RESEARCH LETTER

The deubiquitinating enzyme USP17 regulates c-Myc levels and controls cell proliferation and glycolysis

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The c-Myc oncoprotein is frequently overexpressed in human cancers and is essential for cancer cell proliferation. The dysregulation of ubiquitin-proteasome-mediated degradation is one of the contributing factors to the upregulated expression of c-Myc in human cancers. We herein identified USP17 as a novel deubiquitinating enzyme that regulates c-Myc levels and controls cell proliferation and glycolysis. The overexpression of USP17 stabilized the c-Myc protein by promoting its deubiquitination. In contrast, the knockdown of USP17 promoted c-Myc degradation and reduced c-Myc levels. The knockdown of USP17 also suppressed cell proliferation and glycolysis. Collectively, the present results reveal a novel role for USP17 in the regulation of c-Myc stability and suggest its potential as a therapeutic target for cancer treatment.

Keywords: c-Myc; deubiquitination; glycolysis; proliferation; USP17

c-Myc has been identified as the proto-oncogene product responsible for Burkitt's lymphoma [1] and is involved in a wide variety of biological processes, including cell proliferation and differentiation [2–4]. Additionally, c-Myc has been reported to regulate multiple metabolic pathways, including aerobic glycolysis, termed the Warburg effect [5,6]. c-Myc is a transcriptional factor that regulates both the activation and repression of many target genes [7,8]. The expression of the highly unstable c-Myc protein is almost undetectable in normal quiescent cells. When cells enter the G_1 phase, the expression of the c-Myc protein is rapidly upregulated; however, its half-life is approximately 30 min [9,10]. c-Myc is frequently overexpressed in cancer cells, suggesting a failure in its quantitative regulatory mechanism. The upregulation of *c-Myc* gene expression through the translocation and amplification of the *c-Myc* gene and regulation of mRNA and protein levels has been reported in cancer cells [11–13]. Since c-Myc expression levels correlate with a poor prognosis in human cancers [4,14], c-Myc is expected to become a promising therapeutic target molecule. However, the c-Myc protein does not have a domain structure that may be used as a therapeutic target, and drug discovery targeting c-Myc itself has been challenging; therefore, molecules that contribute to the regulation of c-Myc expression have been attracting attention as drug targets. For example, the transcriptional regulation of

Abbreviations
a.a., amino acid; CHX, cycloheximide; DMEM, Dulbecco's modified Eagle's medium; DUB, deubiquitinase; FBS, fetal bovine serum; Fbw7, F-box and WD repeat domain-containing 7; HK2, hexokinase 2; LDHA, lactate dehydrogenase A; shRNA, short-hairpin RNA; siRNA, short interfering RNA; Ub, ubiquitin; UPS, ubiquitin-proteasome system; USP, ubiquitin-specific protease.
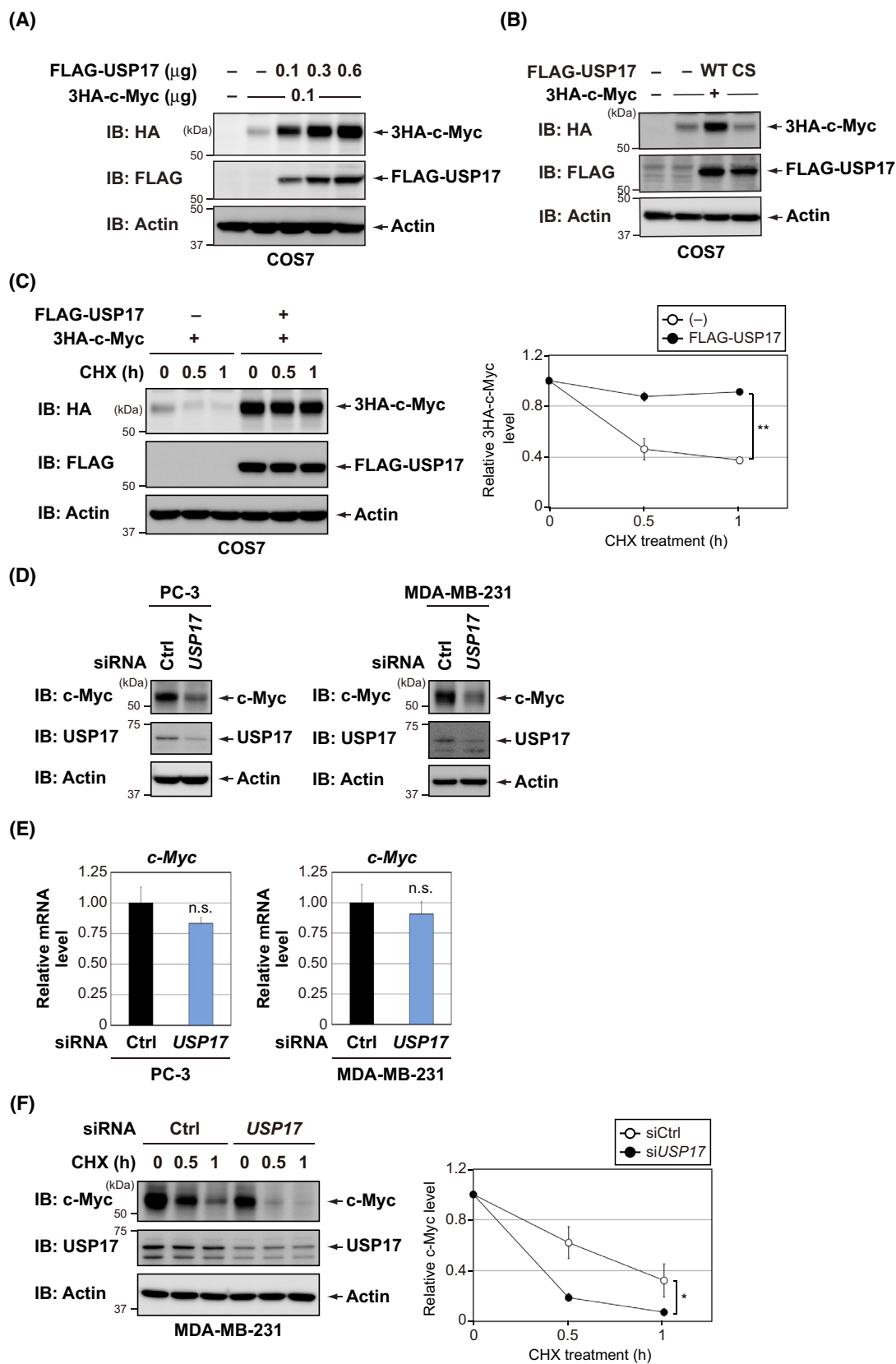
(A)

