Nucleolar protein B23/nucleophosmin regulates the vertebrate SUMO pathway through SENP3 and SENP5 proteases

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Ubiquitin-like protein/sentrin-specific proteases (Ulp/SENPs) mediate both processing and deconjugation of small ubiquitin-like modifier proteins (SUMOs). Here, we show that Ulp/SENP family members SENP3 and SENP5 localize within the granular component of the nucleolus, a subnucleolar compartment that contains B23/nucleophosmin. B23/nucleophosmin is an abundant shuttling phosphoprotein, which plays important roles in ribosome biogenesis and which has been strongly implicated in hematopoietic malignancies. Moreover, we found that B23/nucleophosmin binds SENP3 and SENP5 in Xenopus laevis egg extracts and that it is essential for stable accumulation of SENP3 and SENP5 in mammalian tissue culture cells. After either codepletion of SENP3 and SENP5 or depletion of B23/nucleophosmin, we observed accumulation of SUMO proteins within nucleoli. Finally, depletion of these Ulp/SENP family members causes defects in ribosome biogenesis reminiscent of phenotypes observed in the absence of B23/nucleophosmin. Together, these results suggest that regulation of SUMO deconjugation may be a major facet of B23/nucleophosmin function in vivo.

Introduction

Small ubiquitin-like modifier proteins (SUMOs) are ubiquitin-related proteins that act in many processes through covalent linkage to cellular proteins (Johnson, 2004). Mammalian cells express three major SUMO paralogues: mature SUMO-2 and SUMO-3 are ~95% identical to each other and each is ~45% identical to SUMO-1. (Where they cannot be distinguished, SUMO-2 and -3 will be collectively called SUMO-2/3.) SUMO-1 localizes within the nucleoplasm, nuclear envelope, and nucleolus, whereas SUMO-2 and SUMO-3 are predominantly found in the nucleoplasm (Ayaydin and Dasso, 2004). All newly synthesized SUMOs are processed by ubiquitin-like protein/sentrin-specific proteases (Ulp/SENP family members) and Ulp/SENP family members also deconjugate SUMOylated species (Hay, 2007; Mukhopadhyay and Dasso, 2007). Saccharomyces cerevisiae has two Ulp/SENP family members, Ulp1p and Ulp2p (Li and Hochstrasser, 1999, 2000). Ulp1p is essential and localizes to nuclear pores, whereas Ulp2p is dispensable for growth and localizes to the nucleoplasm. Ulp1p has been particularly implicated in ribosome biogenesis (Panse et al., 2006).

The nucleolus is the site of ribosome synthesis, including preribosomal RNA transcription, ribosomal RNA (rRNA) processing, and ribosomal subunit assembly (Boisvert et al., 2007). B23/nucleophosmin is an abundant 37-kD phosphoprotein that shuttles between the granular component of the nucleolus and cytoplasm. B23/nucleophosmin can become SUMO conjugated under some circumstances (Liu et al., 2007). It has been implicated in many cellular processes, including ribosome biogenesis and export, centrosome duplication, and maintenance of genomic integrity through the Arf–MDM2–p53 pathway (Grigioni et al., 2006; Sherr, 2006). Consistent with these diverse roles,

Supplemental Material can be found at: /content/suppl/2008/11/17/jcb.200807185.DC1.html
granular component (dsRed-B23/nucleophosmin). The signal from GFP-SENP3 and GFP-SENP5 overlapped poorly with UBF staining and only partially with dsRed-fi brillarin, suggesting that they were not concentrated in either the fibrillar centers or the dense fibrillar component (Fig. 1A, top). In contrast, both proteins showed distributions that were very similar to B23/nucleophosmin, which could be observed either by immunostaining or by coexpression of B23/nucleophosmin as a fusion with the dsRed fluorescent protein (Fig. 1A, bottom). We conclude that SENP3 and SENP5 are concentrated within the granular component.

