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Lys-63-specific Deubiquitination of SDS3 by USP17 Regulates HDAC Activity*

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SDS3 is a key component of the histone deacetylase (HDAC)-dependent Sin3A co-repressor complex, serving to maintain its HDAC activity. Here, we report both exogenous and endogenous functional interaction between deubiquitinating enzyme USP17 and human SDS3 by MALDI-TOF-MS, co-immunoprecipitation assay, and GST pull-down assay. In this study, we demonstrated that SDS3 readily undergoes endogenous polyubiquitination, which is associated specifically with Lys-63-branched polyubiquitin chains and not with Lys-48-branched polyubiquitin chains. Further, we also demonstrated that USP17 specifically deubiquitinates Lys-63-linked ubiquitin chains from SDS3 and regulates its biological functions. The deubiquitinating activity of USP17 on SDS3 negatively regulates SDS3-associated HDAC activity. The constitutive expression of USP17 and its substrate SDS3 was involved in the inhibition of anchorage-independent tumor growth and blocks cell proliferation, leading to apoptosis in cervical carcinoma cells. Furthermore, we showed that USP17 and SDS3 mutually interact with each other to regulate cancer cell viability. These data support the possibility that SDS3, being a substrate of USP17, may play an important role in developing a novel therapeutic means to inhibit specific HDAC activities in cancer.

Molecular processes such as phosphorylation, dephosphorylation, acetylation, deacetylation, ubiquitination, and deubiquitination of cellular proteins play an orchestrated role in coordinating homeostasis and contribute to the manifestation of physiological processes in health and disease. The process of ubiquitination is a well established event involving a complex of ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligase (E3) enzymes (1–4). Ubiquitination can be reversed by deubiquitinating enzymes (DUBs)[2] (3, 5). Most DUBs are cysteine proteases and consist of at least five known families: the ubiquitin C-terminal hydrolases, the ubiquitin-specific processing proteases (USP), Jab1/Pab1/MPN domain-containing metallo-enzymes, Otu-domain ubiquitin aldehyde-binding proteins, and Ataxin-3/Josephin (3, 4, 6).

USP17 was previously identified as a human ortholog of DUB-3 that is regulated by the IL-4 and IL-6 cytokines (7). Recent characterization of USP17 as a key regulator of cell proliferation has revealed its critical role in cell growth and survival (7–10). Previously, we showed that USP17 possesses two hyaluronan binding motifs (HABMs) in its C-terminal region and interacts with hyaluronan to regulate cell viability (11). However, the biological significance of these HABMs in USP17 remains to be fully understood.

The Sin3 co-repressor complex has been linked to HDAC enzymatic activity by directly interacting with HDAC1 and HDAC2 within the Sin3-HDAC complex (12, 13). SDS3 is a subunit of the HDAC-dependent Sin3A co-repressor complex and exhibits a critical role in maintenance of mSin3-associated HDAC activity (14, 15). It has a role in transcriptional repression, recruits HDAC activity, and enables HDAC1 catalytic activity *in vivo* (15). mSds3-deficient cells have shown an aberrant association among heterologous chromosomes, chromosomal missegregation, defective karyokinesis, cytokinesis failure, rampant aneuploidy, and cell death (16).

In this study, we demonstrated endogenous and exogenous interactions between USP17 and SDS3 by co-immunoprecipitation assay. Additionally, direct interaction was shown between USP17 and SDS3 by GST pull-down assay, and both of these proteins are seen to co-localize in the nucleus. We demonstrated endogenous polyubiquitination of SDS3 and further showed that SDS3 specifically undergoes Lys-63-linked polyubiquitination. Furthermore, USP17 was shown to be a deubiquitinating enzyme for Lys-63-linked polyubiquitination of SDS3. We also demonstrated that the functional association between USP17 and SDS3 caused an inhibition of cell proliferation and induced apoptosis. Finally, we propose that the deubiquitinating activity of USP17 attenuates SDS3-associated HDAC activity in HeLa cells.

EXPERIMENTAL PROCEDURES

Construction of Expression Vectors—The cDNA encoding the full-length USP17, USP17-N, and USP17 (C89S) in pcDNA3.1 expression vector and pCS4-HA-ubiquitin have been described previously (11). The pEFIRES-HA-ubiquitin, pEFIRES-HA-R48K-ubiquitin, and pEFIRES-HA-R63K-ubiquitin constructs were obtained from Prof. Yossi Yarden (Weizmann Institute, Israel). The USP17-C (aa 399–530) was subcloned into pcDNA3.1-Myc-tagged vector. The cDNAs encoding the full-length SDS3 (SDS3-N, aa 1–170, and SDS3-C, aa 171–328)

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[2] The abbreviations used are: DUB, deubiquitinating enzyme; USP, ubiquitin-specific protease(s); HABM, hyaluronan binding motif; aa, amino acid(s).

were constructed in pCS4-FLAG-tagged expression vector. The full-length USP17 and SDS3 were subcloned into pGEX4T-1 and pet15b, respectively, for GST pull-down assays. The full-length USP17 and SDS3 were constructed in a pTRE-2hyg-2myc vector to establish the inducible Tet-On system (Clontech, Palo Alto, CA, USA). Two shRNA expression vectors for human USP17 were constructed using the vector pSilencer 1.0-U6 (Ambion, Austin, TX). The mRNA target sequences chosen for designing USP17-shRNA are GTC ACC ACT CTC ATG TGA G for USP17-shRNA1 and GAC ACA GAC AGG CGA GCA A for USP17-shRNA2. The mRNA target sequences for SDS3-shRNA are GAC ACT GAG GAT GCT AGT G for SDS3-shRNA1 and GCT AGA TCA GCA GTA CAA AG for SDS3-shRNA2.

Cell Culture and Transfection—HeLa (human ovarian cancer cell line), 293T (human embryonic kidney cell line), and MCF-7 (human breast adenocarcinoma cell line) were grown in DMEM (GIBCO-BRL Rockville, MD) supplemented with 10% FBS and 1% penicillin and streptomycin. Transfection was carried out using polyethyleneimine (Polysciences, Warrington, PA).

Immunoprecipitation, Silver Staining, and Protein Identification by Mass Spectrometry—Myc-USP17-C-transfected 293T cells were immunoprecipitated using an anti-Myc (9E10) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and combined with 30 μl of protein A/G PLUS agarose (Santa Cruz Biotechnology) by rotating for 1 h at 4 °C. The eluents were loaded onto SDS-PAGE gel and silver-stained using a kit (Bioneer, Daejeon, Korea). The differentially expressed distinct band was excised and processed for in-gel trypsin digestion and subjected to MALDI-TOF-MS analysis as described previously (17, 18). The peptide and proteins were identified from the MS/MS spectra using the MASCOT algorithm (Matrix Science, Boston, MA). Peptide mass fingerprinting was carried out using the MASCOT search engine from GPS Explorer software (Applied Biosystems, Foster City, CA). Mass spectra used for manual *de novo* sequencing were annotated with the Data Explorer software (Applied Biosystems).