FLAG-USP17 (μg)   –   –   0.1 0.3 0.6
3HA-c-Myc (μg)   – ┗━ 0.1 ━┛

IB: HA   (kDa)   ← 3HA-c-Myc
          50 ┘

IB: FLAG   ← FLAG-USP17
          50 ┘

IB: Actin 50 ┐
           37 ┘   ← Actin

COS7

(B)

FLAG-USP17 –   –   WT CS
3HA-c-Myc   –   + ┗━━━┛

IB: HA (kDa)   ← 3HA-c-Myc
         50 ┘

IB: FLAG   ← FLAG-USP17
         50 ┘

IB: Actin 37 ┘   ← Actin

COS7

(C)

FLAG-USP17   –   +
3HA-c-Myc   +   +
CHX (h)   0 0.5 1   0 0.5 1

IB: HA (kDa)   ← 3HA-c-Myc
         50 ┘

IB: FLAG   ← FLAG-USP17
         50 ┘

IB: Actin 37 ┘   ← Actin

COS7

(D)

| | PC-3 | | MDA-MB-231 | |
| siRNA | Ctrl | USP17 | Ctrl | USP17 |

IB: c-Myc (kDa) ← c-Myc   IB: c-Myc (kDa) ← c-Myc
            50 ┘   50 ┘

IB: USP17   75 ┘ ← USP17   IB: USP17   75 ┘ ← USP17

IB: Actin   ← Actin   IB: Actin   37 ┘ ← Actin
           37 ┘

(E)

(F)

siRNA   Ctrl   USP17
CHX (h) 0 0.5 1   0 0.5 1

IB: c-Myc (kDa)   ← c-Myc
            50 ┘

IB: USP17   75 ┘   ← USP17

IB: Actin   37 ┘   ← Actin

MDA-MB-231

Fig. 1. USP17 stabilizes the c-Myc protein. (A) 3HA-c-Myc was co-expressed with increasing amounts of FLAG-USP17 in COS7 cells. After 24 h, cell lysates were immunoblotted with the indicated antibodies. (B) 3HA-c-Myc was co-expressed with FLAG-USP17 [WT or the catalytically inactive mutant (C89S)] in COS7 cells. After 24 h, cell lysates were immunoblotted with the indicated antibodies. CS, C89S. (C) COS7 cells were transiently transfected with the indicated constructs. After 24 h, cells were treated with 25 μg·mL^{-1} cycloheximide (CHX) for the indicated periods and harvested for immunoblotting with the indicated antibodies. (D) Cells were transiently transfected with the indicated siRNAs. After 72 h, cell lysates were immunoblotted with the indicated antibodies. Ctrl, control. (E) Cells were transiently transfected with the indicated siRNAs for 72 h. The expression of each gene was assessed by qPCR. The expression level of *c-Myc* was normalized to that of *β-actin* mRNA. Results are shown as the mean ± SD ($n = 3$). (F) Time course of the CHX treatment of MDA-MB-231 cells transiently transfected with the indicated siRNAs. The protein stability of c-Myc was analysed at the indicated times by an immunoblot analysis (left). Quantification of c-Myc protein levels normalized to β-actin. Data represent the means and S.D. of three independent experiments (right). *$P < 0.05$; **$P < 0.01$; *n.s.*, not significant.

c-Myc by bromodomain-containing protein 4 of the bromodomain and extraterminal domain (BET) family has been demonstrated. The antitumour activities of BET inhibitors through the repressed transcription of *c-Myc* and its related genes are currently being examined in clinical trials for the treatment of multiple myeloma and acute myeloid leukaemia. In addition, research on and the development of therapeutics targeting various molecules that regulate the expression of c-Myc at the mRNA and protein levels are being actively pursued [15–18].

The regulation of protein expression by post-translational modifications is known to be important in the dysregulation of c-Myc expression in cancer cells. The stability of c-Myc is regulated by phosphorylation at threonine 58 (T58) and serine 62 (S62) in the Myc Box I (MBI) region of c-Myc [19]. The phosphorylation of S62 on c-Myc by mitogen-activated protein kinase stabilizes c-Myc and promotes cell proliferation [20]. The phosphorylation of S62 triggers the phosphorylation of T58 on c-Myc by glycogen synthase kinase 3β, which promotes the dephosphorylation of S62, resulting in the ubiquitination of c-Myc by the ubiquitin (Ub) ligase F-box and WD repeat domain-containing 7 (Fbw7) [21]. The polyubiquitinatied c-Myc protein is degraded by the 26S proteasome [22]. The T58 mutation in c-Myc has been reported in many human cancers, particularly lymphomas, and has been shown to antagonize Fbw7-mediated c-Myc degradation [23]. In addition to Fbw7, S-phase kinase-associated protein 2, F-box and leucine-rich repeat protein 3, and F-box protein 32 have been reported to regulate the expression of c-Myc [24–27]. The diverse regulation of cell proliferation *via* the controlled expression of c-Myc through the ubiquitin-proteasome system (UPS) has been demonstrated, and dysregulated ubiquitination was identified as a contributing factor to the accumulation of the c-Myc protein in cancer cells [23].

Ubiquitination is a reversible reaction. Deubiquitinating enzymes (DUBs) cleave isopeptide bonds between the substrate protein and ubiquitin [28]. The human genome encodes approximately 100 DUBs, which are classified into eight families: ubiquitin-specific proteases (USPs), ubiquitin carboxy-terminal hydrolase (UCHs), ovarian-tumour proteases, Machado-Joseph disease protein domain proteases, Jab1/Pad1/MPN domain-associated metallopeptidases, the monocyte chemotactic protein-induced protein family, motif interacting with Ub-containing novel DUB family and zinc finger with UFM1-specific peptidase domain protein family [29–31]. DUBs were recently shown to target various oncogenes and tumour suppressor gene products, thereby regulating cancer initiation, promotion and progression. For example, USP7 promotes tumour suppressor p53 degradation by deubiquitinating and stabilizing Mdm2, the ubiquitin ligase of p53 [32]. DUB inhibitors are currently being developed, such as the USP7-specific inhibitor P5091, which has been reported to induce apoptosis in bortezomib-resistant multiple myeloma cells [33].

