Protein phosphatase 2A regulated by USP7 is polyubiquitinated and polyneddylated

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Abstract. Ubiquitin-specific protease 7 (USP7) participates in the ubiquitin-proteasome system (UPS), and is considered an essential regulator of substrate stability in cancers. In a previous study, the substrates that bind to USP7 were separated through two-dimensional electrophoresis (2-DE), which resulted in the identification of protein phosphatase 2A (PP2A) through matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF/MS) analysis. In the present study, GST pull-down assay was performed to determine whether USP7 and PP2A directly bind to each other. Immunocytochemistry assay confirmed that USP7 co-localizes with PP2A in the cytoplasm and nucleus of HeLa cells. Moreover, western blotting and immunoprecipitation were performed to determine whether polyubiquitination and polyneddylation of PP2A were formed. The results of the present study demonstrated that USP7 was a deubiquitinating enzyme of PP2A, and regulated the ubiquitination and stability of PP2A through the K48-linked polyubiquitin chains. Consequently, the knockdown of USP7 reduced the expression of PP2A. The data of the present study revealed the cellular association between USP7 and PP2A, a new substrate of USP7.

Introduction

The ubiquitin-proteasome system (UPS) is a remarkable protein degradation system having diverse known and unknown functions, including degradation of most proteins in eukaryotic cells (1). This system encompasses several mechanisms including ubiquitination, which is mediated by the successive activities of E1, E2, and E3 enzymes. In addition, ubiquitination not only regulates proteasomal degradation, but also cellular functions through single or multiple polyubiquitinations (2). Specifically, ubiquitination is a post-translational modification (PTM) where the ubiquitin binds to a target protein and regulates its proteasomal degradation or cellular functions (3). Reportedly, ubiquitin contains seven lysine residues (K6, K11, K27, K29, K33, K48, and K63) which form a polyubiquitin chain; of these, K48 and K63 are the most well-known polyubiquitination sites (4,5). Notably, numerous potential unknown polyubiquitination sites are being reported currently, and a great amount of research is being focused on identifying them.

Deubiquitination is a reversal process of ubiquitination using enzymes called deubiquitinating enzymes (DUBs), which play an essential role in protein stabilization by removing ubiquitins from the target proteins (6,7). Approximately 100 DUBs have been identified to date, and they are reportedly involved in diverse cellular functions through their special abilities to stabilize or alter the functions of target proteins (8). According to previous research, the DUB family is composed of at least nine classes. In particular, the cysteine protease class contains ubiquitin-specific protease (USP), ovarian tumor protease (OTU), ubiquitin C-terminal hydrolase (UCH), permutated papain fold peptidases of dsDNA viruses and eukaryote (PPPDE), Machado-Joseph disease protease (MJD), motif interacting with Ub-containing novel DUB (MINDY), monocyte chemotactic protein-induced protease (MCPIP), and zinc finger with UFM1-specific peptidase domain protein (ZUFSP) family (9,10). The metalloprotease class belongs to the Jab1/Pab1/MPN metalloenzyme motif protease (JAMM) family (11).

Ubiquitin-specific protease 7 [USP7; also known as herpes virus-associated ubiquitin-specific protease (HAUSP)] is known to play an essential role in various diseases, including cancers. Substrates modulated by USP7 are broadly involved in cellular processes including the cell cycle, DNA repair, chromatin remodeling, and epigenetic regulation (12). The important role of USP7 is to release ubiquitin on substrates that enhance tumorigenesis (12,13). USP7 acts as a negative or positive regulator that modulates the activity of the oncogene or tumor suppressor in numerous cancers (14). Therefore, potential substrates of USP7 could be essential regulators involved in various cellular mechanisms in cancer progression (15,16). Thus, in order to understand the cellular pathway, it is imperative to discover the binding partners of USP7, and explore the cellular and molecular mechanisms of USP7.

In a previous study, substrates related to USP7 were analyzed by two-dimension electrophoresis (2-DE) and

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matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS) analysis, and the analysis confirmed that protein phosphatase 2A (PP2A) is a putative substrate of USP7 (17). PP2A is one of the most abundant cellular enzymes, acting on numerous substrates (18). Furthermore, PP2A regulates diverse cellular functions including cell growth or division (19). Due to multiple cellular processes regulated by PP2A, several different mechanisms are capable of regulating the phosphatase activity, such as association with specific regulatory subunits, or post-translational modifications (PTMs) of PP2A (for example, phosphorylation, carboxymethylation, and ubiquitination) (20). Moreover, PP2A has also been identified as a tumor suppressor (21), wherein it reverses the phosphorylation of oncoproteins such as c-MYC, MEK, and ERK (22,23). The family components of PP2A are reported to be uncommonly mutated or deficient in various cancers (24). Considering all the aforementioned outcomes, PP2A has been determined to play an important role in cancers and is regarded as a significant therapeutic target. Therefore, the intercellular association between USP7 and PP2A was investigated. In addition, confirmation of the role of USP7 as a DUB for PP2A, and elucidation of the regulatory mechanism of PP2A was undertaken.

In the present study, protein-protein interaction between USP7 and PP2A was identified. It is well documented that the UPS regulates PP2A and cellular functions of PP2A are also related to DUBs or E3 ligases. Recent experimental studies have shown that PP2A is a key regulator of progression and metastasis of cancer cells (25). Thus, considering the known functions of USP7 and PP2A, it is suggested that this association between the two proteins is an important feature in cancer progression. Therefore, the association between the two proteins was analyzed by confirming the potential of USP7 as a DUB for PP2A.