The presence of SUMO-2/3–specific proteases within nucleoli suggested that the absence of these SUMO paralogues might be the result of their ongoing removal from nucleolar targets. To test this idea, we depleted SENP3 and SENP5 individually and together by oligonucleotide-mediated RNA interference (RNAi) from HeLa cells (Fig. 2A). Depletion of either protease did not substantially alter the distribution of SUMO proteins. However, nucleolar SUMO-1 increased after codepletion of SENP3 and SENP5 (Fig. 2B and Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200807185/DC1). Significantly, SUMO-2/3 also became concentrated within nucleoli in the absence of both enzymes. Depletion of nuclear pore–associated SENP/Ulps (SENP1 and SENP2) did not increase nucleolar levels of SUMO proteins (unpublished data), arguing that such accumulation is not a general result of SUMO pathway manipulation or of RNAi machinery activation.

SENP3 and SENP5 show robust enzymatic activities against SUMO-2/3–containing substrates (Di Bacco et al., 2006; B23/nucleophosmin binds to nucleic acids, nucleolar components, transcription factors, and histones, as well as to proteins involved in cell proliferation, mitosis, and oncogenic stress responses (Grisendi et al., 2006). It is not clear how these aspects of B23/nucleophosmin may be integrated with each other. Notably, B23/nucleophosmin is often overexpressed in solid tumors and has been strongly linked to hematopoietic malignancies (Grisendi et al., 2006).

We have examined the subnucleolar localization, behavior, and function of two nucleolar Ulp1p-like SUMO proteases, SENP3 and SENP5 (Di Bacco et al., 2006; Gong and Yeh, 2006; Mukhopadhyay and Dasso, 2007). We found that both of these enzymes colocalize and physically interact with B23/nucleophosmin. Moreover, B23/nucleophosmin is essential for their stable accumulation. After either codepletion of these Ulp/SENPs or depletion of B23/nucleophosmin, SUMO proteins accrue within nucleoli. Importantly, depletion of SENP3 and SENP5 causes defects in ribosome biogenesis reminiscent of those observed in the absence of B23/nucleophosmin. Collectively, our findings indicate that regulation of SUMO deconjugation through SENP3 and SENP5 may be a major facet of B23/nucleophosmin function and suggest that at least some of the diverse phenotypes found after disruption of B23/nucleophosmin can be attributed to defects of SUMOylation.

**Results and discussion**

We compared the localization of stably expressed GFP-SENP3 and GFP-SENP5 with components that are characteristic of nucleolar subcompartments, including the fibrillar centers (anti-UBF), the dense fibrillar component (dsRed-fibrillarin), and the granular component (dsRed-B23/nucleophosmin). The signal from GFP-SENP3 and GFP-SENP5 overlapped poorly with UBF staining and only partially with dsRed-fibrillarin, suggesting that they were not concentrated in either the fibrillar centers or the dense fibrillar component (Fig. 1A, top). In contrast, both proteins showed distributions that were very similar to B23/nucleophosmin, which could be observed either by immunostaining or by coexpression of B23/nucleophosmin as a fusion with the dsRed fluorescent protein (Fig. 1A, bottom). We conclude that SENP3 and SENP5 are concentrated within the granular component.

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that defects in ribosome biogenesis were specifically caused by depletion of SENP3 and SENP5. Our results show that SENP3 and SENP5 individually play important roles in ribosome biogenesis (Fig. 3), but depletion of both proteases is required for elevation of SUMO-conjugated species within nucleoli (Fig. 2). Together, these results might suggest that although both SENP3 and SENP5 can act on the bulk of nucleolar substrates, they may each specifically regulate a subset of conjugates that are critical for particular events in the biogenesis pathway.

