Ovarian tumor deubiquitinase 6A regulates cell proliferation via deubiquitination of nucleolin and caspase-7

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Abstract. Most proteins maintain protein homeostasis via post-translational modifications, including the ubiquitin-proteasome system. Deubiquitinating enzymes (DUBs) have essential intercellular roles, such as responses to DNA damage, proteolysis and apoptosis. Therefore, it is important to understand DUB-related diseases to identify DUBs that target abnormally regulated proteins in cells. Ovarian tumor deubiquitinase 6A (OTUD6A) was previously reported as a downregulated DUB in HCT116 cells with p53 knockdown. Therefore, it was expected that the relationship between OTUD6A and p53 would affect cell proliferation. In the present study, putative substrates of OTUD6A related to the p53 signaling pathway were identified. Application of liquid chromatography-tandem mass spectrometry and proteomic analysis led to the identification of nucleolin (known to bind p53) as a binding protein. In addition, immunoprecipitation studies determined that caspase-7, an apoptotic protein, is associated with p53 signaling and is regulated by OTUD6A. It was further identified that OTUD6A regulates the protein stability of nucleolin, but not caspase-7. It was also demonstrated that OTUD6A acts as a respective DUB through the deubiquitination of K48-linked polyubiquitin chain of nucleolin and the K63-linked polyubiquitin chain of caspase-7. Furthermore, overexpression of OTUD6A induced cell proliferation via enhancing cell cycle progression of MCF7 cells. Taken together, OTUD6A may be proposed as a target for anticancer therapy.

Introduction

Humans have a homeostatic effect that maintains a constant internal environment in response to various stimuli, including changes in the external or internal environment. The degradation, repair or stability of damaged proteins affects the efficiency of cellular homeostasis and aging (1). The ubiquitin-proteasome system (UPS) via ubiquitin and the 26S proteasome is essential for protein homeostasis (2). Ubiquitin, a small protein of 76 amino acids, binds to a target protein by interacting with three enzymes: E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin ligase). The E1 enzyme activates ubiquitin and generates a thioester intermediate through ATP-dependent chemical bonding (3). The E2 enzyme receives the activated ubiquitin and the E3 ligase delivers the ubiquitin to the target protein. Ubiquitin forms a polyubiquitin chain such that the c-terminal G76 (glycine) forms an isopeptide bond with the free amino group K (lysine) or M (methionine). The binding sites of ubiquitin are seven lysine residues (K6, K11, K27, K29, K33, K48 and K63) and one methionine residue (M1) (4). The role of ubiquitin varies, depending on the position where it forms the polyubiquitin chain (5). The K6-linked polyubiquitin responds to mitochondrial homeostasis and DNA damage, whereas the K11-linked polyubiquitin regulates the cell cycle (6). The K27-linked polyubiquitin chain responds to DNA damage, K29-linked polyubiquitin regulates lysosomal degradation and kinase modification, and K33-linked polyubiquitin is involved in protein trafficking and kinase modification (7,8). The K48-linked polyubiquitin and K63-linked polyubiquitin are the most studied. The K48-linked polyubiquitin regulates proteasomal degradation (9), whereas the K63-linked polyubiquitin induces autophagic degradation (10,11). The M1-linked polyubiquitin is involved in gene activation, DNA damage response and innate immunity (12). The target proteins tagged with polyubiquitin are directed to the 26S proteasome and the protein separated from the polyubiquitin chain passes through the 26S proteasome and is degraded. This process is called the UPS and the ubiquitin dissociated from the substrates is recycled in the UPS process (13). E3 ligase induces proteasomal degradation by linking the polyubiquitin chain to the substrate protein, but certain enzymes have a contrary role by breaking the ubiquitin bond; these are the deubiquitinating enzymes (DUBs) (14,15). DUBs are classified into 9 subfamilies: Jab1/Pab1/MPN metallo-enzyme motif protease, monocyte chemotactic protein-induced protease, motif interacting with ubiquitin-containing novel DUB family, Machado-Joseph disease protein domain protease, ovarian tumor protease (OTU), permuted papain fold peptidase of dsDNA viruses and eukaryotes, ubiquitin C-terminal

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hydrolases protease, ubiquitin-specific protease (USP) and zinc finger with UFM1-specific peptidase domain protein.

Ovarian tumor deubiquitinase 6A (also called OTU domain-containing protein 6A; OTUD6A) is a member of the OTU subfamily and is known to promote tumorigenesis in various carcinomas. OTUD6A and dynamin-related protein 1 (Drp1), which are upregulated in colorectal cancer tissues, interact with each other. The OTUD6A stabilizes Drp1 by deubiquitination and promotes cancer proliferation and mitochondrial fission (16). OTUD6A acts as a DUB of the cell cycle regulator Aurora kinase-A (Aurora-A), which is overexpressed in malignancies, and stabilizes Aurora-A (17). OTUD6A promotes prostate tumorigenesis via deubiquitinating and stabilizing brahma-related gene 1 androgen receptor (18), and c-Myc (19). OTUD6A also promotes breast cancer proliferation through deubiquitination and stabilization of DNA topoisomerase II binding protein 1 (20). In addition, OTUD6A has a differential expression depending on the presence or absence of p53 in the HCT116 cell line (21). However, the mechanism and effect on cancer cells, which are regulated by interaction between OTUD6A and p53, have not been elucidated. Therefore, in the present study, substrates of OTUD6A related to p53 signaling were discovered through liquid chromatography-tandem mass spectrometry (LC-MS/MS) or immunoprecipitation and it was demonstrated that the substrates regulate cell proliferation in breast cancer cells. Therefore, it is suggested that OTUD6A, which may regulate the p53 signaling pathway, may be a therapeutic target for cancer treatment.

Materials and methods

Construction of expression vector. The full-length cDNA for OTUD6A (cat. no. 61416; Addgene, Inc.) was subcloned into pcDNA3.1-6myc vector (cat. no. V790-20; Invitrogen; Thermo Fisher Scientific, Inc.), pCS4-3XFlag vector (cat. no. 55751; Addgene, Inc.) and pGEX-4T-1 vector (cat. no. 27-4580-01; Pharmacia Biotech) and C152S, a catalytically inactive mutant of OTUD6A, was mutated by PCR with the following primer sequences: Forward, 5'-ACGGCC ACAGCATGTAC-3' and reverse, 5'-TACATGCTGTGG CCGTC-3', Pfu polymerase (cat. no. CMT4002; LaboPass; COSMOGENETECH, Inc) was used and the thermocycling protocol was as follows: Denaturation was carried out at 95˚C for 5 min. Subsequently, 15 cycles of denaturation at 95˚C for 30 sec, annealing at 54˚C for 30 sec, and extension at 72˚C for 5 min and 10 sec were performed. After extension at 72˚C for 5 min, the mixture was maintained at 4˚C. The fragment was subcloned into pcDNA3.1-6myc vector using *BamH*I (cat. no. R003S; Enzynomics, Inc.) and *EcoR*I restriction enzymes (cat. no. R002S; Enzynomics, Inc.). The cDNA for Flag-nucleolin was published in a previous study by our group (22) and caspase-7-Flag (cat. no. 11815; Addgene, Inc.) was used. Furthermore, hemagglutinin (HA)-tagged ubiquitin (wild-type) and mutant constructs (K6, K11, K27, K29, K33, K48 and K63) were used, the details of which were published in a previous study by our group (23).

