SERNP3-mediated De-conjugation of SUMO2/3 from Promyelocytic Leukemia Is Correlated with Accelerated Cell Proliferation under Mild Oxidative Stress*

Received for publication, October 3, 2009, and in revised form, February 19, 2010 Published, JBC Papers in Press, February 24, 2010, DOI 10.1074/jbc.M109.071431

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Small ubiquitin-like modifier (SUMO) 2/3 is known to conjugate to substrates in response to a variety of cellular stresses. However, whether and how SUMO2/3-specific proteases are involved in de-conjugation under cell stress is unclear. Here, we show that low doses of hydrogen peroxide (H2O2) induce an increase of the SENP3 protein, which removes SUMO2/3 from promyelocytic leukemia (PML). Low dose H2O2 causes SENP3 to co-localize with PML bodies and reduces the number of PML bodies in a SENP3-dependent manner. Furthermore, de-conjugation of SUMO2/3 from PML is responsible for the accelerated cell proliferation caused by low dose H2O2. Knocking down PML promotes basal cell proliferation as expected. This can be reversed by reconstitution with wild-type PML but not its mutant lacking SUMOylation, indicating that only the SUMOylated PML can play an inhibitory role for cell proliferation. Thus, SENP3 appears to be a key mediator in mild oxidative stress-induced cell proliferation via regulation of the SUMOylation status of PML. Furthermore, SENP3 is over-accumulated in a variety of primary human cancers including colon adenocarcinoma in which PML is hypo-SUMOylated. These results reveal an important role of SENP3 and the SUMOylation status of PML in the regulation of cell proliferation under oxidative stress.

The conjugation of small ubiquitin-like modifier (SUMO)2 is an important post-translational protein modification. The SUMO family includes SUMO1, SUMO2, and SUMO3. SUMO2 and -3 share only about 46% sequence similarity to SUMO1 but share 96% similarity to each other (1). The majority of SUMO1 is conjugated to substrates. In contrast, SUMO2 and -3, referred to as SUMO2/3, are unconjugated but can rapidly convert to be conjugated in response to a variety of cellular stresses (2–7). The progress of SUMOylation is reversible. De-conjugation or de-SUMOylation reactions are catalyzed by a family of SUMO-specific proteases named SENPs. Among the six kinds of SENPs identified in mammals to date, SENP3, SENP5, and SENP6 have preferences for SUMO2/3 (8–12). Therefore, the conjugation and de-conjugation of SUMO2/3 to a given protein at a given time must be sophisticatedly controlled. However, although the functional study of the SUMO2/3-specific proteases SENP3, SENP5, or SENP6 has recently begun (9, 13–19), the mechanisms by which these proteases work in response to a variety of cellular stresses remain to be elucidated.

Oxidative stress is the most common stress that cells may encounter. An increase in the production of reactive oxygen species (ROS)2 may result not only from oxidants and radiation but also from many extracellular insults that threaten cellular homeostasis, such as changes in temperature, pH, osmotic pressure, oxygen tension, and sugar concentration (20). The extent and duration of ROS increase usually determines the consequences of the cellular adaptive response to the oxidative stress. Although severe oxidative stress causes cell senescence and even cell death, mild oxidative stress paradoxically promotes cell survival and even proliferation, during which global alterations of gene expression and protein post-translational modifications occur (21). We have recently found that SENP3 is rapidly increased in response to a mild oxidative stress, acting as a redox sensor. Moreover, SENP3, acting as an effector, participates in the cellular adaptive response by de-conjugating SUMO2/3 from a number of proteins including p300 (22). It is, therefore, interesting and critical to identify the key substrates for SENP3 and their physiological roles in the cellular behaviors under an oxidative stress context.

Promyelocytic leukemia (PML) protein and its associated nuclear bodies have been intensively studied due to the disruption or alteration of PML nuclear bodies (PML bodies) in several pathogenic conditions, including acute promyelocytic leukemia and viral infections as well as a variety of cellular stresses (23–25). To date, PML has been suggested to play important roles in the control of cell fate as a proapoptotic factor (26, 27).
and as a tumor suppressor (28, 29). Knock-out of PML leads to
the acceleration of cell proliferation (27). On the other hand, it
has been reported that SUMO1 and SUMO2/3 modifications
are required for the stability of PML and its localization in the
nuclear bodies (30, 31). Interestingly, PML has been indicated to
be the first potential substrate for SENP3 (8), and it can also
be regulated by SENP1 (32, 33) and SENP6 (9). We, therefore,
have investigated whether PML is the physiological substrate
for SENP3 under oxidative stress and whether its de-SUMO2/3
is correlated with its role in regulating cell proliferation. We
herein report that PML is modified by SUMO2/3 under the
normal state and that SENP3 removes SUMO2/3 from PML
under mild oxidative stress. The low dose H2O2 can decrease
the number of PML bodies and promote cell proliferation, both
of which depend on the de-conjugation activity of SENP3. We
have also found that SENP3 is accumulated in a variety of pri-
mary human cancers in which an attenuated SUMO2/3 modi-
fication on PML may occur. These results reveal for the first
time the essential role of SENP3-mediated de-SUMOylation of
PML for cell proliferation in response to oxidative stress and its
plausible association with human cancers.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HeLa cells and NIH3T3 were cultured in Dul-
becco’s modified Eagle’s medium (Invitrogen) supplemented
with 10% newborn calf serum (Biochrom AG). Human umbil-
ical vein endothelial cells (HUVEC) were cultured in M200
medium containing the low serum growth supplement (pro-
vided by Cascade, Portland, OR). All media were supplemented
with 100 units/ml penicillin and 100 mg/liter streptomycin.
Cells were maintained at 37 °C in a humidified atmosphere with
5% CO2. Cells were exposed to H2O2 at various doses for dif-
ferent time as indicated.