We observed a protein that strongly and specifically co-precipitated with xSENP3, which mass spectrometric analysis revealed to be the Xenopus homologue of B23/nucleophosmin (xB23/nucleophosmin). We confirmed this interaction by Western blotting of anti-xSENP3 immunoprecipitates (Fig. 1 B). Given the low levels of xSENP5 within XEEs, we used an alternative strategy to determine whether SENP5 might likewise

Budding yeast Ulp1p is required for 60S preribosome maturation and export (Panse et al., 2006). To test whether SENP3 and SENP5 are similarly required, we examined rRNA synthesis and processing through pulse-chase analysis with [3H]uridine (Fig. 3). Although 47S rRNA transcription was not substantially decreased after SENP3 depletion, the production of 28S rRNA from their 32S precursor RNA was inhibited. After a 2-h chase, control cells showed a ratio of labeled 32S versus 28S RNAs that was less than 0.5:1, whereas this ratio was over 2:1 in SENP3-depleted cells (Fig. 3 B). In contrast, loss of SENP5 did not have a strong effect on rRNA processing, but caused the levels of 47S RNA transcription to drop by >60% (Fig. 3 C). Cells depleted of both proteins showed both decreased 47S RNA precursor levels and increased 32S/28S ratios. Relocalization of ribosomal assembly factors further suggested disruption of ribosome biogenesis in the absence of SENP3 and SENP5 (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200807185/DC1). Simultaneous depletion of SENP1 and SENP2 marginally reduced 47S RNA transcription but did not appreciably alter the rate of RNA processing or alter assembly factor localization (unpublished data), arguing that defects in ribosome biogenesis were specifically caused by depletion of SENP3 and SENP5. We found that defects in ribosome biogenesis were specifically caused by depletion of SENP3 and SENP5.
Figure 3. Depletion of SENP3 or SENP5 cause defects in ribosome biogenesis. (A) HeLa cells were transfected with control oligonucleotides (Control) or siRNAs directed against SENP3 (ΔSENP3) and SENP5 (ΔSENP5) or both SENP3 and SENP5 (ΔSENP3/5). 72 h later, the cells were pulsed with [3H]uridine for 25 min, followed by washing and incubation with fresh media supplemented with 1 mg/ml of cold uridine for the indicated times (in hours). At each time point, equal numbers of cells were harvested for analysis of rRNA synthesis and processing. (Bottom) Western blots of cell extracts to monitor SENP3 and SENP5 and Western blotting of actin as a loading control. Ethidium bromide staining shows the amount of total RNA loaded in each lane. (B) The amounts of 28S and 32S RNAs were quantitated for each experiment. Bars represent the mean values calculated from the three experiments ± SD. (C) The amount of 47S pre-rRNA transcript was quantitated at the initiation of the chase period (0 h) within three independent experiments as in A after 2 h. Ratios of 32S to 28S RNAs were calculated for each experiment. Bars represent the mean of normalized values from the three experiments ± SD.

Several observations indicated that SENP3 and SENP5 loss was caused by increased degradation rather than decreased expression. First, GFP-SENP3 and GFP-SENP5 were not transcribed from the native SENP3 and SENP5 promoters, so the drop in their levels upon B23/nucleophosmin depletion (Fig. 4 B) argues that repression of those promoters cannot account for changes in protein levels. Second, the concentrations of SENP3 and SENP5 mRNAs were not substantially altered by B23/nucleophosmin depletion in HeLa cells, as measured by RT-PCR (unpublished data), further arguing against B23/nucleophosmin control of SENP3 and SENP5 transcription or mRNA stability. Third, B23/nucleophosmin-depleted cells did not show a substantial drop in overall protein translation, as measured by incorporation of [35S]methionine (unpublished data), indicating that preexisting ribosomes were largely sufficient to maintain protein synthesis levels. Notably, depletion of a key ribosomal assembly factor, Rrp9 (Venema et al., 2000), did not destabilize SENP3 and SENP5 or cause substantial nucleolar accumulation of SUMO proteins (unpublished data), demonstrating that their loss was not a simple consequence of ribosome synthesis inhibition but may be more specifically linked to the function of B23/nucleophosmin.

Finally, SENP3 and SENP5 levels in B23/nucleophosmin-depleted cells showed substantial, albeit incomplete, recovery upon treatment with the proteasome inhibitor MG132, which is consistent with the notion that they are subject to degradation in the absence of B23/nucleophosmin (Fig. 4 D). Stable cell lines expressing GFP-SENP3 and SENP5 showed a partial redistribution of GFP signals after B23/nucleophosmin depletion and MG132 treatment, with an increase in the ratio of nucleoplasmic to nucleolar distribution of both fusion proteins (unpublished data). This observation might indicate a role for B23/nucleophosmin in retention of these Ulp/SENPs within the nucleolus. However, we noted that the distribution of many other nucleolar proteins was rearranged after MG132 treatment, so it is also possible that relocation of GFP-SENP3 and GFP-SENP5 may reflect alterations of nucleolar structure.