Cell culture and transfection. Human colon cancer cells [HCT116 ($p53^{+/+}$); cat. no. CCL-247; American Type Culture Collection (ATCC)] were grown in RPMI-1640 (cat. no. 31800-022; Gibco; Thermo Fisher Scientific, Inc.) medium containing 10% fetal bovine serum (FBS; cat. no. 12483-020; Gibco; Thermo Fisher Scientific, Inc.) and 1% antibiotic-antimycotic (containing penicillin, streptomycin and amphotericin B; cat. no. 15240-062; Gibco; Thermo Fisher Scientific, Inc.). Human breast cancer cells (MCF7; cat. no. HTB-22; ATCC) were grown in DMEM (cat. no. 12800-017; Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS and 1% penicillin/streptomycin. The cells were grown in a 5% CO_2 incubator at 37˚C. For transfection, 10 mM polyethyleneimine reagent (Polysciences, Inc.), 3-6 μl/μg of DNA and 150 mM NaCl (100 μl/ml of media) were used and incubated at 37˚C. For western blot analysis, immunoprecipitation and glutathione S-transferase (GST) pull-down assay, proteins were purified from harvested cells 48 h after transfection.

Antibodies. Anti-Flag (1:5,000 dilution; cat. no. M185-3L; MBL International Corporation), anti-Myc (1:100 dilution; 9E10 hybridoma cell media; cat. no. CRL-1729; ATCC) and anti-HA (1:1,000 dilution; cat. no. 11 583 816 001; Roche Diagnostics) antibodies were used; furthermore, anti-β-actin (1:1,500 dilution; cat. no. sc-47778), anti-nucleolin (1:1,500 dilution; cat. no. sc-8031), anti-caspase-7 (1:200 dilution; cat. no. sc-56063), anti-poly(ADP ribose) polymerase 1 (PARP1; 1:200 dilution; cat. no. sc-7150), anti-X-linked inhibitor of apoptosis protein (XIAP; 1:200 dilution; cat. no. sc-11426), anti-p53 (1:500 dilution; cat. no. sc-126), anti-p27 (1:200 dilution; cat. no. sc-528) and anti-Bax (1:200 dilution; cat. no. sc-23959) antibodies were purchased from Santa Cruz Biotechnology, Inc. and used for western blot and immunoprecipitation assays.

Western blot analysis, immunoprecipitation and LC-MS/MS. Cells were lysed using a lysis buffer [50 mM Tris-HCl pH 7.5, 1 mM EDTA, 10% glycerol, 300 mM NaCl, 1% Triton X-100, 10% SDS (1:100 dilution), a protease inhibitor cocktail (PIC; 1:100 dilution; cat. no. 11697498001; Roche Diagnostics) and phenylmethanesulfonyl fluoride (PMSF; 1:100 dilution; MilliporeSigma)] and incubated on ice for 20 min after vortexing. Centrifugation was performed at 16,200 x g for 20 min at 4˚C and the supernatants were used as samples. The total protein in the samples was quantified using the Bradford assay, and samples were boiled at 100˚C for 7 min with 2X SDS and used for western blot analysis. For each lane, 30 μg of protein was separated by size by 8% SDS-PAGE and transferred to polyvinylidene fluoride microporous membranes (cat. no. IPVH00010; EMD Millipore) at 120 V for 90 min. Proteins transferred to the membrane were blocked with 20 mM Tris-HCl, 0.05% Tween 20 and 150 mM NaCl (TTBS) containing 5% BSA (cat. no. BSAS 0.1; Bovogen Biologicals) or skimmed milk (cat. no. 232100; Becton, Dickinson and Company) at room temperature for 1 h and then incubated at 4˚C overnight with primary antibodies. The membranes were then washed three times with TTBS for 7 min each and then incubated at room temperature for 1 h with a secondary antibody [mouse IgG (H+L) (1:30,000 dilution; cat. no. 074-1806; LGC SeraCare)]. The membranes are then washed 3 times with TTBS for 7 min each and detected using enhanced chemiluminescence solution (Young-In Frontier).

For immunoprecipitation, 2 mg of cell lysates were incubated at 4˚C on a rotator at 20 rpm overnight with an antibody (1 μg per 500 μg of total protein) and incubated with 30 μl resuspended volume of Protein A/G PLUS-Agarose Beads (cat. no. sc-2003; Santa Cruz Biotechnology, Inc.) at 4˚C on a rotator at 20 rpm for 2 h. The samples were centrifuged at 3,200 x g at 4˚C for 5 min and subsequently, the beads were washed 2 times with wash buffer [50 mM Tris-HCl pH 7.5, 1 mM EDTA, 10% glycerol, 300 mM NaCl, 1% Triton X-100, PIC (1:100 dilution) and PMSF (1:100 dilution)] and then boiled in 2X SDS at 100˚C for 7 min, followed by the western blot procedure.

For LC-MS/MS, pCS4-3XFlag vector or Flag-OTUD6A was transfected into HCT116 (p53+/+) cells. Cells were lysed and immunoprecipitation was performed using anti-Flag antibody. Western blot analysis was performed using 8% SDS-PAGE and gels were stained with Coomassie Brilliant blue R 250 (cat. no. CI 42660; MilliporeSigma) and G 250 (cat. no. CI 42655; MilliporeSigma) solutions at room temperature for 15 min. LC-MS/MS analysis was performed using the sample showing a higher expression in the Flag-OTUD6A-transfected sample compared to the sample transfected with the pCS4-3XFlag vector, which was used as a control.

GST pull-down assay. GST vector (pGEX-4T-1) and GST-OTUD6A were transformed into the BL21 (DE3) bacterial strain (cat. no. C2527H; New England BioLabs) and bacteria grown to an optical density value at 600 nm of 0.6 at 37˚C were incubated in Luria-Bertani broth (cat. no. MB-L4488; KisanBio, Inc.) with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (Promega Corporation) at 18˚C overnight. The protein bound to GST-OTUD6A was boiled at 100˚C with 2X SDS buffer and detected through western blot analysis. The expression of GST and GST-OTUD6A was visualized with Coomassie Brilliant Blue R 250 and G 250 solutions.