Several DUBs, including USP28, USP36 and USP37, have so far been shown to control c-Myc expression [13,34,35]. In the present study, we demonstrated that USP17 is a novel DUB that regulates c-Myc levels and controls cell proliferation and glycolysis. The overexpression of USP17, but not its catalytic-inactive mutant (C89S), stabilized the c-Myc protein. In contrast, the knockdown of USP17 reduced the abundance of c-Myc. In addition, USP17 bound to and deubiquitinated c-Myc in cells and *in vitro*. Moreover, the knockdown of USP17 reduced cell proliferation and glycolysis. Collectively, the present results reveal a novel role for USP17 in the regulation of c-Myc stability and suggest the potential of USP17 as a therapeutic target for cancer treatment.

Materials and methods

Cell lines, plasmids and transfection

COS7 cells and MDA-MB-231 cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM)

(Sigma, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma), 100 U·mL^{-1} of penicillin G and 100 μg·mL^{-1} of streptomycin at 37 °C in the presence of 5% CO_2 [36]. PC-3 cells were cultured in Roswell Park Memorial Institute 1640 medium (Sigma) supplemented with 10% heat-inactivated FBS and penicillin/streptomycin [37].

The original constructs encoding DUBs, c-Myc and Ub were previously described [36,37]. The *USP17* Mission short-hairpin RNA (shRNA) plasmid (TRCN0000038868) was obtained from Sigma.

In DNA transfection, plasmids were transiently transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). In short interfering RNA (siRNA) transfection, siRNAs were transfected using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's protocol. Human *USP17* siRNA (sense, 5′-GCAGGAAGAUGCCCAUGAA-3′) [38], human *USP17* siRNA #2 (sense, 5′-CACAAG CAGGUAGAUCAUCAC-3′) [39] and human *c-Myc* siRNA (sense, 5′-GAGCUAAAACGGAGCUUUU-3′) [40] were purchased from FASMAC (Kanagawa, Japan). Stealth RNAi™ siRNA Negative Control Med GC Duplex was obtained from Invitrogen.

The lentiviral CSII-EF/FLAG-c-Myc vector was generated by subcloning FLAG-c-Myc from pcDNA3/FLAG-c-Myc [37] into CSII-EF, which has an elongation factor 1 α gene promoter for protein expression in mammalian cells [41]. PC-3 cells were infected with viral particles according to standard protocols [42].

RNA extraction, reverse transcription and quantitative PCR (qPCR)

Total RNA was extracted as previously described [43]. cDNA was then synthesized from total RNA using the ReverTra qPCR RT Master Mix (TOYOBO, Osaka, Japan) according to the manufacturer's instructions. qPCR was performed using TB Green Premix Ex Taq II (TaKaRa Bio Inc., Shiga, Japan) and the ABI Prism 7300 sequence detection system (Applied Biosystems, South San Francisco, CA, USA) [36]. The specificities of the detected signals were confirmed by a dissociation curve consisting of a single peak. β-actin was used as the internal control. The primers used are listed in Table S1.

Immunochemical methods and antibodies

Protein solubilization, immunoblotting, immunoprecipitation and Strep-Tactin pull-down were conducted as previously described [36,44]. The following commercially available antibodies were used: anti-c-Myc (AF3696; R&D Systems, Minneapolis, MN, USA), anti-c-Myc (#13987; Cell Signaling Technology, Beverly, MA, USA), anti-K48-linkage Specific Polyubiquitin (D9D5) Rabbit mAb (HRP Conjugate) (#12805; Cell Signaling Technology), anti-K63-linkage Specific Polyubiquitin (D7A11) Rabbit mAb (HRP Conjugate) (#12930; Cell Signaling Technology), anti-c-Myc (67447-1-Ig; Protein Tech, Chicago, IL, USA), anti-USP17/DUB3 (NBP1-79745; Novus Biologicals, Littleton, CO, USA), anti-FLAG (IE6; Fujifilm Wako Pure Chemical, Osaka, Japan), anti-Cyclin A (611268; BD Biosciences, Franklin Lakes, NJ, USA), anti-HA-horseradish peroxidase (HRP) (F-7; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p21 (F-5, Santa Cruz Biotechnology), anti-β-actin-HRP (C4; Santa Cruz Biotechnology) and anti-Ub-HRP (P4D1, Biolegend, San Diego, CA, USA). Mouse immunoglobulin G1 (IgG1) (MB002; R&D Systems) was used as a control.

Recombinant FLAG-USP17 and 3HA-c-Myc were synthesized using the TNT T7 Quick Coupled Transcription/Translation system (Promega, Madison, WI, USA).

In vitro deubiquitination assay

3HA-c-Myc and FLAG-Ub were cotransfected into COS7 cells. After cells had been treated with 20 μm MG132 for 4 h, ubiquitinated c-Myc was immunoprecipitated with anti-FLAG agarose (Sigma), followed by elution with 0.5 mg·mL^{-1} of the 3xFLAG peptide (Sigma). In a parallel experiment, Strep-USP17 (WT or C89S) was transfected into COS7 cells, and Strep-USP17 was also pulled down with Strep-Tactin Sepharose (IBA Lifesciences, Goettingen, Germany) followed by elution with 10 mm desthiobiotin (Sigma). In the *in vitro* deubiquitination assay, purified Strep-USP17 WT or the C89S mutant was incubated with ubiquitinated 3HA-c-Myc in deubiquitination reaction buffer [150 mm Tris-HCl (pH 7.5), 50 mm NaCl, 1 mm EDTA and 5 mm DTT] at 37 °C for 2 h. The ubiquitination status of c-Myc was analysed by immunoblotting with anti-HA antibodies.

Cycloheximide (CHX) half-life assay

Cells were transiently transfected with siRNAs. After treatment with 25 μg·mL^{-1} of CHX, cells were collected at the indicated time points. The protein expression of c-Myc was analysed by immunoblotting.