We hypothesized that failure to deconjugate some SUMO species might disrupt ribosome biogenesis in the absence of SENP3 and SENP5. We therefore examined the SUMOylation of mammalian homologues of ribosomal proteins or ribosome assembly factors that have been implicated as SUMO conjugation targets in yeast (Panse et al., 2004, 2006; Wohlschlegel
argued that SENP3 acts upstream of B23/nucleophosmin, causing inhibition of ribosome biogenesis through increased B23/nucleophosmin SUMOylation. In our hands, depletion of SENP3 and SENP5 by RNAi, either singly (not depicted) or in combination (Fig. 5), did not cause a substantial SUMOylation of B23/nucleophosmin. We do not know why our results differ from those of et al., 2004; Denison et al., 2005; Hannich et al., 2005). Using cells that stably expressed His₆-tagged SUMO-1 (His-SUMO1F) or SUMO-2 (His-SUMO2F) proproteins, we examined changes in electrophoretic mobility of potential conjugation targets by Western blotting after codepletion of SENP3 and SENP5. We also purified the SUMO-conjugated fraction from each cell line by affinity chromatography and examined whether SUMOylated forms of the proteins could be found by Western analysis.

A subset of proteins showed clearly altered SUMOylation patterns under these circumstances: GNL2, the mammalian homologue of a 60S preribosomal export factor, showed a supershifted form upon depletion of SENP3/5 (Fig. 5), which was verified as a SUMOylated form through its preferential retention on nickel–nitrilotriacetic acid (Ni-NTA) agarose beads. We observed a supershifted form of the 60S ribosomal subunit RPL37A that was retained on Ni-NTA agarose beads in the absence of SENP3/5 (Fig. 5, top), suggesting that it was likewise subject to enhanced modification. Other nucleolar proteins (fibrillarin, DDX3, DDX17, and NVL2; unpublished data) did not show evidence of SUMOylation in this assay. Although this is an incomplete survey of potential conjugation targets within the 60S ribosomal biogenesis pathway, it demonstrates that multiple pathway components are SUMOylation targets, as in yeast. More importantly, enhanced RPL37A and GNL2 SUMOylation after depletion of SENP3 and SENP5 implicates these proteases in the control of ribosome biogenesis through this modification.

While this manuscript was under revision, Haindl et al. (2008) reported that SENP3 and B23/nucleophosmin physically interact and that loss of SENP3 disrupts rRNA processing. They argued that SENP3 acts upstream of B23/nucleophosmin, causing inhibition of ribosome biogenesis through increased B23/nucleophosmin SUMOylation. In our hands, depletion of SENP3 and SENP5 by RNAi, either singly (not depicted) or in combination (Fig. 5), did not cause a substantial SUMOylation of B23/nucleophosmin. We do not know why our results differ from those of et al., 2004; Denison et al., 2005; Hannich et al., 2005). Using cells that stably expressed His₆-tagged SUMO-1 (His-SUMO1F) or SUMO-2 (His-SUMO2F) proproteins, we examined changes in electrophoretic mobility of potential conjugation targets by Western blotting after codepletion of SENP3 and SENP5. We also purified the SUMO-conjugated fraction from each cell line by affinity chromatography and examined whether SUMOylated forms of the proteins could be found by Western analysis.