Protein stability assay. MCF7 cells were transfected with pCS4-Flag vector or Flag-OTUD6A and incubated for 24 h after transfection. After 24 h, cycloheximide (CHX; cat. no. 01810; MilliporeSigma) was treated at a concentration of 50 μg/ml in a 5% CO_2 incubator at 37˚C. The cells were then harvested at different time-points (0, 6, 12 and 24 h) and cell lysates were used for western blot analysis.

Ubiquitination and deubiquitination assays. For the ubiquitination assay, Flag-nucleolin (or caspase7-Flag) and HA-Ub were co-transfected into MCF7 cells. For the deubiquitination assay, Myc-OTUD6A, Flag-nucleolin (or caspase-7-Flag) and HA-Ub were co-transfected into MCF7 cells. MG132 (cat. no. F1100; Ubiquitin Proteasome Biotechnologies, LLC) was used to confirm the proteasomal-dependent degradation of the substrate. Cells were treated with 10 mM concentration of MG132 for 4 h before harvest and cultured in a 5% CO_2 incubator at 37˚C. Both assays were performed using an ubiquitination assay kit (cat. no. UBAK-100; D&P Biotech, Inc.) according to the manufacturer's protocol. The cell lysates were immunoprecipitated with an anti-Flag antibody. Western blot analysis was performed using immunoprecipitated cell lysates according to the immunoprecipitation procedure.

Colony-formation assay. pCS4-3XFlag vector or Flag-OTUD6A-transfected MCF7 cells ($1.5x10^3$ cells/100 mm culture dish) were seeded 24 h after transfection and cultured at 37˚C in a 5% CO_2 incubator for 14 days to form colonies. Next, the colonies were stained using crystal violet (cat. no. 27210-0350; Junsei) diluted with PBS at room temperature for 5 min. After washing with PBS, the colonies were counted on each plate. Images were captured using a DUALED Blue/White Transilluminator (cat. no. A6020; Bioneer Corporation) and counting for colony formation analysis was conducted using ImageJ software (version 1.4.3; National Institutes of Health).

Cell cycle assay. MCF7 cells were synchronized in G1/S phase of the cell cycle by double thymidine block. The thymidine block was treated with thymidine (cat. no. T1895; MilliporeSigma) with the final concentration of 2 mM. MCF7 cells were transfected with pCS4-3XFlag vector or Flag-OTUD6A, or pCMV-Flag or Flag-nucleolin. At 24 h after transfection, cells were incubated with thymidine at a final concentration of 2 mM at 37˚C for 18 h. The cells were then washed with PBS and incubated in fresh media at 37˚C for 9 h. For double thymidine block, the second thymidine was applied at a final concentration of 2 mM at 37˚C for 18 h. The synchronized cells were washed with PBS and released with fresh media. Cells were harvested at 0, 3, 6, 12 and 24 h after release. A total of $1x10^6$ cells were fixed with cold 70% ethanol for 30 min at -20˚C and washed with PBS to remove the ethanol. Fixed cells were stained with 500 μl of FxCycle™ PI/RNase Staining Solution (cat. no. F10797; Thermo Fisher Scientific, Inc.) at room temperature for 30 min and analyzed with a CytoFLEX flow cytometer (Beckman Coulter, Inc.) and CytExpert software (version 2.4.0.28; Beckman Coulter, Inc.).

Survival analysis. The survival analysis was performed by Kaplan-Meier Plotter using the log-rank test (https://kmplot.com/analysis/). The Kaplan-Meier plot of survival was based on the high or low OTUD6A expression in patients with breast cancer from the Tang (2018, n=118) dataset (P-value by log-rank test). Patients were divided by the median value and the analysis was performed on 65 patients using the selected dataset.

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 tests using GraphPad Prism version 5 (GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Putative substrates that bind to OTUD6A. Through multiplex reverse transcription-quantitative PCR, our group previously confirmed that OTUD6A is highly expressed in HCT116 ($p53^{+/+}$)

Table I. List of proteins interacting with ovarian tumor deubiquitinase 6A identified by liquid chromatography-tandem mass spectrometry and proteomics analysis.

Sample ID	Protein name	Monoisotopic mass (Da)	*p*I value	Protein sequence coverage, %	Number of matched peptides
a	TSGA10	104514	5.51	1	2
b	EPLIN-β	85630	6.41	2	3
c	Nucleolin	76355	4.59	8	3
d	BAP31	23621	9.57	9	1
d	Porin	38639	6.32	2	1
d	RPS3A	30154	9.75	6	1

*p*I, isoelectric point; TSGA10, testis-specific gene 10 protein isoform; EPLIN-β, epithelial protein lost in neoplasm-β; BAP31, B-cell receptor associated protein 31; RPS3A, v-fos transformation effector protein.

cells as compared to HCT116 ($p53^{-/-}$) cells (21), thereby indicating that OTUD6A is probably affected by p53. However, the regulatory mechanisms of OTUD6A and p53 have not been elucidated. p53 is a well-known tumor suppressor (24). It was expected that the association of OTUD6A with p53 affects cell survival. Therefore, the present study aimed to identify putative substrates for OTUD6A related to p53 in HCT116 (p53+/+) cells via LC-MS/MS (Fig. 1A). LC-MS/MS studies determined that nucleolin is a candidate substrate (Table I). Nucleolin is known to interact with p53 (25,26) and regulates cell proliferation and the DNA damage response by interacting with the herpes virus-associated USP (HAUSP)-p53-MDM2 complex (22). Immunoprecipitation was performed to determine whether nucleolin binds to OTUD6A. The band of nucleolin was detected, indicating that OTUD6A and nucleolin interact with each other (Fig. 1B). In addition, to investigate direct binding between OTUD6A and nucleolin, the GST pull-down assay was performed using the GST-OTUD6A fusion protein. The assay revealed that OTUD6A directly binds to nucleolin (Fig. 1C). It is known that p53 binds to nucleolin through the C-terminal domain (25,26). Therefore, a GST pull-down assay was performed to determine whether OTUD6A, nucleolin and p53 form a complex (Fig. 1D). It was demonstrated that the purified GST-OTUD6A protein binds to nucleolin and p53 in a complex.

In the present study, it was hypothesized that the apoptotic proteins that function similarly to the tumor suppressor p53 are also related to OTUD6A. Therefore, a binding assay between OTUD6A and apoptotic proteins associated with p53 was performed in MCF7 cells. The results revealed that Flag-OTUD6A and caspase-7 or p53 bind to each other (Fig. 1E), thereby demonstrating that OTUD6A binds to caspase-7.

