

Original Article

SENP3 regulates DNA damage repair by downregulating RAP80 SUMOylation

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Abstract

Receptor-associated protein 80 (RAP80) is a key component of the BRCA1-A complex and plays an essential role in recruiting this complex to DNA double strand break (DSB) sites to facilitate homologous recombination (HR) repair. The function of RAP80 in DNA damage repair is tightly regulated by post-translational modifications (PTMs). However, the detailed mechanisms by which PTMs modulate RAP80 activity remain to be fully elucidated. Here, we demonstrate that SENP3 dynamically regulates DSB repair by targeting RAP80. Mechanistically, upon DNA damage, RAP80 undergoes SUMOylation and is recruited to DSB sites, promoting subsequent BRCA1 recruitment. SENP3 may facilitate the timely dissociation of RAP80 from DNA damage sites during the later stage, thereby promoting proper repair progression and ensuring timely termination of the DNA damage response. Dysregulation of RAP80 SUMOylation due to *SENP3* depletion impairs HR repair and increases cellular sensitivity to irradiation and chemotherapy drugs. Collectively, our findings identify SENP3 as a key regulator of DNA repair through its dynamic control of RAP80-BRCA1 complex formation and DNA damage repair processes.

Key words RAP80, SENP3, SUMOylation, DSB, homologous recombination

Introduction

Genome integrity and stability are constantly challenged by both intrinsic and extrinsic insults, such as replication stress, oxidative damage, ultraviolet light, ionizing radiation (IR) and various genotoxic reagents, which can ultimately lead to DNA damage. The accumulation of improperly repaired DNA damage will lead to diseases such as cancer and aging pathologies. To counteract DNA damage, human cells have evolved an elaborate mechanism, a tightly regulated DNA damage response (DDR) that detects and repairs DNA lesions. One of the most severe types of DNA damage is double strand breaks (DSBs). Classical non-homologous end-joining (c-NHEJ) and homologous recombination (HR) are the two major DSB repair pathways [1,2]. C-NHEJ is a relatively fast and efficient process active throughout interphase, while HR operates with slower kinetics and functions during the late S and G2 phases [3]. Notably, abnormalities in DSB repair are observed in many types of cancers, and mutations in genes encoding proteins involved in DSB repair are responsible for tumor initiation, progression, and therapy resistance [4,5]. Although many proteins have been characterized to play important functions in these repair

pathways, proteins that fine-tune the repair processes are necessary to be identified to fully understand the mechanisms in the DDR.

When DNA damage occurs and is detected, a series of repair proteins sequentially accumulate at the damaged site and function to induce signal transduction pathways that serve to initiate the subsequent repair of DSBs [6]. Reversible post-translational modifications (PTMs), including acetylation, phosphorylation, methylation and ubiquitination, have been demonstrated to play crucial roles in DDR pathways. In addition to acetylation, phosphorylation, methylation and ubiquitination, which have been relatively well investigated [7], SUMOylation via the small ubiquitin-related modifier (SUMO) system has been recognized to be involved in many biological processes [8,9]. Compared with the aforementioned types of modifications, research on the role of SUMOylation in the dynamic process of DSB repair is relatively insufficient. Deregulation of the SUMO pathway has been found to correlate with various cancers [10]. Therefore, direct targeting of SUMOylation could be helpful for the diagnosis, prognosis, and treatment of cancers. Under DNA damage or replication stress, components of the SUMO pathway, including E2 UBC9 and E3

PIASs, accumulate at DSB sites within the nucleus [11–13], which orchestrate protein-protein interactions on damaged chromosomes and the recruitment of the key DNA repair factors BRCA1 and 53BP1 to DSBs [14].

SUMO-specific protease 3 (SEN3) is a member of the sentrin/SUMO-specific protease family that preferentially removes SUMO2/3 modifications from substrate proteins, thereby regulating SUMOylation homeostasis in cells [15]. Unlike other SENP family members, SEN3 is highly sensitive to cellular stress signals and has been reported to accumulate and become activated under conditions such as oxidative stress, hypoxia, and inflammatory stimulation [15,16]. Nevertheless, how SEN3 coordinates SUMO homeostasis at DSB damage sites and how its deSUMOylation activity impacts specific repair pathways remain incompletely understood.

In the present study, we demonstrate that SEN3 is involved in the late stage of DDR by inhibiting RAP80 SUMOylation. DNA damage-induced BRCA1 recruitment and the choice of DNA repair pathways are fine-tuned by SEN3. These findings suggest that SEN3 is a potential target for overcoming resistance to chemotherapy and radiotherapy.

Materials and Methods

Cell lines

HEK293T, U2OS and HeLa cells (purchased from ATCC, Manassas, USA) were cultured in DMEM (MA0212; MeilunBio, Dalian, China) supplemented with 10% fetal bovine serum (PWL001; MeilunBio) at 37°C with 5% CO₂.

All knockout cell lines used in this study were generated using the CRISPR-Cas9 gene editing approach. For CRISPR/Cas9 knockout of human *SEN3* in HEK293T and HeLa cells, the following two sgRNAs were used: sgSEN3-1: 5'-CGACTCAAGTCAGGTGGAGGG-3' and sgSEN3-2: 5'-CGAGCCATGAGAGCCTTCCGG-3'. A non-targeting sgRNA (5'-GCGAGGTATTCGGCTCCGCG-3') was used as a control. The sgRNA sequences were cloned and inserted into the U6-Cas9 plasmid (a gift from Dr Wensheng Wei at Peking University). The sgRNA/Cas9 expression constructs were transiently transfected into HeLa, U2OS, or HEK293T cell lines. The *SEN3*-knockdown cell lines used in this study were generated using two different shRNA lentiviruses: shSEN3-1: 5'-CCGGCTTGCTCTTCCCACTCACTTCTCGAGAAGTGAGTGGGAAGAGGCAAGTTTTT-3'; and shSEN3-2: 5'-CCGGCTCGCTGACATTCCTGGAAGTCCAGTCCAGTGGGAATGTGACGAGGTTTTT-3'. The control shRNA sequence was 5'-TTCTCCGAACGTGTACGT-3'. All gene knockout cells were validated and confirmed by western blot analysis.

Antibodies

The following antibodies were used in our studies: mouse monoclonal anti-Flag (F3165; Sigma-Aldrich, St Louis, USA), mouse monoclonal anti-HA (H9658; Sigma-Aldrich), rabbit monoclonal anti-RAP80 (14466T; Cell Signaling Technology, Beverly, USA), rabbit monoclonal anti-SEN3 (5591T; Cell Signaling Technology), rabbit polyclonal anti-BRCA1 (BS6423; Bioworld, Bloomington, USA), polyclonal anti-γH2AX (BS4760; Bioworld), mouse monoclonal anti-β-actin (BE3566; EASYBIO, Beijing, China), and anti-α-Tubulin (BE3212-10; EASYBIO).

Immunofluorescence staining

The recruitment of repair proteins to damage sites at different time

points after IR treatment was investigated in U2OS cells and isolated bone marrow cells. Cells were treated with ionizing radiation (10 Gy) and collected after resting for 1 h or 12 h, or bleomycin (2.5 μM, 2 h; HY-17565; MedChemExpress, Shanghai, China) or etoposide (40 μM, 2 h; HY-13629; MedChemExpress). Immunofluorescence staining was then carried out following previously described procedures [17]. Bone marrow cells were adhered to glass slides by a Thermo Scientific™ Cytospin™ 4 Cyto centrifuge (Thermo Fisher Scientific, Waltham, USA). Images were visualized by a confocal laser-scanning microscope (Zeiss LSM710 NLO and DuoScan, Oberkochen, Germany) using a 63× oil objective lens. The number and intensity of nuclear foci were quantified using Imaris 7.6 software (Bitplane, London, UK). Nuclear DNA was stained with DAPI (5 μg/mL).

Histology and immunohistochemistry (IHC)

Spleens excised from mice were fixed in 10% paraformaldehyde overnight and embedded in paraffin. The maximum cross sections (3 μm thick) were picked and stained using primary anti-Ki67 (28074-1-AP; Proteintech, Wuhan, China) and anti-γH2AX antibodies followed by incubation with HRP-conjugated secondary antibodies (RGAR011; Proteintech), and the signal was visualized using DAB. Apoptosis was detected by TdT-mediated dUTP nick-end labeling (TUNEL) using an *in situ* cell death detection kit (C1086; Beyotime, Shanghai, China) following the manufacturer's instructions [18].