Materials and methods

Cell culture and transfection. HeLa (cat. no. CRM-CCL2), MCF-7 (cat. no. HTB-22), and 293T (cat. no. CRL-3216; all from ATCC) cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic reagent (containing penicillin, streptomycin, and amphotericin B; cat. no. 15240-062; Gibco: Thermo Fisher Scientific, Inc.) at 37˚C in a 5% CO_2 incubator. A549 (cat. no. CRM-CL-185; ATCC) cells were incubated in Roswell Park Memorial Institute 1640 (RPMI-1640; Gibco; Thermo Fisher Scientific, Inc.) medium containing 10% FBS, 1% antibiotic-antimycotic reagent (as previously aforementioned) at 37˚C in a 5% CO_2 incubator. Transfection was performed using 150 mM NaCl and 10 mM linear polyethyleneimine (PEI; Polysciences, Inc.) and harvested after 48 h.

Plasmid DNA and antibodies. *PP2A* gene was subcloned into the pCS4-Flag vector and pcDNA3.1-Myc vector. *USP7* and *USP7* (C223S, a catalytically inactive mutant) genes were subcloned into the pcDNA3.1-Myc vector (12) or pCS4-Flag vector. *USP7* gene was subcloned into the pGEX-4T-1 vector for GST pull-down assay (GST-*USP7*). The HA-tagged *ubiquitin* (K48 and K63) was produced using site-directed point mutagenesis (26). HA-K48 is an HA-tagged ubiquitin in which other lysines (K6, K11, K27, K29, K33, and K63) are mutated to an arginine except for K48. While HA-K63 is an HA-tagged ubiquitin in which other lysines (K6, K11, K27, K29, K33, and K48) are substituted with an arginine except for K63. The purpose of HA-K48 and HA-63 is to specifically identify an HA-K48-linked- or HA-K63-linked ubiquitin chain, respectively. Anti-β-actin (cat. no. sc-4778; Santa Cruz Biotechnology, Inc.), anti-HA (cat. no. 12CA5; product code 11583816001; Roche Diagnostics), anti-Flag (cat. no. M185-3L; MBL International Corporation), anti-Myc (cat. no. sc-40; Santa Cruz Biotechnology, Inc.), anti-USP7 (cat. no. CSB-PA849973LA01HU; Cusabio Technology LLC), anti-USP7 (cat. no. sc-137008; Santa Cruz Biotechnology, Inc.), and anti-PP2A (cat. no. sc-6110; Santa Cruz Biotechnology, Inc.) antibodies were used.

Western blotting and immunoprecipitation (IP). Harvested cells were lysed using a lysis buffer (50 mM Tris-HCL, 300 mM NaCl, 1 mM EDTA, 10% Glycerol, 1% Triton X-100) and were incubated for 20 min on ice. Cell lysates were centrifuged at 16,200 x g at 4˚C for 20 min, and the supernatant was boiled with 2X SDS buffer at 100˚C. The protein amounts (30 μg) were determined by Bradford assay using Bio-Rad protein assay dye reagent (cat. no. 5000006; Bio-Rad Laboratories, Inc.). The samples were run in an 8% SDS-page gel and transferred onto microporous polyvinylidene fluoride membranes (cat. no. IPVH00010; EMD Millipore; Merck KGaA). The membranes were then blocked with 5% skim milk in TBS buffer, including 0.05% Tween-20 for 30 min at room temperature, and incubated at 4˚C overnight with a primary antibody [β-actin (1:3,000), HA (1:1,000), Flag (1:5,000), Myc (1:100), USP7 (1:500; cat. no. sc-137008; Santa Cruz Biotechnology, Inc.), and PP2A (1:5,000)] in 2% skim milk with TBS buffer, including 0.05% Tween-20. The probed membranes were incubated in 1% skim milk with TBS buffer including 0.05% Tween-20 with KPL peroxidase-labeled antibody to mouse IgG (H+L) (cat. no. 074-1806; 1:30,000; LGC SeraCare) and mouse anti-goat IgG-HRP (cat. no. sc-2354; 1:30,000; Santa Cruz Biotechnology, Inc.) at room temperature for 1 h. The proteins were detected using an ECL reagent solution (Young In Frontier). For immunoprecipitation, 2 mg of cell lysates was incubated with antibodies [Flag, Myc, USP7, and PP2A (1 μg per 500 μg of total proteins)] at 4˚C on a rotator overnight. A resuspended volume (30 μl) of Protein A/G PLUS-agarose beads (cat. no. sc-2003; Santa Cruz Biotechnology, Inc.) was then added to the cell lysates and incubated at 4˚C on a rotator for 2 h. The samples were then washed with washing buffer [50 mM Tris-HCl, 300 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, a protease inhibitor cocktail (PIC; cat. no. 11697498001; Roche Diagnostics), and phenylmethanesulfonyl fluoride (PMSF; cat. no. P7626; Sigma-Aldrich; Merck KGaA)]. Subsequently, the samples were centrifuged at 3,200 x g at 4˚C for 5 min, and then boiled in 35 μl of 2X SDS buffer at 100˚C for 7 min. The supernatant (25 μl) was then loaded onto 8% SDS-PAGE gel. The remaining steps were the same as those described for the western blotting.

Ubiquitination and deubiquitination assays. For ubiquitination assay, Flag-*PP2A* and HA-*ubiquitin* (HA-*Ub*) (*K48 or K63*) were transfected into 293T cells. The HA-tagged

lysine mutants have been generated by substituting each lysine site with an arginine except one corresponding lysine site. After 48 h, cells were harvested and lysed using a lysis buffer (50 mM Tris-HCL, 300 mM NaCl, 1 mM EDTA, 10% Glycerol, 1% Triton X-100). Cell lysates were used for immunoprecipitation with anti-Flag antibody (1 μg per 500 μg of total proteins). The ubiquitination level of Flag-PP2A was detected through western blotting. For the deubiquitination assay, Flag-*PP2A*, HA-*Ub* (*K48 or K63*)and Myc-*USP7* were transfected into 293T cells. To confirm whether proteasome-dependent degradation of substrates, cells were treated with 10 mM concentration of MG132 [a proteasome inhibitor, cat. no. F1100; Ubiquitin Proteasome Biotechnologies, LLC (UBPbio)] for 4 h before harvest and cultured at 37˚C in a 5% CO_2 incubator. The cells were harvested after 48 h and immunoprecipitation was performed with anti-Flag antibody. Both assays were performed using an ubiquitination assay kit according to the manufacturer's manual (cat. no. UBAK-100; D&P Biotech Inc.; http://www.dnpbiotech.com).