OTUD6A regulates the protein stability of nucleolin. Next, the effect of OTUD6A on the protein stability of nucleolin and caspase-7 was investigated. The expression level of nucleolin was increased in a dose-dependent manner, but no significant difference was obtained in the protein expression level of caspase-7 in MCF7 cells transfected with varying concentrations of Flag-OTUD6A (Fig. 2A). The overexpression effect of Flag-OTUD6A on the protein half-life of nucleolin and caspase-7 was further investigated using CHX, a protein synthesis inhibitor. The expression level of nucleolin decreased by 25% from 0 to 24 h in the mock control and decreased by 15% from 0 to 24 h in Flag-OTUD6A-transfected cells. The expression of caspase-7 protein was decreased by 19% from 0 to 24 h in the mock control and decreased by 22% from 0 to 24 h in Flag-OTUD6A-transfected cells, but there was no significant difference. The half-life of nucleolin was significantly extended in Flag-OTUD6A-transfected cells (Fig. 2B).

OTUD6A deubiquitinates nucleolin and caspase-7 through the UPS. In order to investigate the function of OTUD6A as a DUB, K mutants of ubiquitin remaining at only one of the K sites were used for ubiquitination and deubiquitination assays (K6, K11, K27, K29, K33, K48 and K63) (Fig. 3A). To investigate whether nucleolin is regulated by proteasomal degradation, the ubiquitination assay was performed using a proteasome inhibitor, MG132 (Fig. 3B). The ubiquitination level of nucleolin was increased on the WT, K6-, K11-, K29-, K33-, K48 and K63-linked polyubiquitin chains, but not on the K27-linked polyubiquitin chain. The binding between OTUD6A and nucleolin was confirmed by the GST pull-down assay (Fig. 1C). Therefore, the deubiquitination assay was performed with Myc-OTUD6A (C152S), a catalytically inactive form of OTUD6A, to examine whether OTUD6A functions as a DUB for nucleolin. The expression level of the polyubiquitin chain of nucleolin decreased in Myc-OTUD6A-transfected MCF7 cells, but no alterations were observed in the Myc-OTUD6A (C152S)-transfected MCF7 cells (Fig. 3C). The binding between OTUD6A and caspase-7 was also demonstrated (Fig. 1D). Therefore, a deubiquitination assay was performed to check the ubiquitination level of caspase-7 mediated by OTUD6A. When OTUD6A was overexpressed, the ubiquitination level of caspase-7 decreased, whereas the ubiquitination level of caspase-7-Flag remained unchanged in the OTUD6A (C152S)-transfected MCF7 cells (Fig. 3D). These results suggest that OTUD6A is a DUB for nucleolin and caspase-7. In addition, the deubiquitination assay was performed using HA-Ub lysine mutants to determine which lysine site-linked polyubiquitin chains were removed by OTUD6A. The expression level of the K63-linked polyubiquitin chain of caspase-7 was observed to be decreased, but not the K48-linked