Laser-induced DNA damage and live cell imaging

Laser micro-irradiation was performed to observe DSB protein recruitment as previously described [17]. Briefly, HeLa cells were grown in a glass-bottomed petri dish and topically irradiated with a 365-nm pulsed nitrogen laser (16 Hz pulse, 41% laser output) generated from a micropoint system (Andor, Belfast, UK). The time-lapse images were captured every 10 s for the indicated time.

Metaphase spread preparation

Cells were treated with 10 ng/mL nocodazole (M1404-2MG; Sigma-Aldrich) for 12 h and fixed in fixative solution (ice cold 75% methanol). After three washes in fixative solution, the cells were spread on glass slides, steam treated for 5 s, heat dried and stained with mounting media containing DAPI. Spreads were imaged using a Nikon E1000 epifluorescence microscope (Nikon, Tokyo, Japan). At least 30 spreads were analyzed in each experimental group.

Co-immunoprecipitation (Co-IP) and mass spectrometry

Cells were cultured in 10-cm dishes and transfected with the indicated plasmids. After 48 h, the cells were washed with 10 mL of pre-chilled PBS and lysed in 1 mL of NP-40 lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, and 1% NP-40, pH 7.4) supplemented with 10 μL of protease inhibitor cocktail (MF182-10; Mei5 Biotechnology, Beijing, China) for 60 min. The lysates were incubated with 1 μg of antibody or control IgG for 4 h to allow binding to the bait proteins, followed by incubation with 30 μL of protein G beads (20397; Thermo Fisher Scientific) for an additional 3 h. The beads were then washed three times with 1 mL of NP-40 lysis buffer and boiled for 10 min at 96°C in 30 μL of 2× SDS loading buffer. Samples were analyzed by SDS-PAGE and western blot analysis.

For immunoprecipitation coupled with tandem mass spectro-

metry (IP-MS), HEK293T cells were lysed in NP-40 lysis buffer, sonicated, and precleared with protein G beads. The supernatants were incubated with anti-Flag affinity beads at 4°C for 4 h, and bound proteins were eluted with Flag peptide. The eluates were precipitated with TCA and subjected to mass spectrometric analysis.

KEGG pathway enrichment analysis for proteins identified by IP-MS was performed using the Metascape online platform (<https://metascape.org>). The list of candidate interacting proteins was submitted with *Homo sapiens* selected as the reference organism. Enriched pathways were determined through a hypergeometric test with Benjamini-Hochberg correction, and pathways with an adjusted $P < 0.05$ were considered statistically significant.

Denaturing immunoprecipitation (dIP)

Cells were lysed in denaturing lysis buffer (1% SDS and 50 mM Tris-HCl, pH 7.5) and boiled at 95°C for 10 min to disrupt protein-protein interactions. The lysates were then subjected to immunoprecipitation with the indicated antibodies overnight at 4°C. Protein G beads were then added and incubated for an additional 3–4 h. The beads were washed three times with NP-40 lysis buffer, and bound proteins were eluted by boiling in SDS loading buffer and analyzed by western blot analysis.

Cell cycle synchronization by double thymidine block

HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37°C with 5% CO₂. To synchronize cells at the G1/S boundary, they were treated with 2 mM thymidine (HY-N1150; MedChemExpress) for 18 h, released into fresh medium for 8 h, and then subjected to a second thymidine block (2 mM; HY-N1150; MedChemExpress) for an additional 16 h. Cells collected immediately after the second thymidine block were considered synchronized at the G1/S-phase. For S/G2-enriched populations, cells were released from the second thymidine block into fresh medium and harvested at the indicated time points (6 h for S phase and 10 h for S/G2 phase). Asynchronous cells were collected in parallel without synchronization treatment. Synchronization efficiency was verified by immunoblotting of cell cycle markers.

In vitro SUMOylation assay

For *in vitro* SUMOylation, His-tagged RAP80 protein was purified from *E. coli*, diluted in SUMOylation buffer, and immunoprecipitated with Mg-ATP, SUMO E1, SUMO E2 and SUMO 2/3 compounds. The mixture was incubated at 37°C for 1 h, following the operation manual (Abcam, Cambridge, UK). Following the reaction, SUMOylated His-RAP80 was enriched by Ni-NTA pulldown under denaturing conditions to minimize non-covalent interactions. The bound proteins were extensively washed and eluted with SDS loading buffer, then analyzed by SDS-PAGE and western blot analysis.

Neutral comet assay

A neutral comet assay was performed to detect DSB-induced genomic instability as previously described [18]. Cells subjected to radiation (5 Gy, 1 h or 12 h of recovery) were harvested and resuspended in ice-cold PBS. The neutral single-cell agarose gel was put in electrophoresis running buffer to allow DNA helix uncoiling. The amount of damaged DNA was visualized by single-cell agarose gel electrophoresis. Observations were performed under an

epifluorescence microscope (Olympus, Tokyo, Japan). Quantitation was performed on 60–100 nuclei from each gel using Comet Assay IV software (Perceptive Instruments, Suffolk, UK). Olive tail moment was used as the parameter of this assay instead of tail length or tail DNA content.

Proximity ligation assay

Proximity ligation assay (PLA) was performed using the Rabbit PLUS and Mouse MINUS Duolink *in situ* PLA kits (Sigma-Aldrich) according to the manufacturer's protocol. Images were recorded by a Zeiss LSM710 confocal microscope system using a 63× lens. Quantification was performed using ImageJ software.

DNA repair assay

In HR assay, cells were co-transfected with DR-GFP, an I-SceI expression vector, and a DsRed plasmid, all kindly provided by Prof. Jiadong Wang (School of Basic Medical Science, Peking University, Beijing, China). Cells were harvested and washed with 1× PBS 48 h after transfection. Green (EGFP) and red (DsRed) fluorescence was measured by FACS on a FACSVerse instrument (BD Biosciences, San Diego, USA). The ratio of EGFP and DsRed double-positive cells to DsRed-positive cells was taken as the repair efficiency. Samples were analyzed using FlowJo software to determine GFP-positive cells relative to cells expressing DsRed. U2OS-DR-GFP cells that were only transfected with DsRed but lacked I-SceI were considered the negative control (background level of HR). Repair frequencies were normalized to those of control cells.

Cell apoptosis assay

The cells were treated with bleomycin for 48 h. Apoptosis was then assessed using an Annexin V-FITC/7-AAD apoptosis detection kit (MA0428; Meilunbio) according to the manufacturer's instructions. Briefly, cells were resuspended in 1× binding buffer and stained with Annexin V-FITC and 7-AAD for 15 min at room temperature in the dark. The stained cells were analyzed by flow cytometry within 1 h. Late apoptotic cells (Annexin V-FITC positive, 7-AAD positive) and viable cells (double negative) were determined.

Clonogenic survival assay

First, 200–500 cells were seeded in 6-well plates in triplicate. After 48 h, the cells were treated with bleomycin or oxaliplatin (HY-17371; MedChemExpress) for 24 h, followed by two washes with DMEM. After an additional 12 days of culture, the cells were washed with PBS, fixed with pre-cooled methanol at –20°C for 10 min, and stained with crystal violet (0.1%; w/v) for 15 min. The number of colonies was counted, and the survival fraction was calculated by normalizing to the number of colonies in untreated cells.

Radio sensitivity of mouse embryonic fibroblasts and mice

Mouse embryonic fibroblast (MEF) cells isolated from *Senp3*^{+/+} and *Senp3*^{+/–} mice were plated into 6-well plates in triplicate. After 10 h, the plates were exposed to IR (0, 2, 4, or 8 Gy). Survival rates were calculated 12 days after IR. Wild-type, *Senp3*^{flp/flp} C57BL/6 mice, CMV-Cre C57BL/6 mice and *Senp3* conditional knockout C57BL/6 mice in bone marrow (*Senp3* cKO), all kindly provided by Prof. Jing Yi (College of Basic Medical Sciences, Shanghai Jiao Tong University, Shanghai, China), were housed in specific pathogen-

free barrier facilities. All experiments were approved by the IACUC of the Center for Experimental Animal Research (China) and Peking University Laboratory Animal Center (IACUC No. LSCZhengX-2-1) and performed in accordance with the 'Guide for the Care and Use of Laboratory Animals'.

