Supplementary Information for

ATM controls the extent of DNA end resection by eliciting sequential posttranslational modifications of CtIP

Jinhua Han[a,b,1], Li Wan [a,b,1,2], Guixing Jiang[c], Liping Cao[c], Feiyu Xia[d], Tian Tian[a,b], Xiaomei Zhu[d], Mingjie Wu[2c], Michael S.Y. Huen[e,f], Yi Wang[g], Ting Liu[c,d,3], and Jun Huang[a,b,c,3]

[a]The MOE Key Laboratory of Biosystems Homeostasis & Protection, Life Sciences Institute, Zhejiang University, Hangzhou 310058, China
[b]Innovation Center for Cell Signaling Network, Life Sciences Institute, Zhejiang University, Hangzhou 310058, China
[c]Department of General Surgery, Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, Hangzhou 310058, China
[d]Department of Cell Biology, Zhejiang University School of Medicine, Hangzhou 310058, China
[e]Department of Anatomy, The University of Hong Kong, Hong Kong, China
[f]Center for Cancer Research, The University of Hong Kong, Hong Kong, China
[g]Department of Chinese Medicine Science & Engineering, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310058, China
[6]Present address: Mechanisms of Transcription Laboratory, The Francis Crick Institute, 1 Midland Road, London, NW1 1AT, UK

[1]J.H. and L.W. contributed equally to this work.
[2]Present address: Mechanisms of Transcription Laboratory, The Francis Crick Institute, NW1 1AT London, United Kingdom.
[3]To whom correspondence may be addressed. Email: Liuting518@zju.edu.cn or jhuang@zju.edu.cn.

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Supplementary Materials and Methods

Antibodies

Polyclonal anti-RNF4 and anti-Ubc9 were generated by immunizing rabbits with glutathione S-transferase (GST)-RNF4 and GST-Ubc9 fusion proteins expressed and purified from *Escherichia coli* (Hangzhou HuaAn Biotechnology Co., Ltd). Antisera were affinity-purified using the AminoLink Plus immobilization and purification kit (Thermo Fisher Scientific). Anti-CtIP (61141, clone: 14-1, western blot [WB] dilution: 1:1000) and anti-RPA2 (ab2175, immunostaining dilution: 1:2000) antibodies were purchased from Active Motif and Abcam, respectively. Anti-PIAS4 (4329S, WB dilution: 1:1000), anti-p-CHK1 (S317) (2344S, WB dilution: 1:1000) and anti-HA (3724S, WB dilution: 1:1000) antibodies were purchased from Cell Signaling Technology. Anti-MRE11 (GTX70212, WB dilution: 1:1000) and anti-CBX4 (A6221, WB dilution: 1:1000) antibodies were purchased from Gene Tex and ABclonal, respectively. Anti-p-KAP1 (S824) (A300-767A, WB dilution: 1:5000) and anti-p-DNA-PKcs (S2056) (ab18192, WB dilution: 1:2000) antibodies were purchased from Bethyl Laboratories and Abcam, respectively. Anti-GAPDH (MAB374, WB dilution: 1:5000) and anti-H3 (04-928, WB dilution: 1:5000) antibodies were purchased from EMD Millipore. Anti-Flag (M2, WB dilution: 1:5000) antibody was purchased from Sigma-Aldrich. Anti-β-tubulin (M20005S, WB dilution: 1:5000), anti-GFP (M20004M, WB dilution: 1:1000) and anti-Myc (M20002S, WB dilution: 1:5000) antibodies were purchased from Abmart. Anti-His (GNI4110-HS, WB dilution: 1:1000) and anti-ubiquitin (05-944, clone: P4D1-A11, WB dilution: 1:1000) antibodies were purchased from GNI GROUP LTD and EMD Millipore.

