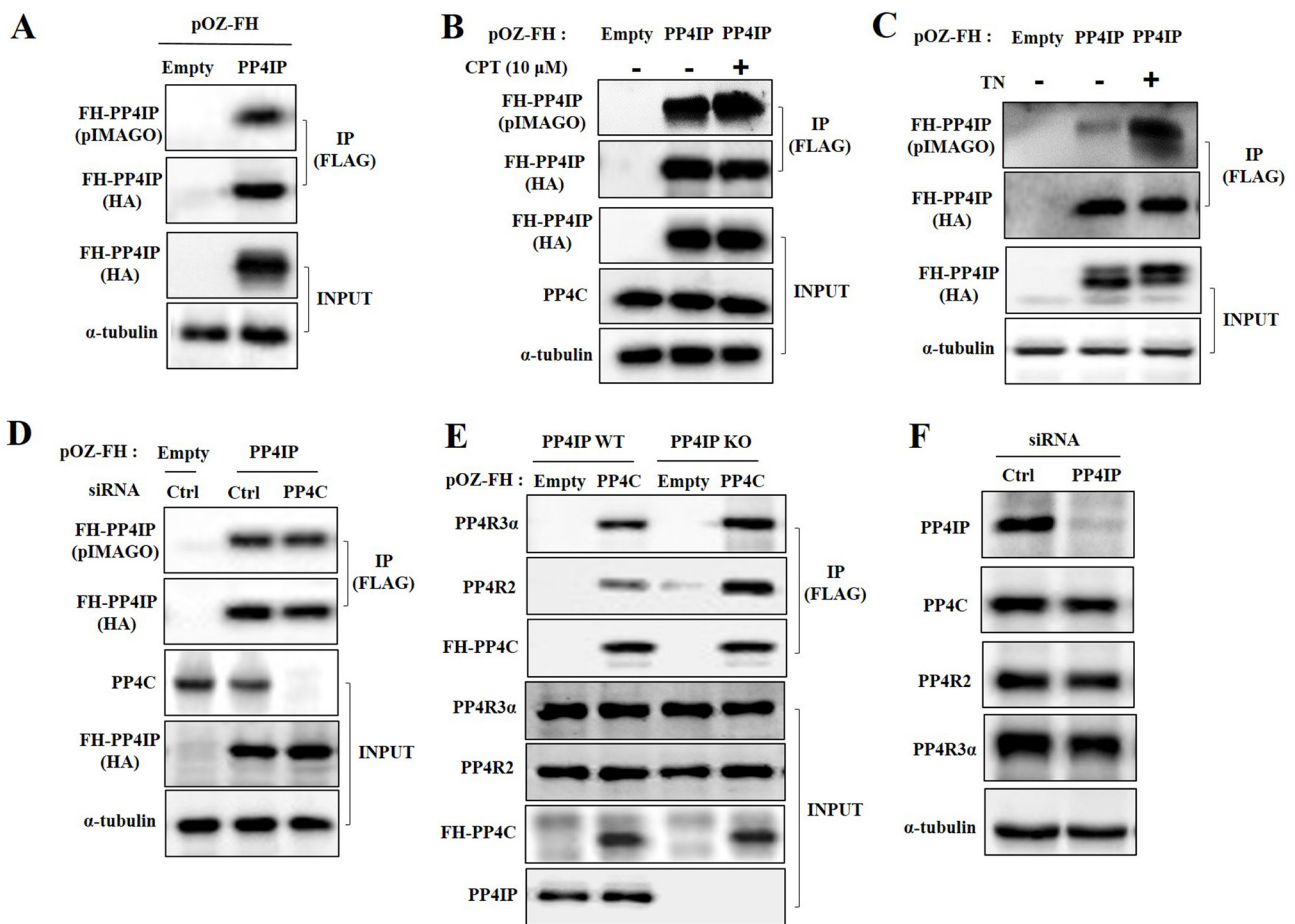
#### PP4IP as a new regulator of PP4

PP4 interacts and dephosphorylates various proteins as substrates in response to DNA damage. Since PP4 interacts with PP4IP, we reasoned that PP4IP might be a substrate for dephosphorylation by PP4. To address this issue, we performed the phosphorylation detection assay to assess the phosphor-

ylation of PP4IP. We found that PP4IP is phosphorylated independent of DNA damage and even hyper-phosphorylated in cell arrested at mitosis (Figs. 4A-4C). Next, to investigate whether the phosphorylation of PP4IP is regulated by PP4, the phosphorylation status of PP4IP is monitored in cells depleted of PP4. Against our expectation, the depletion of PP4 did not cause any notable changes of PP4IP phosphorylation, suggesting PP4IP as a novel binding partner of PP4, not as a substrate for dephosphorylation (Fig. 4D).