Co-immunoprecipitation for Binding, Ubiquitination, and Deubiquitination Assays—HeLa cells were lysed and immunoprecipitated with either an anti-USP17 or an anti-SDS3 and immunoblotted with respective antibodies for detecting endogenous interaction between USP17 and SDS3. To identify the region of interaction between USP17 and SDS3, 293T cells were transfected with expression vectors encoding Myc-USP17-N, Myc-USP17-C, Myc-USP17-Full, FLAG-SDS3-N, FLAG-SDS3-C, and FLAG-SDS3-Full. For ubiquitination and deubiquitination assays, expression vectors encoding Myc-USP17, Myc-USP17 (C89S), pSilencer-USP17-shRNA1, pSilencer-USP17-shRNA2, FLAG-SDS3, HA-ubiquitin, HA-R48K-ubiquitin, HA-R63K-ubiquitin, and Myc-USP7 were transfected. The cells were cultured for 48 h. The harvested cells were lysed in lysis buffer (50 mM Tris (pH 7.6), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) supplemented with protease inhibitor mixture (Roche Diagnostics). Cell lysates were incubated with the corresponding antibody (2 μg) at 4 °C overnight and then incubated with 30 μl of protein A/G PLUS agarose (Santa Cruz Biotechnology) at 4 °C for 1 h. The samples were washed with lysis buffer and resuspended in SDS sample buffer, and co-immunoprecipitated proteins were detected using Western blot analysis. The antibodies used for immunoblotting are polyclonal USP17 antibody (Biomeditech, Seoul, Korea), anti-SDS3 (Abcam, Cambridge, UK), anti-Myc (9E10, Santa Cruz Biotechnology), anti-FLAG (Sigma), and anti-HA antibody (Santa Cruz Biotechnology).

Pull-down Assay—Bacterial lysate expressing His-tagged SDS3 protein was purified using HisTrap Kit (Amersham Biosciences). Purified His-tagged SDS3 was incubated with GST-USP17 immobilized on 100 μl of glutathione-Sepharose beads (GE Healthcare). Beads were extensively washed with Buffer A (20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 150 mM NaCl, 1% Triton X-100) containing 1× protease inhibitor mixture. The bound proteins were eluted by boiling in the SDS sample buffer for 10 min and immunoblotted with an anti-SDS3 antibody.

Localization Study—HeLa cells were seeded at 5×10^4 cells per well into a 4-well cell culture dish (SPL Lifesciences, Gyeonggi-do, Korea). Cell staining procedure was followed as described previously (11). Cell images were captured using Nikon Eclipse 50i microscopy.

Soft Agar Colonogenic Assay—HeLa cells were transfected with pcDNA3-Myc vector (empty vector), Myc-USP17, FLAG-SDS3, SDS3-shRNA1, and SDS3-shRNA2. Stably transfected cells were counted, and a soft agar assay was performed as described previously (19).

FACS Analysis—HeLa-Tet-On cells expressing USP17 and SDS3 constructs were harvested and washed with 1× filtered PBS, and the pellet was resuspended in 100 μl of binding buffer (10 mM Hepes/NaOH (pH 7.4), 140 mM NaCl, 2.5 mM $CaCl_2$). 5 μl of annexin V-PE (BD Biosciences) and 10 μl propidium iodide (50 μg/ml, BD Biosciences) were mixed and added to the samples. FACS analysis was performed as described previously (11).

Cell Counting Kit-8 Assay—100 μl of transfected HeLa cell suspension having 50,000–100,000 cells/ml was added to a 96-well plate. The protocol was followed according to the instructions provided by the company (Dojindo Molecular Technologies, Rockville, MD). Absorbance (*A*) was measured at 450 nm to determine the cell viability in each well.

HDAC Assay—The measurement of HDAC activity was performed using a commercially available kit (BioVision, Mountain View, CA). 1000 μg of cell lysates was subjected to immunoprecipitation using an anti-SDS3 antibody or anti-FLAG antibody overnight. 20 μl of Protein A/G PLUS agarose beads was added and rotated for another 2 h at 4 °C. Beads were spun down at 2000 rpm for 2 min. Supernatant was discarded and beads were washed three times using wash buffer. Finally, beads were resuspended with 85 μl of double-distilled water. The reaction was initiated by the addition of HDAC substrate to the samples. The beads were gently agitated to resuspend well with the substrate and then incubated at 37 °C for 1 h. The reaction was then terminated and measured using Tecan Genios Pro (Tecan, Bubendorf, Switzerland) at a 405-nm wavelength.

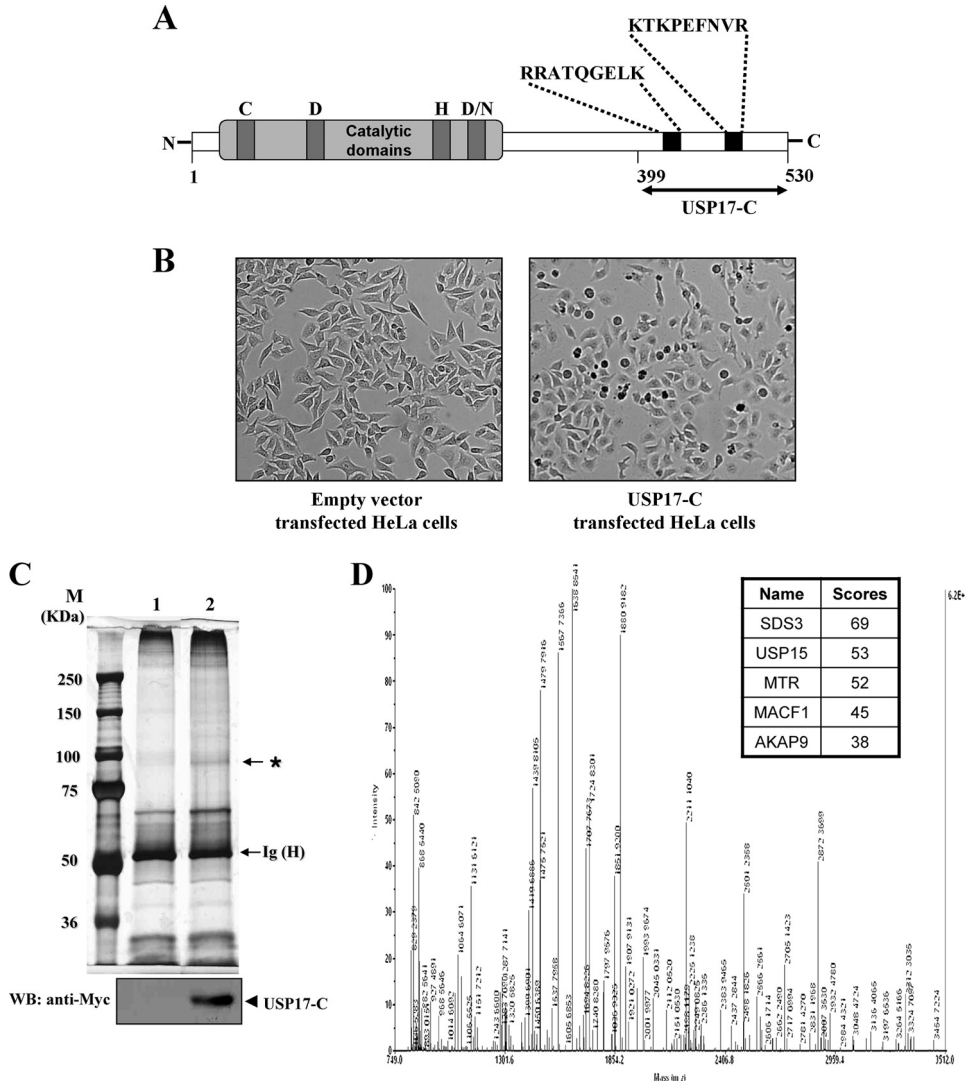


FIGURE 1. **Identification of the USP17-C-interacting proteins.** *A,* schematic representation of USP17 showing the functional catalytic domain region and the C-terminal region (aa 399 to 530) containing two HABMs. *B,* photographic images of HeLa cells transfected with pcDNA3-Myc and pcDNA3-Myc-USP17-C. *C,* samples were immunoprecipitated using an anti-Myc antibody and silver-stained. The *asterisk* indicates the protein band that was examined by mass spectrometric analysis. Also shown is the Western blot analysis for whole cell lysate (*WCL*) from HeLa cells transfected with pcDNA3-Myc (*lane 1*, *lower panel*) and pcDNA3-Myc-USP17-C (*lane 2*, *lower panel*). *D,* mass spectrometric analysis of the band obtained by immunoprecipitation showed different putative binding substrates for USP17-C.

