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The SUMO-Specific Protease SENP5 Is Required for Cell Division

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Posttranslational modification of substrates by the small ubiquitin-like modifier, SUMO, regulates diverse biological processes, including transcription, DNA repair, nucleocytoplasmic trafficking, and chromosome segregation. SUMOylation is reversible, and several mammalian homologs of the yeast SUMO-specific protease Ulp1, termed SENPs, have been identified. We demonstrate here that SENP5, a previously uncharacterized Ulp1 homolog, has SUMO C-terminal hydrolase and SUMO isopeptidase activities. In contrast to other SENPs, the C-terminal catalytic domain of SENP5 preferentially processed SUMO-3 compared to SUMO-1 precursors and preferentially removed SUMO-2 and SUMO-3 from SUMO-modified RanGAP1 in vitro. In cotransfection assays, SENP5 preferentially reduced high-molecular-weight conjugates of SUMO-2 compared to SUMO-1 in vivo. Full-length SENP5 localized to the nucleolus. Deletion of the noncatalytic N-terminal domain led to loss of nucleolar localization and increased de-SUMOylation activity in vivo. Knockdown of SENP5 by RNA interference resulted in increased levels of SUMO-1 and SUMO-2/3 conjugates, inhibition of cell proliferation, defects in nuclear morphology, and appearance of binucleate cells, revealing an essential role for SENP5 in mitosis and/or cytokinesis. These findings establish SENP5 as a SUMO-specific protease required for cell division and suggest that mechanisms involving both the catalytic and noncatalytic domains determine the distinct substrate specificities of the mammalian SUMO-specific proteases.

Posttranslational modification of proteins by the small ubiquitin-like modifier SUMO is an important mechanism to regulate numerous biological processes, including nucleocytoplasmic trafficking, transcription, and DNA repair and replication, as well as mitotic and meiotic chromosome behavior (13, 16, 20). SUMO is covalently attached to lysine residues in substrate proteins in a process similar to ubiquitination (for review, see reference 19). SUMO conjugation requires an E1-activating enzyme (Aos1/Uba2) and an E2-conjugating enzyme (Ubc9), and SUMOylation of specific substrates may be stimulated by the action of diverse E3 ligases (8, 9, 34). Covalent modification of proteins by SUMO is reversible, and a number of SUMO-specific proteases have been predicted based on homology to yeast Ulp1, the first identified SUMO-specific protease (23). The SUMO-specific proteases have dual roles in the SUMOylation pathway. First, they are responsible for the initial processing of SUMO precursors to generate a C-terminal diglycine motif required for conjugation. Second, these proteases execute the deconjugation reaction that removes SUMO from high-molecular-weight SUMO conjugates. At least seven Ulp1 homologs have been identified in mammals (termed SENP1 to -3 and SENP5 to -8), which share a conserved cysteine protease domain at their C termini (12, 14, 22, 30, 46). Four of these, SENP1, -2, -3, and -6, have been reported to have SUMO-specific C-terminal hydrolase and/or isopeptidase activity. In contrast, the Ulp homolog SENP8 has deconjugation activity specific for the ubiquitin-like modifier Nedd8 (12, 18, 27, 43). Activity of the mammalian Ulp1 homologs SENP5 and SENP7 has not yet been reported.

In vertebrates, there are four SUMO family proteins, SUMO-1, -2, -3, and -4. SUMO-2 and SUMO-3 share only about 46% identity to SUMO-1 but are highly related to each other, sharing 96% sequence identity. SUMO-4 is more similar to SUMO-2/3; however, it is not clear that SUMO-4 forms conjugates in vivo (6, 31). The extent of functional overlap or divergence among SUMO family proteins remains an unresolved question. The majority of SUMO-1 present in cells is found conjugated to substrates. In contrast, there is a more significant pool of unconjugated SUMO-2/3 that is rapidly converted to conjugated high-molecular-mass species in response to a variety of cellular stresses (33). SUMO-2 and -3 have a consensus SUMO acceptor site and, somewhat analogous to ubiquitin, may form chains (40). Current data indicate that different SUMO family proteins are preferentially conjugated to different substrates. For example, RanGAP1 is preferentially modified by SUMO-1 (33) and topoisomerase II is preferentially modified by SUMO-2/3 (2, 3), whereas the promyelocytic leukemia protein (PML), has been reported to be conjugated with both SUMO-1 (21, 28, 37) and SUMO-2/3 (11). At present, neither the mechanisms that support differential conjugation of SUMO paralogs nor its functional significance is fully understood.

Analysis of strains bearing mutations in the yeast SUMO-specific proteases Ulp1 and Ulp2 has revealed that these enzymes have distinct biological functions. Ulp1 is essential and ulp1 mutants arrest cell cycle progression at G_2/M (23). In contrast, Ulp2 is not essential for cell cycle progression, but ulp2 mutants have pleiotropic phenotypes, including temperature-sensitive growth, decreased chromosome stability, and increased sensitivity to DNA-damaging agents (25). Notably, the

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cell growth defects in *Schizosaccaromyces pombe* ulp1 mutants were not rescued by expression of processed mature SUMO, indicating that de-SUMOylation of some substrates is required for normal cell cycle progression (41). The two SUMO-specific proteases in yeast have distinct substrate specificities in vivo, as evidenced by dissimilar patterns of SUMO conjugates that accumulate in ulp1 and ulp2 mutant strains. Ulp1 and Ulp2 have different subcellular localizations, which have been suggested to contribute to the differential substrate selection and biological activities of these enzymes (24).

The physiological roles of the SUMO-specific proteases in mammals are not as well described. Recent studies have shown that mouse SENP1 (also called SuPr-2) is required for embryonic development (45), supporting some nonredundant roles for these enzymes. It is striking that the subcellular distribution of SUMO-specific proteases in mammals is quite diverse: SENP1 is localized in the nucleus and in nuclear dots, SENP2 is localized at the nuclear pores, and SENP3 is detected predominantly in the nucleolus (4, 14, 15, 30, 46). The noncatalytic N terminus of SENP1, SENP2, and SENP3 has been shown to be required for proper localization. Moreover, mutants of SENP1 and SENP2 lacking the N terminus were more active at de-SUMOylation of bulk SUMO-1 conjugates than the wild-type enzymes in vivo (4, 15, 23). The observation that the N terminus of the SENPs regulates subcellular localization and limits enzymatic activity has led to the view that subcellular localization of SUMO-specific proteases is a major factor in determining substrate selection in vivo.