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Figure 4. B23/nucleophosmin regulates SENP3 and SENP5 abundance. (A) HeLa cells were transfected with control oligonucleotides (left lane), siRNAs for codepletion of SENP1 and SENP2 (second lane), siRNAs for codepletion of SENP3 and SENP5 (third lane), or siRNAs for depletion of B23/nucleophosmin (right lane). After 72 h, the cells were harvested and immunoblotted with the indicated antibodies, where actin serves as a loading control. (B) U2OS cells stably expressing either GFP-SENP3 (top) or GFP-SENP5 (bottom) were transfected with control or B23/nucleophosmin siRNA, as indicated. After 72 h, the cells were washed and fixed with 4% paraformaldehyde and stained with antibodies against B23/nucleophosmin (red). Signals from the GFP-SENP3 and GFP-SENP5 fusion proteins are shown in green. (C) As in A, HeLa cells were transfected with control oligonucleotides or with siRNAs for depletion of B23/nucleophosmin. After 72 h, the cells were stained with antibodies against B23/nucleophosmin (red) and against either SUMO-1 or SUMO-2/3 (green), as indicated. (D) 48 h after transfection with control or B23/nucleophosmin siRNAs, HeLa cells were treated for 6 h with 20 μM of proteasome inhibitor MG132 (+) or DMSO (−). The cells were harvested and subjected to Western blotting analysis with antibodies against SENP3, SENP5, B23/nucleophosmin, and actin, as indicated.

Figure 5. Enhanced SUMO modification of RPL37A and GNL2 after depletion of SENP3 and SENP5. U2OS stably expressing full-length His-tagged versions of SUMO-1 and SUMO-2 (His-SUMO1F and His-SUMO2F) were transfected with siRNAs for codepletion of SENP3 and SENP5 or with control oligonucleotides. After 72 h, the cells were harvested and His₆-SUMO-modified proteins were isolated. Total cell extracts and His₆ affinity fractions were analyzed by Western blotting with antibodies against RPL37A (top), GNL2 (middle), or B23/nucleophosmin (bottom).
Haindl et al. (2008), but suspect that the relatively low levels of His-tagged SUMO proteins expressed in our stable cell lines may mimic physiological conditions more closely than conditions of transient expression. In any case, our results do not suggest that B23/nucleophosmin is itself the primary target of SENP3 or SENP5. As an alternative model, we note that B23/nucleophosmin interacts with ribosomes (Grisendi et al., 2006), so that it might act as an adaptor for targeting of SUMO proteases to ribosomal proteins. Consistent with this idea, 60S ribosomal components Rpl26 and RplP0 are coprecipitated from XEEs with xSENP3 in a xB23/nucleophosmin-dependent manner (Fig. 1 C).

The requirement for B23/nucleophosmin in ribosome synthesis has been proposed to include both action as an endonuclease (Savkur and Olson, 1998) and as a chaperone (Szebeni and colleagues, 1999). Our data show that regulation of SUMOylation through SENP3 and SENP5 is another major aspect of B23/nucleophosmin function in this process. The capacity of xB23/nucleophosmin to link xSENP3 with ribosomes suggests that it may recruit SUMO proteases to maturing ribosomal particles. Interestingly, B23/nucleophosmin has been implicated in a variety of apparently unrelated cellular processes (Grisendi et al., 2006), many of which are also dependent on the SUMO conjugation (Hay, 2005). We therefore speculate that altered SUMOylation may be a simple, unifying mechanism that underlies much of the apparent plurality of B23/nucleophosmin functions.

Materials and methods

Chemicals and reagents

Human testis QUICK-Clone cDNA, human multiple tissue cDNAs, pEGFP, and pDsRed were obtained from Clontech Laboratories, Inc. Oligofectamine, Rabbit IgG tricolor labeling kit, and genetin were obtained from Invitrogen. siRNA oligonucleotides (SEN1, AACTCTCTCCAGACAGITTTT; SENP2, AACTGCTGAAAACCCTGGTAAAT; SENP3, AACTCTCAGTACCAAGGGTTAT; SENP5, AAGTCCACTGTCTCTCATT; control, AACTCTCAGACACGATTGAT; SENP3, AAGTCCACTGTCTCTCATT; control, AACTCTCAGACACGATTGAT; SENP5, AAGTCCACTGTCTCTCATT; control, AACTCTCAGACACGATTGAT; SENP3, AAGTCCACTGTCTCTCATT; control, AACTCTCAGACACGATTGAT) were obtained from Qiagen. NHS-Sepharose, protein A-Sepharose, and HRP-conjugated secondary antibodies were obtained from GE Healthcare. Mouse monoclonal anti–FLAG antibody were obtained from Sigma-Aldrich unless otherwise stated.