Target specificity and dephosphorylation activity of PP4 is entirely dependent on its regulatory subunits that directly interact with PP4C (Lee and Chowdhury, 2011; Pereira et al., 2011; Shi, 2009). To identify whether PP4IP influences

the interaction of PP4C with regulatory subunits, PP4R3 $\alpha$  and PP4R2, we ectopically expressed PP4C in PP4IP-depleted cells and performed the immunoprecipitation assay to monitor the interaction with regulatory subunits. Relative to the expression level of PP4C, significantly more robust interaction is detected between PP4C and regulatory subunits in PP4IP-depleted cells than that in PP4IP-expressing cells (Fig. 4E). This result suggests that PP4IP as an inhibitor impedes the interaction between PP4C and the regulatory subunits. In addition, the depletion of PP4IP had no significant effect on the expression of PP4 subunits (Fig. 4F). Taken together, our results indicate that PP4IP adversely affect PP4 activity and therefore serves as a regulatory protein to PP4 rather than as



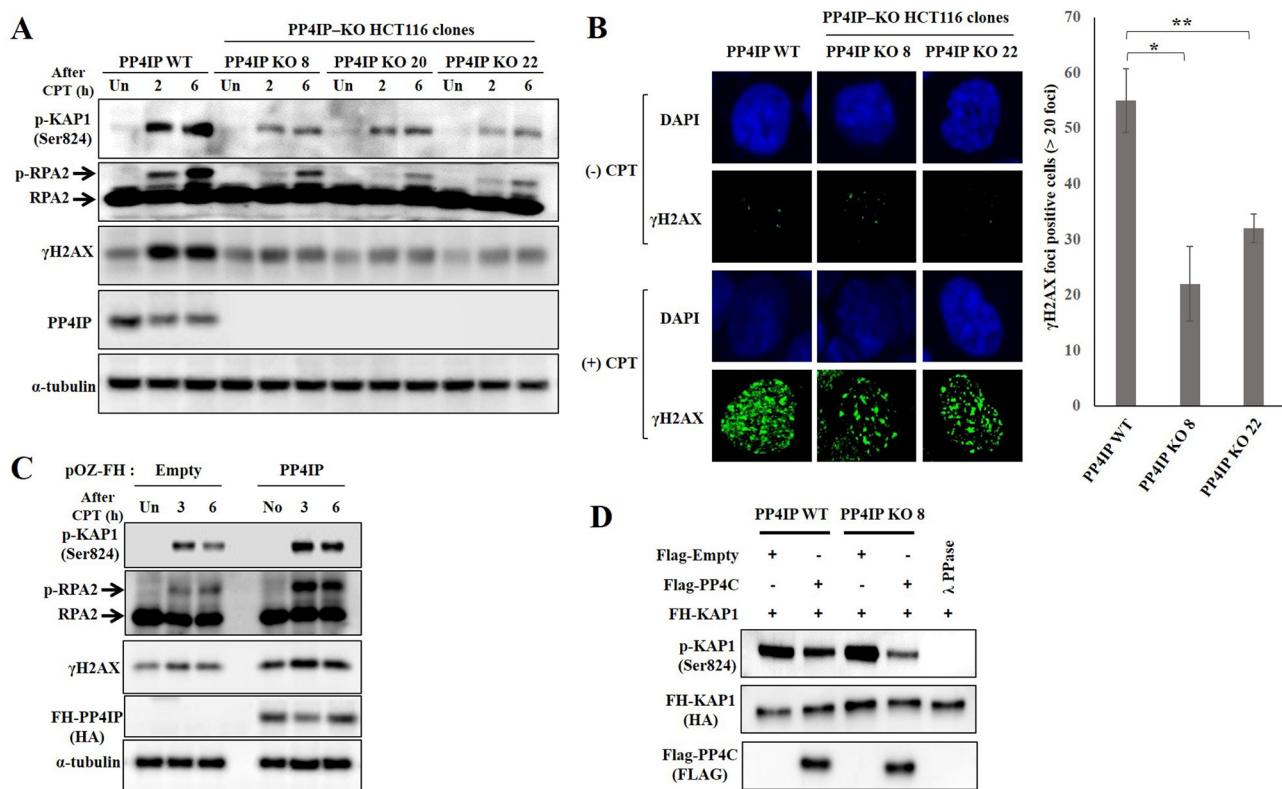
**Fig. 4. Phosphorylation of PP4IP is not regulated by PP4.** (A-D) HeLa S3 cells stably expressing FH-empty or FH-PP4IP were lysed and FH-PP4IP immunoprecipitated by Flag antibody was probed with pIMAGO-biotin and subsequently avidin-HRP for phosphorylation detection. Expression of FH-PP4IP was assessed by antibody against HA. (A) PP4IP was phosphorylated in unperturbed cells. Immunoprecipitates from untreated cells were blotted and phosphorylation of PP4IP was assessed with antibodies indicated. (B) CPT treatment did not significantly augment the phosphorylation of PP4IP. Cells were treated with CPT to induce DSBs. (C) Phosphorylation of PP4IP increased in cells arrested at mitosis. Cells were treated with thymidine and nocodazole, and phosphorylation of PP4IP was assessed. TN, thymidine-nocodazole. (D) Phosphorylated PP4IP is not a substrate of PP4. Cells were transfected with PP4C or control siRNAs and harvested after 48 h. Phosphorylation status of PP4IP was monitored. Ctrl, control. (E) The depletion of PP4IP makes PP4 complex more stable. PP4IP-knockout cells were transfected with FH-empty or FH-PP4C vector, immunoprecipitated with anti-Flag agarose beads. Immunoprecipitates were resolved in SDS-PAGE and probed with antibodies against PP4IP, PP4R2, and PP4R3 $\alpha$ . (F) PP4IP depletion did not affect the stability of PP4 subunits. HCT116 cells were transfected with siRNAs against PP4IP. Lysates were resolved in SDS-PAGE and probed with antibodies against PP4IP, PP4C, PP4R2, and PP4R3 $\alpha$ .

a substrate.