In this paper, we investigate the biochemical and biological activities of the human Ulp homolog SENP5. We show that SENP5 is a SUMO-specific protease, which displays both C-terminal hydrolase and isopeptidase activities. We show that SENP5 has activity toward SUMO-1 and SUMO-2/3, but SENP5 discriminates between these SUMO family proteins. The catalytic domain of SENP5 shows higher efficiency for processing SUMO-3 over SUMO-1 precursors in vitro. Furthermore, in vitro SENP5 more efficiently removes SUMO-2 and SUMO-3 than SUMO-1 from SUMO-modified RanGAP1. Thus, the biochemical activities of SENP5 are distinct from those described for SENP1 and SENP2 (32, 44). In vivo, SENP5 was found to have isopeptidase activity toward both SUMO-1 and SUMO-2 conjugates; however, SENP5 had enhanced activity on SUMO-2 conjugates. Green fluorescent protein (GFP)-conjugated SENP5 was found to localize predominantly to the nucleolus. Deletion of the noncatalytic N-terminal domain led to loss of nucleolar localization and increased de-SUMOylation activity. Inhibition of endogenous SENP5 by RNA interference (RNAi) demonstrates the requirement of this SUMO-specific protease for cell division. Knockdown of SENP5 led to an increase in cells with more than one nucleus or aberrant nuclear structure consistent with defects in mitosis and cytokinesis. Our results demonstrate that the SUMO-specific protease SENP5 has nonredundant functions in vivo, likely due to distinct substrate specificities in SUMO maturation and deconjugation that are regulated by both the catalytic and noncatalytic domains of the enzyme.

MATERIALS AND METHODS

Plasmids and constructs. The SENP5 clone (IMAGp998L0910735Q) was purchased from the RZPD Deutsches Ressourcenzentrum fur Genomforschung GmbH. SENP5 coding sequence was amplified via PCR using the following primers: forward, 5′-A CGC GTC GAC AAA AAA CAG AGG AAA ATT CTA TGG AGG-3′; and reverse, 5′-A TAA GAA TGC GGC CGC TCT AGA TCA GTC CAT GAG CCG GCA CTC ACA TA-3′. The PCR fragment was cloned into pEGFP-C1 (Clontech) to generate GFP-SENP5 and into p3×Flag-CMV-7.1 (Sigma) to generate 3×Flag-SENP5. SENP5-CD (amino acids 567 to 752) was amplified using the forward primer 5′-GCG GGA TCC CAC ATG CTG GAT ATG GAC GAC-3′ and reverse primer 5′-CCC AAG CTT TCA GTC CAT GAG CCG GCA CTC-3′) and subcloned into pGEX-4T-1, pET28a, p3×Flag-CMV-7.1, and pEGFP-C3 to generate glutathione *S*-transferase (GST)-SENP5-CD, His_6-SENP5-CD, 3×Flag-SENP5-CD, and GFP-SENP5-CD, respectively. Mutation of the active site cysteine (C713S) was generated by PCR and verified by sequencing.

Labeling with SUMO, ubiquitin, and other UbL-VS-modified probes. The synthesis of ubiquitin and ubiquitin-like vinyl sulfone (UbL-VS) derivatives has been described; each of the vinyl sulfone derivatives used here was confirmed to be active (7, 18). In vitro transcription and translation (IVT) of His_6-SENP5-CD were performed using the TNT-T7 Quick reticulocyte lysate system (Promega), and labeling was performed with [^{35}S]methionine. Aliquots of the IVT reaction mixture were treated with RNase B (1 mg/ml; Sigma) and then incubated with the various probes (0.2 to 0.4 μg/10 μl IVT lysate) at room temperature for 30 min. Binding was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography.

Protein expression and purification. His_6-SUMO-1-A_{10} and His_6-SUMO-3-A_{10} (46) were induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C for 4 h and purified using Ni-nitrilotriacetic acid (NTA) affinity purification (QIAGEN). GST-SENP5-CD was induced by 0.02 mM IPTG at 18°C for 15 h and purified using glutathione Sepharose 4B affinity purification (Pharmacia).

C-terminal hydrolase assay. The reaction was set up in a total volume of 20 μl of mixture containing the following: 20 mM Tris-HCl, pH 8.0; 100 mM NaCl; 1 mM dithiothreitol (DTT); 3 μg His_6-SUMO-1-A_{10} or His_6-SUMO-3-A_{10}; and from 0.2 μg to 1.6 μg of GST, GST-SENP5-CD, or GST-SENP5-CD-C713S. After incubation at 30°C for 2 h, 20 μl 2× SDS sample buffer was added to stop the reaction. Six microliters of each sample was analyzed by 18% SDS-PAGE and Coomassie staining.

In vitro RanGAP1-SUMO-1 cleavage assay. Mature mouse SUMO-1(1–97), cloned into pET28a (Novagen) with an N-terminal hemagglutinin (HA) tag and a TEV cleavage site, was expressed in *Escherichia coli* and purified using an Ni-NTA column (QIAGEN) and thrombin cleavage (EMD Biosciences). Mouse RanGAP1 (wild type and K526R mutant) cDNAs were kindly provided by Paul Anderson (Brigham and Women's Hospital, Harvard Medical School) and subcloned into pET28a. IVT of RanGAP1 was performed as described for His_6-SENP5-CD. For SUMO conjugation, 50 μl of the IVT mixture was diluted to 150 μl in a solution (50 mM Tris, 150 mM NaCl, pH 7.4) containing 0.5 μg of human SUMO E1 and E2 enzymes, 2 mM ATP, 1 mM DTT, and 25 μg HA-SUMO-1 and incubated at 30°C for 1 h. The reaction was stopped by addition of 1 mM *N*-ethylmaleimide (NEM), and after 15 min, excess NEM was blocked with 3 mM L-cysteine. To assay protease activity, the mixture containing radioactive SUMO-1-modified RanGAP was incubated with GST-SENP5-CD at 30°C for 2 h. The reaction was stopped by boiling in sample buffer, separated by SDS-PAGE, and analyzed by autoradiography.

Isopeptidase assay with RanGAP1-SUMO-1/2/3. The C-terminal fragment of RanGAP1 modified by SUMO-1, -2, and -3 was prepared as described previously (32). Substrate (3 μM) was incubated in a reaction mixture containing 25 mM Tris, pH 8, 150 mM NaCl, 0.1% Tween 20, and 2 mM DTT. At different time points after 160 nM GST-SENP5-CD was added, aliquots were removed and the reaction stopped by adding 2× SDS sample buffer. Protein samples were resolved by 15% PAGE, fixed, and stained with SYPRO Ruby (Invitrogen). Gels were scanned with Typhoon Trio (GE Healthcare) at an excitation wavelength 488 nm. The fraction of SUMO-1/2/3-RanGAP1 remaining was quantified with Image Quant TL (GE Healthcare).

In vitro protein binding/pulldown assay. One microgram of GST or GST-SENP5-CD-C713S and 1 μg of His_6-SUMO-1 or His_6-SUMO-2 ("GG"-mature form) were incubated in 0.5 ml pulldown buffer consisting of 20 mM Tris, pH 7.6, 100 mM NaCl, 0.1 mM EDTA, 0.01% NP40, 5% glycerol, and 1 mM DTT. The samples were clarified by centrifugation, and 10 μl 40% prewashed glutathione Sepharose-4B beads was added. Proteins remaining on the beads after washing were resolved on 15% PAGE and detected with anti-His_6 antibody (Santa Cruz Biotech).