**Cell Proliferation Assay**—Cells were seeded at 20% conflu-
ence and were treated with H2O2 once a day. Proliferation was
assayed using two approaches for assessing living cell numbers.
Cell viability was first analyzed using the 3-(4,5-dimethylthi-
azol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sango,
China) assay, as previously described (34). Living cell number
was double-checked based on trypan blue exclusion using a
hemocytometer. In addition, to eliminate the possibility that
apoptosis might contribute to the decrease in living cell num-
ber, Hoechst 33342/propidium iodide fluorescence staining
was performed to visualize the apoptotic cells, and the result
excluded apoptosis under the contexts in the present study.
The results of the MTT assay and cell counting were, thus, used
to reflect cell proliferation.

**Bromodeoxyuridine (BrdUrd) Incorporation Assay**—Cell cul-
ture medium was replaced by medium containing 2% serum for
a 24-h culture to synchronize cell proliferation. Cell monol-
yers were exposed to H2O2 for 6 h and then labeled with 30 μM
BrdUrd for 1 h. Cells were then fixed with 4% paraformalde-
hyde for 30 min, permeated in 4 M hydrochloric acid for 15 min,
and blocked with 5% bovine serum albumin for 30 min before
incubation with the mouse monoclonal antibody against
BrdUrd (1:50, Boster Bio-Technology, BM0201) at 4 °C over-
night. Subsequently, the cells were incubated with fluorescent
isothiocyanate-conjugated anti-mouse antibody (DAKO A/S)
for 2 h at room temperature. Nuclei were counterstained with
4,6-diamidino-2-phenylindole (DAPI). Cells were then exam-
inied under an Axioplan 2 fluorescent microscope (Zeiss, Ger-
many), and the BrdUrd incorporation rate was quantified by
automatically counting the BrdUrd-positive cells in DAPI-
stained cells within four random fields (×40) using Zeiss KS400
software (Version 2.2).

**Cell Cycle Analysis**—Cells were exposed to H2O2 for 6 h and
then trypsinized and washed before being fixed by absolute eth-
anol for 1 h at 4 °C. Cells were centrifuged and resuspended
in phosphate-buffered saline containing 50 μg/ml propidium
iodide. RNase was added and incubated for another 3 h at 4 °C.
The intensity of propidium iodide staining of DNA was meas-
ured, and the cell cycle was analyzed by flow cytometry on a
FACSCalibur (Becton Dickson).

**Constructs, Transient and Viral Transfections**—The con-
structs for wild-type SENP3 were prepared as previously
described (8). Various tagged constructs were generated using
standard techniques by cloning the full-length cDNA into the
pcDNA3-tag vectors. The RGS-His (RH)- and HA-tagged PML
constructs encoded PML isoform IV. The HA-tagged PML
mutant construct designed to lack all three SUMOylation sites
(PML-SM) was made by site-directed mutagenesis based on the
RH-PML wild-type construct (WT) using a QuikChange
mutagenesis kit (Stratagene, La Jolla, CA). The three sites of
mutation (K65R, K160R, K490R) were selected according to a
previous study (35). siRNA-insensitive PMLs were generated by
mutagenesis based on the cloned HA-PML WT and PML-SM
constructs. The SENP3 viral expression construct was made by
subcloning the conventional pcDNA3-SENP3 into the pTER
vectors (36). SENP3 shRNA viral construct was made by insert-
ing an shRNA oligonucleotide into RNAi-Ready pSIREN-
RetroQ (Clontech Laboratories, Inc., Mountain View, CA). The
PML viral constructs were made by subcloning into the pLXSN
vectors (37) to form pLXSN-PML WT and pLXSN-PML SM.

The non-viral constructs were transiently transfected or co-
transfected into cells using Lipofectamine 2000 (Invitrogen)
following the manufacturer’s instructions. The viral constructs
were introduced to cells using previously described packaging
cells and related methods (36, 37).

**Reverse Transcription and Quantitative PCR**—Reverse tran-
scription was carried out using the routine method. Quanti-
tative real-time PCR was performed on the ABI Prism 7300 sys-
tem (Applied Biosystems, Foster City, CA) using SYBR Green
and following the manufacturer’s instructions. The primers for
mouse PML were 5’-TCGACAGTACGCCAGTGTTCAATA-3’
and 5’-TCGACAGTACGCCAGTGTTCAATA-3’. The primers
for mouse β-actin were 5’-ACCACTGAGGAGGGATGAG-
AGAAA-3’ and 5’-TAGCACAGCGCCTGATAACAGTA-3’.

**Immunoblotting (IB)**—IB was performed using the routine
methods. The antibodies against pan-PML (Santa Cruz Bio-
technology, Santa Cruz, CA; sc-705 for human cells, sc-5621 for
mouse cells), myc (CellSignaling Technology, Beverly, MA,
#2276), proliferating cell nuclear antigen (ABcam, ab29),
cyclinD1 (Medical and Biological Laboratories, K0062-3S),
SUMO-2/3 (Santa Cruz; sc-26969), RH (Qiagen, 34610), HA
(ABcam, ab9110-100), FLAG (Sigma; F1804), and β-actin
(ABcam, ab6276-100) were used. The antibody against SENP6
SENP3 and PML De-SUMOylation in Cell Proliferation