RNA interference

The small interfering RNAs used in this study were designed and synthesized by RuiBo (Guangzhou, China). Their sequences are as follows: non-targeted control siRNA: 5′-UUCAAUAAAUUCUUGAGGUUU-3′; CtIP siRNA: 5′-GCUAAAACAGGAACGAAUC dTdT-3′; PIAS4 siRNA#1: 5′- GCUGAAGCCCACCGAAUUAdTdT-3′; PIAS4 siRNA#2: 5′-GCUCUACGGAAAGUACUUAdTdT-3′; CBX4 siRNA: 5′-GACGCAUCGUGAUCGUGAU dTdT-3′; RNF4 siRNA#1: 5′-CUCAGGUACUGUCAGUUGUdTdT-3′; RNF4 siRNA#2: 5′-CCAUCUGCAUGGACGGAUAdTdT-3′; Ubc9 siRNA#1: 5′-UGCGCCAUUCCAGGAAAGA dTdT-3′; and Ubc9 siRNA#2: 5′-GUAAAUUCGAACCACCAUUdTdT-3′. siRNA-resistant wild-type and mutant CtIP plasmids were constructed by substituting eight nucleotides into the CtIP siRNA-targeted region (G132A, A135T, A138G, G141A, A144G, C145A, A147G, and C150T). siRNA transfection was repeated twice at an interval of 24 h according to the manufacturer's instructions using RNAiMAX (13778075; Thermo Fisher Scientific).

Plasmids and transfection

Plasmids expressing HA-Flag-tagged CtIP, HA-Flag-tagged RNF4, SFB-tagged-RNF111, GFP-tagged-CtIP, and Myc-tagged PIAS1/2α/2β/3/4 were generated using Gateway technology (Thermo Fisher Scientific). 3×Flag-tagged CtIP, 10×His-tagged SUMO1/2/3, 10×His-tagged Ub, HA-tagged SUMO2, and 10×His-HA-tagged SUMO2 were cloned into a Lentivirus vector using MultiF Seamless Assembly Mix (ABclonal). Point mutations in CtIP (K578R and E580A), RNF4 (ΔSIM and ΔRING), SUMO2-ΔGG, and Ub-ΔGG were introduced by PCR-based site-directed mutagenesis and verified by sequencing. Transient plasmid transfection was performed using PEI (polyethylenimine, 23966; Polysciences).

Cell culture
Human HEK293T, U2OS, and HeLa cells were purchased from ATCC and cultured in Dulbecco's modified Eagle's medium (Gibco C11995500BT) containing 10% fetal bovine serum (GIBCO 10270-106) and 1% penicillin/streptomycin (Hyclone SV30010) at 37℃ under 5% CO_2. U2OS cells with DR-GFP integration were a kind gift from Dr. Maria Jasin of Memorial Sloan-Kettering Cancer Center. All cells were routinely tested for mycoplasma contamination and maintained under mycoplasma-free conditions.

Lentivirus packaging and infection
To establish stable cell lines expressing His-tagged SUMO1/2/3, His-HA-tagged SUMO2, His-tagged Ub, GFP-tagged CtIP, or Flag-tagged CtIP, lentiviral vectors were produced in HEK293T cells by co-transfection of the lentiviral-based construct with the packaging and envelope plasmids pMD2G and pSPAX2 (kind gifts from Dr. Songyang Zhou, Baylor College of Medicine). Lentiviral supernatants were collected after 48 h and used to infect HeLa or DR-GFP cells using 8 μg/mL Polybrene (Sigma-Aldrich). Stable cell lines were selected on medium containing 2 μg/mL puromycin (Sigma-Aldrich).

Retrovirus production and infection
RNF4 wild-type and ΔSIM mutant were cloned into the Gateway-compatible retroviral destination vector pEF1A-HA-Flag. Retroviruses were produced in HEK293T cells through co-transfection of the retrovirus-expressing plasmid with the packaging plasmid pCL-ECO and envelope plasmid VSV-G. After 48 h, the viral supernatants were collected and used for infection of HeLa cells with 8 μg/mL Polybrene (Sigma-Aldrich). Cells stably expressing HA-Flag-tagged wild-type or ΔSIM mutant RNF4 were established by selection on medium containing 2 μg/mL puromycin (Sigma-Aldrich).