In vivo isopeptidase activity. HEK293T cells were transfected with plasmids expressing HA-SUMO-1 or HA-SUMO-2 together with either wild-type 3×Flag-SENP5 (3×Flag-SENP5 wt) or C/S, 3×Flag-SENP5-CD wt or C/S, or 3×Flag vector using Lipofectamine 2000 (Invitrogen). For each well in a six-well plate,

0.2 μg HA-SUMO-1 or HA-SUMO-2 and 2 μg SENP5 construct were used. Forty-eight hours after transfection, total protein extracts were collected by addition of 1× SDS protein sample buffer with 50 mM NEM directly to the cells. Twenty micrograms of total protein was resolved by SDS-PAGE and detected with anti-HA antibody (Covance), stripped, and reprobed with anti-Flag antibody (M2; Sigma). Anti-mouse–horseradish peroxidase (HRP) conjugate antibodies (Promega) were used as the secondary antibodies. In order to detect free SUMO and high-molecular-weight conjugates, the protein separating gel was 15% polyacrylamide at the bottom and 7.5% polyacrylamide at the top.

Immunofluorescence. For localization of GFP-SENP5, HeLa cells were plated at 80,000 cells/coverslip and transfected with 1 μg of GFP-SENP5 or GFP-SENP5-CD. After 24 h, cells were washed with phosphate-buffered saline and fixed with 4% paraformaldehyde for 10 min. For colocalization studies, cells were fixed with methanol-acetone (1:1) for 20 min at −20°C and blocked with a serum mixture containing donkey serum (1:10), 0.1% Tween 20, 0.1% Triton X-100, and 3% bovine serum albumin. Polyclonal anti-C23 antibody (1:200) (Santa Cruz Biotechnology, Inc.) was applied, and the mixture was incubated at room temperature for 1 h or at 4°C overnight. Coverslips were washed and then incubated with Cy3-conjugated AffiniPure donkey anti-rabbit immunoglobulin G (1:2,000) (Jackson ImmunoResearch Laboratories, Inc.). For analysis of RNAi-infected cells, cells were fixed in methanol at −20°C for 15 min and then blocked with goat serum (1:10), 0.1% Triton X-100, 0.1% Tween 20, and 3% bovine serum albumin. Anti-α-tubulin (1:3,000) (Sigma) was incubated for 1 h at room temperature, and cells were washed and then incubated with Cy2-conjugated goat anti-mouse antibody (1:500) (Jackson ImmunoResearch Laboratories, Inc.). In all cases, coverslips were washed, stained with DAPI (4′,6′-diamidino-2-phenylindole) (1:20), and mounted with Vectashield mounting medium (Vector Laboratories, Inc.).

RNAi experiments. Short hairpin RNA (shRNA)-expressing vectors were generated as described previously (38). The SENP5 sequences targeted for siRNA were SENP5 RNAi 133 (5′-GAA AGC TAA GCT GGG AAG GCA-3′) and SENP5 RNAi 1111 (5′-GG GAG TGT ACA GAG CTG ATT A-3′). RNAi-mediated knockdown was validated by cotransfection of the SENP5 RNAis with 3×Flag-SENP5 (ratio, 10:1). Nuclear extracts were prepared 72 h posttransfection and analyzed using anti-Flag M2 (Sigma) (1:1,000) and anti-GFP (BD Bioscience) (1:400). The two siRNAs for SENP5 and a control siRNA (scrambled version of Sp3 siRNA) were subcloned into retroviral vector pMSCV-puro, packaged into retroviruses using BOSC cells, and used to infect HeLa cells. Stable SENP5 siRNA- or control siRNA-expressing cells were selected with 3 μg/ml puromycin and analyzed 72 h postinfection or postcotransfection with pBabe. For immunoblotting, SUMO-1-conjugated proteins were detected using anti-SUMO-1 antibody (1:500) (Zymed) or anti-SUMO-2/3 antibody (1:2,000) (ABCAM). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:5,000) (CHEMICON) was used as a control for equal loading. Anti-mouse and anti-rabbit HRP-conjugated antibodies (Promega) were used as secondary antibodies.

RT-PCR. For reverse transcription-PCR (RT-PCR), RNA was prepared using the RNeasy Mini kit (QIAGEN) and 2 μg of RNA was retrotranscribed using Superscript II RNase H reverse transcriptase (Invitrogen). Specific primers for SENP5, SENP3, and GAPDH were used for PCR, and different amounts of cDNA template were tested in order to reach the linear range. Quantitation was performed using Quantity One software.

Statistical analysis. HeLa cells infected with either Senp5 RNAis (no. 1111 and 133) or control RNAi were scored for number of cells with more than one nucleus or a nucleus with aberrant morphology. For each sample, an average of 1,500 cells from five independent experiments were used. An unpaired *t* test was applied for statistical analysis of the samples.

RESULTS

SENP5 binds specifically to SUMO. SENP5 belongs to the Ulp family of SUMO-specific proteases based on homology within the catalytic domain, which contains the conserved amino acids that make up the catalytic triad (histidine, aspartic acid, and cysteine) (Fig. 1A). Up to now, only four of the seven human Ulp homologs (SENP1, SENP2, SENP3, and SENP6) have been shown to have SUMO-specific protease activity, while the Ulp homolog SENP8 was found to be a Nedd8-specific protease (12, 18, 27, 43). Activity of the Ulp1 homologs SENP5 and SENP7 has not been described. Phylogenetic analysis revealed that SENP5 shares the highest homology with

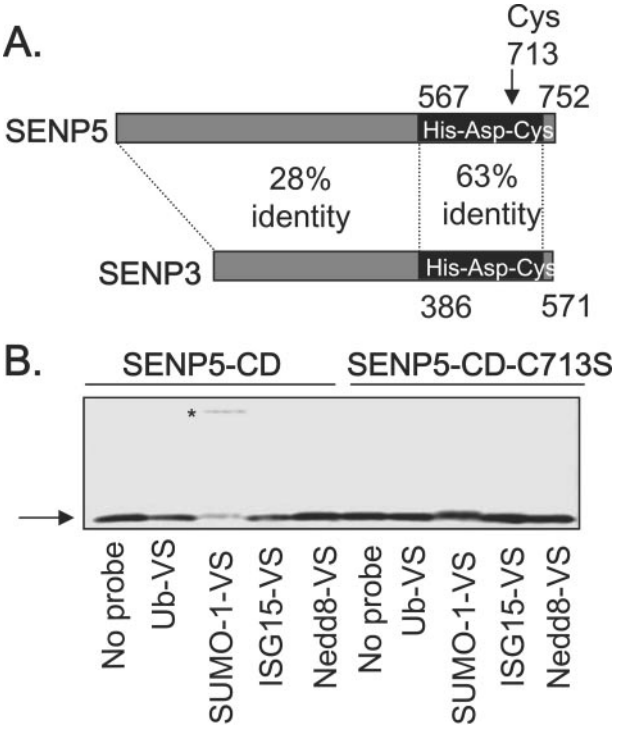

FIG. 1. SENP5 binds specifically to SUMO. (A) Schematic representation of SENP5 protein (positions 1 to 755) and its homology to SENP3 (positions 1 to 574), the most closely related member of the SUMO-specific protease family. Critical residues of the catalytic triad, including SENP5 cysteine 713, are indicated. The position of the SENP5 C-terminal domain (CD) fragment used in these studies (amino acids 567 to 752) is indicated. (B) The His-SENP5-CD wt and catalytic mutant (C713S) were in vitro translated and incubated with SUMO-1, ubiquitin, Nedd8, and ISG15 VS-modified probes as described in Materials and Methods. Only following incubation with HA-SUMO-1-VS was a higher-molecular-weight conjugate of His-SENP5-CD detected. The reactivity of the Ub-, Nedd8-, and ISG-15-vinyl sulfone conjugates was confirmed in separate studies (data not shown). No high-molecular-weight conjugate was observed when the SENP5 C713S mutant was used in the assay. An asterisk indicates the high-molecular-weight His-SENP5-CD/SUMO-1 conjugate, while an arrow indicates unconjugated His-SENP5-CD wt and C713S.