### Alteration of PP4IP expression affects PP4-mediated dephosphorylation.

To demonstrate the inhibitory role of PP4IP on PP4, we constructed the PP4IP-knockout cells using CRISPR/Cas9 genome editing and monitored the phosphorylation of KAP1, H2AX and RPA2, all of which acted as PP4 substrates for dephosphorylation in response to DNA damage (Chowdhury et al., 2008; Lee et al., 2010; 2012). Compared to control cells expressing PP4IP, the phosphorylation of KAP1, RPA2 and H2AX after CPT treatment was largely impeded in PP4IP-knockout cells after CPT treatment (Fig. 5A). Consistent with the results presented in Figure 5A, cytological analysis indicated that there are more intense  $\gamma$ H2AX foci in

control cells than that in PP4IP-knockout cells (Fig. 5B). Thus, these data imply that PP4 could dephosphorylate its target proteins more actively in the absence of PP4IP. In contrast, cells ectopically over-expressing PP4IP show the hyper-phosphorylation of KAP1, RPA2 and H2AX, compared to control cells (Fig. 5C). Previously, we showed that recombinant PP4C efficiently dephosphorylates phospho-KAP1 in *in vitro* dephosphorylation reaction (Lee et al., 2012). Therefore, we were wondering if PP4IP affects the dephosphorylational activity of PP4C. To test this, we immunopurified FH-KAP1 from CPT-treated 293T cells and Flag-PP4C from PP4IP- knockout or -expressed cells and performed the dephosphorylation assay as described earlier (Chowdhury et al., 2008; Lee et al., 2012; 2014). We observed that PP4C from PP4IP-knockout cells dephosphorylates phospho-KAP1 more efficiently,



**Fig. 5. PP4IP inhibits PP4-mediated dephosphorylation.** (A) PP4IP knockout impedes the phosphorylation of KAP1, RPA2, and H2AX induced by DNA damage. PP4IP-KO HCT116 clones were treated with 1  $\mu$ M CPT for 1.5 h and collected at the indicated time points. The lysates were immunoblotted with antibodies against KAP1 pSer824, RPA2,  $\gamma$ H2AX, PP4IP, and  $\alpha$ -tubulin. (B) Formation of  $\gamma$ H2AX foci in PP4IP-KO HCT116 cells is impaired. PP4IP-knockout HCT116 cells were incubated with 1  $\mu$ M CPT for 1.5 h and fixed after 2 h. The cells were subjected to immunostaining for  $\gamma$ H2AX (green). Quantification of  $\gamma$ H2AX foci-positive cells after CPT treatment was shown on right graph. More than 100 cells were quantified per condition at each of three independent experiments. Data are expressed as mean  $\pm$  SD (n = 3). \*P = 0.0026, \*\*P = 0.0129. (C) PP4IP overexpression increases the phosphorylation of RPA2 and H2AX. U2OS cells were transfected with FH-empty or FH-PP4IP vector, followed by 1  $\mu$ M CPT treatment for 1.5 h. The cells were collected at the indicated time points from release and immunoblotted with antibodies against RPA2,  $\gamma$ H2AX, PP4IP, and  $\alpha$ -tubulin. (D) PP4C derived from PP4IP-KO cells dephosphorylates phospho-KAP1 more efficiently than PP4C from PP4IP-expressed cells in *in vitro*. PP4IP-expressed or knockout HCT116 cells were transfected with Flag-empty or Flag-PP4C plasmids and harvested after 48 h. In a parallel experiment, 293T cells were transfected with FH-KAP1. After 2 days, cells were incubated with 10  $\mu$ M CPT for 1 h to form phospho-KAP1 and collected after 1 h from release. Cells were subjected to immunoprecipitation with anti-Flag agarose beads. Immunoprecipitates (Flag-PP4C and FH-KAP1) were mixed for phosphatase reaction and subsequently immunoblotted with antibody against KAP1 pSer824. The amount of FH-KAP1 or Flag-PP4C was assessed. Lambda ( $\lambda$ ) phosphatase (PPase) serves as positive control for the reaction.

compared to PP4C from cells expressing PP4IP. Lambda ( $\lambda$ ) protein phosphatase served as a control (Fig. 5D). In combination, these results strongly suggest that PP4IP impedes the activity of PP4 and therefore functions as an inhibitor.