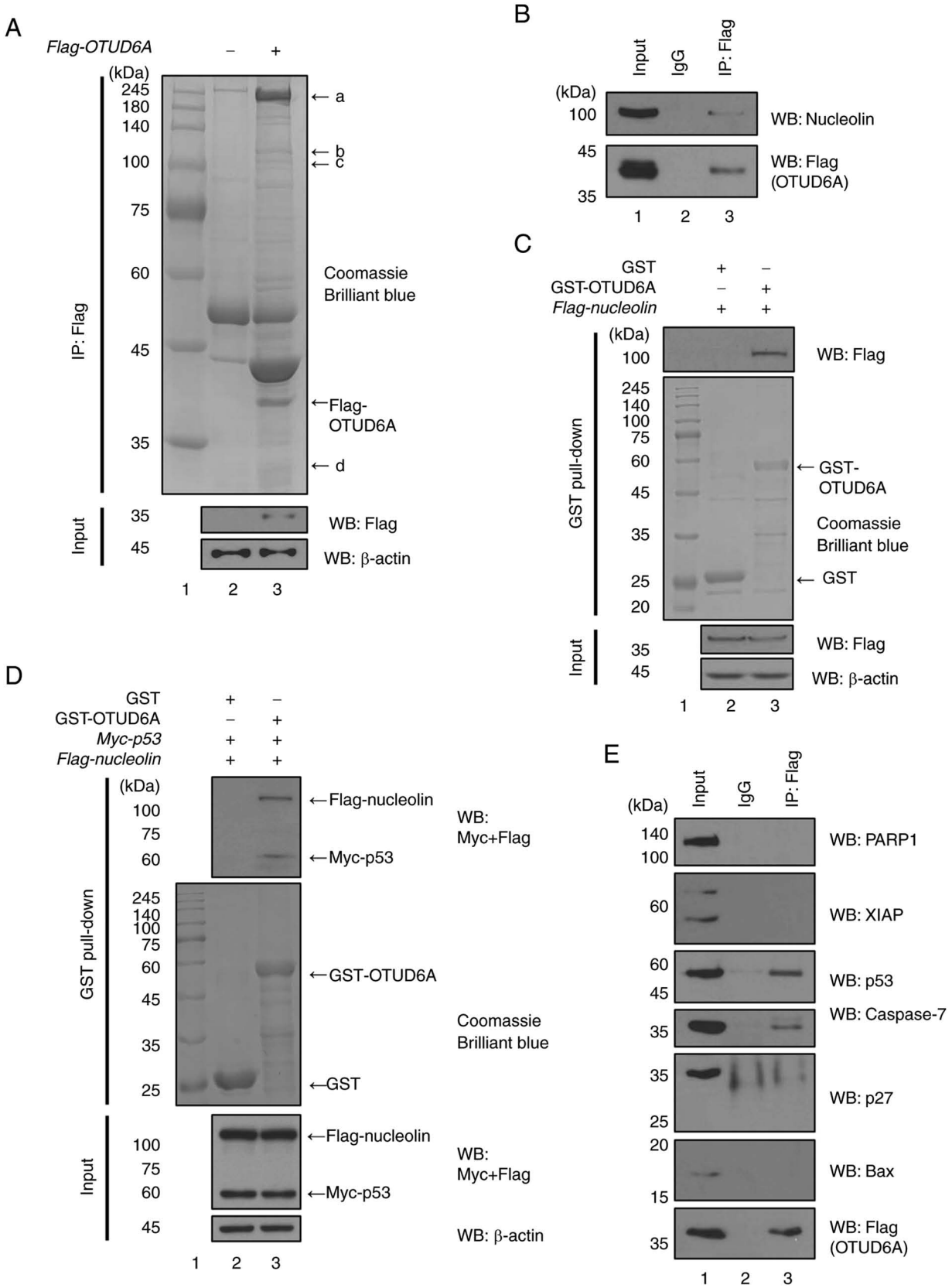

Figure 1. Putative substrates that bind to OTUD6A. (A) Flag-OTUD6A-transfected HCT116 (p53+/+) cell lysates were immunoprecipitated with anti-Flag antibody. Proteins were separated by size using 8% SDS-PAGE. The 1-dimensional electrophoresis gels were used for the liquid chromatography tandem mass spectrometry assay. (B) Flag-OTUD6A-transfected MCF7 cell lysates were immunoprecipitated with an anti-Flag antibody. Bands were detected with anti-Flag or anti-nucleolin antibody. (C) GST and GST-OTUD6A fusion proteins were inducted with 0.5 mM β-D-1-thiogalactopyranoside at 18˚C for 16 h. GST and GST-OTUD6A fusion proteins were incubated with lysates of Flag-nucleolin-transfected MCF7 cells and WB was performed. Flag-tagged nucleolin was blotted with anti-Flag antibody. (D) GST and GST-OTUD6A fusion proteins were incubated with lysates of Flag-nucleolin- and Myc-p53-transfected MCF7 cells and WB was performed. (E) Flag-OTUD6A-transfected MCF7 cell lysates were immunoprecipitated with anti-Flag antibody. Bands were detected with anti-PARP1, anti-XIAP, anti-p53, anti-p27, anti-Bax, anti-caspase-7 or anti-Flag antibody. OTUD6A, ovarian tumor deubiquitinase 6A; PARP, poly (ADP ribose) polymerase; XIAP, X-linked inhibitor of apoptosis protein; GST, glutathione S-transferase; IP, immunoprecipitation; WB, western blot.

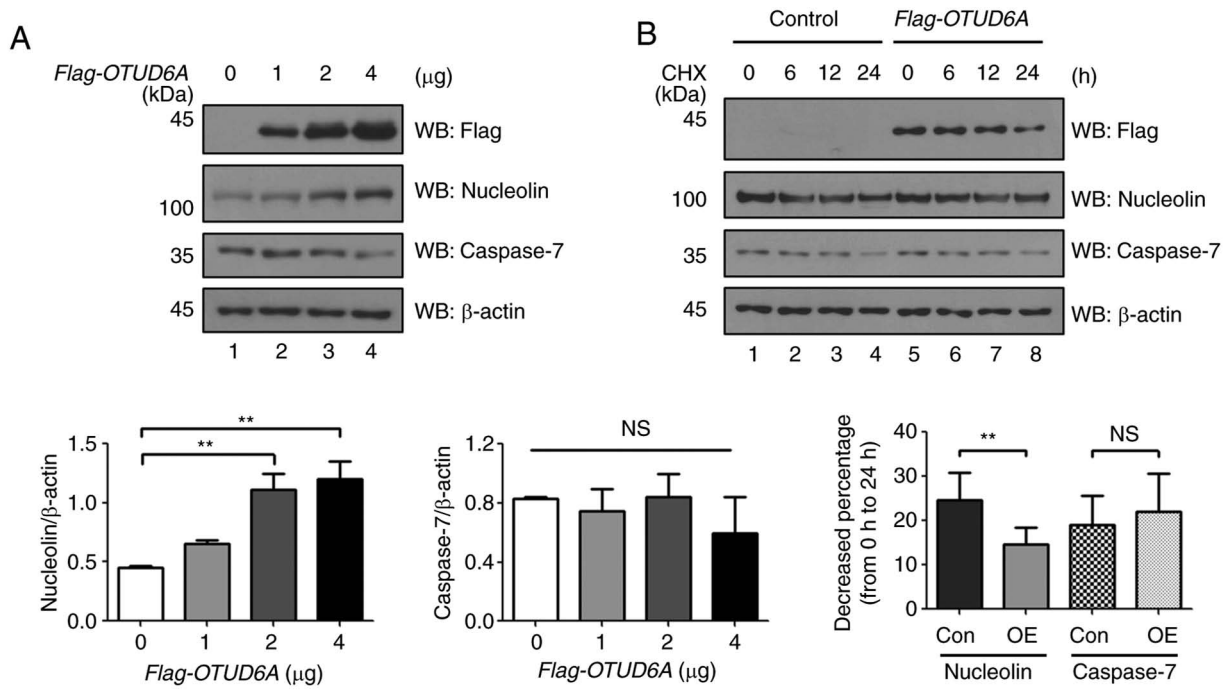

Figure 2. OTUD6A regulates the protein stability of nucleolin. (A) MCF7 cells were transfected with Flag-OTUD6A in a dose-dependent manner and WB was performed with lysates. Nucleolin was detected with anti-nucleolin antibody and caspase-7 was detected with anti-caspase-7 antibody. (B) MCF7 cells were transfected with pCS4-3XFlag vector or Flag-OTUD6A and treated with the protein synthesis inhibitor CHX (50 μg/ml) for 24 h after transfection. Cells were harvested in a time-dependent manner at 0, 6, 12 and 24 h after CHX treatment. WB was performed with lysates and nucleolin or caspase-7 was detected with anti-nucleolin or anti-caspase-7 antibody. The protein expression levels are presented as the mean ± standard deviation from three independently repeated experiments. **P<0.01; NS, not significant. OTUD6A, ovarian tumor deubiquitinase 6A; OE, overexpression; Con, control; WB, western blot; CHX, cycloheximide.

polyubiquitin chain (Fig. 3E). These results indicate that the binding of OTUD6A to caspase-7 affects cancer cells through the removal of the K63-linked polyubiquitin chain, but not the K48-linked polyubiquitin chain associated with proteasomal degradation. Furthermore, the ubiquitination level of nucleolin was found to be decreased on the K6-, K11-, K27-, K33- and K48-linked polyubiquitin chains, but not on the K29- and K63-linked polyubiquitin chains (Fig. 3F). This indicates that OTUD6A regulates the protein stability of nucleolin via deubiquitination of the K48-linked polyubiquitin chain, which is well known to regulate proteasome-mediated protein degradation (9).

OTUD6A regulates cell proliferation. It was investigated whether OTUD6A stabilizes the protein stability of nucleolin through deubiquitination of the K48-linked polyubiquitin chain (Fig. 3F). Nucleolin is known to regulate oncogenes in several cancers (27). It has been reported that nucleolin is upregulated by β-crystallin B2 (CRYβB2) in breast cancer to induce tumorigenesis (28). In addition, caspase-7 is known as an apoptotic protein (29). Therefore, the effect of OTUD6A in cancer cells was investigated through deubiquitination of nucleolin or caspase-7. First, a survival analysis for patients with breast cancer was performed using Kaplan-Meier Plotter. Higher expression of OTUD6A was associated with an increased risk and poor survival (Fig. 4A). Next, a colony formation assay was performed to investigate cell proliferation of cells with high expression of OTUD6A. It was determined that the colony formation ability was increased in Flag-OTUD6A-overexpressing cells, as compared to the mock control (Fig. 4B). It was previously reported that downregulated nucleolin protein is related to cell cycle arrest in ADP-induced cells (30). Therefore, flow cytometric analysis was performed to demonstrate cell cycle progression. The results indicated that cell cycle progression was faster in MCF7 cells with overexpression of OTUD6A (Fig. 4C). In order to prove that the promotion of cell cycle progression is due to upregulation of nucleolin through overexpression of OTUD6A, cell cycle analysis was performed with overexpression of nucleolin. Similar to the effect of overexpression of OTUD6A in Fig. 4C, the cell cycle progression of the MCF7 cells transfected with Flag-nucleolin was promoted (Fig. 4D). These data suggest that OTUD6A regulates cell proliferation by promoting cell cycle progression in breast cancer (Fig. 5).