SENP3 (63% identity in the catalytic domain and 28% identity in N-terminal domain) (Fig. 1A). SENP3 has been reported to have SUMO-specific isopeptidase, but not C-terminal hydrolase, activity in vitro (30), prompting us to investigate the enzymatic activities of SENP5.

Modified UbL proteins that function as active site-directed probes have been successfully used to discover and characterize Ub/UbL-specific proteases (7, 18). These probes are formed by ubiquitin and UbL proteins modified with a C-terminal electrophilic trap, VS, which reacts with the active site cysteine of the proteases generating a covalent bond. We expressed the SENP5 C-terminal domain (CD; residues 567 to 752) wild type and C713S mutant (mutated in the predicted active site cysteine) and tested binding to several UbL-VS probes. As shown in Fig. 1B, SENP5-CD reacted selectively with SUMO-1-VS probe but not with the Ub, Nedd8, or ISG15 probes, as seen by the appearance of a high-molecular-weight conjugate. The Ub-, Nedd8-, and ISG15-vinyl sulfone conju-

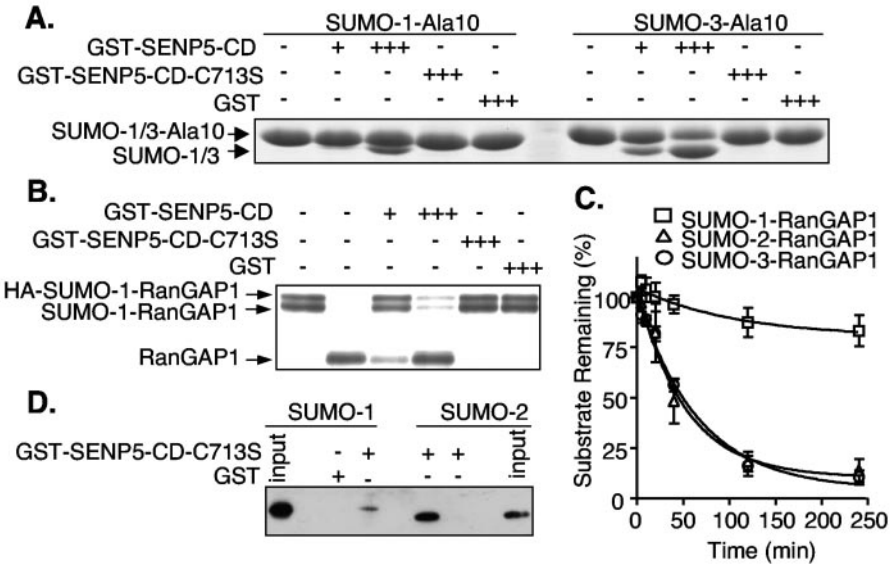

FIG. 2. SENP5 has in vitro SUMO C-terminal hydrolase and isopeptidase activities that discriminate between SUMO family proteins. (A) SUMO-1-10Ala and SUMO-3-10Ala were used as substrates to test the C-terminal hydrolase activity of SENP5. Increasing amounts of GST-SENP5-CD, (+, 0.2 μg; and +++, 1.6 μg) were incubated with SUMO-1 and SUMO-3 precursors. No cleavage was observed with 1.6 μg of GST-SENP5-CD C713S or GST alone. The position of SUMO-1/3 with or without the C-terminal extension is indicated. (B) In vitro-SUMOylated RanGAP1 was used as a substrate to test the isopeptidase activity of SENP5. In lanes 1 and 3 to 6, GST-SENP5-CD at 0.04 μg (+) or 0.35 μg (+++) or GST alone was added to SUMO-1-modified RanGAP1 as indicated. Lane 2 has RanGAP1 K526R, which cannot be SUMOylated. Positions of SUMO-1-modified and -unmodified RanGAP1 are indicated. (C) Time course of SENP5 isopeptidase activity toward different SUMO family proteins. The C-terminal domain of RanGAP1 conjugated to SUMO-1, SUMO-2 and SUMO-3 in vitro (32) was incubated with 160 nM of GST-SENP5-CD, and the fraction of substrate remaining at the indicated time points is indicated. (D) GST-SENP5-CD C713S was incubated with purified mature His-SUMO-1 or His-SUMO-2, and proteins bound to the GST affinity resin were detected with anti-His antisera. Five percent of the input was loaded.

gates were confirmed to be reactive with other proteases (A. Catic and H. Ploegh, data not shown). Covalent binding to the SUMO-1-VS probe was abolished when the predicted active site cysteine of SENP5 was mutated to serine (C713S). These data indicate that SENP5 selectively binds to SUMO and not other UbL proteins.

SENP5 has SUMO-specific protease activity and discriminates between SUMO paralogs in vitro. We investigated whether SENP5 had C-terminal hydrolase activity in vitro using purified recombinant SENP5 C-terminal domain (GST-SENP5-CD). To make it easier to distinguish the SUMO precursors from their mature forms, SUMO-1 and SUMO-3 precursor derivatives bearing an additional 10 alanines at the C terminus were employed as substrates (46). As shown in Fig. 2A, SENP5 hydrolyzed both SUMO-1 and SUMO-3 precursors. When low levels of GST-SENP5-CD wt (0.2 μg) were added to the substrate, a discrete amount of SUMO-3 was hydrolyzed, while hydrolysis of SUMO-1 C terminus was barely detectable. When an increased amount of GST-SENP5 CD wt was added (1.6 μg), hydrolysis of SUMO-1 was evident, although more than half of the SUMO-3 was hydrolyzed under these conditions. The same amount (1.6 μg) of GST-SENP5-CD C713S or GST alone did not hydrolyze SUMO-1 or SUMO-3 (Fig. 2A). These results reveal that SENP5 is able to process both SUMO-1 and SUMO-3 precursors in vitro, but with different efficiencies. The activity of SENP5 catalytic domain is notably different from that reported for SENP1 and SENP2 catalytic domains, which both process nascent SUMO-1 more efficiently than SUMO-3 (32, 44). The different maturation efficiencies catalyzed by SENP5 may regulate the availability of different SUMO family proteins for conjugation.