Cell viability assay and BrdU incorporation assay

Cell viability was measured using Cell Counting Kit-8 according to the manufacturer's instructions (Dojindo, Kumamoto, Japan) [45]. Cells were transiently transfected with the indicated siRNAs. Twenty-four hours after transfection, cells were trypsinized and replated at a concentration of 5×10^3 cells per well in a 96-well plate. After 48 h, the WST-8 reagent was added, and cells were incubated at 37 °C for 3 h in a humidified atmosphere of 5% CO_2. Absorbance at 450 nm of the medium was measured.
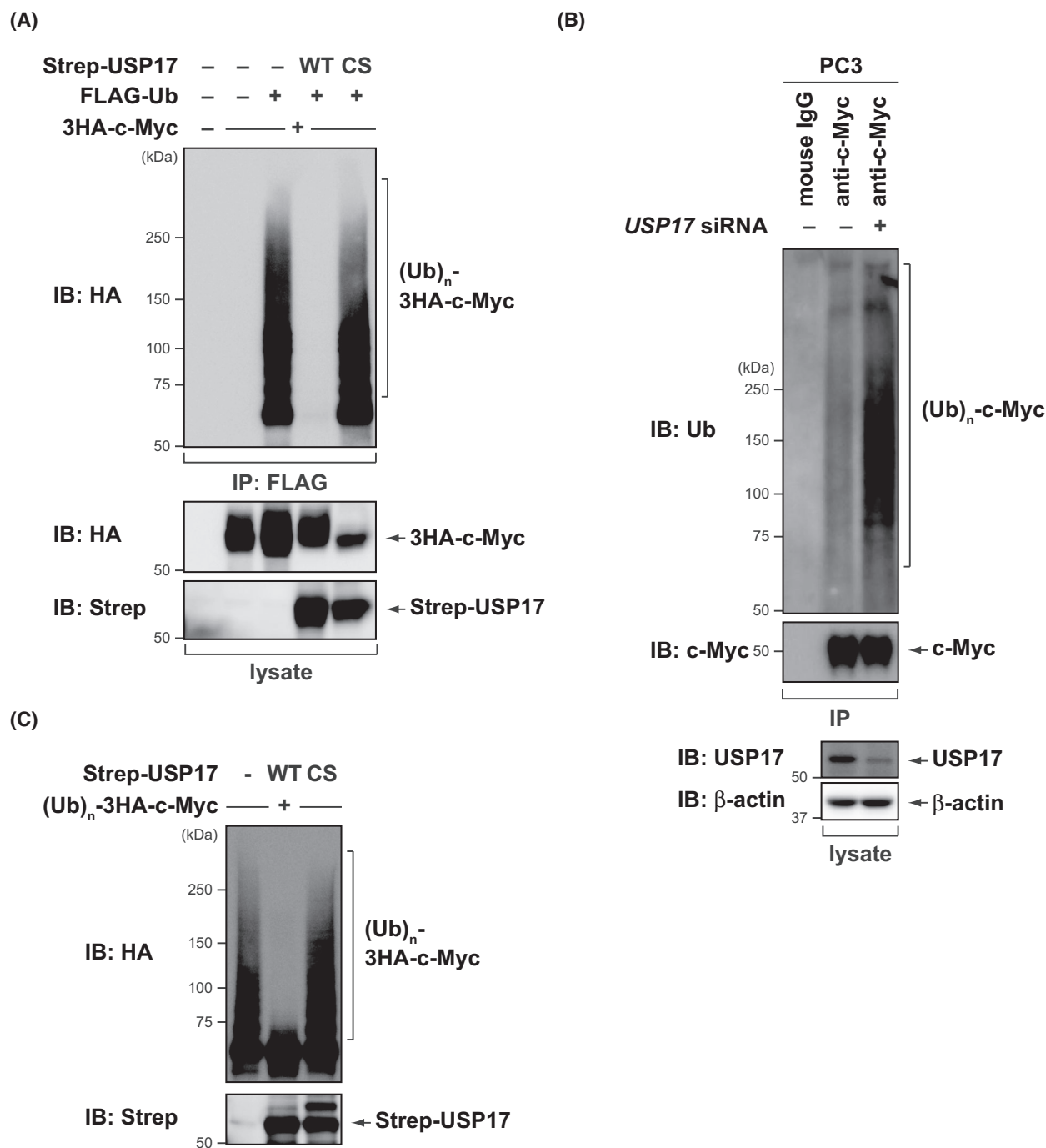
Fig. 2. USP17 deubiquitinates the c-Myc protein. (A) An empty vector or plasmid expressing USP17 WT or C89S was co-expressed with 3HA-c-Myc and a FLAG-Ub plasmid into COS7 cells. Cells were treated with 20 μM MG132. After 4 h, cell lysates were analysed by a pull-down assay and immunoblotting with the indicated antibodies. CS, C89S. (B) PC-3 cells transfected with control or *USP17* siRNA were treated with 10 μM MG132 for 6 h. Cell lysates were subjected to immunoprecipitation with an anti-c-Myc antibody, and the polyubiquitination of c-Myc was assessed by immunoblotting using an anti-ubiquitin antibody. (C) Ubiquitinated c-Myc was purified from MG132-treated COS7 cells transfected with 3HA-c-Myc and FLAG-Ub. Ubiquitinated 3HA-c-Myc was incubated with purified Strep-USP17 (WT or C89S) and the polyubiquitination of c-Myc was analysed by immunoblotting with the indicated antibodies.
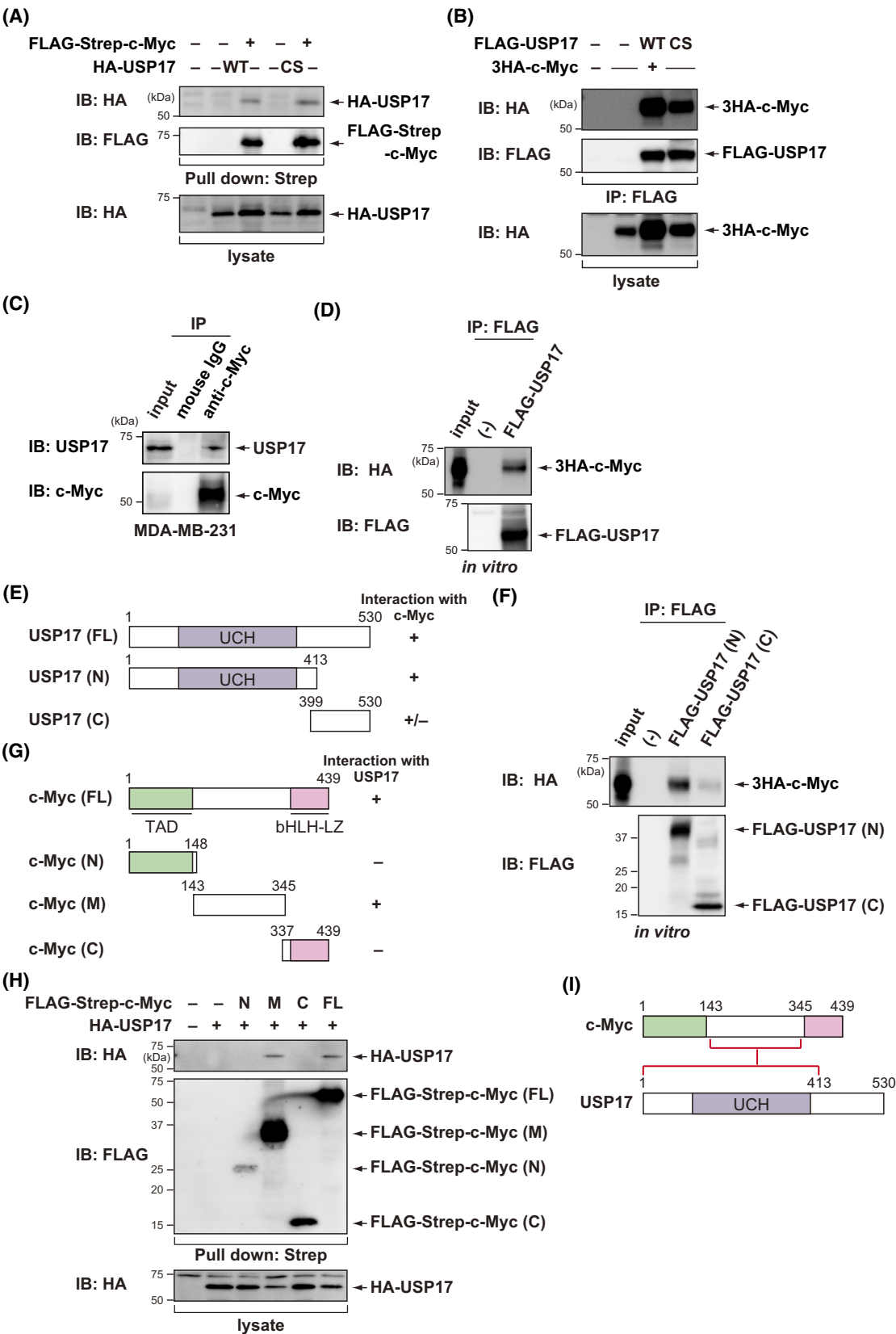
Fig. 3. USP17 interacts with c-Myc. (A) COS7 cells were transiently transfected with the indicated constructs. After 24 h, cell lysates were subjected to the Strep-Tactin pull-down assay and immunoblotting with the indicated antibodies. (B) COS7 cells were transfected with the indicated constructs. After 24 h, cell lysates were immunoprecipitated with an anti-FLAG antibody and then immunoblotted with the indicated antibodies. (C) Endogenous c-Myc was immunoprecipitated from MDA-MB-231 cells and bound endogenous USP17 was examined by immunoblotting. (D) *In vitro* binding assay using recombinant FLAG-USP17 and 3HA-c-Myc. *In vitro* translated FLAG-USP17 and 3HA-c-Myc were used for the *in vitro* binding assay. (E) Schematic of full-length and deletion mutants of USP17. UCH, ubiquitin C-terminal hydrolase. (F) *In vitro* translated FLAG-USP17 (N) or FLAG-USP17 (C), and 3HA-c-Myc were used for the *in vitro* binding assay. (G) Schematic of full-length and deletion mutants of c-Myc. TAD, transactivation domain, bHLH-LZ, basic helix-loop-helix leucine zipper. (H) COS7 cells were transiently transfected with the indicated plasmids. After 24 h, cell lysates were subjected to the Strep-Tactin pull-down assay and immunoblotting with the indicated antibodies. (I) Schematic of protein–protein interactions between c-Myc and USP17.