cDNA constructs

Human SENP3 and SENP5 complete coding sequences were amplified from a testis cDNA library and subcloned into the BamHI-Sall sites of the BamHI–Sall sites of pET28a. Recombinant fusions encoded by these cDNAs were expressed in E. coli (BL21[DE3]) and purified over Ni-NTA agarose beads. Polyclonal antibodies against human SENP3 and SENP5 and against xSENP3 were generated in rabbits (Covance). Anti-xSENP3/nucleophosmin antibodies were generated in rabbits (Pacific Biotechnology, Inc.). Anti-senP3 and anti-senP5 antibodies were purified using the antigens coupled to NHS-activated Sepharose 4 Fast Flow beads (GE Healthcare).

Cell culture, stable cell lines, and siRNA treatment

Hela and U2OS cells were grown at 37°C in a humidified atmosphere of 5% CO2 in DMEM supplemented with 2 mM glutamine, 10% fetal bovine serum, and antibiotics. U2OS cells were transfected with plasmids expressing His-SUMO-1 or SUMO-2/3 or using Effectene reagent, and stable cell lines were selected and maintained with medium containing 0.5 μg/ml geneticin. siRNAs were transfected into HeLa cells using Oligofectamine.

Detection of SUMO-modified substrates

Analysis of SUMOylated substrates (Fig. 5) was performed as described previously (Rodriguez et al., 1999). In brief, U2OS-derived cells stably expressing His-tagged full-length SUMO-1 (His-SUMO1F) or SUMO-2 (His-SUMO2F) were depleted of SENP3 and SENP5 by siRNA. 72 h later, the cells were harvested, lysed in lysis buffer (6 μM guanidine-HCl, 0.1 M Na2HPO4/NaH2PO4, 0.01 M Tris-HCl, pH 8.0, and 10 mM β-mercaptoethanol), plus 5 mM imidazole, total cell lysates were mixed with 50 μl of (Ni-NTA) agarose beads and incubated for 2 h at 23°C. The beads were washed with lysis buffer, followed by buffer A (8 M Urea, 0.1 M Na2HPO4/NaH2PO4, 0.01 M Tris-HCl, pH 8.0, 10 mM β-mercaptoethanol, and 0.2% Triton X-100). After washing, the beads were eluted with 200 μM imidazole in 5% SDS, 0.15 M Tris-HCl, pH 6.7, 30% glycerol, and 0.72 M β-mercaptoethanol. The eluted fraction was subjected to SDS-PAGE and Western blotting with anti-RPA37A, -GNL2, or -B23/nucleophosmin antibodies.

Interactions of B23/nucleophosmin with SENP3 or SENP5

Interphase XEEs were prepared as described previously (Orjalo et al., 2006). For Fig. 1 B (top), anti-senP3 and -xSENP5 antibodies or rabbit IgG were conjugated to protein A Dynabeads (Invitrogen). The XEE was supplemented with 10 μg/ml each of leupeptin, pepstatin, and chymostatin and with 20 μM Ran-GQ69L (Orjalo et al., 2006) and incubated with antibody-coated beads for 1 h at 23°C. The beads were washed with buffer A (250 mM sucrose, 2.5 mM MgCl2, 1 mM DTT, 100 mM KCl, and 10 mM Heps, pH 7.7) plus 0.00001% digitonin and protease inhibitors and eluted with 25 μl of 1 M glycine, pH 2.3. For Fig. 1 B (bottom), protease inhibitors (2.5 mM MgCl2, 1 mM DTT, 50 mM KCl, and 1 mM Heps, pH 7.7, and 0.00001% digitonin) were included by centrifugation. Mouse anti-SUMO antibody (M2, Sigma-Aldrich), rabbit anti-xB23, non-specific mouse IgG, or non-specific rabbit IgG were added to aliquots of the clarified supernatant and incubated for 1 h on ice before addition of 15 μl of preblocked protein G-Sepharose (GE Healthcare). After 1 h at 4°C with rotation, the beads were pelleted, washed with buffer A plus 0.00001% digitonin, and eluted with 25 μl of 1 M glycine, pH 2.3.