In order to investigate the SUMO-specific isopeptidase activity of SENP5, we first tested the ability of purified GST-SENP5 CD to cleave SUMO-1 from in vitro-SUMOylated RanGAP1. Different amounts of GST-SENP5-CD wt or C713S mutant were incubated with SUMO-1-modified RanGAP1 in vitro, and products of the reaction were analyzed. As shown in Fig. 2B, addition of increasing amounts of GST-SENP5 CD wt resulted in progressive de-SUMOylation of the SUMO-1-modified RanGAP1 protein. No isopeptidase activity was detected when the same amount of GST-SENP5-CD C713S was used in the assay. Thus, SENP5 has SUMO isopeptidase activity and cysteine 713 is required for catalysis.

We next compared SENP5 isopeptidase activities for different SUMO family proteins. For these assays, we used a much smaller concentration of enzyme relative to substrate. We incubated a limiting amount of GST-SENP5-CD together with SUMO-1, SUMO-2, and SUMO-3 conjugated to the C-terminal domain of RanGAP1 as described by Reverter and Lima (32). Time course assays of the cleavage reaction showed that the C-terminal domain of SENP5 removes SUMO-2 and SUMO-3 more efficiently than SUMO-1 from SUMOylated RanGAP1 in vitro (Fig. 2C). A titration of SENP5-CD revealed a similar preference for cleavage of SUMO-2 and SUMO-3 compared to SUMO-1 from this substrate (data not shown). This is in striking contrast to the catalytic domain of

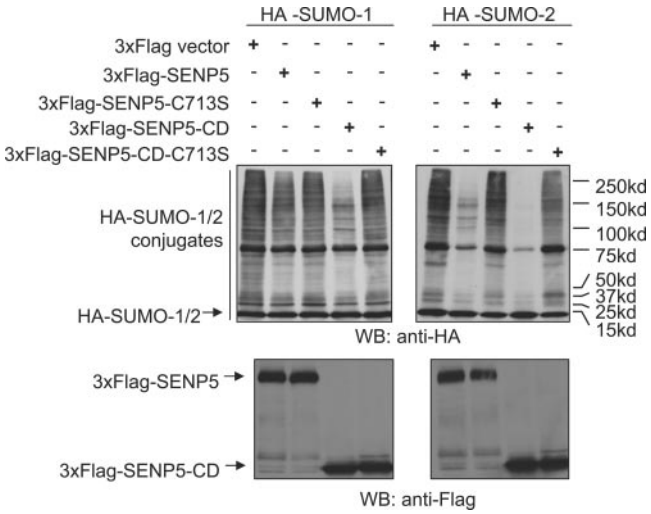

FIG. 3. SENP5 has isopeptidase activity in vivo. 293T cells were cotransfected with HA-SUMO-1 or HA-SUMO-2 together with either 3×Flag vector, 3×Flag-SENP5 full-length, C-terminal domain (CD), wild type, or C713S mutant constructs, and cytomegalovirus (CMV)-GFP. SUMO conjugates were analyzed by Western blotting (WB) using anti-HA antibody. HA-SUMO-1/2 conjugates and free HA-SUMO-1/2 are indicated. Comparable levels of 3×Flag-SENP5 derivatives were confirmed by anti-Flag Western blotting.

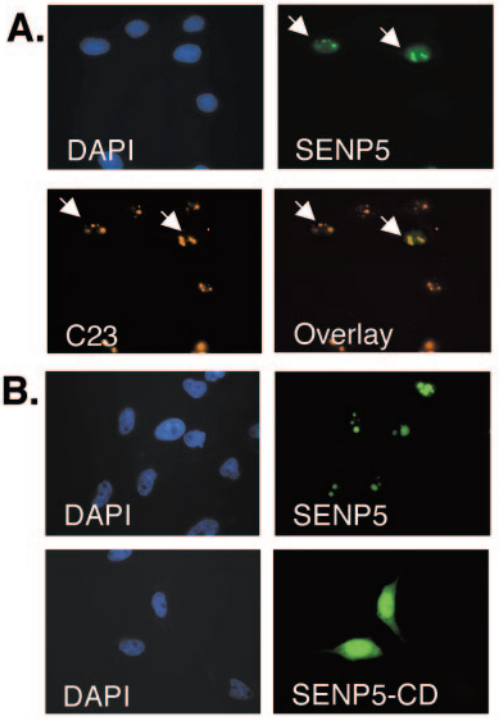

FIG. 4. SENP5 localizes to the nucleolus. HeLa cells were transiently transfected with GFP-tagged SENP5 wild type or GFP-SENP5-CD, lacking the N-terminal domain. (A) GFP-SENP5 colocalized with the nucleolar marker C23. Twenty-four hours after transfection, cells were fixed and probed with antinucleolin antibody C23, a nucleolar marker. (B) The deletion mutant GFP-SENP5-CD had a diffuse nuclear localization, indicating that the N terminus of SENP5 is required for nucleolar localization. Note that the different fixation conditions used in panels A and B account for the increased nuclear staining of wild-type GFP-SENP5 in panel A.

SENP2, which was found to remove SUMO-1, SUMO-2, and SUMO-3 from RanGAP1 with similar efficiencies (32). Using GST-SENP5-CD as an affinity resin, we found that SENP5-CD (wild type and C713S) bound mature SUMO-2 better than mature SUMO-1 (Fig. 2D) (data not shown). Thus, differential binding of the SENP5 catalytic domain to the SUMO cores may contribute to the observed difference in both hydrolase and isopeptidase catalytic activities of this enzyme. These data reveal that although the C-terminal catalytic domain of SENP5 has isopeptidase activity toward SUMO-1, -2, and -3, under limiting conditions, SENP5 discriminates between different SUMO family proteins in vitro, raising the possibility that SENP5 may contribute to differential regulation of SUMO-1 versus SUMO-2/3 conjugates in vivo.

SENP5 has SUMO isopeptidase activity in vivo. SENP5 and various derivatives were assayed for SUMO isopeptidase activity in vivo. 293T cells were cotransfected with HA-epitope-tagged SUMO-1 or SUMO-2 and either the SENP5 wild type or mutant (full-length or catalytic domain only), and the profile of high-molecular-weight SUMO-modified proteins was determined. As shown in Fig. 3, cotransfection with full-length wild-type SENP5 led to a reduction in the level of high-molecular-weight SUMO-1 and SUMO-2 conjugates. Expression of the deletion mutant SENP5-CD wt led to a more dramatic reduction of both SUMO-1 and SUMO-2 conjugates, compared to full-length SENP5. Notably SENP5, full length or C-terminal domain alone, led to a more significant reduction of HA-SUMO-2 conjugates compared to HA-SUMO-1. As expected, cysteine 713 was required for de-SUMOylation since expression of the SENP5 C713S mutant failed to promote the loss of either SUMO-1 or SUMO-2 conjugates. It should be noted that overexpression of HA-SUMO-1 and HA-SUMO-2 may lead to modification of nonphysiological substrates. For example, the patterns of HA-SUMO-1 and HA-SUMO-2 conjugates are strikingly similar, in contrast to endogenous patterns of SUMO-1 and SUMO-2 conjugates (see Fig. 5). Equal expression levels of the SENP5 derivatives were verified by anti-Flag Western blotting (Fig. 3). These results show that SENP5 preferentially catalyzes removal of SUMO-2 relative to SUMO-1 in vivo as well as in vitro and reveal that the N terminus of SENP5 restricts activity on many SUMO substrates in vivo.