Mild oxidative stress accelerates cell proliferation in a SENP3-dependent manner. A, three types of cells were treated with the indicated concentrations of H$_2$O$_2$ for 1 h. B, three types of cells were seeded in 24-well plates with 20% confluence and treated with H$_2$O$_2$ once a day from the second day. The concentrations of H$_2$O$_2$ were 10 $\mu$M for HUVEC and NIH3T3 cells and 50 $\mu$M for HeLa cells. Cell proliferation was assessed and is indicated by a $\times$-fold increase in cell number based on the results of MTT and cell counting. The values are the means ± S.D. of three independent experiments. SENP3 protein was analyzed by IB in a parallel setting every day after H$_2$O$_2$ addition for 1 h. C, HUVEC were cultured with 2% serum for 24 h and exposed to 10 $\mu$M H$_2$O$_2$ treatment for 6 h before labeling with 30 $\mu$M BrdUrd for 1 h. The BrdUrd incorporation rate was quantified by automatically counting the BrdUrd-positive cells among DAPI-stained cells within four random fields ($\times$40) using Zeiss KS400 software (upper panel). D, NIH3T3 cells were transfected with the 100 nM oligonucleotides for NS siRNA or SENP3 siRNA for 24 h and then subcultured for an additional 4 days. The efficiency of the SENP3 siRNA was determined by IB (upper left panel). Cell proliferation was assessed (upper right panel). Cells with intact or knocked-down SENP3 were treated with 10 $\mu$M H$_2$O$_2$ for 6 h, and cell cycle analysis was assessed by propidium iodide staining (bottom panel).

(H00026054-M01) was purchased from Abnova (Taiwan, China). The antiserum against other SENPs were prepared using the method similar to that for SENP5 as previously described (8, 22).

Immunoprecipitation (IP), Ni-IDA and Talon Bead Pulldown Assay—IP and pulldown assays were performed with the previously described methods (22) using PML antibody to pull down endogenous PML and Ni-IDA beads or Talon beads to pull down RH-PML. N-Ethylmaleimide at 20 mM was included in the IP buffer to ensure that SUMOylation was conserved during manipulation.

For the IP of endogenous PMLs in colon tissues, after the connective tissue and the necrotic tissue was carefully removed, 0.1 g of each colon carcinoma or the adjacent tissue was homogenized using the above IP buffer and sonicated on ice for 60 s followed by the IP procedure.

Immunofluorescence—Cell monolayers were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and blocked with 5% bovine serum albumin before incubation with various primary antibodies at 4 °C overnight. The antibodies against PML, SENP3, and HA were similar to those for IB. The antibody against fibrillarin was purchased from Abcam (ab5821-100). The second antibodies were fluorescent isothiocyanate-conjugated antibody (DAKO A/S, Denmark) and rhodamine-conjugated antibody (DAKO A/S). Nuclei were stained with DAPI. Cells were then examined under a LSM 510 fluorescent microscope (Zeiss, Germany). PML body number was quantitatively analyzed in 50 cells using Zeiss KS400 Version 2.2 software.

siRNA—siRNA oligonucleotides were synthesized (RIBOBIO). The sequences of the siRNA oligonucleotides for mouse SENP3 were 5'-CUG-GAAAGGUCUACUAAdTdT-3' and 5'-UUUGUAACCUUCCAGdTdT-3'. The sequences for homo-SENP3 were 5'-CAAUAAGGAGCUACUGCUAdTdT-3' and 5'-UAGCAGUCUACUAUUGdTdT-3'. The sequences for mouse pan PMLs were 5'-CGAUUUGUGAGGAUUCAGCAGdCdA-3' and 5'-CUGCUGAUCUCUACCAAUAUCGdCdA-3'. Cells were transfected with siRNA oligonucleotides using Lipofectamine 2000.

SENP3 Stable-expressing Cells, Tumor Xenograft Generation and Examination—HUVEC and HeLa cells were stably transfected with pcDNA3 or SENP3 using electroporation followed by G418 selection.

FIGURE 1. Mild oxidative stress accelerates cell proliferation in a SENP3-dependent manner. A, three types of cells were treated with the indicated concentrations of H$_2$O$_2$ for 1 h. IB was performed as indicated. B, three types of cells were seeded in 24-well plates with 20% confluence and treated with H$_2$O$_2$ once a day from the second day. The concentrations of H$_2$O$_2$ were 10 $\mu$M for HUVEC and NIH3T3 cells and 50 $\mu$M for HeLa cells. Cell proliferation was assessed and is indicated by a $\times$-fold increase in cell number based on the results of MTT and cell counting. The values are the means ± S.D. of three independent experiments. SENP3 protein was analyzed by IB in a parallel setting every day after H$_2$O$_2$ addition for 1 h. C, control. For the fourth day, $p < 0.01$. C, HUVEC were cultured with 2% serum for 24 h and exposed to 10 $\mu$M H$_2$O$_2$ treatment for 6 h before labeling with 30 $\mu$M BrdUrd for 1 h. The BrdUrd incorporation rate was quantified by automatically counting the BrdUrd-positive cells among DAPI-stained cells within four random fields ($\times$40) using Zeiss KS400 software (upper panel). IB was performed after H$_2$O$_2$ treatment for 6 h as indicated (bottom panel). D, NIH3T3 cells were transfected with the 100 nM oligonucleotides for NS siRNA or SENP3 siRNA for 24 h and then subcultured for an additional 4 days. The efficiency of the SENP3 siRNA was determined by IB (upper left panel). Cell proliferation was assessed (upper right panel). Cells with intact or knocked-down SENP3 were treated with 10 $\mu$M H$_2$O$_2$ for 6 h, and cell cycle analysis was assessed by propidium iodide staining (bottom panel).
HeLa cells stably transfected with pcDNA3 or SENP3 were injected subcutaneously into the mice to generate tumor xenografts. Five mice were used for each group. After the tumor xenografts were visible in 3 weeks, the mice were sacrificed following an institution-approved protocol.

Tumors were examined for weight and PML SUMOylation status. A mixture of 100 mg of tumor tissue evenly derived from each xenograft was homogenized as described previously (22). The homogenates were further sonicated on ice for 40 s and centrifuged at 20,000 × g for 30 min at 4 °C. Fifty microliters of supernatant were taken, and IP with anti-PML antibody was performed followed by IB with antibodies against PML and SUMO2/3.