Co-immunoprecipitation and western blotting
Cells were washed with 1× PBS and lysed with NETN buffer (20 mM Tris HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40) containing Benzonase nuclease (EMD Millipore), protease inhibitors (20 mM NaF and 1 μg/mL each of aprotinin and leupeptin), and 2 mM N-Ethylmaleimide (Sigma Aldrich) for 30 min at 4℃. Whole-cell lysates were then clarified by centrifugation at 12,000 rpm for 10 min at 4℃, and the resulting supernatants were collected and incubated with either S-protein agarose beads (EMD Millipore) or protein A–Sepharose beads combined with 1 μg of the indicated antibodies for 2 h at 4℃ with gentle rocking. Subsequently, the resin containing bound proteins was washed three times in NETN buffer, boiled in 2× sodium dodecyl sulfate (SDS) loading buffer, resolved using SDS polyacrylamide gel electrophoresis (SDS-PAGE), and subjected to Western blotting.

Indirect immunofluorescence
To visualize the cellular localization of CtIP, HeLa cells grown on coverslips were treated with 1 μM CPT for 1 h or left untreated. Cells were then washed with phosphate-buffered saline (PBS), fixed in 3% paraformaldehyde for 10 min, and permeabilized in 0.5% Triton X-100 solution for 5 min at room temperature. For RPA2 staining, HeLa cells stably expressing siRNA-resistant Flag-tagged wild-type CtIP or SUMOylation-deficient mutants were transfected with CtIP siRNAs. After 48 h, cells were treated with 1 mM CPT for 1 h, pre-extracted using 0.5% Triton

X-100 solution for 5 min, and fixed with 3% paraformaldehyde for 10 min at room temperature. Primary antibodies were then added to the cells, which were incubated for 20 min at room temperature, followed by incubation with secondary antibodies for 20 min at room temperature. Subsequently, cells were stained with 4′,6-diamidino-2-phenylindole for 1 min at room temperature to visualize nuclear DNA. Images were acquired using the Nikon Eclipse 80i fluorescence microscope equipped with a Plan-Fluor 60× oil-immersion objective (NA 0.5–1.25; Nikon) and a camera (CoolSNAP HQ^2; PHOTOMETRICS). RPA2 foci were analyzed and quantified using ImageJ software. Data were derived from analysis of at least 100 cells in each experiment and are presented as mean ± SEM.

Recombinant protein purification

RNF4 wild-type, ΔSIM, and ΔRING mutants were cloned into the pCold-GST vector and transformed into BL21 *E. coli* for expression of the GST-tagged fusion protein. PIAS4, wild-type CtIP and the K578R and E580A mutants were cloned into the pCold-MBP vector for expression of the MBP-tagged fusion protein in BL21 *E. coli*. Cells were grown at 37℃ to the log phase and then induced using 0.2 mM isopropyl β-d-1-thiogalactopyranoside (IPTG) at 16℃ for 16 h. The cells were harvested and resuspended in lysis buffer (20 mM Tris-HCl [pH 8.0], 300 mM NaCl, 1% Triton X-100, 2 mM DTT, and 1 μg/mL each of leupeptin, aprotinin, and pepstatin). After sonication, the lysates were centrifuged at 12,000 rpm for 40 min. The supernatant was collected and incubated with glutathione–Sepharose resin (Thermo Scientific) or amylose resin (New England Biolabs) for 4 h at 4℃ to purify GST- or MBP-tagged proteins, respectively. The protein-bound beads were washed three times with washing buffer A (20 mM Tris-HCl [pH 8.0], 500 mM NaCl, 0.5% NP-40, 2 mM DTT, and 1 μg/mL each of leupeptin, aprotinin, and pepstatin) and once with washing buffer B (20 mM Tris-HCl [pH 8.0], 50 mM NaCl, 2 mM DTT, and 1 μg/mL each of leupeptin, aprotinin, and pepstatin). GST-tagged proteins were eluted with washing buffer B containing 20 mM reduced glutathione and then subjected to dialysis prior to the *in vitro* ubiquitination assay.