Statistical analysis

The exact number of biological replicates (n) for each experiment is specified in the corresponding figure legends. All results are presented as the mean $\pm$ SEM unless otherwise stated. P values were obtained via Student's t test (two-tailed) or one-way ANOVA using GraphPad Prism 5.0 software. Survival curves were obtained from Kaplan-Meier estimates and validated with the log-rank test. $P < 0.05$ indicated statistical significance.

Results

SENP3 safeguards genome stability

Senp3-knockout mice were generated using homologous recombination. Crossbreeding of heterozygous mice yielded heterozygous and wild-type (WT) offspring at an approximate 2:1 ratio, whereas no homozygous *Senp3*^{-/-} mice were obtained (Figure 1A,B). This non-Mendelian segregation pattern indicates embryonic lethality resulting from homozygous *Senp3* deletion, suggesting that SENP3 plays a critical role in normal growth and development. Next, clonogenic survival assays were performed using primary cultured MEF cells isolated from pregnant *Senp3*^{+/+} and *Senp3*^{+/-} mice. Compared with MEF cells from *Senp3*^{+/+} mice, MEF cells with the *Senp3*^{+/-} genotype exhibited increased sensitivity to IR (Figure 1C). Moreover, *Senp3*^{+/-} mice showed decreased cell proliferation, as indicated by a decrease in Ki67-positive cells, along with elevated levels of γ H2AX (phosphorylation of histone H2AX at Ser 139), a well-established marker of DNA DSBs (Figure 1D). These findings suggest that *SENP3* deficiency results in increased DNA damage accumulation.

Furthermore, we constructed *SENP3*-knockout (*SENP3*^{-/-}) HEK293T cells using the CRISPR-Cas9 system, and the depletion of *SENP3* was confirmed by DNA sequencing (Figure 1E). *SENP3* WT and *SENP3*^{-/-} HEK293T cells were treated with or without 5 Gy IR, and then neutral comet assays were performed to assess the role of SENP3 in maintaining genome stability. Compared with untreated controls, both WT and *SENP3*^{-/-} cells exhibited a marked increase in comet tail length at 1 h post-IR, confirming effective induction of DNA damage and genome instability. Notably, under basal conditions and after a 12-h recovery following IR exposure, *SENP3*^{-/-} cells displayed longer comet tail lengths than WT cells (Figure 1F), indicating compromised genome stability upon *SENP3* depletion. In agreement with the results of the neutral comet assay, metaphase analysis revealed a higher frequency of chromosomal breaks and fusion in myeloid cells isolated from myeloid-specific *Senp3*-knockout mice (*Senp3*^{fllox/fllox} *Lyz2*-Cre) (Figure 1G). Taken together, these data demonstrate that SENP3 plays an essential role in maintaining genomic integrity.

SENP3 is recruited to DSB sites and interacts with RAP80

To explore whether SENP3 is involved in the DDR, we employed a laser micro-irradiation system to generate localized DNA damage in cells. Endogenous SENP3 was observed to co-localize with γ H2AX along the laser-induced DNA damage tracks (Figure 2A), indicating recruitment of SENP3 to the damage sites. Furthermore, we

assessed the subcellular localization of SENP3 by immunofluorescence staining in HeLa cells treated with the DNA damaging reagents bleomycin (2.5 μ M) and etoposide (40 μ M). In cells exposed to either agent, SENP3 predominantly formed discrete foci that co-localized with γ H2AX foci (Figure 2B). These findings indicate that SENP3 is recruited to DSB damage sites in response to DNA damage.

To elucidate the mechanism by which SENP3 regulates the DDR, we performed IP-MS to identify potential SENP3-interacting proteins in cells. The results revealed that SENP3 is likely associated with several proteins known to function in DNA repair, including the BRCA1-A complex subunit RAP80 and BRCA1-associated ATM activator 1 (BRAT1) (Figure 2C). To further investigate the functional relevance of these interactions, KEGG pathway enrichment analysis revealed that SENP3 is involved in the pathways of DNA damage repair and cellular stress response (Figure 2D). Co-immunoprecipitation (Co-IP) assay confirmed an interaction between endogenous SENP3 and RAP80 (Figure 2E). Taken together, these results indicate that SENP3 is recruited to DSB sites and interacts with RAP80.

RAP80 is SUMOylated at lysine 52 by SUMO3 and is negatively regulated by SENP3

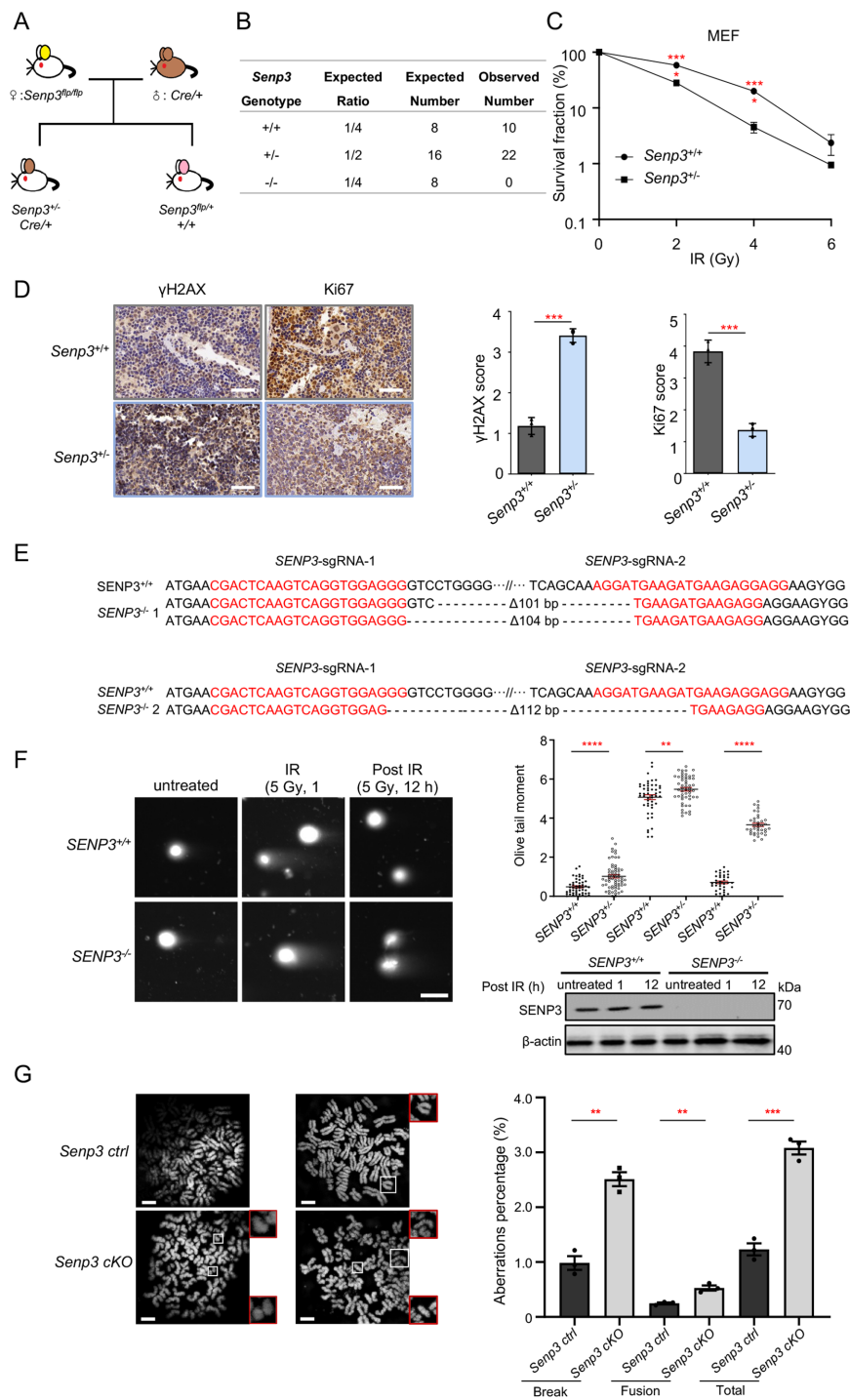
Given that SENP3 is a SUMO-specific protease, we first sought to determine whether RAP80 itself undergoes SUMOylation. Co-IP assay confirmed the SUMOylation of endogenous RAP80 mediated by SUMO3 (Figure 3A). To further validate this modification in a controlled system, GST-tagged RAP80 proteins were expressed and purified from *E. coli* and subjected to an *in vitro* SUMOylation assay, which confirmed that RAP80 could be directly SUMOylated by SUMO3 (Figure 3B).