RESULTS

The C Terminus of USP17 Regulates Cell Proliferation—We have shown previously that USP17 is involved in the regulation of cell viability and that it has two HABMs at its C terminus that are able to bind with polymeric HA. Thus, we hypothesized that this region may be involved in the activation of apoptosis in cancer cells (11). To analyze its apoptotic effect, we subcloned the C-terminal region of USP17 (aa 399–530) into a myc-tagged expression vector, termed it USP17-C (Fig. 1*A*), and transfected HeLa cells with this construct. Interestingly, HeLa cells transfected with USP17-C showed signs of apoptosis (Fig. 1*B*). These observations suggest that the C terminus of USP17 may interact with other substrates to regulate cell viability. Thus, we wished to identify the substrates for USP17-C that are plausibly involved in the regulation of cell proliferation.

Identification of Binding Proteins with USP17-C—We performed immunoprecipitation to identify novel interacting proteins for the C terminus of USP17. USP17-C-transfected HeLa cells were immunoprecipitated and loaded onto SDS-PAGE followed by silver staining (Fig. 1*C*). The differentially expressed distinct band was analyzed by MALDI-TOF-MS and several binding proteins were identified (Fig. 1*D*). Of these, a protein known as suppressor of defective silencing 3 homolog (*Saccharomyces cerevisiae*) (SDS3) had a high MS score. Pertinent to the aim of our current study, SDS3 can be identified as a recessive oncogene regulating cell viability and is involved in tumor suppressor pathways (16, 20). Thus, we decided to conduct further functional studies on USP17 interaction with SDS3 and its biological role in regulation of apoptosis.

USP17 Interacts with SDS3—The interaction between USP17-C and SDS3 was validated by co-immunoprecipitation assay. USP17-C co-immunoprecipitated with SDS3 in 293T cells either with an anti-Myc or an anti-FLAG antibody (Fig. 2*A*). This result suggests that the USP17-C-containing HABMs

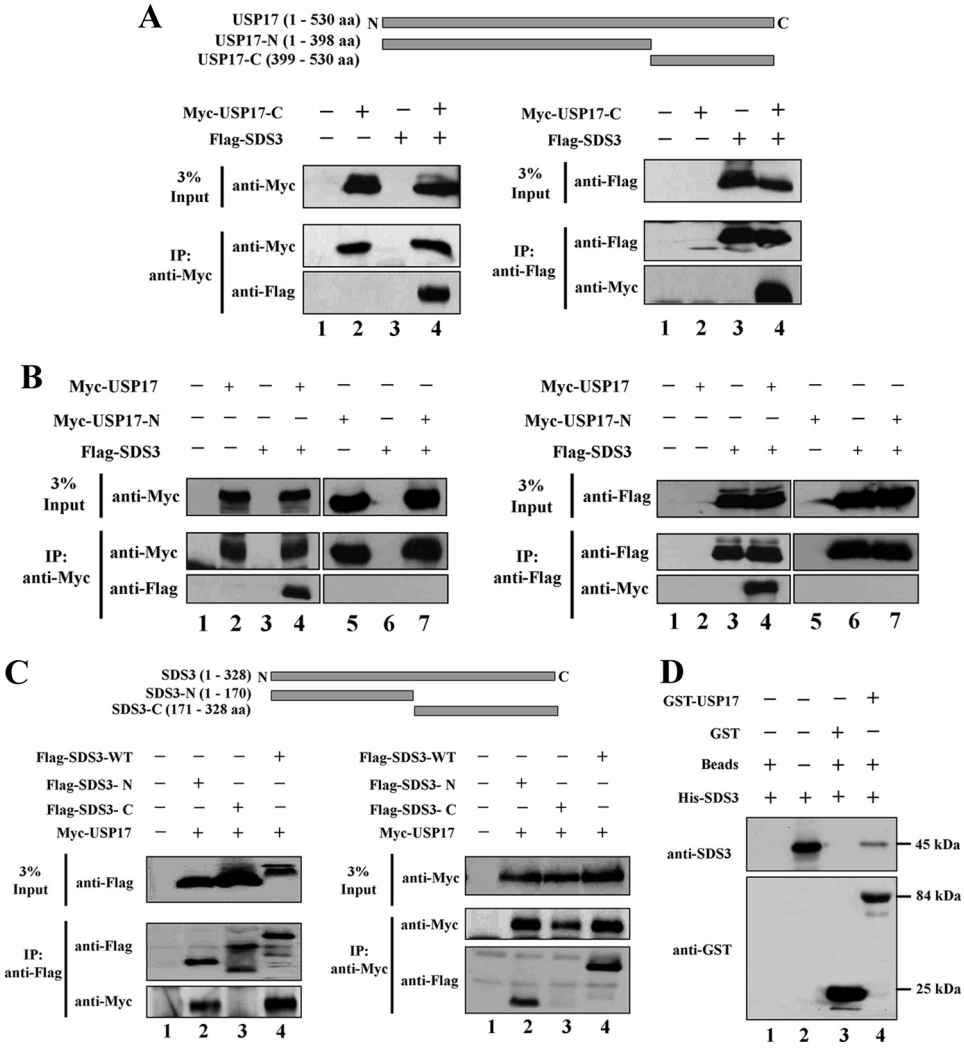


FIGURE 2. **USP17 interacts with SDS3.** *A,* interaction between USP17-C and SDS3. Interaction between USP17-C and SDS3 was confirmed by co-immunoprecipitation (*IP*) with either an anti-Myc antibody or an anti-FLAG antibody. *B,* SDS3 binds to the C-terminal region of USP17. Full-length USP17 and USP17-N were co-expressed with the full-length SDS3 in 293T cells. Immunoprecipitation was performed with either an anti-Myc or an anti-FLAG antibody. *C,* USP17 binds to the N-terminal region of SDS3. Full-length USP17 was co-expressed with the N- or C-terminal region of SDS3 in 293T cells. Immunoprecipitation was carried out either with an anti-Myc or an anti-FLAG antibody. *D,* purified SDS3 was incubated with GST or GST-USP17 coupled to GSH-Sepharose. The bound proteins were analyzed by immunoblotting with the indicated antibodies.