BrdU incorporation assays

HeLa cells stably expressing siRNA-resistant wild-type CtIP or the SUMOylation-deficient mutants were transfected twice with the indicated siRNAs for an internal 24 h. 48 h after the second transfection, 20 μM BrdU was added into the medium for 40 min. Cells were then trypsinized, washed with PBS, and fixed with ice-cold 70% ethanol overnight. Once centrifuged, cells were washed with PBS and the DNA was denatured in 2.5 M HCl for 1 h at room temperature. After washing with PBS, cells were incubated with anti-BrdU antibody (555627, BD PharmingenTM) for 12 h at room temperature. Cells were then wash with blocking buffer (0.1% Triton X-100 and 5% BSA in PBS) containing 500 mM NaCl for three times. Subsequently, cells were incubated with FITC-conjugated goat anti-mouse IgG (115-095-146, Jackson ImmunoResearch Laboratories) for 4 h at room temperature. After washing with blocking buffer containing 500 mM NaCl, cells were resuspended in PBS containing 20 μg/mL propidium iodide (PI) and 100 μg/mL RNase A at 37°C for 20 min. Cell cycle distribution was analyzed on Beckman CytoFlex S.

Colony formation assay

HeLa cells stably expressing siRNA-resistant wild-type CtIP or SUMOylation-deficient mutants were transfected with CtIP siRNAs for 48 h and then seeded into 6-well plates at a density of

5×10^2/well. After 24 h, cells were treated with CPT at the indicated concentrations or IR at the indicated doses. After a further 24 h, the medium containing CPT was replaced with fresh medium, and cells were incubated for an additional 10 days to allow colony formation. The resulting colonies were fixed, stained with Coomassie blue, and counted. Data are presented as means ± standard error of mean (SEM) of three independent experiments.

HR assay

DR-GFP U2OS cells stably expressing siRNA-resistant wild-type CtIP or SUMOylation-deficient mutants were transfected with CtIP siRNAs for 48 h. The cells were then electroporated with 12 μg pCBASce plasmid (an I-SceI expression vector) at 270 V and 975 μF using the BioRad Genepulsar II. At 48 h after electroporation, cells were harvested and subjected to flow cytometric analysis to determine the percentage of GFP-positive cells. HR frequencies are presented as the mean ± SEM of three independent experiments.

Laser micro-irradiation and live-cell imaging

U2OS cells stably expressing siRNA-resistant GFP-tagged wild-type CtIP or SUMOylation-deficient mutants were transfected with CtIP siRNAs for 48 h. The cells were then seeded into 35-mm glass-bottomed dishes. After 24 h, laser micro-irradiation was performed using the Nikon Eclipse Ti-E inverted microscope (60× oil-immersion objective) equipped with the computer-controlled MicroPoint laser ablation system (Photonics Instruments; 365 nm, 20 Hz). Time-lapse images were acquired using MetaMorph software. Mean fluorescence intensity was quantified using ImageJ as the difference between the average fluorescence intensity in micro-irradiated areas versus the average fluorescence intensity from adjacent undamaged areas in the same nuclei. Mean intensity of more than 20 cells for each condition was quantified.