GST pull-down assay. GST-*USP7* was transformed into the BL21 strain. After IPTG treatment, GST-*USP7*-transformed cells were cultured in an 18˚C shaking incubator. The cells were then precipitated and released with phosphate-buffered saline (PBS) containing Triton X-100, PIC, and PMSF and then the cells were lysed using a sonicator. The Flag-*PP2A*-transfected 293T cell lysates (2 mg) were incubated with GST-USP7-fastened Glutathione Sepharose beads (300 μg per 2 mg of total protein; cat. no. GE17-0756-01; GE Healthcare; Cytiva). The beads were then washed with a lysis buffer (20 mM Tris-HCl, 1 mM EDTA, 1 mM dithiothreitol, 150 mM NaCl, 1% Triton X-100). After removing the lysis buffer, 35 μl of 2X SDS buffer was added and boiled at 100˚C for 7 min. The supernatants (25 μl) were then loaded onto 8% SDS-PAGE gel. The remaining steps were the same as those described for the western blotting. A portion of the sample was confirmed for protein expression through western blotting, and the expression of GST-USP7 using the remaining samples was confirmed through Coomassie Brilliant Blue (CBB) staining. The gels (1.5 mm thickness) were stained with 0.1% Coomassie Brilliant blue G 250 (cat. no. 1.15444; Sigma-Aldrich; Merck KGaA) at room temperature for 15 min. Subsequently, the gels were washed by destaining buffer (40% methanol and 10% acetic acid). Gel images were captured using a DUALED Blue/White Transilluminator (cat. no. A6020; Bioneer Corporation). Western blotting was performed to validate the results.

Immunocytochemistry. HeLa, MCF-7, and A549 cells were plated on a sterile glass and were washed with PBS. The cells (80% confluence) were treated with 4% paraformaldehyde at room temperature for 10 min and were then treated with 0.2% Triton X-100 in PBS at room temperature for 1 min. Cells were washed with PBS and were blocked in 1% bovine serum albumin (BSA) in PBS for 1 h. The samples were incubated with primary antibodies, PP2A (1:200) and USP7 (1:200; cat. no. CSB-PA849973LA01HU), at 4˚C overnight. Cells were washed with PBS including 0.5% Tween-20 at room temperature for 10 min and were incubated with Alexa-Fluor-488-conjugated goat anti-mouse (cat. no. A32723; 1:500) and with Alexa-Fluor-568-conjugated goat anti-rabbit (cat. no. A11011; 1:500; Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 1 h. Following incubation, nuclei were stained using DAPI (Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 10 min. Cell images were obtained by a confocal microscope (Zeiss LSM880; Carl Zeiss Microscopy GmbH).

Protein stability assay. The sequences of small interfering RNA negative control (*siNC*) and *siUSP7* were 5'-UUCUCC GAACGUGUCACGUTT-3' and 5'-CAUGCACAAGCAGUG CUGAAGAUAA-3', respectively. *siNC* or *siUSP7* was transfected into HeLa cells, and then cells were cultured in a 60-mm dish at 37˚C in a 5% CO_2 incubator for 24 h. Transfection with *siUSP7* was performed as previously described (27). After 24 h, cycloheximide (CHX) (cat. no. 01810; Sigma-Aldrich; Merck KGaA) was added to a final concentration of 100 μg/ml in *siUSP7*-transfected cells. Next, the cells were harvested in a time-dependent manner (0, 12 and 24 h) and cell lysates were used for western blotting.

Statistical analysis. For all measured data, the values for all samples obtained from at least three independent experiments were averaged, and the standard deviation or standard error was subsequently calculated. Statistical analysis was performed using the unpaired t-test and one-way analysis of variance followed by Tukey's multiple comparisons post hoc tests using GraphPad Prism version 5 (GraphPad Software, Inc.). Densitometric analysis was conducted using ImageJ software (version 1.4.3; National Institutes of Health).

Results

USP7 interacts with PP2A. In a previous study, 2-DE and MALDI-TOF/MS analysis determined that PP2A is a putative substrate of USP7 (17), thereby suggesting the possibility that PP2A could physically bind to USP7. To confirm the interaction between PP2A and USP7, a binding assay using 293T cells was performed. The results revealed binding between PP2A and USP7 (Fig. 1A), indicating the formation of an interaction between endogenous or exogenous PP2A and USP7. Additionally, in order to investigate the binding between these two proteins, the binding of PP2A was investigated with the expression of the differential amount of Myc-USP7. As a result, it was determined that as the concentration of Myc-USP7 increased, the amount of Myc-USP7 binding to PP2A also increased (Fig. 1B). Thereafter, the GST pull-down assay was performed to identify the occurrence of direct binding between the two proteins. GST-USP7 was incubated with overexpressed Flag-PP2A in 293T cell lysates, and subsequently subjected to Western blotting, which revealed that the two proteins bind directly each other (Fig. 1C). Furthermore, immunocytochemical analysis revealed that USP7 and PP2A are co-localized in the cytoplasm and the nucleus in HeLa cells, MCF-7 cells, and A549 cells. (Fig. 1D). Collectively, these results demonstrated the direct and indirect interplay between USP7 and PP2A in the cytoplasm and the nucleus.