Next, to identify the specific SUMOylation sites on RAP80, we first predicted the potential SUMOylation sites using SUMOplot™ (<https://www.abcepta.com/sumoplot>) and GPS-SUMO (<https://sumo.biocuckoo.cn/userguide.php>) tools. The full-length RAP80 protein sequence was retrieved and analyzed using both platforms under default settings. SUMOplot™ predicts SUMOylation sites by identifying consensus motif and assigns scores that reflect the probability of SUMO conjugation. In contrast, GPS-SUMO utilizes a position-specific scoring matrix combined with a machine learning-based algorithm to predict both consensus and non-consensus SUMOylation sites. Lysine residues predicted with high confidence by both tools were selected as candidates for further analysis. Lysine 52 was found to reside within a conserved SUMO consensus motif across multiple species (Figure 3C).

We mutated this lysine (lysine 52 substituted with arginine, K52R) and examined its effect on RAP80 SUMOylation by His-SUMO pulldown assays. The results showed that the K52R mutation significantly reduced SUMOylation levels compared to wild-type RAP80 (Figure 3D). Consistent with these findings, DIP assays further confirmed a marked decrease in SUMOylation of the RAP80 K52R mutant (Figure 3E). Importantly, overexpression of SENP3 significantly decreased the SUMOylation level of RAP80 (Figure 3F). Collectively, these findings demonstrate that RAP80 is SUMOylated at lysine 52 and that this modification is negatively regulated by SENP3.

SUMOylation of RAP80 at the lysine 52 residue is critical for its recruitment to DSB sites

To assess the functional significance of RAP80 SUMOylation



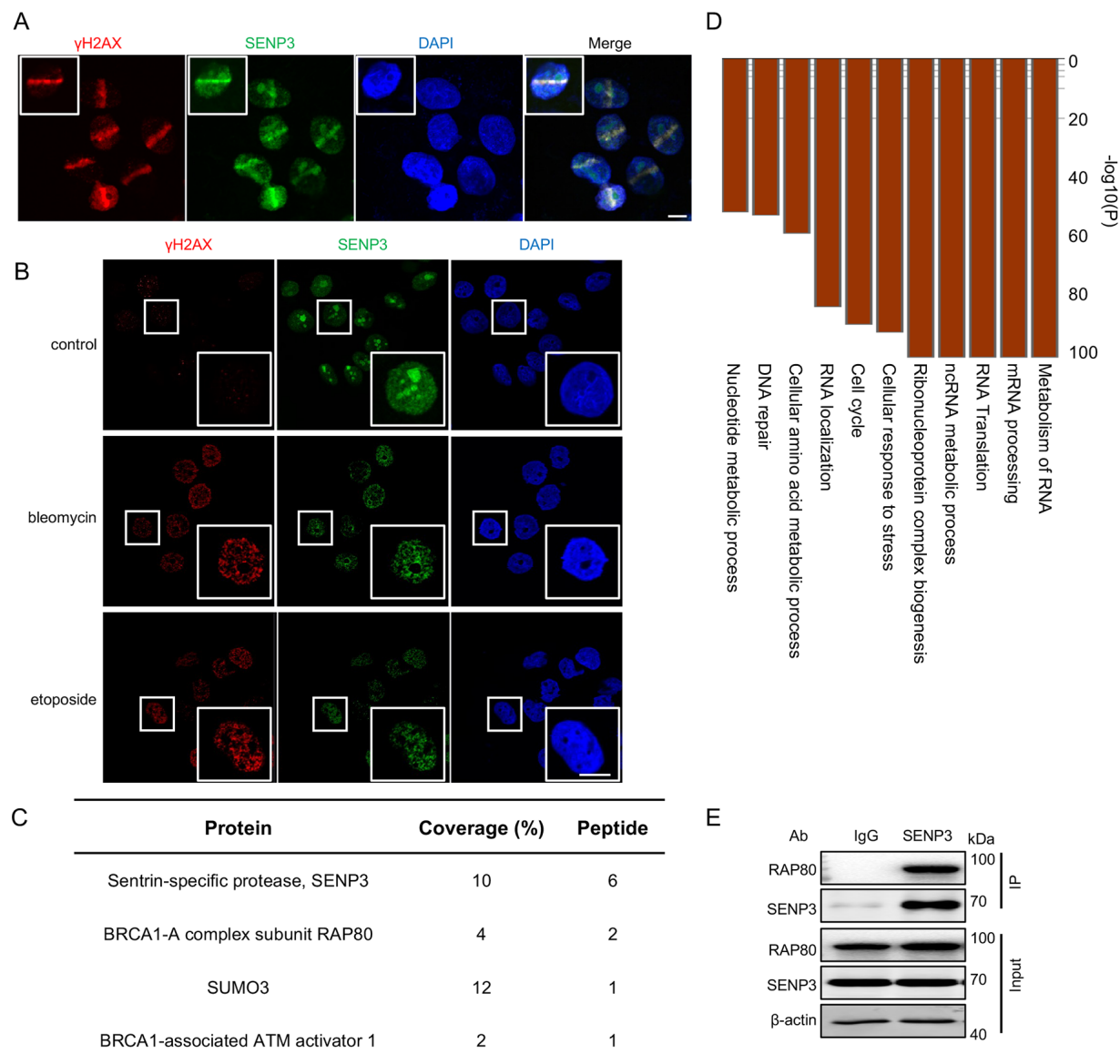


Figure 2. SENP3 is involved in DNA damage repair pathways and interacts with RAP80 (A) HeLa cells were subjected to laser micro-irradiation to generate DSBs in a linear pattern. After 30 min of recovery, recruitment of SENP3 to DSB sites was detected using an anti-SENP3 antibody. Scale bar, 5 μ m. (B) Immunofluorescence analysis of the subcellular localization of SENP3 and γ H2AX foci in HeLa cells treated with bleomycin (2.5 μ M, 2 h) or etoposide (40 μ M, 2 h). Scale bar, 5 μ m. (C) Co-immunoprecipitation and mass spectrometry were performed using HEK-293T cells transfected with Flag-SENP3. Flag-SENP3 was immunopurified with anti-Flag affinity beads and eluted with Flag peptide. The eluates were separated by SDS-PAGE and visualized by silver staining, followed by mass spectrometric analysis. Major proteins identified by mass spectrometry are shown in the [Supplementary Table S1](#). (D) Enriched biological pathway analysis of potential SENP3-interacting targets identified by mass spectrometry. A bar graph of enriched terms across SENP3 targets was generated using Metascape. GO analysis revealed that the interaction partners of SENP3 are enriched in pathways related to the DNA damage response. (E) Immunoprecipitation using a SENP3-specific antibody demonstrated an interaction between endogenous SENP3 and RAP80.

at lysine 52, we detected the recruitment of wild-type RAP80 and the K52R mutant to DSB sites using laser micro-irradiation. Live-cell fluorescence imaging showed a marked reduction in the recruitment of the RAP80 K52R mutant to damage sites (Figure 4A), indicating that SUMOylation of RAP80 is important for its recruitment to DSB sites. GFP-RAP80 was efficiently recruited to laser-induced DNA damage sites in both SENP3-proficient and SENP3-deficient cells. Notably, while the initial recruitment kinetics were comparable, RAP80 displayed markedly prolonged retention and delayed dissociation in SENP3-deficient cells (Figure

4B), indicating that SENP3 is required for efficient release of RAP80 at DSB damaged sites. Consistent with these observations, PLA and subsequent quantitative analysis demonstrated that the K52R mutant exhibited a decreased level of interaction with histone H3 compared to wild-type RAP80 (Figure 4C,D). Additionally, dIP assays revealed an increase in RAP80 SUMOylation following etoposide-induced DNA damage (Figure 4E). Together, these results indicate that SUMOylation of RAP80 at the lysine 52 residue is essential for its efficient recruitment to DSB sites.