**Tissue Chips and Immunohistochemistry**—Tissue chips containing 198 pieces of surgically dissected cancer and non-cancer specimens derived from 9 types of tissues were provided by Shanghai Biochip Center. Colon carcinoma and the adjacent normal colon tissues were collected from Ren Ji Hospital following an institute-approved protocol. Normal colon tissues were the tissues 3 cm adjacent to the edges of surgical dissections having normal appearance. The tissues were processed into paraformaldehyde-fixed and paraffin-embedded specimens. Sections were incubated overnight at 4 °C with primary antibodies against SENP3 followed by biotinylated secondary antibodies. Immunohistochemical reactions were visualized with the peroxidase-conjugated streptavidin, in which 3,3′-diaminobenzidine was used as a chromogen. Sections were finally counterstained with hematoxylin and then examined under an Axioplan 2 fluorescent microscope. The ratio of the positive area for SENP3 was quantified by Zeiss KS400 software.

**Reduced Glutathione (GSH) Assay**—Tissues were prepared and analyzed according to the manufacturer’s instructions (Jiancheng...
Bioengineering, Nanjing, China). Briefly, the frozen tissues were sonicated on ice, and the homogenates were mixed with the kit-provided reagents and then centrifuged at 4000 \( \times \) g for 10 min. Five hundred microliters of supernatant were mixed sequentially with the kit-provided reagents and incubated at room temperature for 5 min before being read at \( A_{420} \). A relative GSH level was determined by comparison of \( A_{420} \) absorbance with a standard curve and normalization by the total protein contents.

**Statistical Analysis**—SPSS11.5 software was used for statistical analysis. Analysis of variance was applied for single- or multifactor variance analysis. A Student-Newman-Keuls test was used for the comparison of mean. A value of \( p < 0.05 \) was considered significant.

**RESULTS**

**Mild Oxidative Stress Accelerates Cell Proliferation in a SENP3-dependent Manner**—When non-tumor cells (HUVEC and NIH3T3) and a tumor cell line (HeLa) were exposed to the various low concentrations of \( H_2O_2 \), SENP3 protein levels rapidly increased in a dose-dependent manner (Fig. 1A). These identical low doses of \( H_2O_2 \) promoted the proliferation of these cells in 1 day (Fig. 1B). The increase of cell proliferation was confirmed by an increased percentage of BrdUrd-incorporated cells as well as up-regulated proliferating cell nuclear antigen (PCNA) and cyclinD1 proteins (Fig. 1C). Interestingly, when SENP3 was knocked down by siRNA, basal cell proliferation was markedly repressed, and \( H_2O_2 \)-mediated cell proliferation could be completely blocked (Fig. 1D, upper). Cell cycle analysis revealed that \( H_2O_2 \) caused a shift from G1 phase to S phase, which relied on endogenous SENP3 (Fig. 1D, bottom). These results strongly indicate that low \( H_2O_2 \)-stimulated cell proliferation is dependent on SENP3.

**Mild Oxidative Stress Decreases the PML Bodies via SENP3**—Because PML is reported to be predominantly modified by SUMO3 in the nuclear or PML bodies (31) and is also indicated as the first potential substrate for SENP3 (8), we investigated whether the \( H_2O_2 \)-induced increase of SENP3 could affect the PML bodies.

![FIGURE 3. SENP3 de-conjugates SUMO2/3 from PML under mild oxidative stress. A, NIH3T3 cells were transfected with RH-PML. After transfection for 48 h and then the addition of 10 \( \mu \)M \( H_2O_2 \) for 1 h as indicated, cells were lysed, and pulldown assay was performed with Talon beads. IB was performed as indicated. B, HUVEC were treated with 10 \( \mu \)M \( H_2O_2 \) for 1 h as indicated. Immunofluorescence was performed with PML and SENP3 antibodies. Cell nuclei were stained with DAPI. Scale bar, 5 \( \mu \)m (left panel). Quantitative data show the percentage of PML bodies that co-localized with SENP3 versus all PML bodies counted in 30 cells in each group (right panel). C, NIH3T3 cells were transfected with RH-PML alone or plus Myc-SENP3 or Myc-SENP3 mutant as indicated for 48 h. Nickel bead pulldown assays and IB were performed as indicated. D, after transfection of SENP3 siRNA or NS siRNA for 72 h and then the addition of 10 \( \mu \)M \( H_2O_2 \) for 1 h as indicated, IP was performed with PML antibody, and IB was performed as indicated in NIH3T3 cells.](https://example.com/figure3.png)
Normally, SENP3 resided in the nucleoli, as it colocalized with fibrillarin, a nucleolus marker (Fig. 2A, upper), whereas PML existed in PML bodies, which were perinucleolar (Fig. 2A, bottom). We found that the number of PML bodies was markedly decreased when HUVEC were exposed to low doses of H₂O₂ (Fig. 2B). Likewise, the number of PML bodies was decreased in HUVEC stably overexpressing SENP3 (Fig. 2C). In contrast, when endogenous SENP3 was knocked down, the number of PML bodies was dramatically increased and could no longer be decreased by H₂O₂ treatment (Fig. 2D).