In Fig. 1 C, interphase XEEs were depleted with protein A Dynabeads coupled with anti-xSENP3/nucleophosmin antibodies or mock treated with rabbit IgG-coupled control beads. Resultant XEEs were supplemented with protease inhibitors and 20 μM Ran-GQ69L, and immunoprecipitations with anti-xSENP3 antibody or rabbit IgG were performed as described in the previous paragraph. Bound proteins were eluted with 0.1 M glycine, pH 2.3.

In all cases, equal elution volumes were subjected to SDS-PAGE and Western blotting. In Fig. 1, protein A–HRP was used for visualization of xSENP3. Anti-mouse IgG–HRP was used for FLAG epitope visualization. Anti-rabbit HRP was used for visualization of all other antibodies.

Immunofluorescence microscopy and Western blotting

Cells were grown on LabTek II coverslip-bottomed chambers or glass slide coverslips. 72 h after transfection with siRNAs, cells were fixed for 5 min with 4% formaldehyde in PBS and permeabilized for 5 min with 0.5% Triton X-100 in PBS. The cells were blocked for 1 h in PBS containing 1% bovine serum albumin. After washing with PBS, the cells were incubated with primary antibodies diluted 1:200 in blocking solution for 1 h. The coverslips were washed and incubated for 1 h with Alexa-488-conjugated
goat anti-rabbit or AlexaFluor 568–conjugated goat anti-mouse secondary antibodies (Invitrogen) diluted 1:500 in blocking solution. All the incubations were performed at 23°C. After antibody incubations, the cells were washed twice with PBS and Hoechst 33258 was added to the final washing solution for 1 min to stain the DNA. Coverslips were mounted in Vectashield (Vector Laboratories). Fluorescence images were captured using a confocal microscope (LSM510 META; Carl Zeiss, Inc.) equipped with a 40× Plan Neofluar objective, with confocal microscopy software (SP2 version 3.2; Carl Zeiss, Inc.). Images were analyzed using Photoshop 7.0 (Adobe).

Western blotting was performed using standard protocols (Gallagher et al., 3.2; Carl Zeiss, Inc.). Images were analyzed using Photoshop 7.0 (Adobe).

shield (Vector Laboratories). Fluorescence images were captured using a 6 min to stain the DNA. Coverslips were mounted in Vecta.