Discussion

It has been reported that DUBs function to stabilize the protein by dissociating the ubiquitin bond of the polyubiquitin chain and inducing protein degradation (31). Another important function of DUB family members is to regulate cancer progression by inhibiting the degradation and stabilizing cancer-related proteins (32). Certain DUBs have been identified to be involved in cancer chemoresistance (33) and metastasis (34). Therefore, identifying cancer-related DUBs would be beneficial for anticancer therapy. p53 is a well-known tumor suppressor (35). However, p53 is frequently mutated in human cancers and the frequency varies depending on the tissue and type of cancer (36). Identifying the DUBs for the p53 signaling pathway may

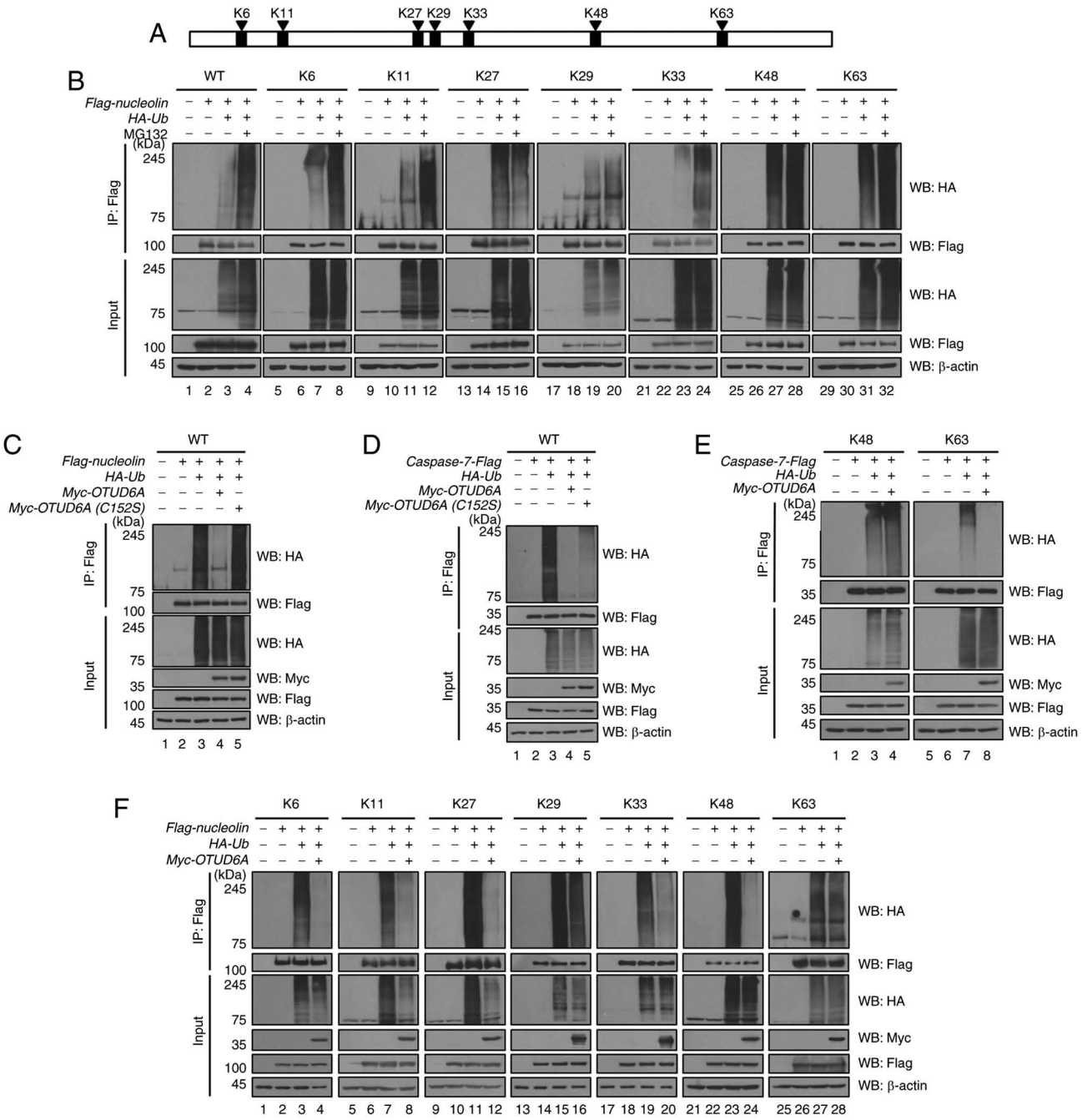

Figure 3. OTUD6A deubiquitinates nucleolin and caspase-7 through UPS. (A) HA-ubiquitin lysine mutants contain only one lysine residue among the seven lysine sites of ubiquitin. (B) The ubiquitination assay of Flag-nucleolin was performed in MCF7 cells with MG132. MCF7 cells were transfected with Flag-nucleolin along with HA-Ub^{+K6}, HA-Ub^{+K11}, HA-Ub^{+K27}, HA-Ub^{+K29}, HA-Ub^{+K33}, HA-Ub^{+48} or HA-Ub^{+K63} and treated with MG132. (C) Flag-nucleolin was co-transfected with HA-Ub, Myc-OTUD6A or Myc-OTUD6A (C152S) into MCF7 cells. (D) Caspase-7-Flag was co-transfected with HA-Ub, Myc-OTUD6A or Myc-OTUD6A (C152S) into MCF7 cells. (E) MCF7 cells were transfected with caspase-7-Flag or Myc-OTUD6A along with HA-Ub^{+48} or HA-Ub^{+K63}. (F) MCF7 cells were transfected with Flag-nucleolin or Myc-OTUD6A along with HA-Ub^{+K6}, HA-Ub^{+K11}, HA-Ub^{+K27}, HA-Ub^{+K29}, HA-Ub^{+K33}, HA-Ub^{+48} or HA-Ub^{+K63}. Cells were lysed and WB was performed. Lysates were immunoprecipitated with an anti-Flag antibody and polyubiquitin chains were detected with an anti-HA antibody. OTUD6A, ovarian tumor deubiquitinase 6A; IP, immunoprecipitation; WB, western blot; HA, hemagglutinin-tag protein; Ub, ubiquitin.

be helpful as a potential target for anticancer therapy. A previous study by our group reported on the changes in the expression level of OTUD6A in the presence and absence of p53 (21). OTUD6A is known to promote cell proliferation in several carcinomas, such as cervical cancer, colorectal carcinoma (16) and prostate cancer cell lines (18,19). However, the mechanism of the association between OTUD6A and p53 is yet to be elucidated. In the present study, it was hypothesized that OTUD6A controls cell proliferation through substrates associated with p53.