SENP3 De-conjugates SUMO2/3 under Mild Oxidative Stress—We then examined whether PML was a substrate for SENP3 under a mild oxidative stress. Pull-down assays showed that PML could physically interact with endogenous SENP3 upon H₂O₂ treatment but not with SENP1, SENP2, SENP5, or SENP6, which also had de-SUMOylation activity toward SUMO2/3 conjugates. And endogenous SUMO2/3 modification of PML was decreased upon H₂O₂ treatment, whereas global SUMO2/3 conjugations were increased (Fig. 3A). Immunofluorescence was then conducted in cells exposed to H₂O₂ treatment to visualize the spatial correlation of endogenous SENP3 with endogenous PML. The results showed that SENP3, which originally resided in the nucleoli, accumulated in the nucleoplasm and in part colocalized with PML bodies upon H₂O₂ treatment, indicating that SENP3 interacted with PML in response to mild oxidative stress (Fig. 3B). Furthermore, the co-IP result based on overexpression of PML and SENP3 showed that SENP3 was able to remove endogenous SUMO2/3 from PML, whereas the SENP3 mutant was not (Fig. 3C). The removal of SUMO2/3 from endogenous PML was executed by H₂O₂, but the de-conjugation was markedly blocked in cells with SENP3 knockdown (Fig. 3D).

**FIGURE 4.** SENP3-mediated de-conjugation of SUMO2/3 from PML is responsible for the accelerated cell proliferation and decreased PML bodies under mild oxidative stress. A, HUVEC that were stably transfected with RH-SENP3/m or pcDNA3 vector were cultured. Cell proliferation was assessed. B, after NIH3T3 cells were transfected with non-specific siRNA or PML siRNA for 24 h, cells were subcultured and treated with H₂O₂ once a day as indicated. Cell proliferation was assessed (left panel). The efficiency of PML siRNA was determined by quantitative PCR (right panel). The differences between the paired values for proliferation at day 4 were statistically assessed (see “Results”). C, after NIH3T3 cells were transfected with PML siRNA for 24 h, cells were subcultured and reintroduced with the viral constructs for WT-PML and SUMOylation mutant (SM)-PML and the empty vector (V) containing HA tags. The viral constructs for SENP3 were simultaneously transfected as indicated. Cell proliferation was assessed 48 h post-viral transfections (bottom panel). IP and IB were performed as indicated (upper panel). D, HUVEC were transfected with HA-PML WT or HA-PML SM for 48 h and then were treated with 10 μM H₂O₂ for additional 1 h as indicated. Immunofluorescence was performed with HA antibody. Cell nuclei were stained with DAPI. Scale bar = 5 μm.
upon H2O2 treatment, global SUMO2/3 conjugation was increased, whereas SUMO2/3 modification of PML was decreased in cells with intact SENP3. These data indicate that SENP3 can specifically de-conjugate SUMO2/3 from PML under mild oxidative stress.

**SENP3-mediated De-conjugation of SUMO2/3 from PML Is Responsible for Accelerated Cell Proliferation and Decreased PML Bodies under Mild Oxidative Stress**—The rate of cell proliferation under the basal condition was higher in cells overexpressing SENP3 but not in cells overexpressing SENP3 mutant that lost de-SUMOylating activity compared with that in control (Fig. 4A). PML was then silenced by siRNA (Fig. 4B, right). PML silencing could also promote cell proliferation (Fig. 4B, left, compare PML siRNA with nonspecific (NS) siRNA at day 4, p < 0.01), confirming that PML normally serves an inhibitory role in cell proliferation. In addition, cell proliferation was enhanced by H2O2 treatment in control cells with nonspecific siRNA (Fig. 4B, left, compare NS siRNA with NS siRNA + H2O2 at day 4, p < 0.01) but was no longer enhanced by H2O2 in cells with PML siRNA (Fig. 4B, left, compare PML siRNA with PML siRNA + H2O2 at day 4, p > 0.05), implying that PML might be involved in the effects of H2O2.

To determine whether the SUMOylation status of PML correlated with its inhibitory role in cell proliferation and whether this might be affected by SENP3, we established a system to compare the phenotypes caused by PML with and without SUMOylation in both SENP3-overexpressing and -depleting contexts. Endogenous PML was knocked down using a siRNA irrelevant to exogenous PML, and then either a PML mutant lacking three sites for SUMOylation or its wild-type counterpart were reintroduced into the cells. The efficiency of PML knockdown was first verified, and the mutation of the SUMOylation sites in PML was confirmed (data not shown). In addition, SENP3 was overexpressed or depleted in some of the samples. Each sample was determined for SENP3 expression, PML expression, and its SUMO2/3 modification state (Fig. 4C, upper). The results of cell proliferation assay showed that in control cells with intact
SENPs (Fig. 4C, bottom, left group), the reintroduction of wild-type PML could reverse the enhancement of cell proliferation caused by PML silencing (compare WT with vector) but PML mutant could not (compare SM with vector). In SENP3-depleted cells, proliferation was largely suppressed regardless of the presence or absence of PML (Fig. 4C, bottom, middle group). In SENP3-overexpressing cells, the reintroduction of wild-type PML could no longer reverse the enhancement of cell proliferation caused by PML silencing (Fig. 4C, bottom, right group). This indicates that only SUMOylated PMLs play an inhibitory role in cell proliferation. After comparing the function of the wild-type PML in SENP3 intact cells with SENP3-overexpressing cells, it is clear that SENP3 abrogates the inhibitory effect of PML on proliferation, further suggesting that the role of SUMOylated PML is overridden by SENP3. Moreover, PML silencing has no additive proliferative effect upon SENP3 overexpression, supporting that SENP3-enhanced proliferative effect is dependent on PML.

We also assessed the correlation of de-SUMOylation of PML with the decrease of the PML bodies under mild oxidative stress by overexpression of wild-type or SUMOylation mutant PML. The results of immunofluorescence demonstrated that wild-type PML was well localized at the PML bodies, and H_2O_2 treatment decreased their number, which was similar to the effect in cells with native PML (Fig. 4D, left; compare with Fig. 2B). However, in cells introduced with mutant PML, PML failed to form normal PML bodies; instead, it appeared as huge patches in the nucleoplasm, and H_2O_2 treatment could not decrease their number (Fig. 4D).