### PP4IP affects DNA DSBs repair

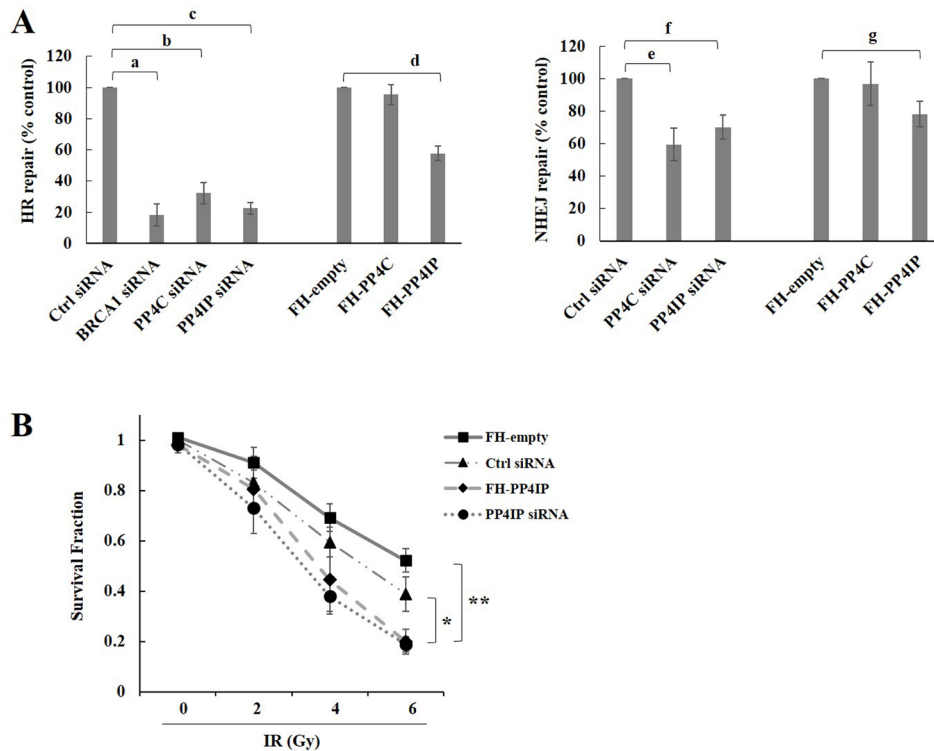
From previous studies, we and others clearly demonstrated that PP4 is essential for DNA DSBs repairs, HR and NHEJ (Lee et al., 2010; 2012; Liu et al., 2012; Nakada et al., 2008). Therefore, we reasoned that PP4IP might influence the repair efficiency of DSBs. To address this issue, we expressed rare-cutting I-SceI endonuclease in HeLa cells containing DR-GFP or EJ5-GFP reporters (Lee and Lee, 2014) and monitored HR and NHEJ rates in cells either overexpressing or depleted of PP4IP. As expected, the efficiency of HR and NHEJ is significantly decreased in PP4IP-overexpressing cells, compared to controls (Fig. 6A). Interestingly, we observed that the depletion of PP4IP also largely hampers both HR and NHEJ. This implies that the formation of phospho-signal of PP4 substrates prior to the action of PP4 as a phosphatase is necessary for cells to properly respond the DNA damage. Defects in the efficiency of DSB repair would be expected to be biologically relevant, and indeed, cells, where PP4IP is either

overexpressed or depleted, had lower viability than control cells at all tested dose of IR (Fig. 6B). Altogether, these results suggest that PP4IP negatively regulates the ability of PP4 to dephosphorylate proteins, but it is also required for cells to secure enough time to form the phosphorylation of PP4 substrates and then properly process DSB repair.

### DISCUSSION

In the light of fact that PP4 has crucial roles in various cellular functions, it is feasible that study on seeking novel molecules, besides regulatory subunits in complex, is worth exploring.

The recombinant C19ORF43, referred to as human telomerase RNA component interacting RNase (hTRIR) from a recent study, has RNase activity according to *in vitro* nuclease assay (Xie et al., 2017). However, the cellular role of hTRIR *in vivo* still remains to be elucidated. Here, we identified the role of hTRIR as a novel inhibitor of PP4 and accordingly designated it as PP4IP. PP4IP interacts with PP4 subunits, PP4C, PP4R2, and PP4R3 $\alpha$  as a regulator with inhibitory role rather than as a substrate. Based on the fact that PP4 complex is tightly inactivated through the phosphorylation of PP4R2 and PP4R3 by CDK in cells arrested in mitosis (Voss et al.,