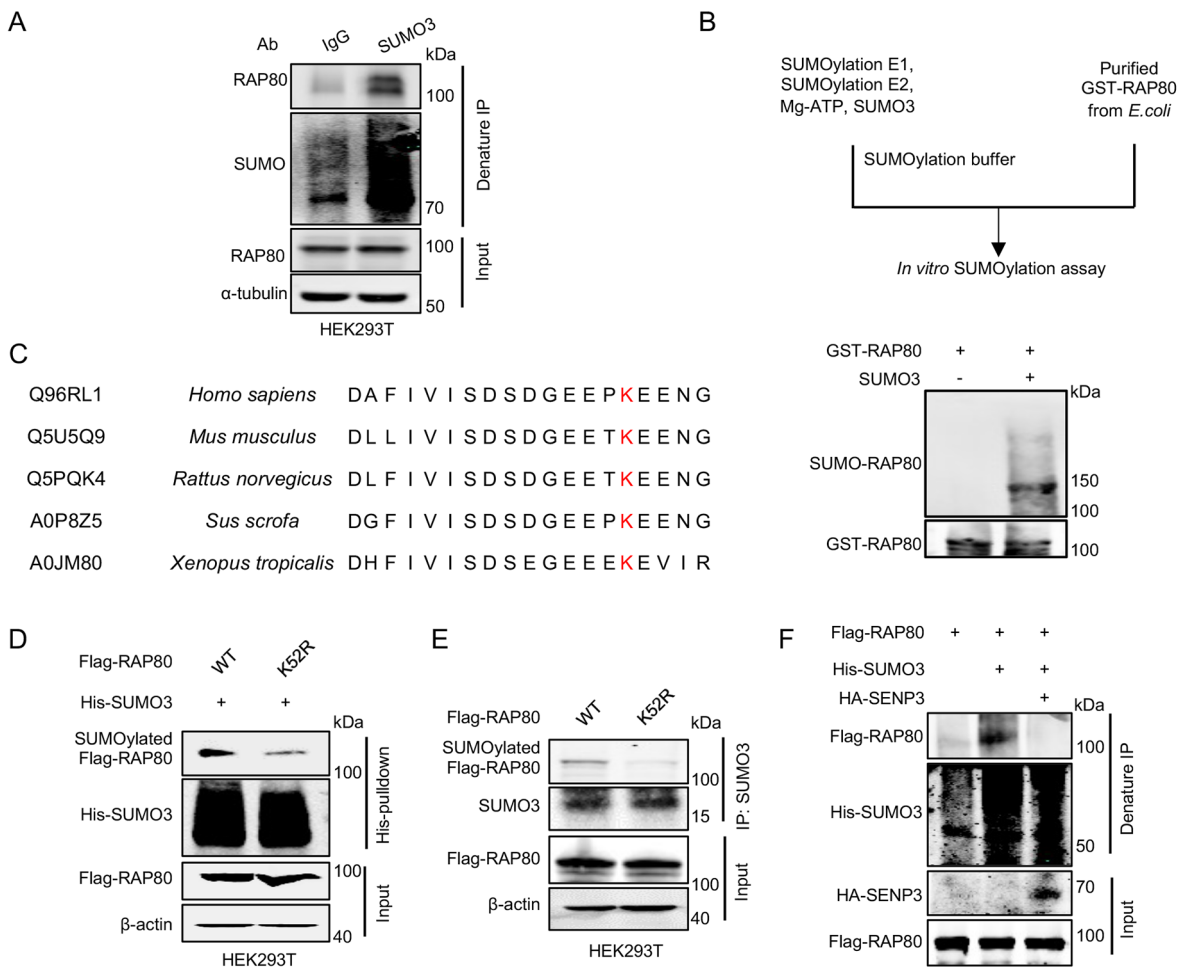


Figure 3. RAP80 undergoes SUMOylation mainly at lysine 52 (A) Denaturing immunoprecipitation was performed using a SUMO3-specific antibody to enrich SUMO3-modified proteins from HEK293T cell lysates, followed by western blot analysis to detect SUMOylated RAP80. IgG was used as a negative control. (B) *In vitro* SUMOylation assays were performed using RAP80 protein purified from *E. coli* to assess RAP80 SUMOylation. (C) Alignment of various RAP80 (UIMC1) sequences from different species as indicated at the consensus SUMOylation site, with the conserved lysine in mammalian species shown in red. (D) HEK293T cells were transfected to overexpress His-SUMO3 together with wild-type RAP80 or the K52R mutant. His-pulldown assays were then performed to assess the SUMOylation of RAP80. (E) Denaturing immunoprecipitation was performed in HEK293T cells using a SUMO3-specific antibody to detect the SUMOylation of wild-type RAP80 or the K52R mutant. IgG was included as a negative control. (F) The effect of SENP3 overexpression on RAP80 SUMOylation was examined by denaturing immunoprecipitation in HEK293T cells.

Lysine 52 SUMOylation of RAP80 contributes to HR repair efficiency

Given that RAP80 is a key component of the BRCA1-A complex, we next investigated whether SUMOylation of RAP80 at lysine 52 influences its interaction with BRCA1. The results of PLA showed that the *in situ* interaction between the RAP80 K52R mutant and BRCA1 was diminished (Figure 5A,B). This finding was further supported by Co-IP assays (Figure 5C). To directly assess the effect of RAP80 SUMOylation on its interaction with BRCA1, purified RAP80 protein was SUMOylated *in vitro* and subsequently used in pulldown assays with BRCA1. These experiments showed that SUMOylation significantly enhanced the protein-protein interaction between RAP80 and BRCA1 (Figure 5D). Because homologous recombination mainly occurs during the S/G2 phases of the cell cycle, we next examined whether the association between SENP3 and RAP80 is cell cycle dependent. Cells were synchronized in G1 or S/G2 phase, and the interaction between SENP3 and RAP80 was

examined by Co-IP. Notably, SENP3 bound RAP80 much more strongly in S/G2-phase cells than in G1-phase cells, indicating that the SENP3-RAP80 interaction is enhanced during HR-permissive phases (Figure 5E).

Given the critical role of RAP80 and the BRCA1-A complex in HR repair, we employed a fluorescence-based reporter assay to quantitatively evaluate HR repair efficiency in cells. The results showed that overexpression of the K52R mutant failed to restore HR repair activity in *RAP80*-deficient cells (Figure 5F). Consistent with these observations, *SENP3* depletion impaired HR repair, and overexpression of a catalytically inactive *SENP3* mutant (catalytically inactive mutant with cysteine 532 substituted by alanine, C532A) was unable to rescue HR efficiency in *SENP3*-deficient cells (Figure 5G). Together, these results demonstrate that SUMOylation of RAP80 is required for efficient assembly of the RAP80-BRCA1-A complex and for the proper functioning of HR repair.