(aa 399–530) interact with SDS3. To determine the USP17 binding site with SDS3, we conducted co-immunoprecipitation between SDS3 and the full-length USP17 or USP17-N. SDS3 could co-immunoprecipitate with the full-length USP17 but not with USP17-N (Fig. 2*B*). The SDS3 protein has a coiled-coil domain (aa 66–171) at its N-terminal region which is responsible for formation of homodimerization of the SDS3 protein. Its C-terminal region has an mSin3 interaction domain (aa 188–226) that plays an essential role in associating with the full-length mSin3 protein and is responsible for HDAC activity. Mapping the region of SDS3 required for USP17 binding revealed that SDS3-N (aa 1–170) containing a coiled-coil domain is critical for the interaction between SDS3 and USP17 (Fig. 2*C*). These results indicate that the N-terminal region of SDS3 binds to the C-terminal region of USP17. Then we investigated the interaction between USP17-SDS3 by GST pull-down assay. For this purpose, we performed bacterial recombinant expression of GST-USP17 and immobilized it on a glutathione-Sepharose matrix. Binding between USP17 and SDS3 was established in a GST pull-down assay, wherein the GST-USP17 fusion protein was incubated with purified His-SDS3 protein. GST alone was used as a control sample in this experiment. His-SDS3 protein was retained by GST-USP17 but not by GST alone, indicating a direct interaction between USP17 and SDS3 (Fig. 2*D*).

Endogenous USP17 Associates with SDS3—Endogenous interaction between USP17 and SDS3 was demonstrated by co-immunoprecipitation assay using an anti-USP17 antibody that could co-precipitate endogenous SDS3. Conversely, an anti-SDS3 antibody co-precipitated endogenous USP17 (Fig. 3*A*). To investigate cellular localization of USP17 and SDS3, we performed immunofluorescent staining of endogenous USP17 and SDS3, and co-staining together in HeLa cells. USP17 was predominantly expressed in the nucleus and distributed throughout the cell (Fig. 3*B*, *left panel*). We confirmed SDS3 localization using an anti-SDS3 antibody and observed strong signals in the nucleus (Fig. 3*C*, *left panel*). USP17 and SDS3 showed endogenous co-localization in the nucleus (Fig. 3*D*, *Merge*).

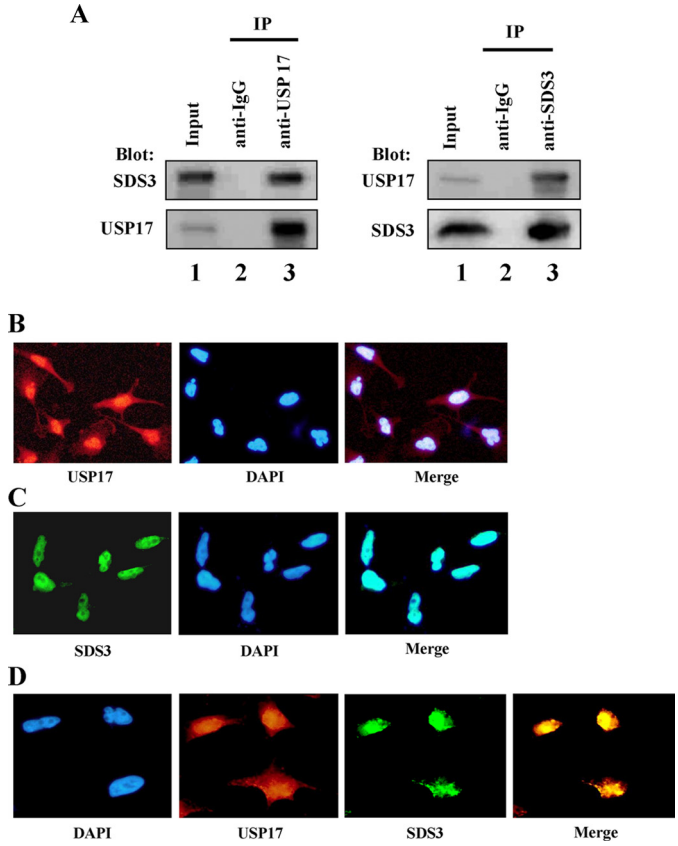
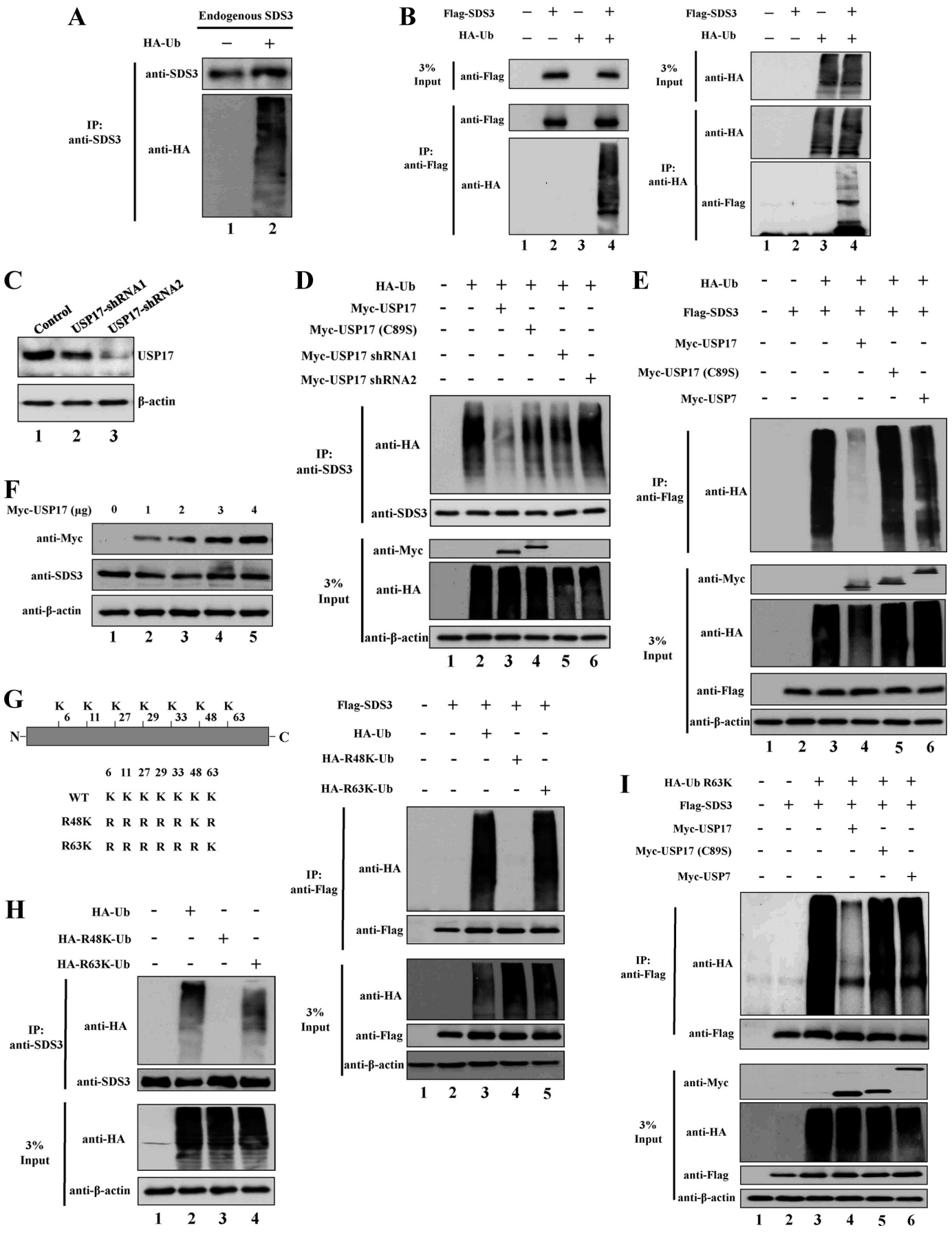