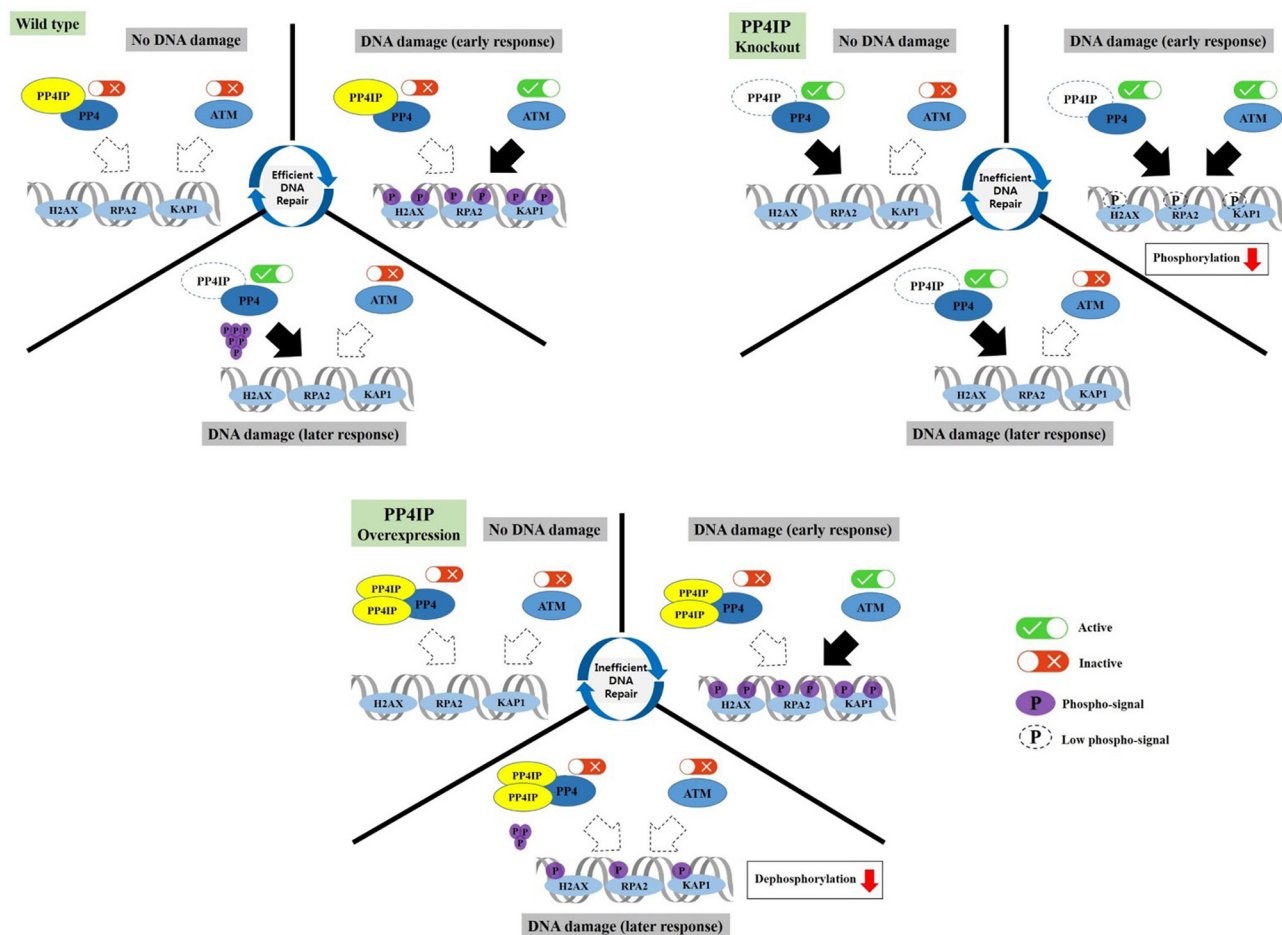
**Fig. 6. Altered expression of PP4IP affects DNA DSBs repair and cell survival.** (A) DNA DSBs repairs are impaired by either the depletion or overexpression of PP4IP. HeLa-DRGFP (HR) and -EJ5GFP (NHEJ) cells depleted of BRCA1, PP4C, or PP4IP by siRNAs were transfected with I-SceI endonuclease. In a parallel experiment, cells expressing FH-empty, FH-PP4C, or FH-PP4IP were transfected with I-SceI endonuclease vector. GFP-positive cells were quantified using flow cytometry. siRNAs against BRCA1 and PP4C serve as control. Data are expressed as mean  $\pm$  SD (n = 3). <sup>a</sup>P = 0.0024, <sup>b</sup>P = 0.003, <sup>c</sup>P = 0.0007, <sup>d</sup>P = 0.004, <sup>e</sup>P = 0.020, <sup>f</sup>P = 0.019, <sup>g</sup>P = 0.042. (B) Radiosensitivity of cells with altered expression of PP4IP. HeLa cells were transfected with siRNAs against PP4IP for silencing or with FH-PP4IP for overexpression. At 72 h after transfection, cells were irradiated at the indicated doses, and viability was evaluated by clonogenic survival assay. Data are expressed as mean  $\pm$  SD (n = 3). \*P = 0.0216, \*\*P = 0.0012.