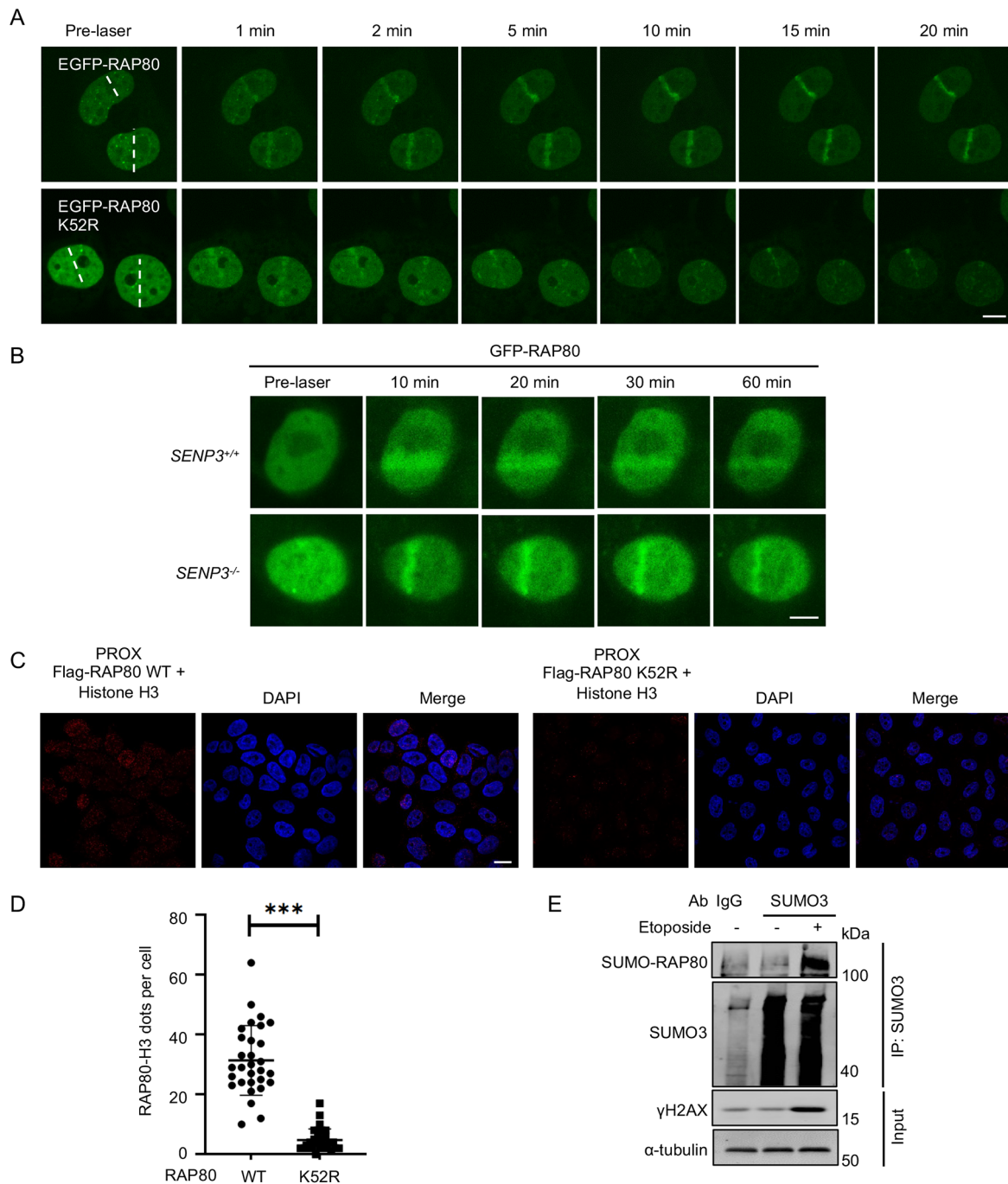
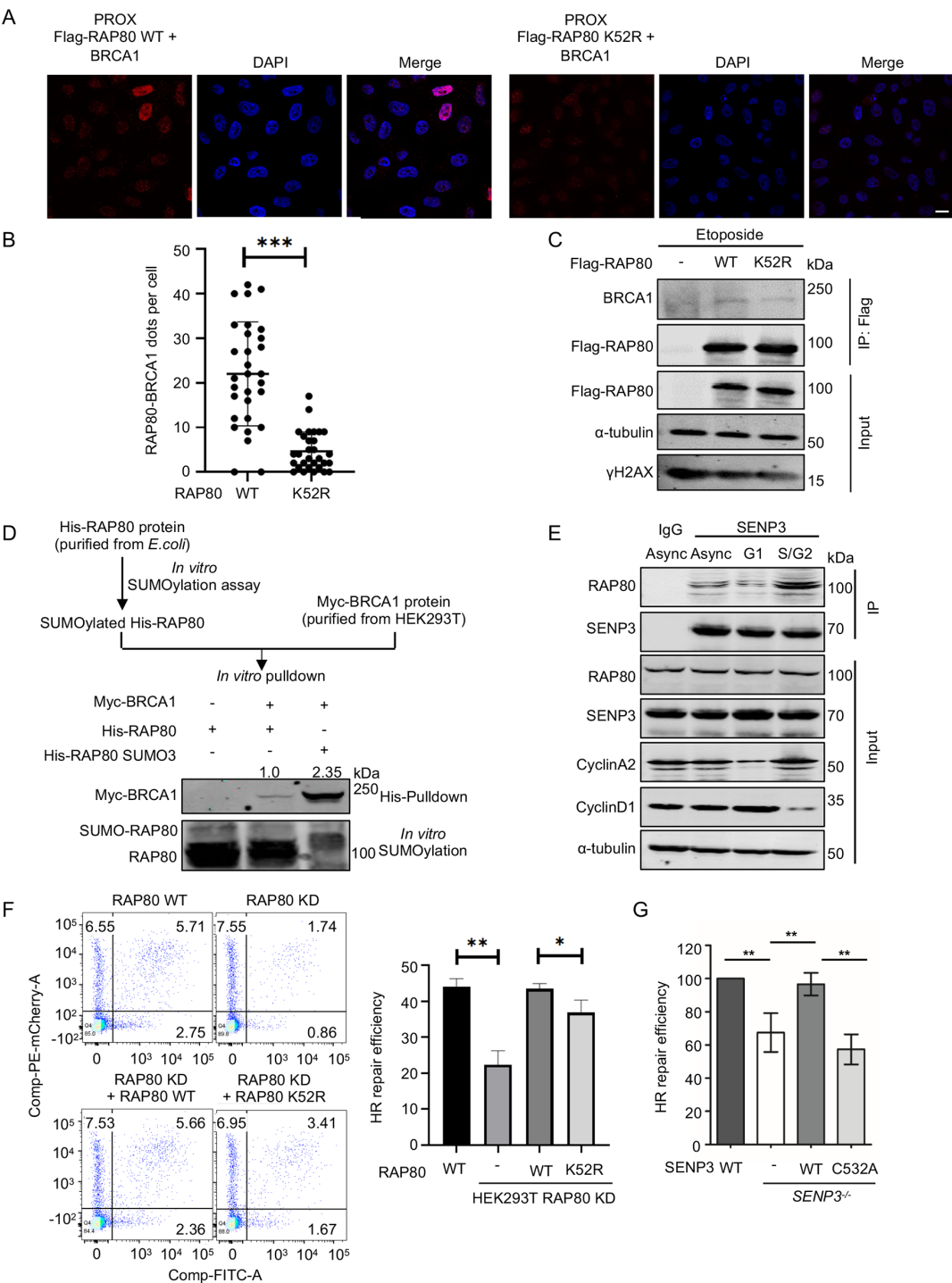


Figure 4. RAP80 SUMOylation is critical for its function in DNA DSB repair (A) HeLa cells transfected with wild-type RAP80 or the K52R mutant were subjected to a laser micro-irradiation assay. GFP fluorescence was monitored by fluorescence microscopy at the indicated time points. Scale bar, 5 μ m. (B) *SENP3*^{+/+} and *SENP3*^{-/-} HEK293T cells transfected with wild-type RAP80 were analyzed using laser micro-irradiation. GFP fluorescence was monitored by fluorescence microscopy at the indicated time points. Scale bar, 5 μ m. (C) PLA was conducted to examine the *in situ* interaction between WT RAP80 or the K52R mutant and histone H3 in HeLa cells. Scale bar, 10 μ m. (D) Quantification of PLA signals per cell from panel (C). Statistical analysis was performed using Student's *t* test (****P* < 0.001) (*n* = 30). (E) SUMOylation of RAP80 was analyzed by denaturing immunoprecipitation using a SUMO3-specific antibody after etoposide treatment (40 μ M, 2 h) in HeLa cells.