In order to identify the putative substrates, LC-MS/MS was first performed using overexpression of Flag-OTUD6A in HCT116 (p53$^{+/+}$) cells. Among the potential candidates identified, nucleolin that binds to HAUSP and p53 was selected (22). MicroRNA (miRNA/miR)-577 regulates testis-specific gene 10 protein isoform (TSGA10), and TSGA10

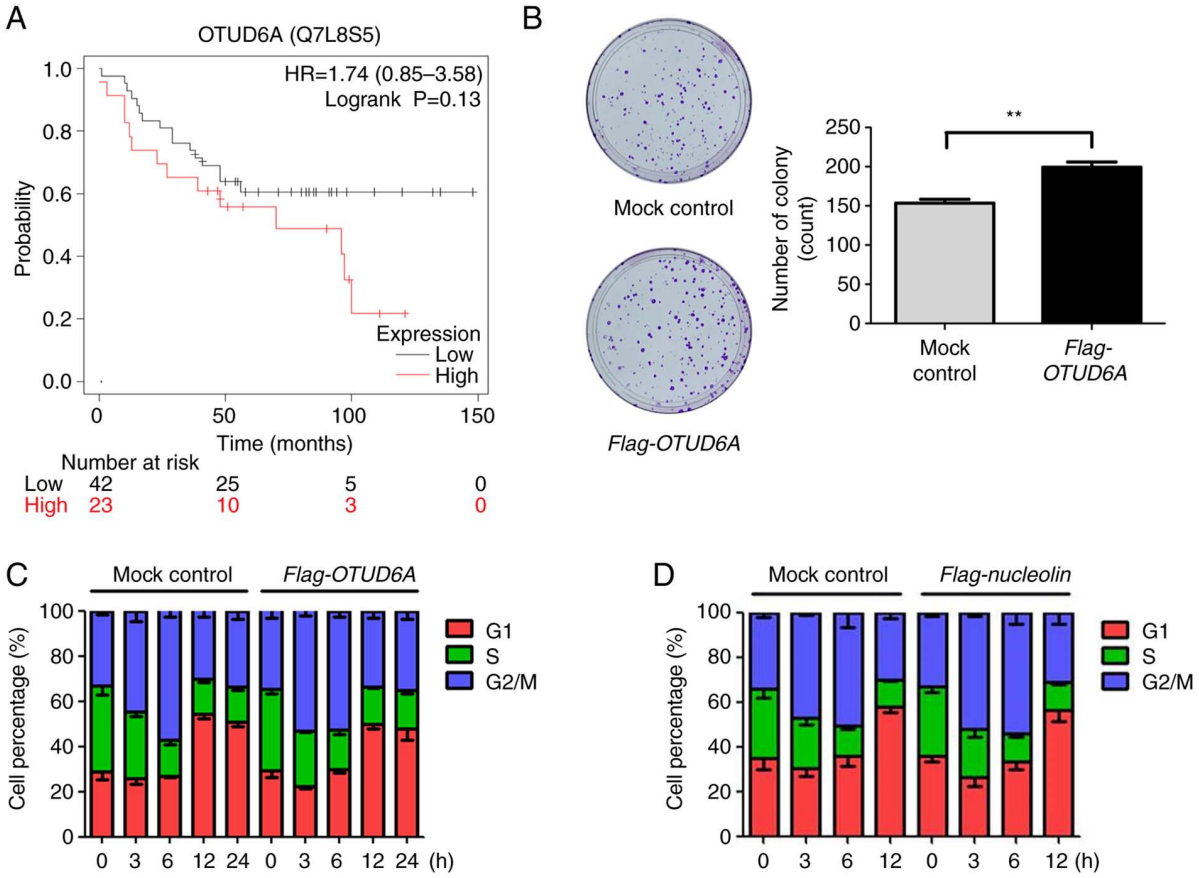

Figure 4. OTUD6A regulates cell proliferation. (A) Kaplan-Meier plot of survival based on high or low OTUD6A expression in patients with breast cancer. (B) pCS4-3XFlag- or Flag-OTUD6A-transfected MCF7 cells were seeded ($1.5x10^3$ cells/100 mm culture dish) after 24 h of transfection. The number of colonies was determined as the mean ± standard deviation from three independently repeated experiments. **P<0.01. (C) pCS4-3XFlag- or Flag-OTUD6A-transfected MCF7 cells were synchronized in G1/S phase of the cell cycle by double thymidine block. Synchronized cells were harvested at 0, 3, 6, 12 and 24 h after release and stained with PI. Flag-OTUD6A-transfected MCF7 cells exhibited a significant increase in the G2/M phase for 3 h after release, as compared to the mock control. (D) pCMV-Flag- or Flag-nucleolin-transfected MCF7 cells were synchronized in G1/S phase of cell cycle by double thymidine block. Synchronized cells were harvested at 0, 3, 6 and 12 h after release and stained with propidium iodide. Flag-nucleolin-transfected MCF7 cells exhibited a significant increase in G2/M phase for 3 h after release as compared to the mock control. The progression of the cell cycle was analyzed by flow cytometry. The results were determined as the mean ± standard deviation from three independently repeated experiments. OTUD6A, ovarian tumor deubiquitinase 6A; HR, hazard ratio.

restores the expression of p53 reduced by miR-577 (37). The LIM domain and actin-binding 1 (LIMA1) gene encoding epithelial protein lost in neoplasm (EPLIN) is transcribed by p53 and knockdown of LIMA1 partially suppressed the cell invasion inhibitory effect of p53 (38). In addition, EPLIN has different roles depending on the isoform (39). However, it is not known whether TSGA10, EPLIN-β, B-cell receptor associated protein 31, porin and v-fos transformation effector protein directly bind to p53. On the other hand, the interaction between nucleolin and p53 is known. It has been demonstrated that nucleolin binds to p53 through the C-terminal GAR domain of nucleolin (645-707 aa) (25) and p53 binds to nucleolin through the C-terminal domain of p53 (320-393 aa) (26). Therefore, p53-binding nucleolin was selected as a putative target of OTUD6A. Immunoprecipitation and GST pull-down assays revealed the direct binding between OTUD6A and nucleolin, and interaction in the OTUD6A-nucleolin-p53 complex. In addition, it was hypothesized that OTUD6A would interact with apoptotic proteins associated with p53, which would function as a tumor suppressor. Therefore, immunoprecipitation was performed with Flag-OTUD6A-transfected cell lysate and western blot analysis was performed by applying antibodies of apoptotic proteins. The results confirmed that both caspase-7 and p53 bind to OTUD6A. Caspase-7 is a protease that regulates inflammation and apoptosis (40). The p53-caspase-7-PARP signaling pathway is known to be associated with cordycepin-induced apoptosis (41). In addition, the first intron of human and mouse caspase-7 is involved in binding to p53 (42). Therefore, nucleolin and caspase-7 were set as new target candidates for OTUD6A.