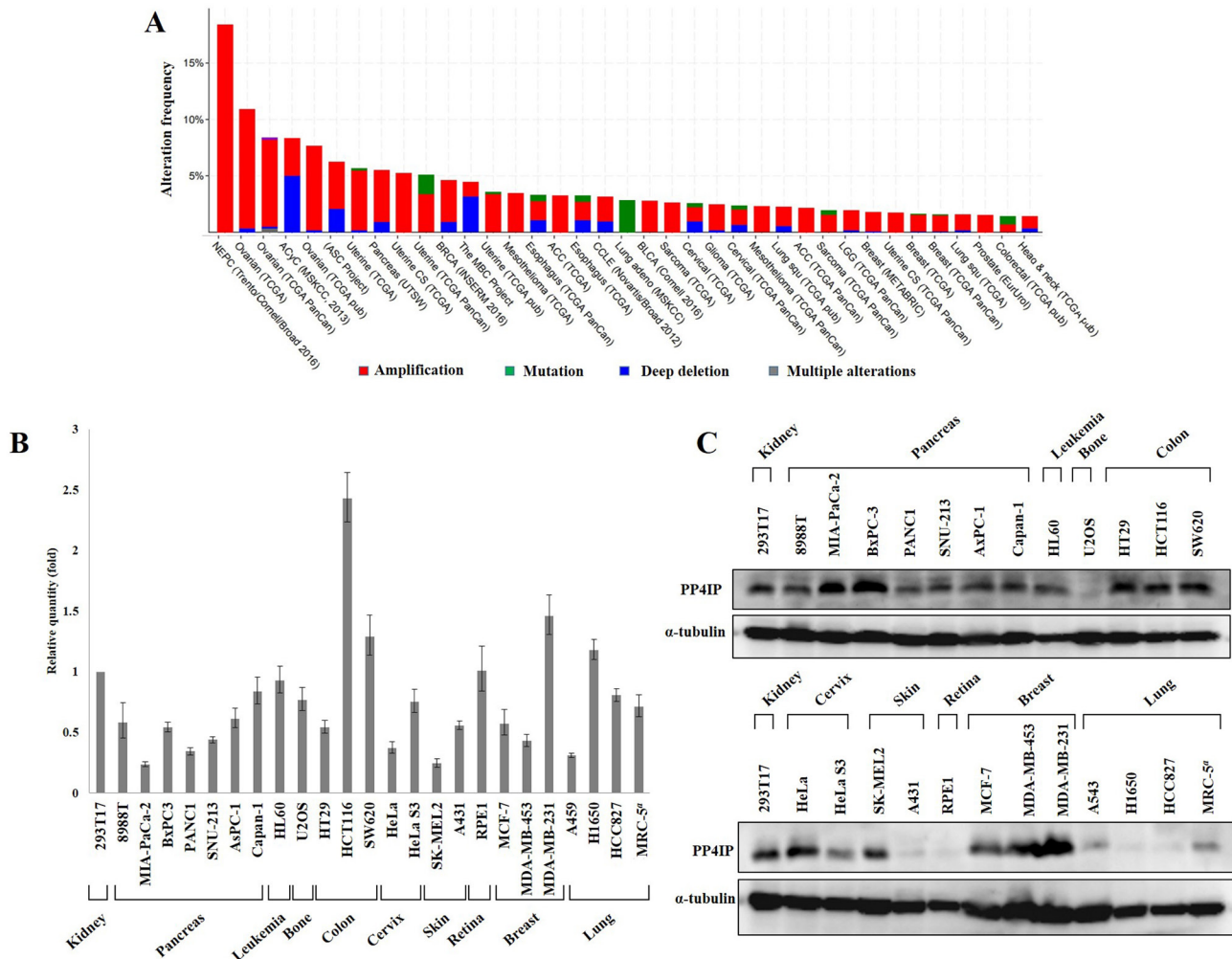
2013), the steep increase in interaction between PP4IP and PP4C at mitosis allows us to predict that PP4IP play a role in inhibiting PP4. Indeed, we clearly demonstrated that PP4 activity is significantly suppressed in cells overexpressing PP4IP, whereas the depletion of PP4IP promotes PP4C-mediated dephosphorylation of its target substrates, such as  $\gamma$ H2AX, phospho-KAP1, and phospho-RPA2. Furthermore, by *in vitro* dephosphorylation assay, we revealed that PP4C in cells depleted of PP4IP dephosphorylates phospho-KAP1 more efficiently, compared to the cells expressing PP4IP. However, we observed that the depletion of PP4IP also significantly impairs the efficiency of DSBs repairs and cell survival, which is compatible to cells expressing PP4IP. Why is PP4IP as an inhibitor of PP4 still a requisite for cells? Previous studies showed that timely phosphorylation of H2AX, RPA2 or KAP1 in repair of DSBs is critical for cells to transmit the signal to downstream proteins involved in DDR process (Chowdhury et al., 2008; Lee et al., 2010; 2012; 2014; 2015; Nakada et al., 2008). But, the early activation of PP4 by PP4IP knockout even prior to the induction of DNA damage impedes the proper for-

mation of phosphorylational signal during the early phase of DNA repair and consequently reduces the repair efficiency of DSBs. This result implies that PP4 should be suppressed for certain time period in order to generate a sufficient phosphorylation on primary proteins in DDR. Interestingly, PP4 activation following DNA damage might be strongly associated with a corresponding decrease in the expression of PP4IP (Figs. 1 and 5-7). Taken together, PP4IP is a novel inhibitor of PP4 and suppresses PP4-mediated cellular functions.

Due to its frequent overexpression in various types of cancer, PP4C has been considered as an oncoprotein (Li et al., 2015; 2016; Mohammed et al., 2016; Wang et al., 2008; Weng et al., 2012; Wu et al., 2015). Data extracted from Cancer Genome Atlas shows that PP4IP is amplified in 36 out of 37 different cancer tissues (Fig. 8A) and the total amount of mRNA and protein of PP4IP varies in cancer cell lines (Fig. 8B and 8C). To address its association with cancer, but not limited to, the study regarding expressional profiles and functional role of PP4IP in various tumors should be followed. From another perspective, a synthetic inhibitor, which is spe-



**Fig. 7. Working model of PP4-PP4IP complex during DNA repair.** In absence of PP4IP, prematurely inadequate activation of PP4 by depletion of PP4IP weakens phospho-signaling by DNA repair kinase (ATM), which is critical for initiation of DNA repair. However, overexpression of PP4IP also impairs DNA repair since prolonged inactivation of PP4 by PP4IP (longer than it should be) is not able to dephosphorylate major repair proteins during DDR, which is also required for DNA repair (Lee and Chowdhury, 2011).



**Fig. 8. Alteration frequency of TRIR (PP4IP) gene in various cancers and the expression of PP4IP in cancer cell lines.** (A) Thirty-six out of thirty-seven cancer tissues showed amplifications in TRIR (PP4IP) gene. Data extracted from cBioportal (<http://www.cBioportal.org>). (B) Assessment of PP4IP mRNA from a variety of human cancer cell lines using quantitative PCR. Data are expressed as mean  $\pm$  SD (n = 3). (C) Immunoblotting of PP4IP from extracts prepared from various human cancer cell lines. <sup>a</sup>MRC-5 is only non-cancerous cell derived from normal lung tissue.

cific to PP4C, can be developed using the domain of PP4IP required for physical interaction with PP4. Inhibitors have been used in the studies on PP4 also affects other phosphatases, meaning that there is no PP4-specific inhibitor developed. The specific inhibitor to PP4 should be useful for studies on phosphatase and cancer therapy as well. Future studies will be followed.