SUMOylation of RAP80 at lysine 52 promotes chemotherapy resistance and enhances cell survival

Unrepaired or improperly repaired DSB damage often leads to apoptosis. To further evaluate the role of RAP80 SUMOylation in cellular DNA damage repair, we examined apoptosis in *RAP80*-deficient cells reconstituted with wild-type RAP80 or the K52R

mutant following bleomycin treatment. Flow cytometric analysis and quantification revealed that *RAP80*-deficient cells exhibited increased sensitivity to bleomycin-induced apoptosis. Overexpression of wild-type RAP80 restored resistance to bleomycin, whereas reconstitution with the K52R mutant only partially rescued such resistance (Figure 6A,B). Clonogenic survival assays further



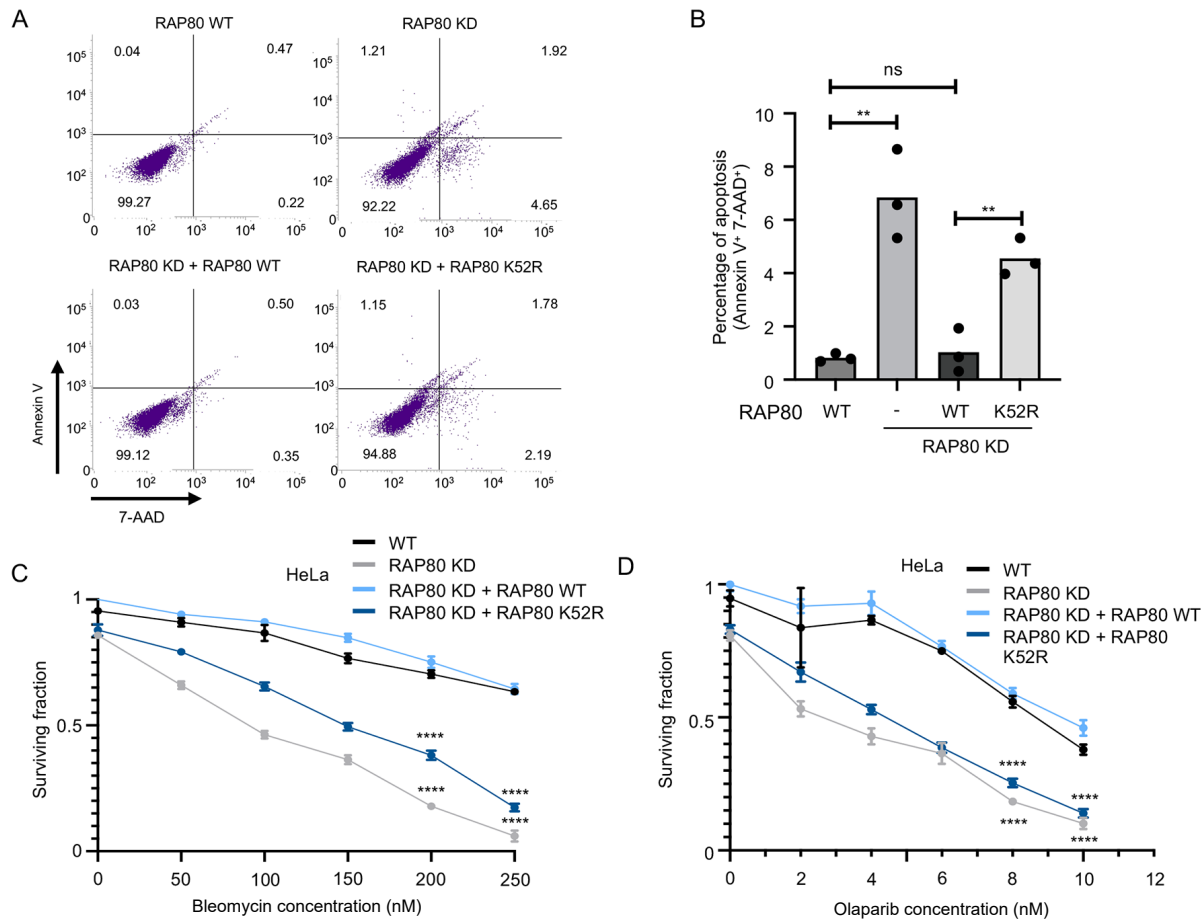


Figure 6. Loss of RAP80 SUMOylation sensitizes cells to DSB-inducing chemotherapeutic agents (A) The effect of RAP80 SUMOylation on apoptosis was examined in WT cells, *RAP80*-deficient cells, and *RAP80*-deficient HeLa cells rescued with either RAP80 WT or K52R mutant. Representative flow cytometry plots are shown for the indicated conditions. (B) Quantification of cell apoptosis based on flow cytometry analysis ($n = 3$). Statistical analysis was performed using Student's t test (** $P < 0.01$; ns, not significant). (C,D) Clonogenic survival assay was performed to examine the effect of *RAP80* depletion and RAP80 SUMOylation on sensitivity to bleomycin and olaparib in WT cells, *RAP80*-deficient cells, and *RAP80*-deficient cells reconstituted with either WT RAP80 or the K52R mutant. Statistical analysis was performed using Student's t test (**** $P < 0.0001$) ($n = 3$).

demonstrated that cells overexpressing the RAP80 K52R mutant exhibited increased sensitivity to both bleomycin (Figure 6C) and olaparib (Figure 6D) compared to those expressing wild-type RAP80. Together, these results indicate that SUMOylation of RAP80 at lysine 52 is required for efficient DNA damage repair and promotes cell survival under genotoxic stress.

Discussion

DSBs pose a severe threat to genome integrity, and their timely and accurate repair relies on highly coordinated networks of PTMs [9,19]. Among these, SUMOylation has emerged as a critical regulatory mechanism that dynamically modulates protein interactions and chromatin-associated signaling during the DNA damage response [19,20]. In this study, we identified RAP80 as a DNA damage-responsive SUMOylated protein and demonstrated that SUMOylation of RAP80 at lysine 52 is essential for its efficient recruitment to DSB sites, assembly of the BRCA1-A complex, and proper HR repair. Our findings highlight a previously unrecognized role for SUMOylation-dependent regulation of RAP80 in maintaining genome stability.

Accumulating evidence indicates that SUMO modification func-

tions as a reversible molecular switch that fine-tunes protein behavior in multiple biological processes, including the DNA damage response [9,21,22]. Consistent with this concept, we demonstrate that RAP80 SUMOylation is dynamically regulated by DNA damage, as etoposide treatment significantly increases the SUMOylated pool of RAP80. Importantly, mutation of lysine 52 markedly impairs RAP80 SUMOylation and compromises its recruitment to DSBs. These results suggest that SUMOylation at K52 promotes the recruitment of RAP80 to DSB sites. Impaired RAP80 SUMOylation likely underlies the observed defects in HR efficiency, increased apoptosis, and heightened sensitivity to DNA-damaging agents such as bleomycin and etoposide. Notably, residual SUMOylation observed in the K52R mutant (Figure 3D) suggests that RAP80 may harbor additional SUMOylation sites, including other lysine residues. Nevertheless, the marked reduction in SUMOylation upon mutation of K52 supports the conclusion that K52 represents a major SUMOylation site in RAP80.

A key finding of this study is the identification of SENP3 as a negative regulator of RAP80 SUMOylation. *SENP3* depletion resulted in increased DNA damage, impaired repair capacity, and reduced genome stability, while *SENP3* overexpression markedly

decreased RAP80 SUMOylation levels. These findings support a model in which SENP3 contributes to SUMOylation homeostasis at DSB sites by counterbalancing damage-induced SUMOylation of RAP80. This dynamic regulation likely ensures both efficient recruitment and timely release of RAP80 during DNA repair, thereby preventing aberrant or prolonged signaling at damaged chromatin. These results are consistent with previous studies demonstrating that SENP family proteases fine-tune SUMO signaling during cellular stress responses and genome maintenance [23,24]. The enhanced IR sensitivity observed in *Senp3*^{+/-} MEFs supports the role of SENP3 in maintaining genome stability. Since *Senp3* knockout results in embryonic lethality, only heterozygous cells could be analyzed. These findings suggest a dosage-dependent requirement for SENP3 in the DNA damage response, wherein reduced SENP3 levels impair SUMOylation dynamics, leading to persistent SUMOylation of RAP80, delayed protein turnover, and compromised homologous recombination. Although we show that SENP3 is recruited to DNA damage sites and co-localizes with γ H2AX, the molecular mechanism underlying its recruitment remains incompletely understood. SENP3 may be recruited through interactions with RAP80 or other DNA damage response factors or indirectly through recognition of SUMOylated substrates that accumulate at sites of DNA damage. Given the dynamic regulation of SUMOylation during the DNA damage response, it is also possible that SENP3 recruitment is temporally regulated and coordinated with local SUMO signaling. Further studies, particularly those employing time-resolved approaches, will be required to elucidate the precise mechanisms and kinetics governing SENP3 recruitment to sites of DNA damage.