PP2A is regulated by ubiquitin and ubiquitin-like molecules. The ubiquitination assay was performed to investigate whether PP2A is regulated by the UPS (Fig. 2A). Results obtained

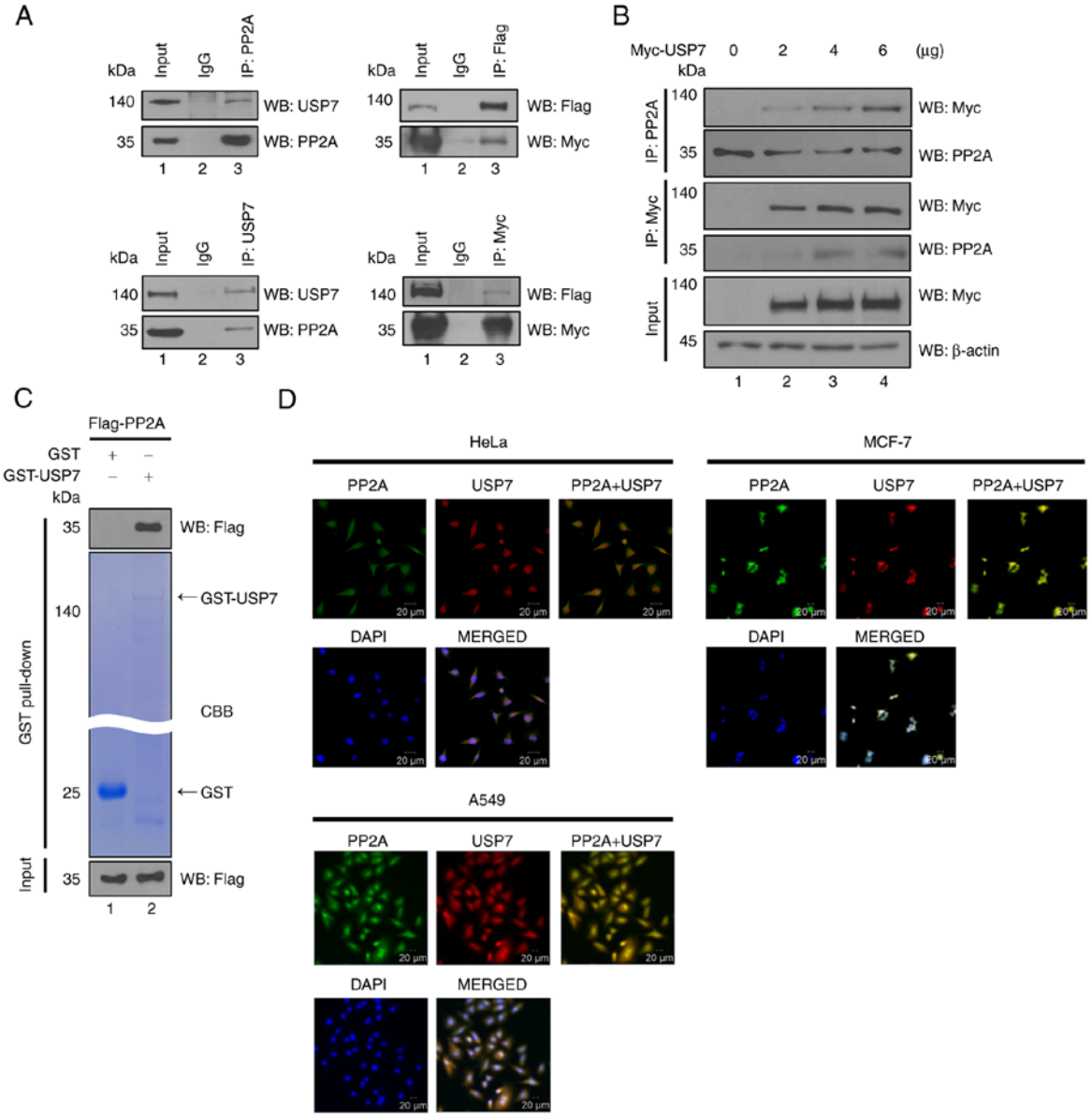

Figure 1. USP7 interacts with PP2A. (A) Endogenous binding assays were performed to confirm the endogenous binding between PP2A and USP7 (left). Exogenous binding assays were also performed to confirm the binding of Flag-PP2A with Myc-USP7, using respective antibodies (right). (B) Myc-*USP7* was transfected into 293T cells in a dose-dependent manner. The immunoprecipitation assay was performed using an anti-Myc or an anti-PP2A antibody. (C) Flag-*PP2A* was transfected into 293T cells for the GST pull-down assay with GST-USP7. (D) Co-localization of USP7 and PP2A in HeLa, MCF-7, and A549 cells, as determined by immunocytochemical analysis (green, PP2A; red, USP7; and blue, DAPI). Scale bar, 20 μm. USP7, ubiquitin-specific protease 7; PP2A, protein phosphatase 2A.

indicated that PP2A is degraded by the ubiquitin-mediated proteasomal degradation. The PTMs involve neddylation, SUMOylation, and ISGylation as well as ubiquitination (28). Moreover, numerous proteins undergo a proteasome-dependent degradation mechanism through ubiquitination, neddylation, SUMOylation, and ISGlyation (29). First, when MG132 was treated with the differential concentration, neddylation of Flag-PP2A was not increased at 2 and 5 μM of MG132 (Fig. 2B); a minimum 10-μM concentration was required to confirm the efficacy of MG132 on neddylation of PP2A. To assume that PP2A would also be modulated by conjugation with ubiquitin as well as ubiquitin-like molecules, IP was performed using co-transfected HA-neuronal precursor cell-expressed developmentally down-regulated protein 8 (HA-*NEDD8*) and Flag-*PP2A* in 293T cells with a proteasome inhibitor, MG132 (Fig. 2C), and it was determined that Flag-PP2A formed neddylation. However, SUMOylation and ISGylation on PP2A were not formed (data not shown). These results suggested that PP2A could also degrade via neddylation as well as ubiquitination due to increased expression level of neddylation on PP2A with the treatment of MG132. Therefore, PP2A could be regulated by PTMs including ubiquitination and neddylation. Increased levels of ubiquitinated or neddylated PP2A were observed after exposure to MG132, indicating that PP2A is degraded in a ubiquitination- or neddylation-mediated proteasome dependent manner. In addition, the ubiquitination assay of PP2A was performed with a K48-linked or K63-linked polyubiquitin chain (Fig. 2D). It was demonstrated that PP2A formed the K48-linked and K63-linked polyubiquitin chains. This suggested that PP2A