FIGURE 3. **Endogenous interaction between USP17 and SDS3.** *A,* USP17 interacts with SDS3 in HeLa cells. Proteins from HeLa cells were immunoprecipitated (*IP*) and immunoblotted (*Blot*) with either an anti-USP17 or an anti-SDS3 antibody. *B,* HeLa cells were stained using an anti-USP17 antibody (*left panel*) and DAPI staining for the nucleus (*center panel*). A merged image is also shown (*right panel*). *C,* HeLa cells were stained with an anti-SDS3 antibody (*left panel*) and DAPI staining for the nucleus (*center panel*). A merged image is also shown (*right panel*). *D,* HeLa cells were stained with both an anti-USP17 antibody (*center left panel*) and with an anti-SDS3 antibody (*center right panel*). DAPI staining for the nucleus (*left panel*) and a merged image (*right panel*) are also shown.

USP17 Deubiquitinates SDS3—Interaction between USP17 and SDS3 led us to investigate ubiquitination of SDS3. We performed an *in vivo* ubiquitination assay for SDS3 and found that endogenous SDS3 undergoes polyubiquitination *in vivo* (Fig. 4*A*). A typical high molecular weight smear of the polyubiquitin chains were conjugated with SDS3 (Fig. 4*A*, *lane 2*). We also demonstrated SDS3 polyubiquitination in 293T cells transfected with FLAG-SDS3 and HA-ubiquitin by co-immunoprecipitation assay. A smear was observed when it was immunoblotted for the presence of ubiquitin. Our result demonstrates that SDS3 undergoes polyubiquitination *in vivo* (Fig. 4*B*). To verify the hypothesis that USP17 deubiquitinates polyubiquitinated SDS3 and to check its specificity in this context, we designed two sets of shRNA molecules corresponding to nucleotides 118–138 (shRNA1) and to nucleotides 1192–1210 (shRNA2) of the USP17 cDNA. The shRNA were transfected into HeLa cells to check their effect on endogenous expression levels of USP17. Transfection of shRNA2 efficiently down-regulated the level of USP17, whereas transfection with shRNA1 showed a weak effect (Fig. 4*C*). Then we confirmed the deubiquitination activity of USP17 on both endogenous and overexpressed ubiquitinated SDS3 protein. Interestingly, we found that expression of USP17 significantly reduced the level of polyubiquitination both on endogenous SDS3 (Fig. 4*D*, *lane 3*) and overexpressed SDS3 (*E*, *lane 4*). In contrast, overexpression of USP17-shRNA1 and shRNA2 resulted in a significant increase in the SDS3 ubiquitination level in cells (Fig. 4*D*, *lanes 5* and *6*), indicating specific deubiquitinating activity of USP17 on SDS3. In addition, a catalytic mutant USP17 (C89S) and USP7, which were used as the negative controls, failed to reduce the ubiquitination level of SDS3 (Fig. 4*E*, *lanes 5* and *6*). Thus our results suggest that USP17 is a potential DUB enzyme for SDS3.

To further investigate whether deubiquitinating activity of USP17 stabilizes the endogenous protein level of SDS3, we overexpressed USP17 in a dose-dependent manner and checked endogenous SDS3 expression levels in HeLa cells. Interestingly, endogenous SDS3 protein levels were not increased by USP17 in a dose-dependent manner (Fig. 4*F*), and similar results were observed when the same experiment was performed in 293T cells (data not shown), suggesting that although SDS3 is polyubiquitinated, the ubiquitin-mediated protein degradation may not be the key process responsible for its turnover in the cell. Therefore, we further investigated the type of ubiquitin chains that form on SDS3 using mutant ubiquitin constructs in which all the lysine residues were replaced by arginine residues, except at the Lys-48 or Lys-63 positions (Fig. 4*G*), so that only Lys-48- or Lys-63-branched ubiquitin chains could be conjugated to SDS3 upon co-transfection. The ubiquitination smear on SDS3 was observed exclusively with a mutant that can only form Lys-63-linked ubiquitin chains, but not with a mutant that forms only Lys-48-linked ubiquitin chains (Fig. 4*G*). We also confirmed Lys-63-branched polyubiquitination on endogenous SDS3 in HeLa cells (Fig. 4*H*). Considering the possibility that Lys-63-branched polyubiquitination of SDS3 could be removed by USP17, we performed an immunoprecipitation assay to check USP17 deubiquitinating activity. USP17 showed its deubiquitinating activity on Lys-63-branched polyubiquitination of SDS3 (Fig. 4*I*, *lane 4*). This suggests that SDS3 undergoes K63-branched polyubiquitination and these ubiquitin molecules, which are conjugated to SDS3 protein, can be removed by USP17. Our data indicate that USP17 may not be involved in SDS3 protein stabilization by rescuing it from the protein degradation. Instead, it might play a key role in modulating biological functions of SDS3 in cancer cells.

USP17 and SDS3 Block Cell Proliferation in HeLa Cells—It is known that USP17, a key regulator of cell proliferation, has revealed its critical role in cell growth and survival (7–10). Identification of SDS3 interaction with USP17 led us to investigate whether the inhibitory role of USP17 on cell proliferation and survival is affected by the endogenous expression of SDS3. We therefore examined the effect of USP17-mediated cell proliferation on depleted expression of SDS3 by using two specific shRNAs for SDS3. The designed shRNAs for SDS3 effectively depleted endogenous SDS3 in HeLa cells (Fig. 5*A*). We demonstrated an inhibitory effect of USP17 on cell growth in the presence of SDS3 shRNAs by soft agar assay. HeLa cells transfected with the empty vector control displayed a large number of colonies, whereas those transfected with USP17 presented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1 2 3 4 5 6

reduced colony formation. In addition, USP17 and SDS3 co-transfected cells significantly reduced the size and number of colonies formed on soft agar as compared with USP17 alone. We further wished to investigate USP17-mediated cell viability in presence of shRNAs of SDS3. Interestingly, USP17 and shRNAs of SDS3-transfected cells showed a greater number of colonies than USP17 alone (Fig. 5*B*). The FACS analysis of the propidium iodide- and annexin V-stained cells showed an approximately 3-fold increase in the early stage of apoptosis with USP17-transfected HeLa cells, whereas cells co-transfected with USP17 and SDS3 exhibited an approximately 4-fold increase in apoptosis when compared with empty vector controls. In contrast, USP17-mediated apoptosis was significantly reduced in the presence of shRNAs of SDS3 (Fig. 5*C*). Next, we performed a cell proliferation assay using a cell counting kit-8 assay over 72 h in three separate experiments. Under conditions of SDS3 gene silencing by shRNA, the block in cell proliferation caused by USP17 was greatly reduced (Fig. 5*D*). To confirm this effect in a quantitative manner, we again transfected the same number of HeLa cells with mock, USP17, USP17/SDS3, and USP17 with SDS3-shRNA1 or SDS3-shRNA2 for 7 days, trypsinized, and counted cells using a hemocytometer in three individual experiments. Transfection of USP17 along with SDS3-shRNA1 or SDS3-shRNA2 caused an increase in the cell number compared with the USP17 alone (Fig. 5*E*). Taken together, our data indicate that functional interaction between USP17 and SDS3 is essential for the regulation of cell viability. Endogenous SDS3 increases USP17-mediated cell apoptosis in HeLa cells. Thus, USP17 and SDS3 mutually interact with each other to block cell proliferation.