To measure the levels of DNA synthesis, cells were treated with 10 μM 5-bromo-2′-deoxyuridine (BrdU) at 37 °C for 1 h. BrdU-incorporated cells (S phase) were detected by flow cytometry (FACSVerse, BD Biosciences) after being fixed with paraformaldehyde, permeabilized with saponin and stained with anti-BrdU FITC (FITC BrdU Flow kit, BD Biosciences) according to the manufacturer′s protocol [45].

Measurement of glucose consumption and lactate production

Cells were seeded on 24-well plates, and medium was changed after 24 h to serum-free DMEM. Cells were incubated for 24 h, and the culture medium was then collected for the measurement of glucose and lactate concentrations. Glucose concentrations were assessed using the Glucose Assay Kit-WST (G264, Dojindo). Lactate concentrations were measured using the Lactate Assay Kit-WST (L256,. Dojindo).

Statistical analysis

The significance of differences between two groups was evaluated using a two-tailed Student's *t*-test. In multigroup analyses, significance was assessed using a one-way analysis of variance with the *post hoc* Tukey–Kramer honestly significant difference test.

Results

USP17 regulates the stability of the c-Myc protein

To identify novel DUBs that regulate the c-Myc protein, we expressed 3HA-c-Myc and several DUBs in COS7 cells. 3HA-c-Myc protein levels were assessed by immunoblotting. Among the 22 DUBs, we confirmed that several DUBs, including USP17, increased c-Myc protein levels in COS7 cells in the first screening (Fig. S1A,B). Next, we evaluated these results quantitatively and found that USP17 in particular significantly increased c-Myc protein levels (Fig. S1C,D).

Although USP28 has already been identified as a DUB for c-Myc [13], the increase in c-Myc protein levels by USP17 was more comparable under the present experimental conditions (Fig. S1E). Thus, we attempted to clarify the stabilization mechanism of c-Myc protein by USP17.

We confirmed that the ectopic expression of USP17 increased c-Myc protein levels in a dose-dependent manner (Fig. 1A). In contrast, the expression of catalytic-inactive mutant USP17 C89S [46] failed to stabilize the c-Myc protein (Fig. 1B). The overexpression of WT USP17 extended the protein half-life of c-Myc, suggesting that the accumulation of c-Myc was due to an increase in protein stability (Fig. 1C).

We then silenced USP17 in PC-3 cells and MDA-MB-231 cells and examined its effects on endogenous levels of c-Myc. We found that the knockdown of USP17 reduced the abundance of the c-Myc protein without affecting *c-Myc* mRNA levels in these cells (Fig. 1D,E). Furthermore, a reduction in c-Myc protein levels was observed in cells expressing *USP17* shRNA (Fig. S2). The knockdown of USP17 also shortened the protein half-life of c-Myc in MDA-MB-231 cells (Fig. 1F). Collectively, these results suggest the potential of USP17 as a novel candidate DUB for c-Myc that negatively regulates the degradation of c-Myc.