#### Disclosure

The authors have no potential conflicts of interest to disclose.

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#### REFERENCES

- Chen, M.Y., Chen, Y.P., Wu, M.S., Yu, G.Y., Lin, W.J., Tan, T.H., and Su, Y.W. (2014). PP4 is essential for germinal center formation and class switch recombination in mice. *PLoS One* 9, e107505.
- Chowdhury, D., Xu, X., Zhong, X., Ahmed, F., Zhong, J., Liao, J., Dykxhoorn, D.M., Weinstock, D.M., Pfeifer, G.P., and Lieberman, J. (2008). A PP4-phosphatase complex dephosphorylates gamma-H2AX generated during DNA replication. *Mol. Cell* 31, 33-46.
- Goodarzi, A.A., Kurka, T., and Jeggo, P.A. (2011). KAP-1 phosphorylation regulates CHD3 nucleosome remodeling during the DNA double-strand break response. *Nat. Struct. Mol. Biol.* 18, 831-839.
- Hu, C., Zhang, S., Gao, X., Gao, X., Xu, X., Lv, Y., Zhang, Y., Zhu, Z., Zhang,

- C., Li, Q., et al. (2012). Roles of Kruppel-associated Box (KRAB)-associated Co-repressor KAP1 Ser-473 phosphorylation in DNA damage response. *J. Biol. Chem.* 287, 18937-18952.
- Jackson, S.P. and Bartek, J. (2009). The DNA-damage response in human biology and disease. *Nature* 461, 1071-1078.
- Kim, H., Dejsuphong, D., Adelmant, G., Ceccaldi, R., Yang, K., Marto, J.A., and D'Andrea, A.D. (2014). Transcriptional repressor ZBTB1 promotes chromatin remodeling and translesion DNA synthesis. *Mol. Cell* 54, 107-118.
- Lee, D.H., Acharya, S.S., Kwon, M., Drane, P., Guan, Y., Adelmant, G., Kalev, P., Shah, J., Pellman, D., Marto, J.A., et al. (2014). Dephosphorylation enables the recruitment of 53BP1 to double-strand DNA breaks. *Mol. Cell* 54, 512-525.
- Lee, D.H. and Chowdhury, D. (2011). What goes on must come off: phosphatases gate-crash the DNA damage response. *Trends Biochem. Sci.* 36, 569-577.
- Lee, D.H., Goodarzi, A.A., Adelmant, G.O., Pan, Y., Jeggo, P.A., Marto, J.A., and Chowdhury, D. (2012). Phosphoproteomic analysis reveals that PP4 dephosphorylates KAP-1 impacting the DNA damage response. *EMBO J.* 31, 2403-2415.
- Lee, D.H., Pan, Y., Kanner, S., Sung, P., Borowiec, J.A., and Chowdhury, D. (2010). A PP4 phosphatase complex dephosphorylates RPA2 to facilitate DNA repair via homologous recombination. *Nat. Struct. Mol. Biol.* 17, 365-372.
- Lee, J., Adelmant, G., Marto, J.A., and Lee, D.H. (2015). Dephosphorylation of DBC1 by protein phosphatase 4 is important for p53-mediated cellular functions. *Mol. Cells* 38, 697-704.
- Lee, J. and Lee, D.H. (2014). Leucine methylation of protein phosphatase PP4C at C-terminal is critical for its cellular functions. *Biochem. Biophys. Res. Commun.* 452, 42-47.
- Li, M., Li, X., Xu, S., Xue, P., Li, Q., Lu, Q., Jia, Q., Zhang, L., Li, X., and Li, X. (2016). Protein phosphatase 4 catalytic subunit is overexpressed in glioma and promotes glioma cell proliferation and invasion. *Tumour Biol.* 37, 11893-11901.
- Li, X., Liang, L., Huang, L., Ma, X., Li, D., and Cai, S. (2015). High expression of protein phosphatase 4 is associated with the aggressive malignant behavior of colorectal carcinoma. *Mol. Cancer* 14, 95.
- Liu, B., Wang, Z., Ghosh, S., and Zhou, Z. (2013). Defective ATM-Kap-1-mediated chromatin remodeling impairs DNA repair and accelerates senescence in progeria mouse model. *Aging Cell* 12, 316-318.
- Liu, J., Xu, L., Zhong, J., Liao, J., Li, J., and Xu, X. (2012). Protein phosphatase PP4 is involved in NHEJ-mediated repair of DNA double-strand breaks. *Cell Cycle* 11, 2643-2649.
- Lyu, J., Kim, H.R., Yamamoto, V., Choi, S.H., Wei, Z., Joo, C.K., and Lu, W. (2013). Protein phosphatase 4 and Smek complex negatively regulate Par3 and promote neuronal differentiation of neural stem/progenitor cells. *Cell Rep.* 5, 593-600.
- Martin-Granados, C., Philp, A., Oxenham, S.K., Prescott, A.R., and Cohen, P.T. (2008). Depletion of protein phosphatase 4 in human cells reveals essential roles in centrosome maturation, cell migration and the regulation of Rho GTPases. *Int. J. Biochem. Cell Biol.* 40, 2315-2332.