The present study therefore investigated whether OTUD6A regulates the protein stability of nucleolin or caspase-7. A dose-dependent increase in the protein expression levels of nucleolin was obtained with increasing expression of Flag-OTUD6A. In addition, examination of the half-lives of nucleolin and caspase-7 after exposure to CHX, a protein synthesis inhibitor, revealed that the half-life of nucleolin was increased in Flag-OTUD6A-overexpressing MCF7 cells. However, caspase-7 exhibited no significant change in both protein expression level and half-life.

Ubiquitin has a different role depending on the lysine site at which it forms the polyubiquitin chain. Typically, the K48-linked polyubiquitin chain induces proteasomal degradation through the 26S proteasome and the K63-linked

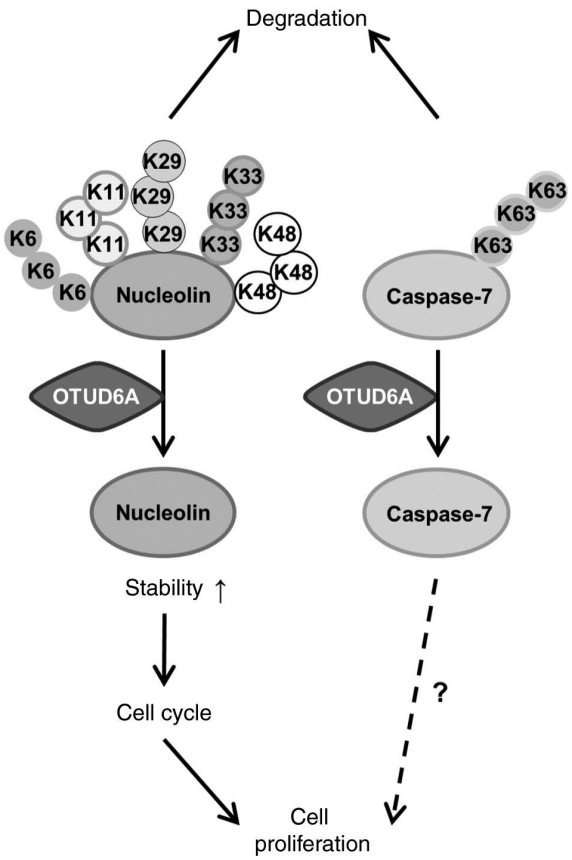

Figure 5. A schematic diagram for the intracellular function of OTUD6A. OTUD6A promotes cell proliferation through deubiquitination of nucleolin on K48-linked polyubiquitin chain and caspase-7 on K63-linked polyubiquitin chain. OTUD6A, ovarian tumor deubiquitinase 6A.

polyubiquitin chain responds to lysosomal targeting, DNA damage and stress response (43,44). OTUD6A regulates the K48-linked polyubiquitin chain of nucleolin, whereas OTUD6A regulates the K63-linked polyubiquitin chain of caspase-7. Based on these results, it is expected that OTUD6A increases the stabilization of the nucleolin by deubiquitination on the K48-linked polyubiquitin chain that induces proteasomal degradation. However, the K63-linked polyubiquitin chain of nucleolin was regulated by MG132. This is a result different from the known proteasomal degradation-independent function of the K63-linked polyubiquitin chain. It is possible that other K sites formed a branch from the K63-linked polyubiquitin chain, leading to an association with proteasomal degradation (45). In addition, no alterations were obtained in the protein expression level of caspase-7, since there is no difference in the level of the K48-linked polyubiquitin chain. Although there was no change in the protein level of caspase-7, it may be expected that it will respond to DNA repair or stress response, which is one of the cellular roles for the K63-linked polyubiquitin chain. In addition, it is suggested that OTUD6A may regulate caspase-7 by responding to lysosomal targets and not by regulating the protein expression level by OTUD6A.

The present study confirmed that OTUD6A acts as a DUB for nucleolin and caspase-7. Nucleolin is known to act as an oncogene in various cancers. Overexpression of nucleolin is a predictor of poor prognosis of lung cancer (46) and prostate cancer (47). Upregulation of nucleolin by CRYβB2 promotes tumorigenesis of triple-negative breast cancer (28), and activation of the nucleolin-AKT pathway by CBP/p300-interacting transactivator with E/D-rich carboxy-terminal domain-2 promotes tumorigenesis of prostate cancer (48). XIAP induces bladder cancer invasion and lung cancer metastasis by enhancing the nucleolin-mediated Rho GDP-dissociation inhibitor 2 stability (49), and nucleolin promotes cervical cancer by activating EGFR signaling involved in tumor growth and invasion (50). The interaction of nucleolin and ErbB2 induced tumorigenesis in ErbB2-positive breast cancer (51), whereas inhibition of nucleolin and ErbB2 inhibited tumorigenesis in ErbB2-positive breast cancer (52). In addition, inhibition of the nucleolin protein by nucleolin antagonist N6L promoted the normalization of tumor vasculature and impaired the progression of pancreatic cancer (53). Caspase-7 is a known apoptotic protein (54). Therefore, flow cytometric analysis was performed to investigate the effect of OTUD6A on cell proliferation and cell cycle progression. Overexpression of OTUD6A induced cell proliferation through the promotion of cell cycle progression. In addition, flow cytometric analysis was performed following overexpression of nucleolin to prove that the promotion of the cell cycle by OTUD6A is due to the upregulation of nucleolin induced by OTUD6A. It was indicated that overexpression of nucleolin promoted cell cycle progression in MCF7 cells. Therefore, the present results suggest that OTUD6A stabilizes nucleolin by deubiquitination and that upregulated nucleolin increases cell proliferation by promoting cell cycle progression.

Taken together, the present results indicate that nucleolin and caspase-7, the substrates of OTUD6A, are potential anticancer therapy targets. However, further studies are required to discover the synergistic effect and regulatory mechanism in the p53 signaling pathway of OTUD6A and nucleolin or caspase-7.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SHK and KHB designed the study and confirm the authenticity of all the raw data. SHK performed most of the experiments and wrote the manuscript. Both authors 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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