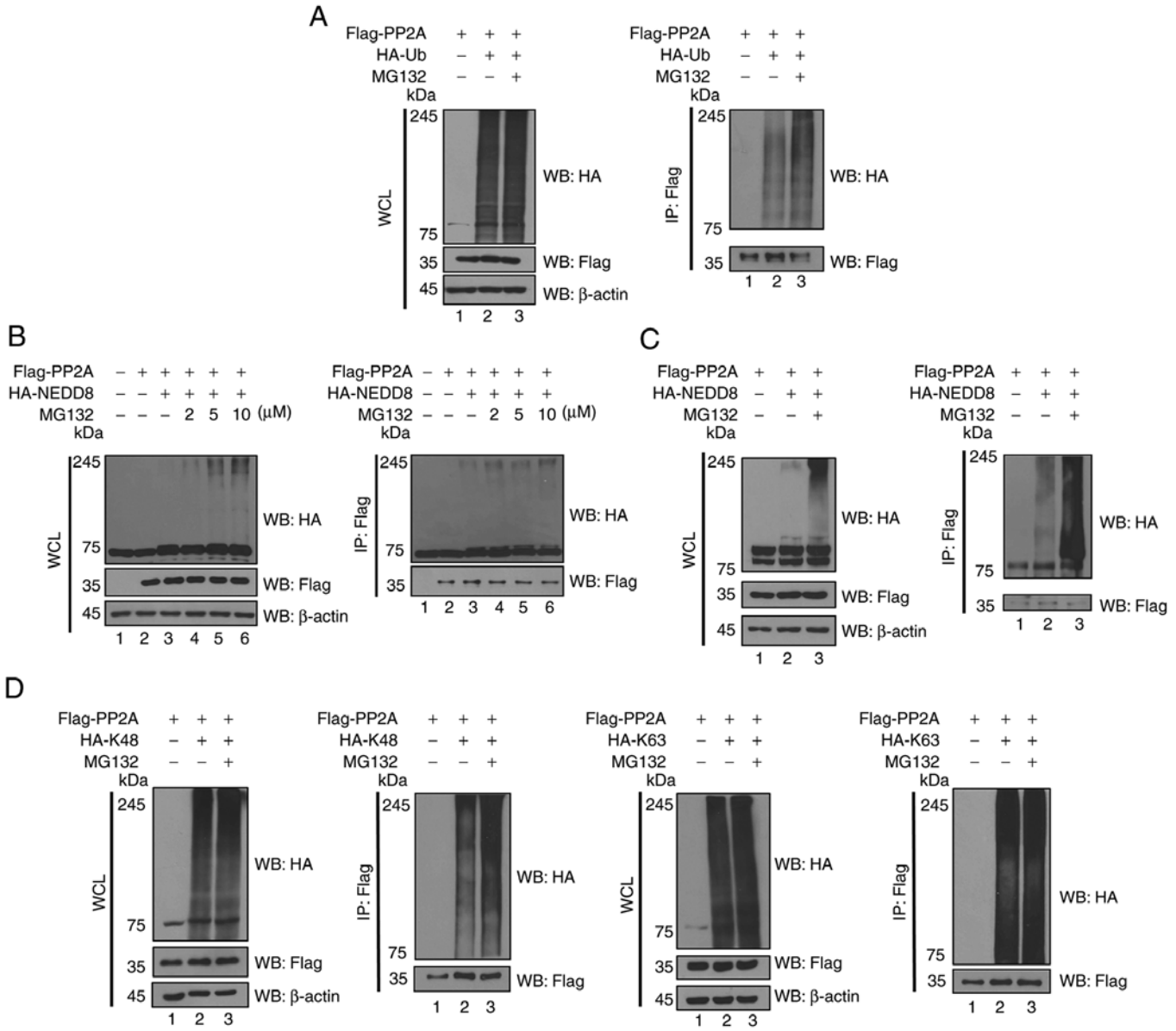

Figure 2. PP2A is regulated by post-translation modifications. (A) HA-*Ub* and Flag-*PP2A* were co-transfected into 293T cells for the ubiquitination assay with MG132 (a proteasome inhibitor). (B) HA-*NEDD8* and Flag-*PP2A*-co-transfected cells were treated with MG132 (2, 5, and 10 μM) in a dose-dependent manner. (C) HA-*NEDD8* and Flag-*PP2A* were co-transfected into cells for the neddylation assay with MG132. (D) HA-*Ub* (K48 and K63) and Flag-*PP2A* were transfected for a ubiquitination assay with the inhibition of proteasomal degradation mediated by MG132. These immunoprecipitation assays were performed using an anti-Flag antibody. PP2A, protein phosphatase 2A; HA-*Ub*, HA-ubiquitin; HA-*NEDD8*, HA-neuronal precursor cell-expressed developmentally down-regulated protein 8.

may be affected by proteasome-dependent degradation associated with K48-linked polyubiquitin chain and intracellular effect by K63-linked ubiquitination (4). K63-linked polyubiquitin chains are known to regulate the proteasome-independent pathways such as signal transduction and endocytosis (5). By contrast, K48-linked polyubiquitin chains are known to regulate the proteasome-dependent pathway (5) Since PP2A formed a K63-linked polyubiquitin chain as shown in Fig. 2D, it is possible that the K63-linked polyubiquitination of PP2A is not related to proteasomal degradation. Furthermore, the reciprocal co-immunoprecipitation assays were performed as ubiquitination assays, as shown in Fig. S1. These results indicated that Flag-PP2A is connected to the HA-tagged polyubiquitin chain or polyneddylated chain.