**SENP3-mediated De-conjugation of SUMOylated PML May Be Involved in Tumor Growth**—To clarify the biological relevance of the above findings, we investigated the correlation of SENP3 and PML SUMOylation status with cell proliferation in an in vivo tumor xenograft mouse model in which tumors were derived from HeLa cells stably overexpressing SENP3. These tumors grew faster than controls (Fig. 5A, left). Endogenous PML that was immunoprecipitated from the SENP3-overexpressing tumor tissues showed a remarkably attenuated SUMO2/3 conjugation (Fig. 5A, right), indicating that SENP3-mediated de-SUMO2/3 of PML might promote the proliferation of tumor cells. Because cancers are considered to have increased ROS, to verify whether SENP3 was truly overexpressed in human primary cancers, SENP3 immunohistochemistry was conducted on tissue chips containing cancers and the paired non-cancer tissues. The image quantitative analysis demonstrated that the ratios of SENP3-positive areas were significantly higher in certain types of cancers than in normal tissues, although some paired samples did not display a statistically significant difference, which was likely due to the limited case numbers (Fig. 5B). As colon carcinomas were among the types with the highest ratio of SENP3 expression and they have been known to have the high ROS levels, we further examined the state of SUMO2/3 modification of PML in two freshly collected colon carcinomas that exhibited the typical high expression of SENP3 shown by immunohistochemistry. The SENP3-positive reaction was predominantly localized in the nuclei of the cancerous epithelial cells, with relatively weak expression in the cytoplasm of these cells. However, the adjacent normal epithelial cells were almost devoid of SENP3 expression (Fig. 5C, left). The PML that was immunoprecipitated from the corresponding cancer tissue exhibited attenuated SUMO2/3 modification compared with the adjacent normal tissue, as detected by antibodies against both PML and SUMO2/3 (Fig. 5C, right). Finally, an assay for reduced glutathione (GSH), a major cellular antioxidant, confirmed that colon carcinomas tissues bore lower GSH level, thus undergoing a mild oxidative stress compared with the adjacent normal tissues (Fig. 5D). These imply that SENP3-mediated de-SUMO2/3 of PML might contribute to the development or progression of human cancers.

**DISCUSSION**

The functional activity of PML is dependent on nuclear bodies (38), the assembly of which relies on SUMO conjugation of PML (25, 31, 39–41). The depletion of SUMO1 affects the formation of PML bodies, but it can be compensated by SUMO2 and SUMO3 (40). In contrast, the depletion of SUMO3 leads to a decrease of PML bodies that can not be rescued by SUMO1 or SUMO2 (31). These facts indicate that PML body formation under a normal state requires PML to be SUMO3-modified. The disassembly of the PML bodies may occur under various stress conditions, such as heat shock, heavy metal exposure, virus infection, and DNA damage (39, 42–45). However, how the SUMOylation status of PML changes under these stress conditions, how this change affects the formation and function of PML bodies, and furthermore, what triggers the change have all yet to be elucidated. We report here that under a mild oxidative stress induced by low doses of H_2O_2, SUMO2/3 modification of PML is de-conjugated by SENP3, which is responsible for the H_2O_2-induced decrease of PML bodies. Although a free pool of SUMO2/3 that becomes conjugated to substrates upon cellular stress is believed to exist (2), the present study provides evidence demonstrating that PML is among the targets subjected to a specific de-conjugation of SUMO2/3 under a mild oxidative stress. Kuo et al. (13) have also reported that the silencing of SENP3 results in a halt of cell proliferation under the basal state, and Klein et al. (14) have shown that SENP3 is involved in mitotic progression, although the effect remains to be clarified. The effect of SENP3 might be multifactorial. How-

**FIGURE 5.** SENP3-mediated de-conjugation of SUMOylated PML may be involved in tumor growth. A, HeLa cells stably transfected with pcDNA3, and SENP3 plasmids were injected subcutaneously into nude mice to form the xenografts. After 3 weeks, the mice were sacrificed. The tumor xenografts were dissected (left panel) and weighted (middle panel). The tumor tissues of the xenografts were homogenized, and IP was performed with PML antibody. IB was performed as indicated (right panel). B, immunohistochemistry for SENP3 was performed in the tissue chips. Image quantification shows the means ± S.D. of the percentage of SENP3-positive areas. n = the number of samples. *, p < 0.05. C, immunohistochemistry for SENP3 was performed in two colon adenocarcinomas and adjacent normal colon tissues. The brown staining represents a positive signal (left panel). Scale bar, 10 μm. Two colon adenocarcinomas and adjacent normal colon tissues were homogenized. IP was performed with PML antibody, and IB was performed as indicated (right panel). D, two colon adenocarcinomas and adjacent normal tissues were homogenized, and the GSH level was assessed. Quantification showed the means ± S.D. of the relative GSH levels.
ever, our evidence delineates at least one of the pathway(s) that links SENP with cell proliferation.

SENP3 is a nucleolar protein (11). PML has been shown as the substrate of SENP3 (8), but how SENP3 is able to interact with PML, a non-nucleolar protein, remains unclear. As we demonstrate here, under mild oxidative stress, SENP3 redistributes from the nucleoli to the nucleoplasm and can colocalize with PML. The PML bodies are normally separated from the SUMO proteases, but they undergo an intensive de-SUMOylation upon a mild oxidative stress. This spatial change of SENP3 may also allow for the de-SUMOylation of other nucleoplasmic proteins, which could coordinate the diverse nuclear events necessary for cellular adaptation to oxidative stress.