Mechanistically, SUMOylation of RAP80 is critical for its interaction with BRCA1 and for maintaining the integrity of the BRCA1-A complex. Both *in vivo* and *in vitro* assays demonstrated that SUMOylation enhances the RAP80-BRCA1 interaction, whereas the K52R mutation weakens this association and disrupts complex assembly. Given the established role of the BRCA1-A complex in modulating the HR repair pathway [25–27], impaired RAP80 SUMOylation underlies the observed defects in HR efficiency, increased apoptosis, and heightened sensitivity to DNA-damaging agents such as bleomycin and etoposide. The relatively modest enhancement observed *in vivo*, compared to *in vitro* assays, may reflect additional layers of regulation [28], including chromatin context, competing interactions, or other PTMs that influence RAP80-BRCA1 binding.

Together, our results support a model in which DNA damage induces SUMOylation of RAP80, thereby promoting its recruitment to DSBs and facilitating assembly of the BRCA1-A complex. This cascade enables efficient homologous recombination repair and promotes cell survival. Conversely, SENP3-mediated deSUMOylation counteracts this process to maintain SUMOylation homeostasis and preserve genome integrity. Disruption of this regulatory balance, whether through loss of SENP3 or impaired RAP80 SUMOylation, leads to defective DNA repair and increased cellular sensitivity to chemotherapeutic agents. These findings highlight the importance of dynamic SUMO modification in the DNA damage response and suggest that dysregulated SUMO homeostasis may contribute to genome instability and affect chemotherapeutic resistance.

Supplementary Data

Supplementary data are available at *Acta Biochimica et Biophysica*

Sinica online.

Data Availability

The mass spectrometry proteomics data generated in this study have been deposited in the iProX database under accession number PXD075308 (<https://www.iprox.cn/page/project.html?id=IPX0015995000>). The complete list of proteins identified by IP-MS analysis is provided in [Supplementary Table S1](#). All other data supporting the findings of this study, including raw data and original images, are available from the corresponding author upon reasonable request.

Author Contributions

J.F., M.W., R.X., and X.Z. designed this study. J.F., M.W., and R.X. performed the experiments, analyzed the data and wrote the manuscript. X.Z. supervised this study and wrote the manuscript.

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Conflict of Interest

The authors declare that they have no conflict of interest.

References

1. Ceccaldi R, Rondonelli B, D'Andrea AD. Repair pathway choices and consequences at the double-strand break. *Trends Cell Biol* 2016, 26: 52–64
2. Shibata A. Regulation of repair pathway choice at two-ended DNA double-strand breaks. *Mutat Res-Fundamental Mol Mech Mutagenesis* 2017, 803–805: 51–55
3. Buisson R, Boisvert JL, Benes CH, Zou L. Distinct but concerted roles of ATR, DNA-PK, and Chk1 in countering replication stress during S phase. *Mol Cell* 2015, 59: 1011–1024
4. Samadder P, Aithal R, Belan O, Krejci L. Cancer TARGETases: DSB repair as a pharmacological target. *Pharmacol Ther* 2016, 161: 111–131
5. Pearl LH, Schierz AC, Ward SE, Al-Lazikani B, Pearl FMG. Therapeutic opportunities within the DNA damage response. *Nat Rev Cancer* 2015, 15: 166–180
6. Morris JR. More modifiers move on DNA damage. *Cancer Res* 2010, 70: 3861–3863
7. Huen MS, Chen J. The DNA damage response pathways: at the crossroad of protein modifications. *Cell Res* 2008, 18: 8–16
8. Bartek J, Hodny Z. SUMO boosts the DNA damage response barrier

- against cancer. *Cancer Cell* 2010, 17: 9–11
9. Flotho A, Melchior F. Sumoylation: a regulatory protein modification in health and disease. *Annu Rev Biochem* 2013, 82: 357–385
 10. Haindl M, Harasim T, Eick D, Muller S. The nucleolar SUMO-specific protease SENP3 reverses SUMO modification of nucleophosmin and is required for rRNA processing. *EMBO Rep* 2008, 9: 273–279
 11. Galanty Y, Belotserkovskaya R, Coates J, Polo S, Miller KM, Jackson SP. Mammalian SUMO E3-ligases PIAS1 and PIAS4 promote responses to DNA double-strand breaks. *Nature* 2009, 462: 935–939
 12. Morris JR, Boutell C, Keppler M, Densham R, Weekes D, Alamshah A, Butler L, *et al.* The SUMO modification pathway is involved in the BRCA1 response to genotoxic stress. *Nature* 2009, 462: 886–890
 13. Driscoll JJ, Pelluru D, Lefkimiatis K, Fulciniti M, Prabhala RH, Greipp PR, Barlogie B, *et al.* The sumoylation pathway is dysregulated in multiple myeloma and is associated with adverse patient outcome. *Blood* 2010, 115: 2827–2834
 14. Fattah F, Lee EH, Weisensel N, Wang Y, Lichter N, Hendrickson EA, Pearson CE. Ku regulates the non-homologous end joining pathway choice of DNA double-strand break repair in human somatic cells. *PLoS Genet* 2010, 6: e1000855
 15. Long X, Zhao B, Lu W, Chen X, Yang X, Huang J, Zhang Y, *et al.* The critical roles of the SUMO-specific protease SENP3 in human diseases and clinical implications. *Front Physiol* 2020, 11: 558220
 16. Huang C, Han Y, Wang Y, Sun X, Yan S, Yeh ETH, Chen Y, *et al.* SENP3 is responsible for HIF-1 transactivation under mild oxidative stress via p300 de-SUMOylation. *EMBO J* 2009, 28: 2748–2762
 17. Xu R, Yu S, Zhu D, Huang X, Xu Y, Lao Y, Tian Y, *et al.* hCINAP regulates the DNA-damage response and mediates the resistance of acute myelocytic leukemia cells to therapy. *Nat Commun* 2019, 10: 3812
 18. Yang C, Zang W, Tang Z, Ji Y, Xu R, Yang Y, Luo A, *et al.* A20/TNFAIP3 regulates the DNA damage response and mediates tumor cell resistance to DNA-damaging therapy. *Cancer Res* 2018, 78: 1069–1082
 19. Jackson SP, Durocher D. Regulation of DNA damage responses by ubiquitin and SUMO. *Mol Cell* 2013, 49: 795–807
 20. Psakhye I, Jentsch S. Protein group modification and synergy in the SUMO pathway as exemplified in DNA repair. *Cell* 2012, 151: 807–820
 21. Gareau JR, Lima CD. The SUMO pathway: emerging mechanisms that shape specificity, conjugation and recognition. *Nat Rev Mol Cell Biol* 2010, 11: 861–871
 22. Hendriks IA, Vertegaal ACO. A comprehensive compilation of SUMO proteomics. *Nat Rev Mol Cell Biol* 2016, 17: 581–595
 23. Zhao X. SUMO-mediated regulation of nuclear functions and signaling processes. *Mol Cell* 2018, 71: 409–418
 24. Nayak A, Müller S. SUMO-specific proteases/isopeptidases: SENPs and beyond. *Genome Biol* 2014, 15: 422
 25. Li S, Tang M, Xiong Y, Feng X, Wang C, Nie L, Huang M, *et al.* Systematic investigation of BRCA1-A, -B, and -C complexes and their functions in DNA damage response and DNA repair. *Oncogene* 2024, 43: 2621–2634
 26. Guzzo CM, Berndsen CE, Zhu J, Gupta V, Datta A, Greenberg RA, Wolberger C, *et al.* RNF4-dependent hybrid SUMO-ubiquitin chains are signals for RAP80 and thereby mediate the recruitment of BRCA1 to sites of DNA damage. *Sci Signal* 2012, 5
 27. Sobhian B, Shao G, Lilli DR, Culhane AC, Moreau LA, Xia B, Livingston DM, *et al.* RAP80 targets BRCA1 to specific ubiquitin structures at DNA damage sites. *Science* 2007, 316: 1198–1202
 28. Jansen NS, Vertegaal ACO. A chain of events: regulating target proteins by SUMO polymers. *Trends Biochem Sci* 2021, 46: 113–123