USP17 Regulates SDS3-associated HDAC Enzymatic Activity—It is known that SDS3 is required for the maintenance of mSin3-associated HDAC enzymatic activity in NIH3T3 cells (15). After demonstrating the functional significance of the interaction between USP17 and SDS3 in the regulation of cell viability, we further wished to investigate the role of ubiquitination on SDS3-associated HDAC activity. For this purpose, we transfected empty vector, HA-ubiquitin, HA-R48K-ubiquitin, and HA-R63K-ubiquitin constructs to HeLa cells and performed an HDAC assay on anti-SDS3 immunoprecipitates in three independent experiments. Interestingly, the basal level of SDS3-associated HDAC activity is increased in ubiquitin- and R63K-ubiquitin-transfected cells compared with the mock control (Fig. 6*A*). In contrast, R48K-ubiquitin-transfected HeLa cells did not show any change in SDS3-associated HDAC activity. Thus, our result signifies that Lys-63-branched ubiquitination increases SDS3-associated HDAC activity in HeLa cells. Further, to determine whether the deubiquitinating activity of USP17 on SDS3 could regulate the maintenance of SDS3-associated HDAC activity, we employed an HDAC assay on anti-SDS3 immunoprecipitates in three independent experiments. HeLa cells transfected with empty vector, USP17-shRNA1, and USP17-shRNA2 were subjected to immunoprecipitation using anti-SDS3 antibody, and an HDAC assay was performed. The depletion in endogenous expression of USP17 increased the endogenous SDS3-associated HDAC activity compared with the empty vector-transfected sample (Fig. 6*B*). The result indicates that deubiquitinating activity of USP17 negatively regulates the SDS3-associated HDAC activity. HDAC assay was also detected in anti-FLAG immunoprecipitates of HeLa cells transfected with FLAG-SDS3 along with several constructs such as USP17, USP17 (C89S), USP17-shRNA1, USP17-shRNA2, and scrambled shRNA (SCR-shRNA), which lacks sequence homology to the genome was taken as a negative control. The expression of USP17 significantly reduced the total amount of released deacetylated lysine substrate by 50%, whereas USP17 (C89S) did not affect the total turnover of the substrate (Fig. 6*C*). The expression level of individual constructs was checked by Western blot analysis (Fig. 6*C*, *bottom panel*). In addition, the impact of the reduced USP17 level on SDS3-associated HDAC activity was assessed by transfecting HeLa cells with SDS3 in the presence or absence of USP17-shRNA1 and USP17-shRNA2. As expected, SDS3-associated HDAC activity was reproducibly augmented by the transfection of USP17-shRNA1 and USP17-shRNA2 (Fig. 6*C*). SCR-shRNA, which lacks sequence homology to the genome, could not alter SDS3-associated HDAC activity. The results were consistent over three independent experiments. Taken together, these results indicate that USP17 attenuates SDS3-associated HDAC activity in cancer cells, leading to cell apoptosis.

DISCUSSION

The present study identifies USP17 as an SDS3 deubiquitinating enzyme that regulates its HDAC activity to impact its biological function. Unlike other DUBs, USP17 does not increase the expression level of SDS3 by preventing protea-

FIGURE 4. **USP17 deubiquitinates Lys-63-branched polyubiquitination of SDS3.** *A,* endogenous polyubiquitination of SDS3. HeLa cells transfected with pEFIRES-HA-ubiquitin are shown. Whole cell lysate from HeLa cells (*lane 1*) and HeLa cells transfected with pEFIRES-HA-ubiquitin (*lane 2*) is shown. Ubiquitination of SDS3 was confirmed by co-immunoprecipitation (*IP*) with an anti-SDS3 antibody and immunoblotted with anti-HA antibody. *B,* ubiquitination of SDS3. 293T cells were transfected with FLAG-SDS3 and HA-ubiquitin individually or together. Ubiquitination of SDS3 was confirmed by co-immunoprecipitation with an anti-FLAG antibody or anti-HA antibody, and the counter blot was detected by an anti-HA antibody or anti-FLAG antibody. *C,* the knockdown efficiency of USP17-shRNA1 and shRNA2 was checked by Western blot analysis in HeLa cells. *D,* endogenous deubiquitination of SDS3 by USP17. HeLa cells transfected with pEFIRES-HA-ubiquitin, pcDNA3-myc-USP17, pcDNA3-myc-USP17 (C89S), pSilencerUSP17-shRNA1, and pSilencerUSP17-shRNA2 were immunoprecipitated using an anti-SDS3 antibody and blotted with an anti-HA antibody to check the specificity of the deubiquitinating activity of USP17 on endogenous polyubiquitinated SDS3. *E, in vivo* deubiquitination of SDS3 by USP17. 293T cells were transfected with SDS3, ubiquitin, USP17, USP17 (C89S), and/or USP7. A deubiquitination assay was performed by co-immunoprecipitation with an anti-FLAG antibody and Western blot analysis using an anti-HA antibody. *F,* 293T cells were co-transfected with an increasing amount of pcDNA3-Myc-USP17. Cell lysates were immunoblotted with indicated antibodies to analyze the endogenous expression level of SDS3. *G,* SDS3 undergoes Lys-63-branched polyubiquitination. 293T cells were transfected with SDS3, HA-ubiquitin, HA-R48K-ubiquitin, and HA-R63K-ubiquitin. Ubiquitination of SDS3 was confirmed by co-immunoprecipitation with an anti-FLAG antibody, and the counterblot was detected by an anti-HA antibody. *H,* endogenous SDS3 undergoes Lys-63-branched polyubiquitination. HeLa cells were transfected with HA-ubiquitin, HA-R48-Kubiquitin, and HA-R63K-ubiquitin. Ubiquitination of SDS3 was confirmed by co-immunoprecipitation with an anti-SDS3 antibody, and the counterblot was detected by an anti-HA antibody. *I*, USP17 removes Lys-63-branched ubiquitin chains from SDS3. 293T cells were transfected with FLAG-SDS3, HA-R63K-ubiquitin, USP17, USP17 (C89S), and/or USP7. A deubiquitination assay was performed by co-immunoprecipitation with an anti-FLAG antibody and Western blot analysis using either an anti-HA or an anti-FLAG antibody.