PML has been known to be a negative regulator for cell proliferation (27) and, thus, plays a role in tumor suppression (28, 29). Based on our findings that SUMO2/3-modified PML is de-conjugated by H2O2 and that silencing of SENP3 may abrogate H2O2–stimulated cell proliferation, we speculate that under mild oxidative stress SENP3 executes the de-conjugation of SUMO2/3-modified PML, which disrupts the PML bodies, resulting in the abrogation of the PML functions that negatively regulate cell proliferation. It is noted that both the mRNA level (data not shown) and total protein of PML are not affected by H2O2 treatment or SENP3, indicating that disassembly of PML bodies after de-conjugation of SUMO2/3 is a key factor in the event. Likewise, in cancer the role of PML in tumor suppression may be overridden through a similar mechanism by SENP3. We report for the first time that SENP3 is over-accumulated in a variety of primary human cancers and that an attenuation of the SUMO2/3 modification of PML concomitantly exists in some of these cancers. This suggests that the SENP3-mediated de-SUMOylation of PML might contribute to the development and progression of human cancer.

Because various environmental carcinogens almost exclusively induce ROS generation and because the ROS level is usually increased in many human cancers, the pathogenic role of ROS in cancer initiation and progression is acquiring increasing attention (21, 46–48). Our data suggest that SENP3 may mediate the pathogenic effects of ROS.

Of note, the doses of H2O2 that simultaneously induce an SENP3 increase, PML de-SUMOylation, and cell proliferation are low in two types of non-tumor cells ( 10 μM) and in HeLa cells (about 50 μM). ROS elevation at these magnitudes may happen during various imbalances of homeostasis and would usually lead to an adaptive and positive cellular response rather than deteriorative or life-threatening outcomes such as proliferation arrest, senescence, or apoptosis. Our finding regarding the PML de-conjugation of SUMO2/3 by SENP3 may represent a typical physiological regulation of PML SUMOylation in normal cells in response to a mild and coincidental oxidative stress. However, cancer cells have increased ROS generation due to diverse intra- or extracellular factors, such as oncogene activation, abnormal metabolism, or hypoxia, and are thus experiencing a constant, substantial, but still non-fatal oxidative stress. SENP3 accumulates in cancer cells and causes inappropriate de-SUMOylation of certain proteins to impair their functions. PML appears to be one of the important substrates in this circumstance. PML silencing has no additive proliferative effect upon SENP3 overexpression, suggesting that the SENP3-mediated proliferative effect is, at least in part, dependent on PML and its de-SUMOylation.

Multiple mechanisms have been proposed to explain the phenomenon that mild oxidative stress leads to accelerated cell proliferation (21). ROS can activate the growth factor receptor-MAPK3-MAPK2-MAPK pathway (49), during which the suppression of phosphatase may be involved (50), and the rise of Ca2+ levels and increased protein phosphorylation also play a role (51–53). Additionally, the activation of NF-κB is responsible for the cell proliferation stimulated by ROS or other oxidative insults (54, 55). Based on our findings in the present study, we speculate that the SENP3-mediated de-conjugation of SUMO2/3-modified substrates plays a critical role in these processes.