PP2A is deubiquitinated by USP7. To identify whether USP7 acts as a DUB for PP2A, a deubiquitination assay was performed subsequent to the expression of Myc-USP7, Flag-PP2A, and HA-Ub in 293T cells. USP7 decreased the ubiquitination level of PP2A, and inhibition of the enzymatic activity of USP7 exerted no change in the ubiquitination level of PP2A (Fig. 3A). These results indicated that the catalytic activity of USP7 induced the deubiquitination of PP2A. In addition, the effect of USP7 on the K48-linked or K63-linked polyubiquitin chain on PP2A was investigated (Fig. 3B). It was determined that USP7 deubiquitinated the K48-linked polyubiquitin chain on PP2A, with no associated difference in the K63-linked ubiquitination level on PP2A. This result indicated that USP7 deubiquitinated the K48-linked polyubiquitin chain

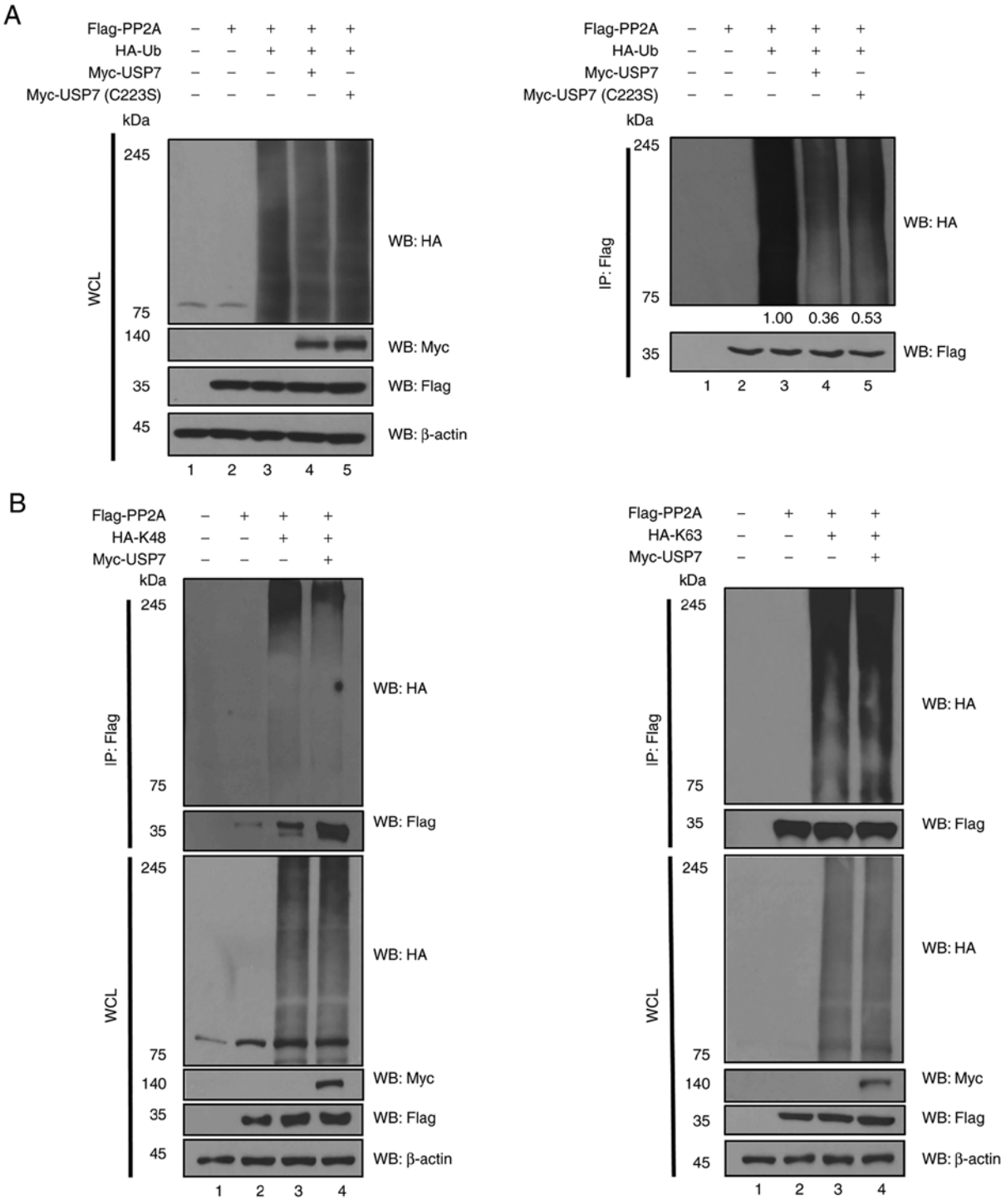

Figure 3. PP2A is deubiquitinated through USP7. (A) The deubiquitination assay was performed using 293T cells transfected with Myc-*USP7*, a catalytically inactive mutant Myc-*USP7* (C223S), Flag-*PP2A*, and HA-*Ub*. Densitometric analysis was performed for densities of HA-Ub relative to the amount of Flag-PP2A. (B) 293T cells were transfected with Myc-*USP7*, Flag-*PP2A*, and specific mutant ubiquitin constructs (HA-*K48* and HA-*K63*). These deubiquitination assays were performed using an anti-Flag antibody. PP2A, protein phosphatase 2A; USP7, ubiquitin-specific protease 7; HA-*Ub*, HA-ubiquitin.

on PP2A, thereby establishing that modulation of proteolysis is proteasome-dependent.