USP17 deubiquitinates c-Myc

To gain insights into the USP17-mediated regulation of c-Myc, we investigated whether USP17 regulates c-Myc protein stability through deubiquitination. We transfected expression vectors encoding 3HA-c-Myc and FLAG-Ub with the WT or C89S mutant of USP17 into COS7 cells. Cells were treated with MG132, Ub-conjugating proteins were purified with the anti-FLAG antibody conjugated to agarose resins, and c-Myc ubiquitination was analysed using the anti-HA antibody. As shown in Fig. 2A, WT USP17, but not USP17 C89S, significantly reduced the ubiquitination of c-Myc. In contrast, the knockdown of USP17
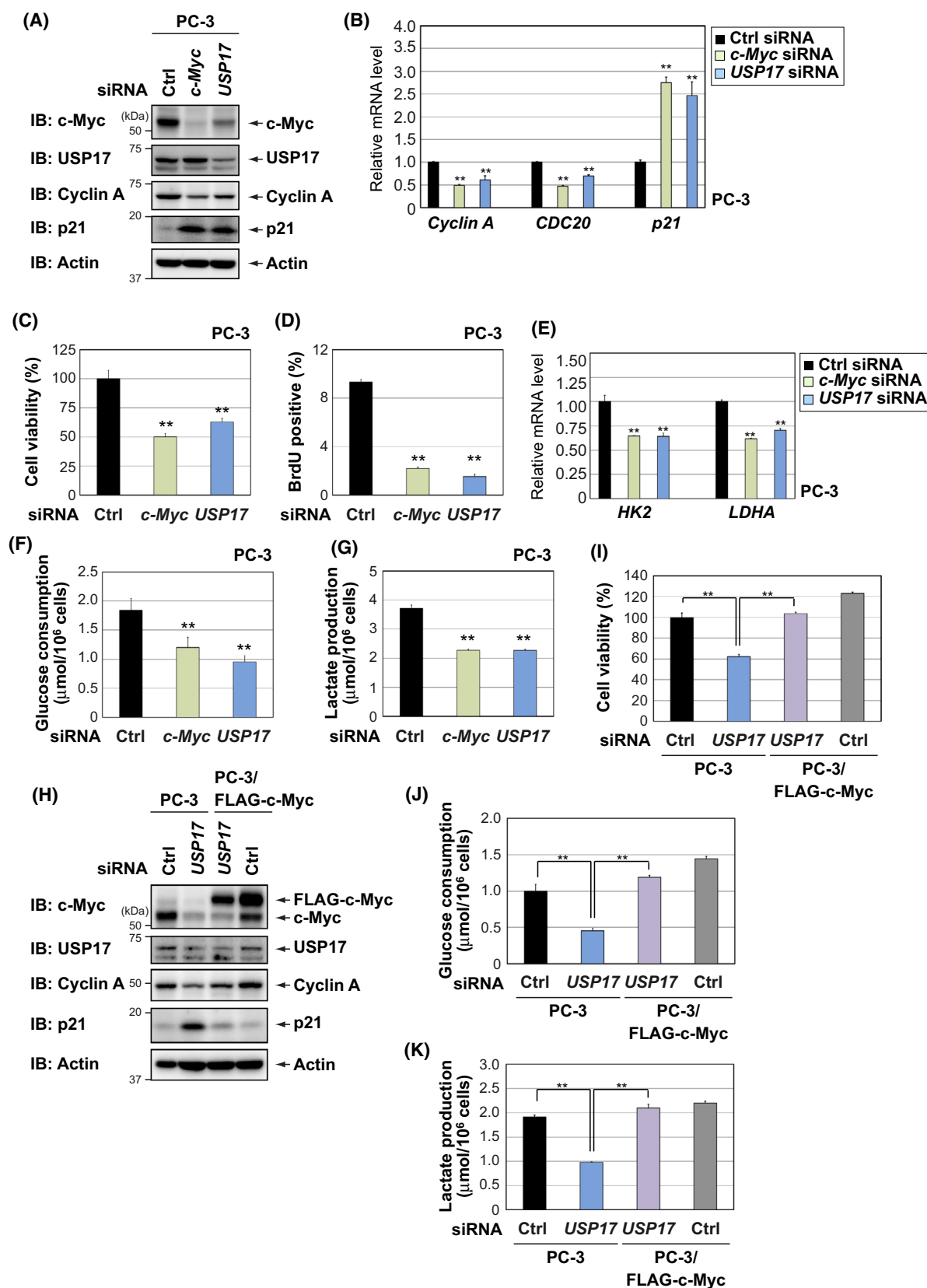
Fig. 4. USP17 regulates c-Myc-dependent gene expression and promotes cell proliferation and glycolysis. (A) PC-3 cells were transiently transfected with the indicated siRNAs. After 72 h, an immunoblot analysis was performed with the indicated antibodies. Ctrl, control. (B) PC-3 cells were transiently transfected with the indicated siRNAs for 72 h. The expression of each gene was assessed by quantitative PCR, and the mRNA levels of the indicated genes were normalized with *β-actin* mRNA. Results are shown as means ± SD (*n* = 3). **P < 0.01. (C) PC-3 cells were transiently transfected with the indicated siRNAs. After 72 h, cell viability was measured by a WST-8 cell proliferation assay. Results are shown as means ± SD (*n* = 3). (D) PC-3 cells were transiently transfected with the indicated siRNAs for 72 h. Cells were treated with 10 μM 5-bromo-2′-deoxyuridine (BrdU) at 37 °C for 1 h. BrdU-incorporated cells were detected by flow cytometry after being fixed with paraformaldehyde, permeabilized with saponin, and stained with anti-BrdU FITC. Results are shown as means ± SD (*n* = 3). (E) PC-3 cells were transiently transfected with the indicated siRNAs for 72 h. The expression of each gene was assessed by quantitative PCR, and the mRNA levels of the indicated genes were normalized with *β-actin* mRNA. Results are shown as means ± SD (*n* = 3). (F, G) The medium from the PC-3 cell culture was collected for the analysis of glucose consumption (F) and lactate production (G). Results are shown as means ± SD (*n* = 3). (H) PC-3 cells and PC-3/FLAG-c-Myc cells were transiently transfected with the indicated siRNAs. After 72 h, an immunoblot analysis was performed with the indicated antibodies. (I) PC-3 cells and PC-3/FLAG-c-Myc cells were transiently transfected with the indicated siRNAs. After 72 h, cell viability was measured by a WST-8 cell proliferation assay. Results are shown as means ± SD (*n* = 3). (J, K) The medium from the PC-3 cell culture or PC-3/FLAG-c-Myc cell culture was collected for the analysis of glucose consumption (J) and lactate production (K). Results are shown as means ± SD (*n* = 3).

increased c-Myc ubiquitination in PC-3 cells (Fig. 2B). As shown in Fig. S3, the knockdown of USP17 increased K48-linked polyubiquitination of c-Myc, but had little effect on K63-linked polyubiquitination of c-Myc. These results support that USP17 regulates c-Myc stabilization *via* cleavage of K48-linked polyubiquitination. The *in vitro* deubiquitination assay showed that c-Myc was a direct substrate of USP17. WT USP17, but not the C89S mutant, reduced the ubiquitinated species of c-Myc (Fig. 2C). These results indicate that USP17 stabilized c-Myc by directly deubiquitinating the c-Myc protein.