In vivo SUMOylation and ubiquitination assays

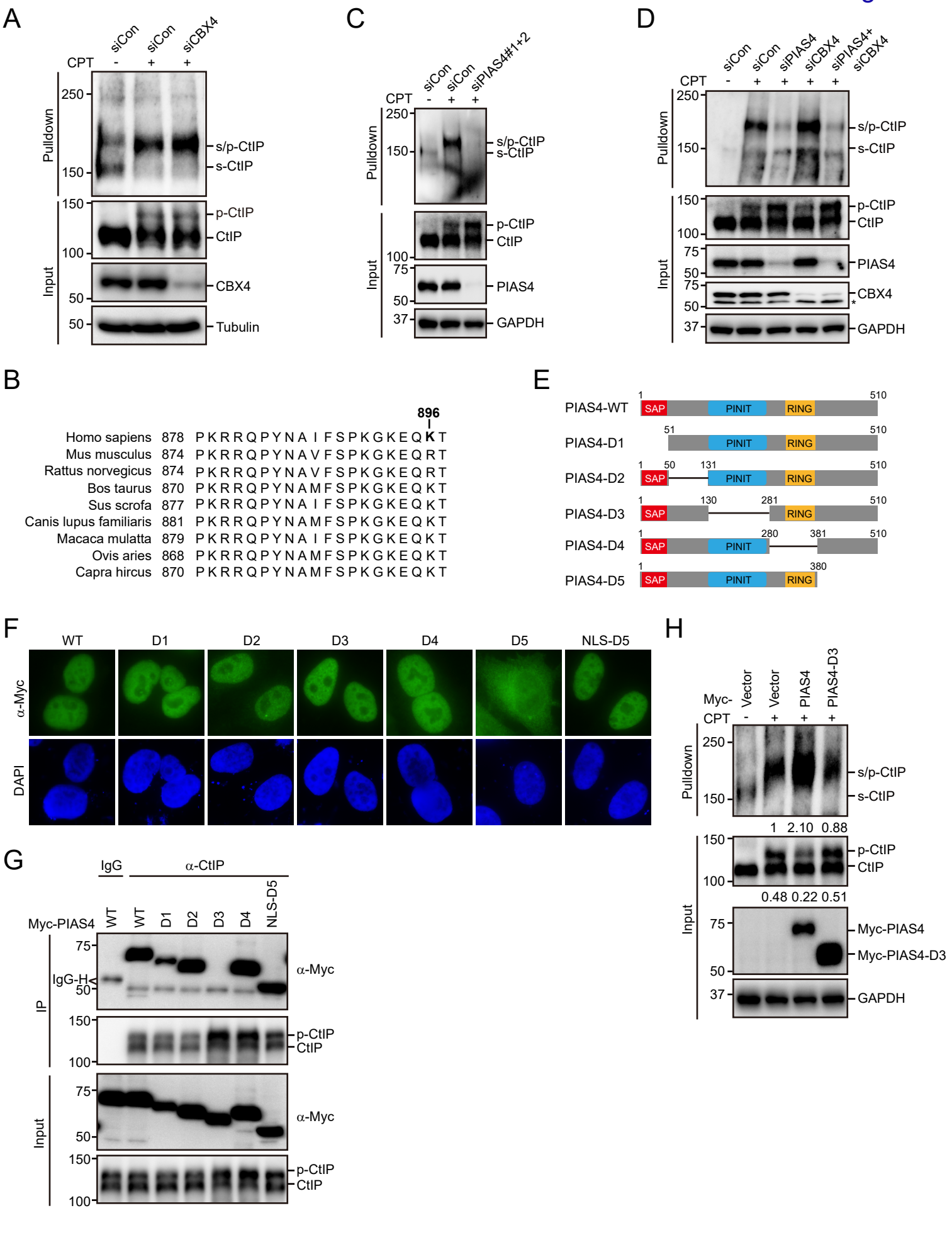
In vivo SUMOylation and ubiquitination assays were conducted using similar techniques. HeLa cells stably expressing His-tagged SUMO/SUMO-ΔGG or Ub/Ub-ΔGG were treated with 1 μM CPT for 1 h or left untreated. For the ubiquitination assay, cells were additionally treated with 10 μM MG132 at 1 h before CPT treatment. The cells were then harvested and lysed in Buffer I (6 M guanidine-HCl, 100 mM NaH_2PO_4/Na_2HPO_4, 10 mM Tris-HCl [pH 8.0], and 5 mM imidazole). After sonication, the lysates were incubated with cobalt resin (Thermo Scientific) at 4℃ overnight. Bound complexes were washed once each in Buffer I, Buffer II (8 M urea, 100 mM NaH_2PO_4/Na_2HPO_4, and 10 mM Tris-HCl [pH 8.0]), Buffer III (8 M urea, 100 mM NaH_2PO_4/Na_2HPO_4, and 10 mM Tris-HCl [pH 6.3]), and Buffer IV (25 mM Tris-HCl [pH 6.8] and 10 mM imidazole). The protein-bound beads were boiled in 2× sodium dodecyl sulfate (SDS) loading buffer supplemented with 250 mM imidazole and then subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE). Western blotting was performed according to standard procedures. For detection of endogenously SUMOylated CtIP, HEK293T cells were left untreated or treated with 1 μM CPT for 1 h. Cells were harvested and lysed in the SDS lysis buffer (150 mM Tris-HCl [pH 8.0], 1% SDS, 30% glycerol), sonicated, and boiled at 100℃ for 10 minutes. Cell lysates were then centrifuged at 12,000 rpm for 10 min at 4℃, and the resulting supernatants were diluted 1:10 in RIPA dilution buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% NP-40, 1 mM (ethylenedinitrilo)tetraacetic acid [EDTA], 0.25% Sodium deoxycholate) containing NEM and protease inhibitors. Lysates were subsequently incubated with protein A beads combined with 2 μg of mouse IgG or α-CtIP antibody for 4 h at 4 ℃ with gentle rocking.