Subcellular localization of SENP5. Previous studies in both yeast and mammalian cells suggested that subcellular localization of the SUMO-specific proteases is important for substrate selection in vivo (4, 15, 24). In order to examine the subcellular distribution of SENP5, plasmids expressing a GFP-SENP5 fusion were transfected into HeLa cells. The GFP-SENP5 fusion protein localized predominantly in the nucleolus, as confirmed by colocalization with the nucleolar marker nucleolin (C23) (Fig. 4A). Mutation of the active site cysteine in SENP5 (C713S) did not change the localization of the protein (data not shown). Nucleolar localization of GFP-SENP5 was also observed in U2OS cells (data not shown). The deletion mutant, GFP-SENP5-CD, failed to localize to the nucleolus, indicating that the N-terminal portion of SENP5 contains sequences essential for nucleolar localization (Fig. 4B). Thus, upon over-

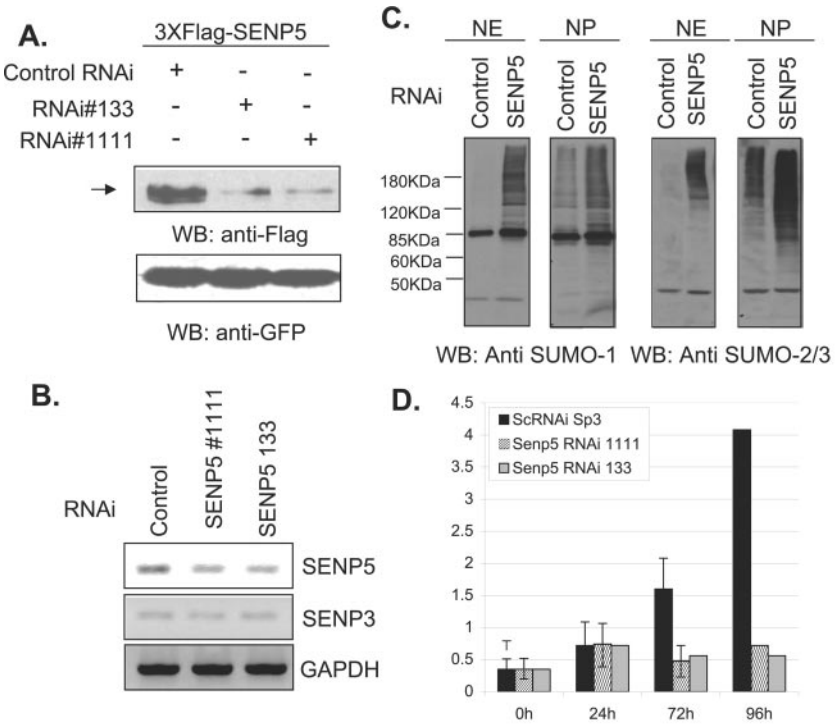

FIG. 5. Loss of SENP5 leads to increased SUMOylation and decreased cell proliferation. (A) Either of two short hairpin RNAs targeting SENP5, but not a control hairpin, reduced expression of cotransfected 3×Flag-SENP5 as revealed by Western blotting (WB) of HeLa cell extracts. GFP expression level was monitored as a loading control. (B) HeLa cells transfected with Senp5 RNAi or control RNAi were selected with puromycin, and RNA was isolated after 72 h. RT-PCR analysis using specific primers for SENP5, SENP3, and GAPDH was performed. SENP5 and SENP3 mRNA levels were quantified and normalized against GAPDH. (C) HeLa cells transfected with Senp5 RNAi 1111 or control RNAi were selected with puromycin, and cell lysates were analyzed by immunoblotting with anti-SUMO-1 and anti-SUMO-2/3 antisera. Anti-GAPDH was used as a control for equal loading (not shown). Knockdown of SENP5 led to a dramatic increase in SUMO-1- and SUMO-2/3-modified proteins in HeLa cells, compared to cells transfected with control RNAi. NE, nuclear extract; NP, nuclear pellet. (D) HeLa cells were infected with SENP5 RNAi or control RNAi retroviruses, and cell number was counted daily. Cells infected by SENP5 RNAi showed a severe reduction in cell growth compared to cells infected with a control RNAi. The bar graph represents the mean ± standard deviation of three independent experiments.

expression, SENP5, like its close relative SENP3, localizes predominantly to the nucleolus. SENP5 may de-SUMOylate substrates in the nucleolus, and/or SENP5 may be sequestered in the nucleolus until it is released to de-SUMOylate substrates in the nucleus.

SENP5 is required for cell proliferation. Mutations in the *Saccharomyces cerevisiae* de-SUMOylating enzymes, Ulp1 and Ulp2, led to defects in cell cycle transition, chromosome stability, and meiosis (24, 25). In order to investigate the physiological role of SENP5, we created two different short hairpin RNA constructs, both of which efficiently knocked down expression of SENP5 protein in cotransfection assays (Fig. 5A). These shRNAs also led to a specific reduction of endogenous SENP5 mRNA in stably transfected HeLa cells (Fig. 5B). SENP5 RNAi 1111 induced 70% reduction, while RNAi 133 induced 80% reduction in the level of endogenous SENP5 mRNA 72 after transfection with the RNAi plasmids. The levels of SENP1, SENP2, and SENP3 mRNA were not reduced in these cells (Fig. 5B) (data not shown).

Immunoblot analysis confirmed that loss of SENP5 led to an increase in the level of SUMO-1 and SUMO-2/3 conjugates compared to that in control cells (Fig. 5C). In these studies, it appeared that SENP5 RNAi promoted a greater relative increase in SUMO-2/3- than SUMO-1-conjugated proteins. It should be noted that the dramatic increase in high-molecular-weight SUMO conjugates in the SENP5 RNAi cells may reflect increased SUMOylation of direct SENP5 substrates as well as indirect effects due to proliferation defects or other alterations in metabolism. Interestingly, knockdown of SENP5 led to a dramatic reduction in the proliferation rate (Fig. 5C). Relative to the control, SENP5 RNAi did not lead to increased cell death as judged by the absence of a sub-G_1 peak in fluorescence-activated cell sorting (FACS) analysis and no observed increase in the number of trypan blue-staining cells (data not shown). By FACS analysis, we did not observe arrest during a specific phase of the cell cycle, but the SENP5 RNAi-treated cells were noticeably larger in size (data not shown). These data suggest that SENP5 controls the de-SUMOylation of substrate proteins required for cell proliferation.