USP17 interacts with c-Myc

We examined whether USP17 physically interacts with c-Myc. FLAG-Strep-c-Myc and HA-USP17 were transfected into COS7 cells, and a Strep-Tactin sepharose pull-down assay showed that USP17 coprecipitated with c-Myc (Fig. 3A). The 3HA-c-Myc protein was also detected when FLAG-USP17 was immunoprecipitated by the anti-FLAG antibody in COS7 cells (Fig. 3B). Furthermore, USP17 C89S interacted with c-Myc, indicating that the catalytic activity of USP17 was not required for the interaction with c-Myc (Fig. 3A,B). The interaction between endogenous c-Myc and USP17 was also demonstrated in MDA-MB-231 cells (Fig. 3C). The immunoprecipitation assay showed that recombinant FLAG-USP17 and recombinant 3HA-c-Myc bound to each other *in vitro*, suggesting a direct interaction (Fig. 3D).

We then investigated the interaction between c-Myc and USP17 in more detail. As shown in Fig. 3E,F, the N-terminal region of USP17 (a.a. 1-413) bound strongly to c-Myc. On the other hand, the C-terminal region of USP17 (a.a. 399-530) bound weakly to c-Myc. We also co-expressed various c-Myc deletion mutants (Fig. 3G) with HA-USP17 in COS7 cells. The Strep-Tactin pull-down assay showed that c-Myc bound to USP17 through its central region (a.a. 143-345) containing the PEST domain (Fig. 3H). A schematic diagram of the protein–protein interaction between c-Myc and USP17 is shown in Fig. 3I.

USP17 regulates c-Myc-dependent gene expression and promotes cell proliferation and glycolysis

Since USP17 prevented the degradation of c-Myc, we examined the effects of USP17 on c-Myc-mediated transcriptional activity. The expression of both the c-Myc target Cyclin A and CDC20 was downregulated by the knockdown of c-Myc (Fig. 4A,B) [47]. In contrast, the cyclin-dependent kinase inhibitor p21, a negative regulator of cell cycle progression in G_1 and S phases, is known to be repressed at the transcriptional level by c-Myc [48,49]. Hence, p21 was significantly increased after the knockdown of c-Myc (Fig. 4A,B). Similar to the knockdown of c-Myc, the knockdown of USP17 decreased the expression of Cyclin A and CDC20 and increased that of p21 in PC-3 cells (Fig. 4A,B and Fig. S4A,B). Therefore, the knockdown of USP17 suppressed the proliferation of PC3 cells, similar to the knockdown of c-Myc (Fig. 4C and Fig. S4C). The BrdU incorporation analysis showed that the knockdown of USP17 induced G_1 arrest in PC-3 cells (Fig. 4D).

c-Myc has been identified as a potent regulator of multiple metabolic pathways that are essential for cancer cell proliferation [5]. c-Myc promotes aerobic glycolysis through constitutive enhancements in the activities of glycolytic enzymes, including hexokinase 2 (HK2) and lactate dehydrogenase A (LDHA) [50,51].

We herein found that the loss of USP17 reduced the mRNA expression levels of *HK2* and *LDHA*, similar to the knockdown of c-Myc, in PC-3 cells (Fig. 4E and Fig. S4D). Furthermore, the knockdown of USP17, similar to the knockdown of c-Myc, reduced glucose consumption and lactate production by PC-3 cells, indicating that it suppressed glycolysis (Fig. 4F, G and Fig. S4E,F). Finally, overexpression of c-Myc largely restored cell proliferation and glucose metabolism by USP17 knockdown in PC-3 cells (Fig. 4H–K, Fig. S5). These results showed that USP17 promoted cell proliferation and glycolysis by controlling c-Myc stability.

Discussion

c-Myc is a potent oncogene that is involved in a wide range of biological processes, including cell proliferation, differentiation and metabolism [1–4]. The overexpression of c-Myc occurs in many cancer cells, and its correlation with a poor prognosis in human cancers has been reported [4,14]. c-Myc expression is regulated by several mechanisms, which are expected to become attractive therapeutic targets for the treatment of cancers. UPS is a regulated mechanism of intracellular protein degradation and turnover and participates in a wide range of biological functions. Several E3 ligases have been reported to play roles in the ubiquitination and degradation of c-Myc [23]. However, it currently remains unclear whether other DUB(s) target c-Myc. In the present study, we identified USP17 as a novel DUB that targets c-Myc. USP17 bound to and deubiquitinated c-Myc in cells and *in vitro*. We also demonstrated that the knockdown of USP17 decreased c-Myc protein levels. These results contribute to our understanding of the diverse mechanisms regulating c-Myc (Fig. 5).