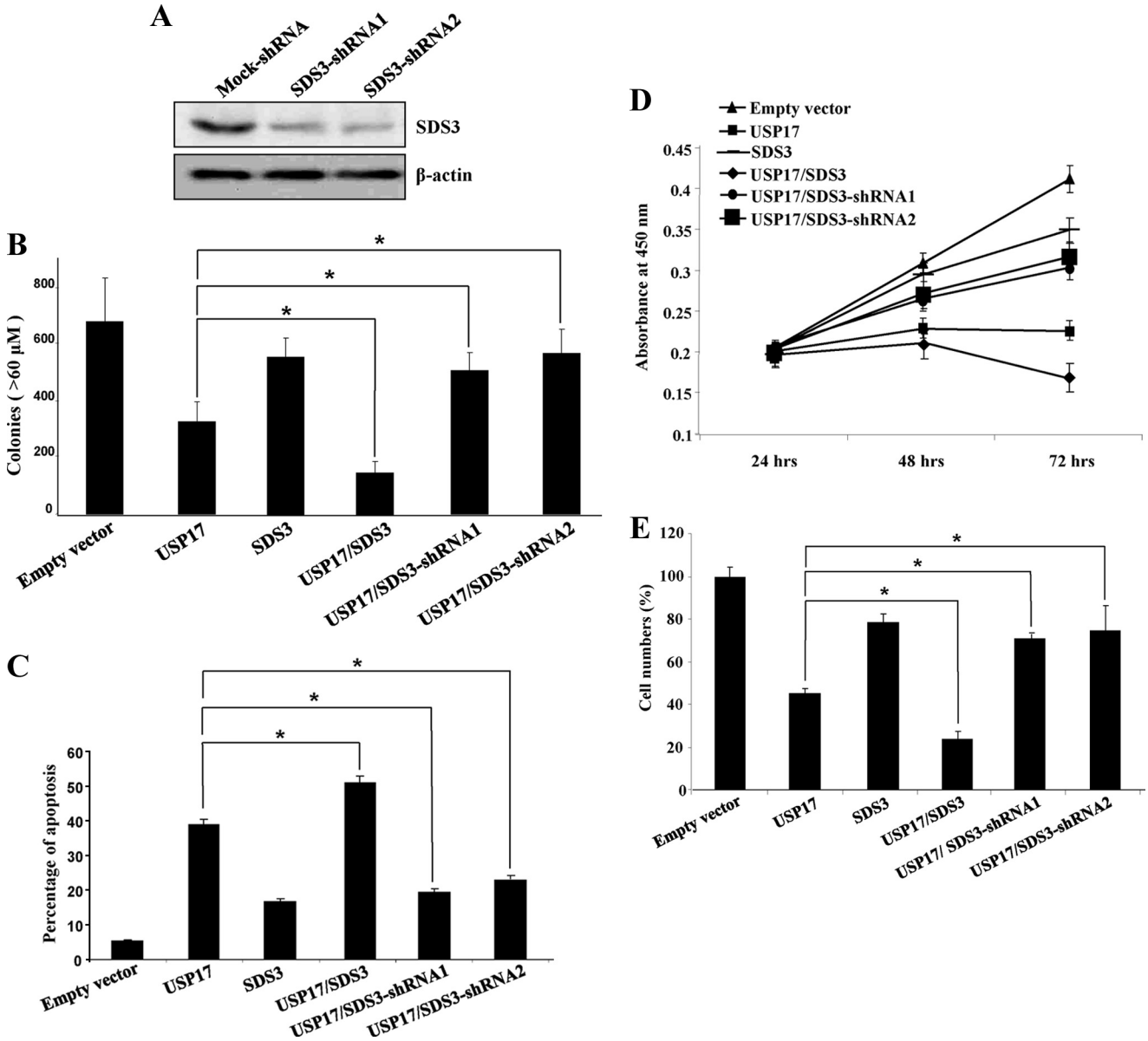

FIGURE 5. **USP17-mediated cell viability is regulated by SDS3.** *A,* the knockdown efficiency of SDS3-shRNA1 and shRNA2 was checked by Western blot analysis in HeLa cells. *B,* empty vector control HeLa cells transfected with USP17, SDS3, USP17/SDS3, USP17/SDS3-shRNA1, and USP17/SDS3-shRNA2 were plated in triplicate. After 14 days, the colonies were stained and counted. Colony numbers were determined by counting colonies from soft agar. The results represent the average number of colonies formed from three individual experiments. *Error bars* represent standard error of the mean. *, $p < 0.05$. *C,* doxycycline-induced HeLa Tet-On cells expressing respective constructs were harvested and stained with annexin V and propidium iodide. The statistical representation of the percentage of apoptosis recorded. The results represent the mean ($n = 3$) of a representative experiment. *Error bars* represent standard error of the mean. *, $p < 0.05$. *D,* HeLa cells were transfected with respective constructs and cell proliferation was assessed by cell counting kit-8 assay over 72 h. Mean ± S.D. of a triplicate experiment is shown. *E,* an equal number of HeLa cells were transfected with respective constructs for 7 days, trypsinized, and the viable cells were counted. The relative number of viable cells is shown as a percentage. Data are the mean of triplicate measurements. *Bars* represent mean ± S.D. ($n = 3$). *, $p < 0.05$.

somal degradation. Our present observation may be justified in the light of previous reports that have established USP17 deubiquitination of Lys-63-linked ubiquitin chains conjugated to Ras converting enzyme 1 (RCE1), which is not involved in the total protein turnover of RCE1 (9). The different types of ubiquitin modification are linked to distinct physiological functions in cells (21). The hypothesis that USP17 could act on the Lys-63-ubiquitin chain conjugated to SDS3 was confirmed by the observation that USP17 could remove Lys-63-linked polyubiquitin chains from SDS3.

The ubiquitin chains can assemble in several ways, depending on the lysine site used to form polyubiquitin chains including lysine Lys-6, Lys-11, Lys-29, Lys-48, and Lys-63. These chains can be formed from all lysine residues on the substrate with various lengths and shapes (22, 23). Among them, polyubiquitin chains formed through Lys-48 and Lys-63 are very well studied (24). Lys-48-branched polyubiquitination is responsible for regulating protein stability and signals for proteasomal degradation of the substrate (25). Lys-29- and Lys-33-branched mixed chains have been implicated in the regulation