REFERENCES

1. Yeh, E. T., Gong, L., and Kamitani, T. (2000) Gene 248, 1–14
2. Saitoh, H., and Hinchey, J. (2000) J. Biol. Chem. 275, 6252–6258
3. Manza, L. L., Codreanu, S. G., Stamer, S. L., Smith, D. L., Wells, K. S., Roberts, R. L., and Liebler, D. C. (2004) Chem. Res. Toxicol. 17, 1706–1715
4. Ting, L., Santoykte, R., Shen, R. F., Teke, E., Wang, G., Yang, D. C., and Chock, P. B. (2006) J. Biol. Chem. 281, 36221–36227
5. Dorval, V., and Fraser, P. E. (2007) Biochim. Biophys. Acta 1773, 694–706
6. Yang, W., Sheng, H., Warner, D. S., and Paschen, W. (2008) Cereb. Blood Flow Metab. 28, 269–279
7. Cimarostro, H., Lindberg, C., Bomholm, S. F., Renn, L. C., and Henley, J. M. (2008) Neuropharmacology 54, 280–289
8. Gong, L., and Yeh, E. T. (2006) J. Biol. Chem. 281, 15869–15877
9. Mukhopadhyay, D., Ayaydin, F., Kolli, N., Tan, S. H., Anan, T., Kametaka, A., Azuma, Y., Wilkinson, K. D., and Dasso, M. (2006) J. Cell Biol. 174, 939–949
10. Lee, J., Lee, Y., Lee, M. J., Park, E., Kang, S. H., Chung, C. H., Lee, K. H., and Kim, K. (2008) Mol. Cell. Biol. 28, 6056–6065
11. Nishida, T., Tanaka, H., and Yasuda, H. (2000) Eur. J. Biochem. 267, 6423–6427
12. Yeh, E. T. (2009) J. Biol. Chem. 284, 8223–8227
13. Kuo, M. L., den Besten, W., Thomas, M. C., and Sherr, C. J. (2008) Cell Cycle 7, 3378–3387
14. Klein, U. R., Haindl, M., Nigg, E. A., and Muller, S. (2009) Mol. Biol. Cell 20, 410–418
15. Haindl, M., Harasim, T., Eick, D., and Muller, S. (2008) EMBO Rep. 9, 273–279
16. Ding, X., Sun, J., Wang, L., Li, G., Shen, Y., Zhou, X., and Chen, W. (2008) Oncol. Rep. 20, 1041–1045
17. Zunino, R., Schauss, A., Rippstein, P., Andrade-Navarro, V., and McBride, H. M. (2007) J. Cell Sci. 120, 1178–1188
18. Di Bacco, A., and Gill, G. (2006) Cell Cycle 5, 2310–2313
19. Di Bacco, A., Ouyang, J., Lee, H. Y., Catic, A., Ploegh, H., and Gill, G. (2006) Mol. Biol. Cell 26, 4489–4498
20. Halliwell, B. (2003) FEBS Lett. 540, 3–6
21. Halliwell, B. (2007) Biochem. J. 401, 1–11
22. Huang, C., Han, Y., Wang, Y., Sun, X., Yan, S., Yeh, E. T., Chen, Y., Cang, H., Li, H., Shi, G., Cheng, J., Tang, X., and Yi, J. (2009) EMBO J. 28, 2748–2762
23. Dellaire, G., and Bazett-Jones, D. P. (2004) BioEssays 26, 963–977
24. Beech, S. J., Lethbridge, K. J., Killnich, N., McGlincy, N., and Leppard, K. N. (2005) Exp. Cell Res. 307, 109–117
25. Salomoni, P., Ferguson, B. J., Wylie, A. H., and Rich, T. (2008) Cell Res. 18, 622–640
26. Quignon, F., De Bels, F., Koken, M., Feunteun, J., Amelisen, J. C., and de Thé, H. (1998) Nat. Genet. 20, 259–265
27. Wang, Z. G., Ruggero, D., Ronchetti, S., Zhong, S., Gabolì, M., Rivi, R., and Pandolfo, P. P. (1998) Nat. Genet. 20, 266–272
28. Le, X. F., Yang, P., and Chang, K. S. (1996) J. Biol. Chem. 271, 130–135
29. Mu, Z. M., Chin, K. V., Liu, J. H., Lozano, G., and Chang, K. S. (1994) Mol. Cell. Biol. 14, 6858–6867
30. Boddy, M. N., Howe, K., Etkin, L. D., Solomon, E., and Freemont, P. S. (1996) Oncogene 13, 971–982
31. Fu, C., Ahmed, K., Ding, X., Lan, J., Yang, Z., Miao, Y., Zhu, Y., Shi, Y., Zhu, J., Huang, H., and Yao, X. (2005) Oncogene 24, 5401–5413
32. Ohbayashi, N., Kawakami, S., Muromoto, R., Togi, S., Ikeda, O., Kamitani, S., Sekine, Y., Honjoh, T., and Matsuda, T. (2008) Biochem. Biophys. Res. Commun. 371, 823–828
33. Gong, L., Millas, S., Maul, G. G., and Yeh, E. T. (2000) J. Biol. Chem. 275, 3355–3359
34. Huang, X. Z., Wang, J., Huang, C., Chen, Y. Y., Shi, G. Y., Hu, Q. S., and Yi, J. (2008) Cancer Biol. Ther. 7, 468–475
35. Shen, T. H., Lin, H. K., Scaglioni, P. P., Yung, T. M., and Pandolfi, P. P. (2006) Mol. Cell 24, 331–339
36. Wang, F., Zhang, Q., Cao, J., Huang, Q., and Zhu, X. (2008) Exp. Cell Res. 314, 213–226
37. Wang, R., Zou, Y., Yuan, Z., Wang, Y., Chen, Y., Mao, Y., Zhu, Z. A., Li, H., Tang, X., Lu, J., and Yi, J. (2009) Anat. Rec. 292, 777–786
38. Salomoni, P., and Pandolfi, P. P. (2002) Cell 108, 165–170
39. Eskiw, C. H., Dellaire, G., Mymryk, J. S., and Bazett-Jones, D. P. (2003) J. Cell Sci. 116, 4455–4466
40. Evdokimov, E., Sharma, P., Lockett, S. J., Lualdi, M., and Kuehn, M. R. (2008) J. Cell Sci. 121, 4106–4113
41. Bernardi, R., Papa, A., and Pandolfi, P. P. (2008) Oncogene 27, 6299–6312
42. Maul, G. G., Yu, E., Ishov, A. M., and Epstein, A. L. (1995) J. Cell. Biochem. 59, 498–513
43. Kamei, H. (1997) Exp. Cell Res. 237, 207–216
44. Ishov, A. M., Sotnikov, A. G., Negorev, D., Vladimirova, O. V., Neff, N., Kamitani, T., Yeh, E. T., Strauss, J. F., 3rd, and Maul, G. G. (1999) J. Cell Biol. 147, 221–234
45. Nefkens, I., Negorev, D. G., Ishov, A. M., Michaelson, J. S., Yeh, E. T., Tanguay, R. M., Müller, W. E., and Maul, G. G. (2003) J. Cell Sci. 116, 513–524
46. Messina, M. I. (1991) Free Radic. Biol. Med. 10, 175–176
47. Galas, D., Skiada, V., and Barbouti, A. (2008) Cancer Lett. 266, 21–29
48. Laviano, A., Meguid, M. M., Preziosa, I., and Rossi Fanelli, F. (2007) Curr. Opin. Clin. Nutr. Metab. Care 10, 449–456
49. Matsuzawa, A., and Ichijo, H. (2008) Biochim. Biophys. Acta 1780, 1325–1336
50. Raingeaud, J., Gupta, S., Rogers, J. S., Dickens, M., Han, J., Ulevitch, R. J., and Davis, R. J. (1995) J. Biol. Chem. 270, 7420–7426
51. Schieven, G. L., Kirihara, J. M., Myers, D. E., Ledbetter, J. A., and Uckun, F. M. (1993) Blood 82, 1212–1220
52. Bianchini, L., Todderud, G., and Grinstein, S. (1993) J. Biol. Chem. 268, 3357–3363
53. Qin, S., Inazu, T., Takata, M., Kurosaki, T., Homma, Y., and Yamamura, H. (1996) Eur. J. Biochem. 236, 443–449
54. Natarajan, R., Fisher, B. J., Jones, D. G., and Fowler, A. A., 3rd (2002) Free Radic. Biol. Med. 33, 962
55. Takada, Y., Mukhopadhyay, A., Kundu, G. C., Mahabeleshwar, G. H., Singh, S., and Aggarwal, B. B. (2003) J. Biol. Chem. 278, 24233–24241