USP7 affects the stability of PP2A. Since USP7 affects the protein stability by deubiquitinating the K48-linked polyubiquitin chain on PP2A, the protein levels of PP2A were investigated to determine whether USP7 controls the PP2A stability in a dose-dependent manner of *siUSP7*. First, the *siUSP7* was used as previously determined (Fig. 4A) (27); the experimental protocol to confirm a decrease in the expression of USP7 was performed as previously described (Fig. 4B) (27). The expression of PP2A was dose-dependently reduced after exposure to varying concentrations of *siUSP7* (Fig. 4C). Since the expression of PP2A was decreased subsequent to inhibition of USP7, it was further investigated whether *siUSP7* also affects the ubiquitination of PP2A (Fig. 4D). The results

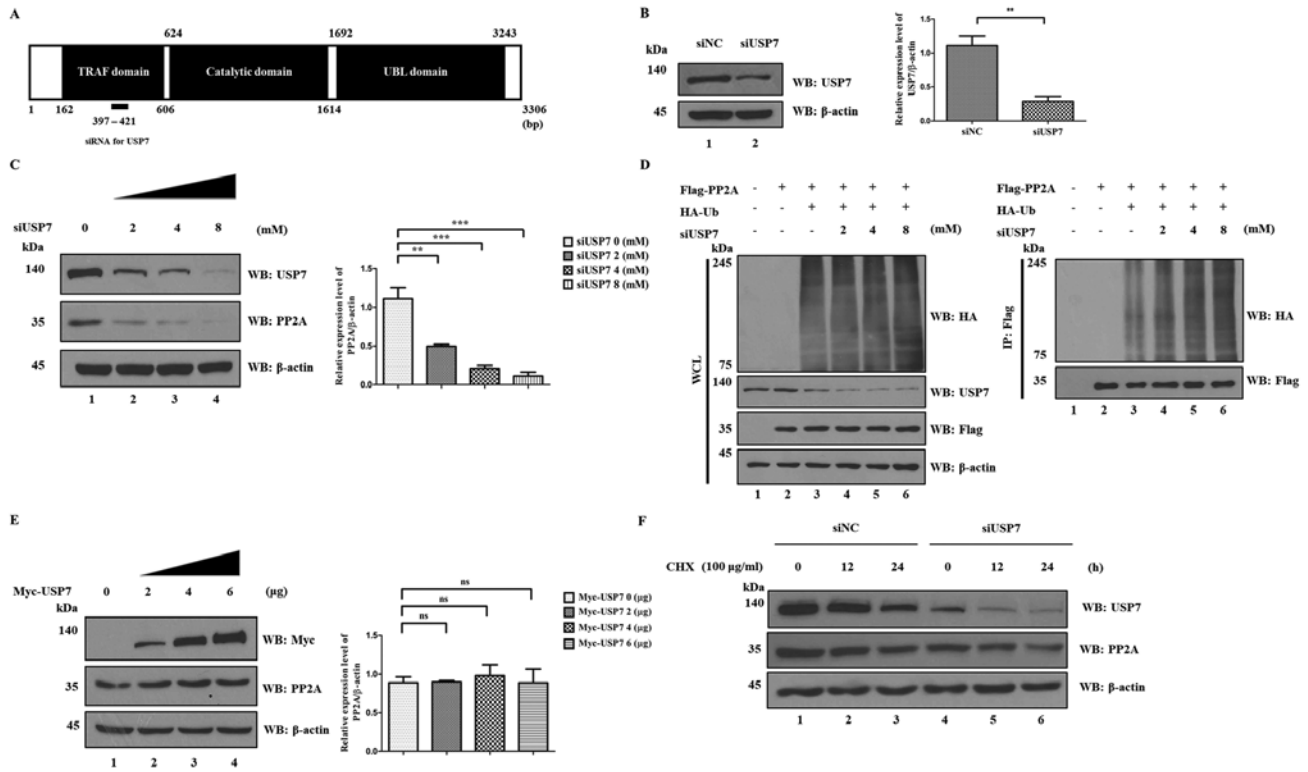

Figure 4. USP7 regulates the stability of PP2A. (A) A schematic diagram of the siRNA of *USP7*. (B) MCF-7 cells were transfected with *siUSP7* or *siNC*. Percentage of PP2A/β-actin from separate experiments (n=3; **P<0.01). (C) *siUSP7* was transfected into MCF-7 cells at varying concentrations (0, 2, 4, and 8 mM). The expression levels of PP2A/β-actin were analyzed by at least three independent experiments (n=3; **P<0.01 and ***P<0.001). (D) HA-*Ub* and Flag-*PP2A* were co-transfected into 293T cells, followed by transfection with different concentrations of *siUSP7* (0, 2, 4, and 8 mM). (E) Myc-*USP7* was transfected into HeLa cells in a dose-dependent manner (0, 2, 4, 6 μg). Percentage of PP2A/β-actin from three independent experiments (n=3; ns). (F) The half-life of PP2A was investigated with *siUSP7*, with time-dependent exposure to CHX. USP7, ubiquitin-specific protease 7; PP2A, protein phosphatase 2A; siRNA, small interfering RNA; *siNC*, siRNA negative control; HA-*Ub*, HA-ubiquitin; ns, not significant; CHX, cycloheximide.

confirmed that a dose-dependent increase in the ubiquitination level of PP2A subsequent to *siUSP7* exposure indicated that the absence of USP7 may fail to induce deubiquitination of PP2A. Collectively, these experimental results revealed that the deubiquitination of PP2A was suppressed following inhibition of USP7, and PP2A stability was reduced by UPS. Conversely, overexpression of USP7 had no effect on the expression of PP2A (Fig. 4E); if the protein expression is too strong, the band is affected by saturation (30,31). Therefore, it appears that saturation of the PP2A protein signal in the western blotting may hinder the ability to observe an increase in the expression level of PP2A induced by USP7. Furthermore, the half-life of PP2A tended to be modulated by *siUSP7* in a time-dependent manner of CHX exposure (Fig. 4F). This indicated that inhibition of USP7 reduced the protein stability of PP2A.