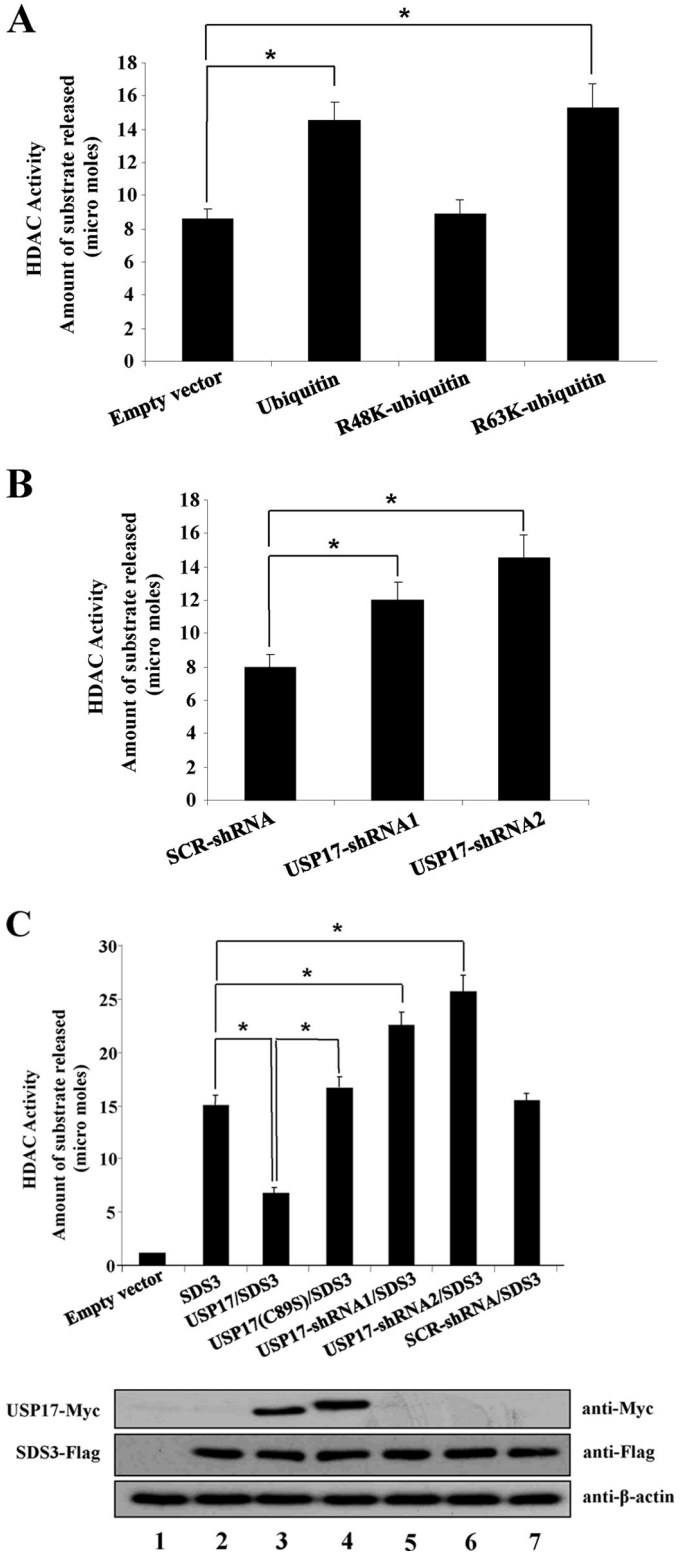

FIGURE 6. **USP17 attenuates SDS3-associated HDAC activity.** *A*, HeLa cells were transfected with empty vector, HA-R48K-ubiquitin, and HA-R63K-ubiquitin. HDAC activity on anti-SDS3 immunoprecipitates was determined by measuring released deacetylase substrate using an HDAC assay kit. Data are the mean of triplicate measurements. *Bars* represent mean ± S.D. ($n = 3$). *, $p < 0.05$. *B,* HeLa cells were transfected with either scrambled shRNA (*SCR-shRNA*) or pSilencer vectors expressing USP17-shRNA1 and USP17-shRNA2. HDAC activity on anti-SDS3 immunoprecipitates was determined by measuring released deacetylase substrate using an HDAC assay kit. Data are the mean of triplicate measurements. *Bars* represent mean ± S.D. ($n = 3$). *, $p < 0.05$. *C,* HeLa cells were transfected with either empty pCS4-FLAG vector (*lane 1*) or FLAG expression vector expressing SDS3 (*lane 2*), along with other constructs such as USP17/SDS3 (*lane 3*), USP17 (C89S)/SDS3 (*lane 4*), USP17-shRNA1/SDS3 (*lane 5*), USP17-shRNA2/SDS3 (*lane 6*), and SCR-shRNA/SDS3 (*lane 7*). HDAC activity on anti-FLAG immunoprecipitates was determined using an HDAC kit. Data are the mean of triplicate measurements. *Bars* represent mean ± S.D. ($n = 3$). *, $p < 0.05$. Western blot analysis to determine the efficiency of USP17, USP17 (C89S), and SDS3 transfection is also shown (*bottom panel*).

of AMP-activated protein kinase-related kinases (26). Recently, it has been reported that Lys-29-branched ubiquitin chains promote the proteasomal and lysosomal degradation of proteins (27–29), whereas Lys-63-branched polyubiquitination plays a key role in the regulation of endocytosis, DNA repair, protein kinase activation (30, 31), signal transduction (32), intracellular trafficking of membrane proteins (32), and stress responses (33). For example, CYLD regulates NF-κB activation by removing Lys-63-linked ubiquitin chains from TRAF2, TRAF6, and IκB kinase γ (34–37). USP17 deubiquitinates both Lys-48- and Lys-63-linked ubiquitin chains of RCE1, resulting in modulation of Ras processing and activation by negatively regulating RCE1 activity (9).

mSds3 is a key component of the mSin3-HDAC complex, sharing physical and functional properties of its yeast ortholog and sharing 99.6% identity with human SDS3. The impact of mSds3 haploinsufficiency on cancer development, along with p53-dependent checkpoint control, reveals that mSds3 accelerates tumor onset and increased tumor burden in the p53 null background. mSds3 promotes functional HDAC1 activity in the mSin3 complex, whereas RNAi-mediated knockdown decreases HDAC1 enzymatic activity (15). Deregulation of acetylation and deacetylation plays a causative role in the aberrant changes in gene expression in various cancers. Our findings suggest that the expression of USP17 could act on SDS3-associated HDAC activity. This was confirmed by the observation that HDAC activity was decreased in the presence of USP17 but not with its catalytic mutant. Knockdown of endogenous USP17 showed an increase in SDS3-associated HDAC activity.

Cytokine-induced DUB enzymes such as murine DUB-1, DUB-1A, DUB-2, and DUB-2A and human DUB-3 are involved in the growth regulation and survival of lymphocytes (6). USP17 reduces the rate of cell proliferation and regulates cell growth and survival (7–10). We reported that USP17 having two HABMs on its C terminus regulates cell viability and induces apoptosis in adenocarcinoma cells (11). Beyond its role in regulating cell viability, the significance of HABMs in USP17 is not yet clear.

Our studies demonstrated that expression of both USP17 and SDS3 results in a significant decrease in number and size of colonies formed by HeLa cells in soft agar. The importance of interaction between USP17 and SDS3 was further strengthened by the observation that USP17-mediated apoptosis is reduced when the endogenous SDS3 protein level is depleted. Recently, the relationship between USP17 and RCE1 was reported with the observations that USP17 could not block proliferation of cells lacking RCE1 (9). These observations strongly support that there could be several substrates that are involved in the regulation of USP17-mediated apoptosis.

In summary, we have shown that USP17 interacts directly with SDS3 and deubiquitinates Lys-63-linked polyubiquitin chains. The C-terminal region of USP17 binds to the N-terminal region of SDS3 and co-localizes in the nucleus. In particular, the constitutive expression of USP17 and SDS3 clearly demonstrates the tumor-suppressive nature, resulting in inhibition of cell growth, induction of apoptosis, and reduced tumor growth in cervical adenocarcinoma cells. Finally, Lys-63-branched polyubiquitination of SDS3 increases its HDAC activity, whereas deubiquitination of SDS3 by USP17 negatively regulates SDS3-associated HDAC activity in HeLa cells. Based on the fact that USP17 is a novel regulator of SDS3 activity and SDS3 is a key component of the mSin3 co-repressor complex maintaining HDAC enzymatic activity, USP17 could be a focus for therapeutic implications. In reality, inhibitors of HDAC activity have been implicated in cancer therapy (38–40). In conclusion, our findings may lead to the development of a novel strategy to trigger apoptosis and block tumor progression by regulating HDAC activity.

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