We further examined the morphology of SENP5 knockdown cells 72 h after infection by immunofluorescence using DAPI and α-tubulin staining. As shown in Fig. 6, cells infected with either SENP5 RNAi showed a statistically significant increase in the number of cells with more than one nucleus and with aberrant nuclear morphology compared to cells infected with control RNAi. The aberrant nuclei observed in cells infected

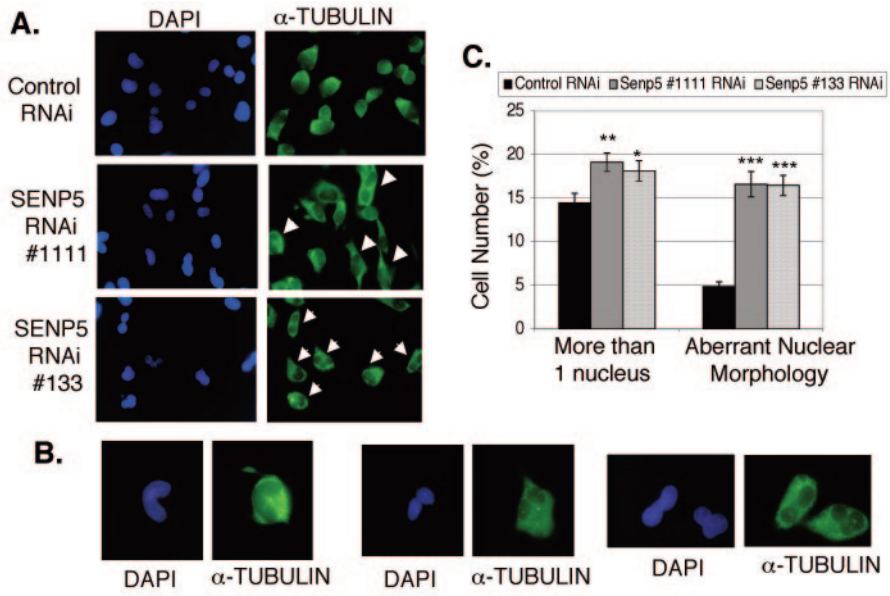

FIG. 6. Knockdown of SENP5 leads to defects in cell division and aberrant nuclear morphology. HeLa cells were infected with retrovirus expressing either of two SENP5 RNAis or control RNAi and then stained with DAPI and α-tubulin antisera 72 h postinfection. (A) Upon infection with either SENP5 RNAi 1111 or 133, an increased number of cells with multiple nuclei or defects in the morphology of the nucleus was observed (indicated by arrows). (B) Examples of SENP5 knockdown cells with multiple nuclei or aberrant nuclear morphology. (C) Quantitation of the number of cells showing multiple nuclei and aberrant nuclear morphology following infection with RNAi retrovirus. Compared to control RNAi-treated cells, SENP5 RNAi resulted in a small but reproducible (25 to 32%) increase in the number of cells with multiple nuclei (predominantly two) and a threefold increase in the number of cells that exhibited defects in nuclear morphology (predominantly bilobed or dumbbell-shape nuclei). An average of 1,500 cells was counted per sample. An unpaired *t* test was used for statistical analysis of the samples, and the bar graph represents the mean ± standard error of five independent experiments. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, *P* value of ≤ 0.0001.

with SENP5 RNAi were predominantly elongated, dumbbell-shape nuclei, which sometimes became fragmented or formed multilobar structures (Fig. 6C). Quantitatively, depletion of SENP5 reproducibly increased the number of cells with more than one nucleus 25 to 32% and increased the number of cells displaying an aberrant nuclear morphology threefold, compared to control RNAi-infected cells (Fig. 6B). Knockdown of the close SENP5 homolog SENP3 by RNAi was not found to increase the appearance of binucleate cells, further supporting a specific role for SENP5 in cell proliferation (J. Ouyang and G. Gill, unpublished data). Dumbbell-shape nuclei have been suggested to arise due to defects in chromosome segregation (26), while binucleate cells arise due to defects in mitosis or cytokinesis (35). The phenotypes of the SENP5 knockdown cells indicates that SENP5 has a nonredundant function in mammalian cell division and suggest a requirement for SENP5 in completion of mitosis and/or cytokinesis.

DISCUSSION

SUMO and SUMOylation pathway enzymes are important regulators of diverse nuclear processes, including transcription, DNA repair, and chromosome segregation (13, 16, 20). We present here an analysis of the biochemical and biological activities of human SENP5, a previously uncharacterized SUMO-specific protease that we show to be important in the control of cell division. SENP5 and SENP3 share the highest homology in sequence among the seven human Ulp homologs (Fig. 1A), and similar to SENP3, GFP-tagged SENP5 localized predominantly to the nucleolus (Fig. 4). Distinct from SENP3, however, for which only isopeptidase activity has been reported (30), SENP5 possesed both C-terminal hydrolase and isopeptidase activities (Fig. 2 and 3). Interestingly, the C-terminal catalytic domain of SENP5 discriminated between SUMO-1 and SUMO-2/3 in both C-terminal hydrolase and isopeptidase assays in vitro. Furthermore, both full-length and the C-terminal catalytic domain of SENP5 more efficiently removed HA-SUMO-2 than HA-SUMO-1 from modified substrates in vivo. The noncatalytic N-terminal domain was found to limit isopeptidase activity of SENP5 in vivo. Knockdown of SENP5 by RNAi revealed a nonredundant function of this SUMO-specific protease in cell proliferation. Our data support the hypothesis that the different mammalian SUMO-specific proteases have unique biological functions dependent on distinct substrate specificities, which are regulated by both the catalytic and noncatalytic domains of these enzymes.

SUMO proteins are synthesized in a precursor form, which must be proteolytically cleaved to expose diglycine residues at the C terminus prior to conjugation. SENP5 was found to hydrolyze both SUMO-1 and SUMO-3 precursors in vitro. Analysis of the C-terminal hydrolase activity of SENP5 revealed a difference in the efficiency with which SENP5 processes these SUMO precursors: SUMO-3 was processed more efficiently than SUMO-1 to its mature form (Fig. 2A). The preferences of SENP5 C-terminal hydrolase activity were notably different from those of other SUMO-specific proteases described to date. In similar studies, the catalytic domain of SENP1 hydrolyzed immature SUMO-1 > SUMO-2 > SUMO-3 and the catalytic domain of SENP2 showed prefer-

ence for SUMO-2 > SUMO-1 > SUMO-3 (32, 44). In the case of SENP1 and SENP2, differences in precursor processing efficiencies were determined by residues in the extension immediately following the double glycine at the C terminus of mature SUMO. The mechanism underlying the preferential hydrolysis of SUMO-3 over SUMO-1 precursors by SENP5 remains to be determined; however, our studies of SENP5 isopeptidase activity and SUMO binding suggest that differences in the SUMO core, where SUMO-1 is only 46% identical to SUMO-2/3, may contribute. Differential SUMO precursor processing by different SUMO proteases suggests a mechanism to regulate the availability of the different SUMO proteins for conjugation that may be of physiological importance.