Discussion

USP7 is one of the most prominent DUBs that has been studied in various cancers (32). USP7 has an N-terminal domain, a tumor necrosis factor receptor-associated factor (TRAF) domain, a catalytic domain (CD), and a C-terminal domain including five ubiquitin-like (UBL) domains (33). The USP7 CD domain contains the Ub hydrolase activity (34). UBL domains are essential for the full activity of USP7 (35). The TRAF domain plays an important role in recognition of target proteins (16). This domain recognizes target proteins through the P/A/E-x-x-S motif. P/A/E-x-x-S motifs allow numerous different interactors capable of binding with USP7, and are probably responsible for the promiscuity of USP7 with other binding partners (36). The secondary binding domain in USP7 is located in UBL-2. UBL-2 interacts with a partner with K-x-x-x-K motifs (37). Interestingly, since PP2A has the P/A/E-x-x-S motif (that binds to the TRAF domain) but no K-x-x-x-K motifs, the possibility of interacting with the TRAF domain in USP7 was analyzed in a previous study (15). This suggests the possibility that PP2A can interact with the TRAF domain in USP7. Moreover, the TRAF domain is known to regulate intracellular signaling such as ubiquitin-dependent degradation (38). Since ubiquitination in substrates occurs in various ubiquitination motifs, further studies are required to determine whether these motifs, in which USP7 and PP2A bind to each other are related to protein activity and stability exerted via ubiquitination. Furthermore, USP7 plays an important role in tumor suppressor function by deubiquitinating both p53 and MDM2, one of the E3 ligases for p53 (39). Conversely, USP7 is also considered as an oncogene, enhancing the stability of c-MYC which contributes to cancer progression (40). Thus, USP7 paradoxically acts either as an oncogene or a tumor suppressor. The substrates targeted by USP7 are therefore considered to affect cancers through diverse functions, making research focusing on substrates regulated by USP7 essential. In a previous study by the authors, PP2A, a substrate

to which USP7 binds, was identified through 2-DE and mass spectrometric analysis (17).

PP2A controls various cell functions, including protein synthesis, cell cycle regulation, and cell death through signaling pathways. Therefore, alterations in the levels of PP2A are considered an interesting topic in cancer research (41). PP2A enhances tumor immunity by inhibiting the MAPK and PI3K pathways (including AKT, MEK, and ERK), as well as inhibition of MYC through dephosphorylation (23). In addition, PP2A contains numerous subunits, including subunit A, B, and C, and mutations of PP2A subunits have also been reported in various cancer types (42). PP2A is known as a critical tumor suppressor, since it functions as a negative regulator of oncoproteins, including c-MYC, BCL2, ERK, and AKT (43). Thus, the PP2A subunits exert numerous roles in cancer cells. Moreover, depending on the cancer cell type, PP2A acts as a tumor suppressor or an oncogene. Thus, there is increasing interest in the role of PP2A in cancer development (44). It has been confirmed that treatment with a PP2A inhibitor affects drug resistance and cancer cell survival in various cancer cell types (45-47). Thus, treatments targeting PP2A are being considered as potential cancer treatments (48). PP2A has long been regarded as a tumor suppressor, but recent arguments have contradicted the original hypothesis that inhibition of PP2A induces cell apoptosis (49). Therefore, treatment with PP2A needs to be considered for both aspects of activity and inhibition, depending on the cell type or type of drugs. As such, PP2A is considered an important target for anticancer therapy (50), and research on anticancer treatment targeting PP2A is in progress. It is expected that understanding of the enzyme targeting PP2A will also be required for complete elucidation of the process. Thus, both USP7 and PP2A play important roles in cancer therapy. The development of small molecules that inhibit the interplay of USP7 and PP2A could be considered as a new anticancer treatment.

The present study aimed to provide an understanding of a new molecular mechanism by elucidating the association between USP7 and PP2A, which are considered important modulators in cancer progression. The direct binding of USP7 and PP2A was determined (Fig. 1C). It was of interest whether PP2A is neddylated, SUMOylated, and ISGylated as well. The experimental results indicated that PP2A can be regulated not only through ubiquitination but also through other processes including neddylation, and further research on the enzymes modulating these processes may be required to understand the regulatory mechanism of PP2A. Results of the deubiquitination assay revealed the deubiquitination of PP2A through USP7 (Fig. 3B), predicting that the PP2A activity and stability could be modulated by the removal of ubiquitins on PP2A by USP7. Furthermore, regulation of the expression level of PP2A by USP7 was also established (Fig. 4A, B, E and F). These results indicated that USP7 is a modulator of PP2A stability via the K48-linked polyubiquitin chain. Since USP7 and PP2A are considered to be tumor suppressors or oncoproteins, the results indicating that both proteins are co-localized in various tumor cell lines, and that USP7 regulates the PP2A stability, suggest that USP7 and PP2A are intricately associated in various tumors. Further studies validating the effect of USP7-mediated PP2A stability changes are required for determining the oncogenic roles of USP7 as a positive or a negative regulator to PP2A. Moreover, the expression levels of PP2A and USP7 in cancer tissues need to be confirmed and verified *in vivo*, thereby validating the potential of USP7 as a therapeutic target for cancer in tumor malignant phenotypes. A previous study demonstrated that USP7 interacts with PP2A and controls the location of PP2A in the cytoplasm (51). In the present study, the direct interaction between USP7 and PP2A was confirmed, and the role of USP7 as a deubiquitinating enzyme was investigated by modulating the K48-linked ubiquitin chain of PP2A. Additionally, it was confirmed that PP2A not only forms polyubiquitin chains but also proteasome-dependent degradation related to polyneddylation. Collectively, the interplay between USP7 and PP2A provides an essential understanding that should be considered for the development of anticancer therapeutics.

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Availability of data and materials

All data generated and/or analyzed during the present study are included in this published article.

Authors' contributions

HAD and KHB designed the study and confirm the authenticity of all the raw data. HAD performed most of the experiments and wrote the manuscript. Both authors have read and agreed to the published version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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