ary antibodies (Invitrogen) diluted 1:500 in blocking solution. All the incu-


Figure S1. Quantitation of SUMO redistribution after SENP3 and SENP5 depletion. Cells prepared as in Fig. 2 B were imaged using a confocal microscope (LSM510; Carl Zeiss, Inc.). (A) Line-scans were made for B23/nucleophosmin (red) and SUMO-1 (top, green) or SUMO-2 (bottom, green) along the indicated paths. The intensities for both signals are shown graphically below each image. (B) The localization of B23/nucleophosmin was used to define the boundary of individual nucleoli (B region). The mean intensity within this region was divided by the mean intensity of line-scan regions outside of it (A regions), to obtain a rough measure of the relative enrichment of SUMO paralogues within nucleoli. These measurements represent averages obtained from analysis of 20 nuclei within each sample. Consistent with earlier findings (Ayaydin, F., and M. Dasso. 2004. Mol. Biol. Cell. 15:5208–5218), SUMO-1 levels were slightly higher within nucleoli than in nucleoplasm. Codepletion of SENP3 and SENP5 caused a further twofold enhancement of SUMO-1 concentration. Also consistent with earlier findings (Ayaydin and Dasso, 2004), SUMO-2/3 levels were lower within nucleoli than in nucleoplasm. However, codepletion of SENP3 and SENP5 caused a substantial concentration of SUMO-2/3 within nucleoli, roughly threefold higher than nucleoplasmic levels. (C) Similar analysis was performed using B23/nucleophosmin-depleted cells. Although quantitation of nucleolar SUMO levels was not possible because nucleolar boundaries were not defined in the absence of B23/nucleophosmin, we observed clear accumulation of all paralogues in structures that appear to be nucleoli in a pattern that appears very similar to the SENP3/5-depleted cells.
Figure S2. **SENP3 and SENP5 paralogue preferences and expression patterns.** (A) To examine the activity of GFP-SENP3 and GFP-SENP5 for deconjugation, we prepared a purified, recombinant C-terminal fragment of Ran GTPase-activating protein 1 (RanGAP1; His6-T7-RanGAP1-C2), which contains its primary acceptor site. We incubated this fragment with purified E1 and E2 enzymes plus SUMO-1 or -2 in vitro to obtain monconjugated species containing each paralogue (Mukhopadhyay, D., F. Ayaydin, N. Kolli, S.H. Tan, T. Anan, A. Kametaka, Y. Azuma, K.D. Wilkinson, and M. Dasso. J. Cell Biol. 2006. 174:939–949). Lysates of U2OS cells stably expressing GFP-SENP3 and GFP-SENP5 were subjected to immunoprecipitation with anti-GFP antibodies. After extensive washing, proteins bound to the antibody beads were incubated with the model substrates for the indicated times at room temperature (in minutes). The reactions containing His6-T7-RanGAP1 C2-SUMO-1 model substrates (top) and His6-T7-RanGAP1 C2-SUMO-2 (middle) were immunoblotted with anti-T7 antibodies. The cleavage product (His6-T7-RanGAP1 C2) should appear as a lower band with the indicated mobility. We also blotted each sample with anti-GFP antibodies to confirm equivalent concentrations of the fusion proteins (bottom). In each set of reactions minus (-) indicates a reaction were the model substrate was incubated for 30 min without the addition of beads. (B) Lysates of U2OS cells stably expressing GFP-SENP3 and GFP-SENP5 were subjected to immunoprecipitation with anti-GFP antibodies. After extensive washing, proteins bound to the antibody beads were incubated with HA-tagged vinyl sulfone derivatives of SUMO-1 and -2 (HA-SU1-VS and HA-SU2-VS) for the indicated times at room temperature. VS reagents derived from ubiquitin-like proteins covalently react with the nucleophilic active site residues of their deconjugation enzymes (Mukhopadhyay et al., 2006). The reactions were terminated with SDS sample buffer and analyzed by Western blotting with the indicated antibodies. Long exposures are shown to demonstrate that both fusion proteins showed little reactivity with HA-SU1-VS, if any. (C) SENP3 and SENP5 were amplified by quantitative PCR from a human multiple tissue cDNA library (Clontech Laboratories, Inc.). (D) 5 µl of interphase XEE was incubated with buffer (control) with SUMO-1-vinyl sulfone (S1VS) or SUMO-2-vinyl sulfone (S2VS) for 30 min at 23°C (top). Reactions were terminated with SDS sample buffer and Western blotted with anti-Xenopus SENP3 (xSENP3) antibodies.
**Figure S3. Mislocalization of ribosomal processing factor NVL (nuclear valosin-containing protein-like protein 2) in the absence of SENP3 and SENPS5.** NVL2 is a nucleolar member of the AAA ATPase family that mediates release of DOB1, a DExD/H-box RNA helicase, from 60S preribosomes at a late step in the maturation pathway (Nagahama, M., T. Yamazoe, Y. Hara, K. Tani, A. Tsuji, and M. Tagaya. 2006. Biochem. Biophys. Res. Commun. 346:1075–1082). HeLa cells were transfected with control oligonucleotides (top) or siRNAs directed against SENP3 and SENP5 (bottom). 72 h after transfection, the cells were stained with antibodies against NVL (middle, red) and against SUMO-2/3 (left, green). Cells that showed extensive relocalization of SUMO-2/3 into nucleoli (asterisks) were assumed to be effectively depleted of SENP3 and SENP5, and they are intentionally imaged in juxtaposition to cells within the same culture that were not effectively transfected. In this case, the accumulation of SUMO-2/3 within the nucleoli of SENP3- and SENPS-depleted cells coincides with loss of NVL2 nucleolar accumulation.