Our finding that the SENP5 isopeptidase activity discriminates between SUMO-1 and SUMO-2/3 substrates represents a significant difference between SENP5 and other mammalian SUMO-specific proteases described to date. Although SENP5 was active on all SUMO family proteins examined, under limiting conditions in vitro, the catalytic domain of SENP5 preferentially removed SUMO-2 and SUMO-3 from RanGAP1. This is in contrast to the catalytic domain of SENP2, which did not discriminate between these substrates (32). In transfection assays, we also found that SENP5 preferentially reduced high-molecular-weight conjugates of HA-SUMO-2 compared to HA-SUMO-1 (Fig. 3). Furthermore, we observed that knockdown of SENP5 led to a significant increase in levels of both SUMO-1 and SUMO-2/3 conjugates in vivo. This is in marked contrast to studies of the SENP1 knockout mouse in which SUMO-1 conjugates, but not SUMO-2/3 conjugates, were observed to increase (45). These findings suggest that SENP5 has a role in regulation of substrates posttranslationally modified by SUMO-2/3 that may not be shared with the SUMO-specific proteases SENP1 and SENP2.

Our studies have revealed that both the C-terminal catalytic domain and the nonconserved N-terminal domain contribute to substrate selection by SENP5. Thus, we found that the C-terminal catalytic domain of SENP5 was sufficient to discriminate between SUMO-1 and SUMO-2/3 conjugates in vitro and in vivo (Fig. 2 and 3). In vivo, deletion of the N-terminal domain dramatically enhanced deconjugation of both SUMO-1 and SUMO-2 (Fig. 3). Thus, the N-terminal domain of SENP5 negatively regulates the isopeptidase activity toward many substrates in vivo. Deletion of the noncatalytic N terminus of SENP1 and SENP2 has also been shown to enhance de-SUMOylation activity of these enzymes on SUMO-1-modified substrates in vivo (4, 15). Analysis of the yeast SUMO-specific protease Ulp1 identified elements in the noncatalytic N terminus that both positively and negatively regulate substrate selection in vivo (24). It remains to be determined whether the role of the SENP5 N-terminal domain in regulating substrate selection is due solely to the role of the N terminus in subcellular localization (see below) or whether the N-terminal domain might have additional functions as well.

Previous studies in yeast and mammals have suggested that subcellular localizations of the different SUMO-specific proteases are important for in vivo activity. Analysis of the subcellular localization of mammalian SUMO proteases has revealed a very complex distribution. SENP1 broadly localizes in the nucleus and in nuclear dots (4, 14); SENP2 is present at the nuclear pore and in nuclear dots (5, 15, 46); SENP3 localizes to the nucleolus (30), and although SENP6 has been reported to be cytoplasmic (22), we have observed diffuse nuclear localization in HeLa cells (A. Di Bacco, H.-Y. Lee, and G. Gill, unpublished data). Although the localization of endogenous SENP5 remains to be determined, we have found that GFP-tagged SENP5 localizes predominantly to the nucleolus, similar to GFP-tagged SENP3, with which it shares high homology (Fig. 4A). The nonconserved N terminus of SENP5 is required for nucleolar localization. Basic residues contribute to nuclear and/or nucleolar signals, and analysis of the N-terminal portion of SENP5 protein revealed the presence of arginine-rich domains, which might be responsible for the nucleolar localization (48). Given the preferential activity of SENP5 toward SUMO-2/3 substrates, it is interesting to note that SUMO-2 and SUMO-3 have been reported to be largely excluded from the nucleolus (1). It is possible that SENP5 activity keeps SUMO-2/3 conjugate levels low in the nucleolus in interphase. It is also possible that SENP5 is sequestered in the nucleolus until it is released to de-SUMOylate substrates in the nucleus, similar to the cell cycle-regulated localization of CDC14 phosphatase (36, 42).

Notable differences in SENP5 enzymatic activity and localization compared with other mammalian SUMO-specific proteases characterized to date are consistent with a unique physiological function for this enzyme. We have shown that RNAi-mediated knockdown of SENP5 led to a marked decrease in cell growth, accompanied by the appearance of cells with more than one nucleus or aberrant nuclear morphology (Fig. 5 and 6). When SENP5 was knocked down, nuclei appeared elongated and often displayed a dumbbell-shape or multilobar morphology. As binuclear cells or nuclei with multiple lobes generally derive from defects in the cytokinesis machinery or as a result of defects in chromosome segregation, these findings suggest a nonredundant function for SENP5 in these pathways (26, 35). In contrast to SENP5, the SUMO E2-conjugating enzyme Ubc9 is essential for cell viability, but it is notable that depletion of Ubc9 in chicken cells was associated with increased numbers of binucleate cells and knockout of Ubc9 in mouse led to aberrant nuclear morphologies and defects in chromosome segregation (17, 29). Taken together, these studies indicate that the proper balance of SUMOylation and de-SUMOylation is required for normal nuclear architecture and cell proliferation in vertebrates.

Previous studies have shown that SUMOylation and de-SUMOylation of many proteins, including PML and topoisomerase II, are regulated during the cell cycle (2, 10). We have observed that knockdown of SENP5 leads, directly or indirectly, to a significant increase in SUMOylation of many cellular proteins, consistent with a requirement for the de-SUMOylation activity of SENP5 in cell proliferation. Additional studies are required to determine which SENP5 substrates or partners are required for proper mitosis and/or cytokinesis. Candidate substrates include proteins such as topoisomerase II whose cell cycle-regulated modification by SUMO-2/3 is required for chromosome segregation in *Xenopus* extracts (3). Additionally, SENP5 could act in a common pathway as the Polo-like kinase-1 (Plk-1), since the SENP5 RNAi phenotype resembles that observed in cells depleted of Polo-like kinase-1 (Plk-1) or expressing a nonphosphorylatable

form of B23/nucleophosmin, a substrate of Plk-1, which can be modified by SUMO (26, 39, 47).

In conclusion, we have described the biochemical functions of a previously uncharacterized nucleolar SUMO-specific protease, SENP5, and revealed its nonredundant role in cell division. We have shown that the catalytic domain of SENP5 discriminates between SUMO-1 and SUMO-2/3 proteins in both C-terminal hydrolase and isopeptidase assays in vitro. SENP5 also discriminates between SUMO paralogs in vivo, leading to a preferential loss of SUMO-2 compared to SUMO-1 conjugates when SENP5 is expressed. Furthermore, the nonconserved N-terminal domain of SENP5 limits deconjugation activity in vivo. The biochemical activities of SENP5 suggest a mechanism for differential regulation of SUMO-1- and SUMO-2/3-modified substrates. Intriguingly, the differential activity of SENP5 on SUMO-2/3 conjugates in vivo suggests a role for this enzyme in regulation and deconjugation of SUMO chains, which are preferentially formed by SUMO-2 and SUMO-3 (40). Additional studies of the complex mechanisms of substrate selection by SENP5 and the other SUMO-specific proteases in mammals will provide further insight into the biological activities of these enzymes in cell growth regulation.

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