- Matsuoka, S., Ballif, B.A., Smogorzewska, A., McDonald, E.R., 3rd, Hurov, K.E., Luo, J., Bakalarski, C.E., Zhao, Z., Solimini, N., Lerenthal, Y., et al. (2007). ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. *Science* 316, 1160-1166.
- Mohammed, H.N., Pickard, M.R., and Mourtada-Maarabouni, M. (2016). The protein phosphatase 4 - PEA15 axis regulates the survival of breast cancer cells. *Cell. Signal.* 28, 1389-1400.
- Mu, J.J., Wang, Y., Luo, H., Leng, M., Zhang, J., Yang, T., Besusso, D., Jung, S.Y., and Qin, J. (2007). A proteomic analysis of ataxia telangiectasia-mutated (ATM)/ATM-Rad3-related (ATR) substrates identifies the ubiquitin-proteasome system as a regulator for DNA damage checkpoints. *J. Biol. Chem.* 282, 17330-17334.
- Nakada, S., Chen, G.I., Gingras, A.C., and Durocher, D. (2008). PP4 is a gamma H2AX phosphatase required for recovery from the DNA damage checkpoint. *EMBO Rep.* 9, 1019-1026.
- Noon, A.T., Shibata, A., Rief, N., Lobrich, M., Stewart, G.S., Jeggo, P.A., and Goodarzi, A.A. (2010). 53BP1-dependent robust localized KAP-1 phosphorylation is essential for heterochromatic DNA double-strand break repair. *Nat. Cell Biol.* 12, 177-184.
- Pereira, S.R., Vasconcelos, V.M., and Antunes, A. (2011). The phosphoprotein phosphatase family of Ser/Thr phosphatases as principal targets of naturally occurring toxins. *Crit. Rev. Toxicol.* 41, 83-110.
- Shi, Y. (2009). Serine/threonine phosphatases: mechanism through structure. *Cell* 139, 468-484.
- Sripathy, S.P., Stevens, J., and Schultz, D.C. (2006). The KAP1 corepressor functions to coordinate the assembly of de novo HP1-demarcated microenvironments of heterochromatin required for KRAB zinc finger protein-mediated transcriptional repression. *Mol. Cell. Biol.* 26, 8623-8638.
- Toyo-oka, K., Mori, D., Yano, Y., Shiota, M., Iwao, H., Goto, H., Inagaki, M., Hiraiwa, N., Muramatsu, M., Wynshaw-Boris, A., et al. (2008). Protein phosphatase 4 catalytic subunit regulates Cdk1 activity and microtubule organization via NDEL1 dephosphorylation. *J. Cell Biol.* 180, 1133-1147.
- Voss, M., Campbell, K., Saranzewa, N., Campbell, D.G., Hastie, C.J., Pegg, M.W., Martin-Granados, C., Prescott, A.R., and Cohen, P.T. (2013). Protein phosphatase 4 is phosphorylated and inactivated by Cdk in response to spindle toxins and interacts with gamma-tubulin. *Cell Cycle* 12, 2876-2887.
- Wang, B., Zhao, A., Sun, L., Zhong, X., Zhong, J., Wang, H., Cai, M., Li, J., Xu, Y., Liao, J., et al. (2008). Protein phosphatase PP4 is overexpressed in human breast and lung tumors. *Cell Res.* 18, 974-977.
- Weng, S., Wang, H., Chen, W., Katz, M.H., Chatterjee, D., Lee, J.E., Pisters, P.W., Gomez, H.F., Abbruzzese, J.L., Fleming, J.B., et al. (2012). Overexpression of protein phosphatase 4 correlates with poor prognosis in patients with stage II pancreatic ductal adenocarcinoma. *Cancer Epidemiol. Biomarkers Prev.* 21, 1336-1343.
- Wu, G., Ma, Z., Qian, J., and Liu, B. (2015). PP4R1 accelerates cell growth and proliferation in HepG2 hepatocellular carcinoma. *OncoTargets Ther.* 8, 2067-2074.
- Xie, J., Chen, Z., Zhang, X., Chen, H., and Guan, W. (2017). Identification of an RNase that preferentially cleaves A/G nucleotides. *Sci. Rep.* 7, 45207.
- Xie, Y., Juschke, C., Esk, C., Hirotsune, S., and Knoblich, J.A. (2013). The phosphatase PP4c controls spindle orientation to maintain proliferative symmetric divisions in the developing neocortex. *Neuron* 79, 254-265.
- Zhao, H., Huang, X., Jiao, J., Zhang, H., Liu, J., Qin, W., Meng, X., Shen, T., Lin, Y., Chu, J., et al. (2015). Protein phosphatase 4 (PP4) functions as a critical regulator in tumor necrosis factor (TNF)-alpha-induced hepatic insulin resistance. *Sci. Rep.* 5, 18093.
- Ziv, Y., Bielopski, D., Galanty, Y., Lukas, C., Taya, Y., Schultz, D.C., Lukas, J., Bekker-Jensen, S., Bartek, J., and Shiloh, Y. (2006). Chromatin relaxation in response to DNA double-strand breaks is modulated by a novel ATM- and KAP-1 dependent pathway. *Nat. Cell Biol.* 8, 870-876.