The human genome encodes approximately 100 DUBs, divided into eight different subfamilies [29–31]. DUBs target various oncogenes and tumour suppressor gene products and have been shown to play a role in cancer development and progression [52]. We identified USP17, belonging to the USP family, as a novel c-Myc DUB. Several DUBs, including USP28, USP36 and USP37, have so far been shown to regulate the expression of c-Myc [13,34,35]. It has been demonstrated that USP17/USP17L2/DUB3 was overexpressed in many types of cancers [53]. Among them, overexpression of *USP17* mRNA has been identified in breast cancer (Fig. S6A) [54], as previously reported [55]. We investigated whether there is a correlation between *USP17* expression and prognosis in human breast cancers [56] using Kaplan–Meier Plotter [57]. In
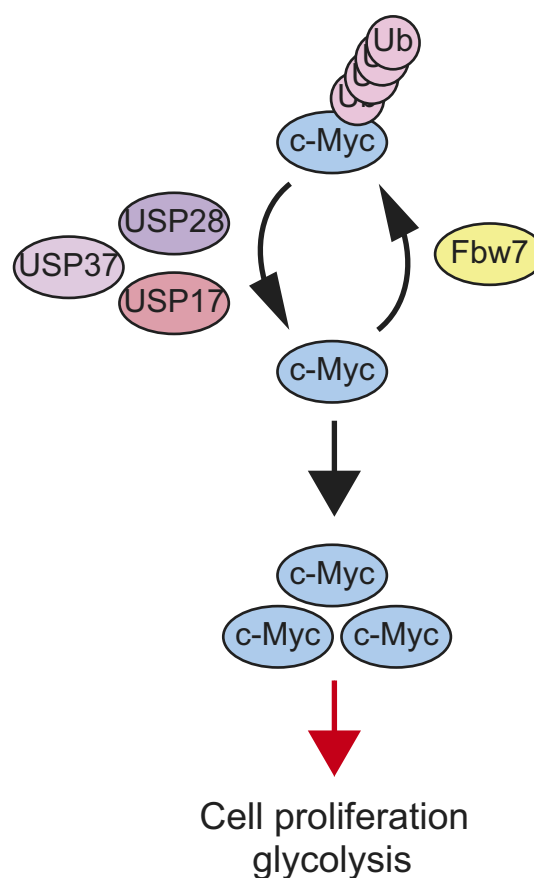

Fig. 5. A schematic illustration of the E3 ligase-mediated ubiquitination and DUB-mediated deubiquitination of the c-Myc protein.

breast cancers, the prognosis of patients with high expression levels of *USP17* was significantly worse ($p = 0.00045$) than that of USP28, USP36 or USP37 (Fig. S6B). Furthermore, bioinformatic analysis by TIMER (Tumor Immune Estimation Resource database) [58] showed that in breast cancer as a whole or in luminal-A breast cancer, the expression level of *USP17* correlated with that of c-Myc target genes such as *carbamoyl-phosphate synthetase 2, aspartate transcarbamylase and dihydroorotase* (*CAD*) and *lysine demethylase 1A* (*KDM1A*), [37,59], but not with that of *c-Myc* itself. Therefore, USP17 may promote tumorigenesis in these cancers through stabilization of c-Myc protein, but further studies are needed. In addition, other subfamilies of DUBs may also contribute to the regulation of c-Myc.

USP17 is an immediate-early gene that is induced in response to cytokines, including IL-4 and IL-6 [60]. It plays roles in tumour proliferation, apoptosis, invasion and migration. For example, USP17 exhibits oncogenic potency by stabilizing proteins that positively

regulate the cell cycle, such as Cdc25A and Cyclin A, thereby contributing to the proliferation of cancer cells [46,61]. Furthermore, previous studies reported that USP17 contributed to cancer metastasis by stabilizing Snail, an EMT-related transcription factor [39,55]. In addition to these proteins, the stabilization effect of the c-Myc protein induced by USP17 may contribute to the malignant transformation of cancer.

We demonstrated that USP17 directly bound to c-Myc and interacted with USP17 through a region containing the Myc box III/PEST domain (Fig. 3G). Pan et al. [35] reported that USP37 stabilized c-Myc and interacted with c-Myc through the Myc box III domain. In the present study, we did not examine the binding region of c-Myc in more detail; nevertheless, it is possible that USP17 binds through Myc box III, similar to USP37, suggesting a role for Myc box III in the binding of c-Myc to DUBs.

We confirmed that USP17 stabilized c-Myc and upregulated its transcriptional regulatory function. The knockdown of USP17 reduced the protein level of Cyclin A, a c-Myc target gene. USP17 has been shown to stabilize Cyclin A through deubiquitination [61]. Therefore, the decrease observed in Cyclin A protein levels by the knockdown of USP17 may be due to a reduction in the stability of the Cyclin A protein itself, in addition to weaker transcriptional activation by reduced c-Myc expression. We also observed an increase in p21 protein levels following the knockdown of USP17. We previously reported that USP17 decreased the expression of p21 by stabilizing methyltransferase SET8 [36]. Therefore, the reduction induced in p21 expression by USP17 may be due to an increase in the stability of both the SET8 and c-Myc proteins. Furthermore, the knockdown of SET8 was found to suppress the expression of c-Myc [62], suggesting that USP17 regulates the expression of p21 through a complex mechanism *via* SET8 and c-Myc.

The present study demonstrated that USP17 contributed to the stability of the c-Myc protein in cancer cells through deubiquitination. Since c-Myc expression has been reported to correlate with a poor prognosis in human cancers [4], USP17 is expected to become an attractive molecular target for cancer therapy. Proteasome inhibitors, such as bortezomib, have been clinically applied as anticancer drugs targeting UPS. However, bortezomib has been associated with the development of drug resistance and side effects, such as dose-limiting peripheral neuropathy, in some patients [63]. Therapeutic strategies using DUB inhibitors allow for the targeting of specific proteins; therefore, they are less likely to trigger associated toxicities. A previous study reported that P5091, a USP7

inhibitor, induced apoptosis in bortezomib-resistant multiple myeloma cells [33]. WP1130, an inhibitor of multiple DUBs, including USP5, USP9X, USP14 and UCHL5, may induce apoptosis by regulating the expression of MCL-1 and p53 [63]. Therefore, DUB inhibitors have potential as cancer therapeutic targets. The present results suggest that the inhibition of USP17 suppressed the proliferation of cancer cells caused by an increase in the expression of c-Myc and also that the inhibition of USP17 has potential as an attractive strategy for the treatment of cancers.

Acknowledgements

Author contributions

YI designed and performed the experiments. MN, MY, CM, MT, HN and MS performed the experiments. DM and NO contributed reagents and tools. HH supervised the study. MN, YI and HH wrote the manuscript. All authors discussed the results and commented on the manuscript.

Data accessibility

The data that support the findings of this study are available from the corresponding author [hhayashi@phar.nagoya-cu.ac.jp] upon reasonable request.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Identification of USP17 as a novel deubiquitinase of c-Myc.

Fig. S2. The depletion of USP17 decreased c-Myc protein levels.

Fig. S3. The depletion of USP17 increased K48-linked polyubiquitination of c-Myc.

Fig. S4. USP17 regulates c-Myc-dependent gene expression and promotes cell proliferation and glycolysis.

Fig. S5. The effect of c-Myc overexpression on the regulation of c-Myc-dependent gene expression by USP17 knockdown.

Fig. S6. *USP17* expression is related to prognosis in breast cancers.

Table S1. Primer sequences used in RT-qPCR.