The resin was washed three times with RIPA buffer containing 0.1% SDS. The protein-bound beads were boiled in 2 × SDS loading buffer and subjected to Western blotting.

Chromatin fractionation

HEK293T cells stably expressing His-SUMO2 were treated with 1 μM CPT for 1 h or left untreated. The cells were then collected, washed once with PBS, and resuspended in cold Buffer A (10 mM Hepes [pH 7.9], 0.1% Triton X-100, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1 mM dithiothreitol [DTT], and 2 mM N-ethylmaleimide) containing 20 mM NaF and protease inhibitors (1 μg/ml aprotinin and leupeptin) for 5 min at 4 ℃. Subsequently, the cell lysates were centrifuged at 1300 × g for 5 min at 4 ℃, and the supernatant of each lysate was collected as the soluble fraction. The resulting crude nuclear pellet was washed once with Buffer A and resuspended in Buffer B (3 mM EDTA, 0.2 mM Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), 1 mM DTT, and 2 mM N-Ethylmaleimide) containing 20 mM NaF and protease inhibitors for 10 min at 4 ℃. This was followed by centrifugation at 1300 × g for 5 min at 4 ℃. The chromatin-enriched pellet was then washed once with Buffer B followed by lysis in Buffer I (6 M guanidine HCl, 100 mM NaH_2PO_4/Na_2HPO_4, 10 mM Tris-HCl [pH 8.0], and 5 mM imidazole) containing protease inhibitors. After sonication, the lysates were incubated with cobalt resin at 4 ℃ overnight. Protein-bound beads were washed sequentially with the following buffers: once each with Buffer I, Buffer II (8 M urea, 100 mM NaH_2PO_4/Na_2HPO_4, and 10 mM Tris-HCl [pH 8.0]), buffer III (8 M urea, 100 mM NaH_2PO_4/Na_2HPO_4, and 10 mM Tris-HCl [pH 6.3]), and Buffer IV (25 mM Tris-HCl [pH 6.8] and 10 mM imidazole). Proteins were eluted in Laemmli sample buffer supplemented with 250 mM imidazole and then boiled.

Figure S1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siCBX4
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CtIP
50
CBX4
50
Tubulin
C
siCon
siCon
siPIAS4#1+2
CPT - + +
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150
s/p-CtIP
s-CtIP
Input
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p-CtIP
CtIP
75
50
PIAS4
37
GAPDH
D
siCon
siCon
siPIAS4
siCBX4
siPIAS4+ siCBX4
CPT - + + + +
Pulldown
250
150
s/p-CtIP
s-CtIP
Input
150
100
p-CtIP
CtIP
75
50
PIAS4
75
50
CBX4
*
37
GAPDH
B
896
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Mus musculus 874 P K R R Q P Y N A V F S P K G K E Q R T
Rattus norvegicus 874 P K R R Q P Y N A V F S P K G K E Q R T
Bos taurus 870 P K R R Q P Y N A M F S P K G K E Q K T
Sus scrofa 877 P K R R Q P Y N A I F S P K G K E Q K T
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Macaca mulatta 879 P K R R Q P Y N A I F S P K G K E Q K T
Ovis aries 868 P K R R Q P Y N A M F S P K G K E Q K T
Capra hircus 870 P K R R Q P Y N A M F S P K G K E Q K T
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1
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SAP
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510
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50
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510
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D4
D5
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DAPI
H
Myc-
CPT
Vector
Vector
PIAS4
PIAS4-D3
- + + +
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150
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Myc-PIAS4-D3
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GAPDH
G
IgG
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Myc-PIAS4
WT
WT
D1
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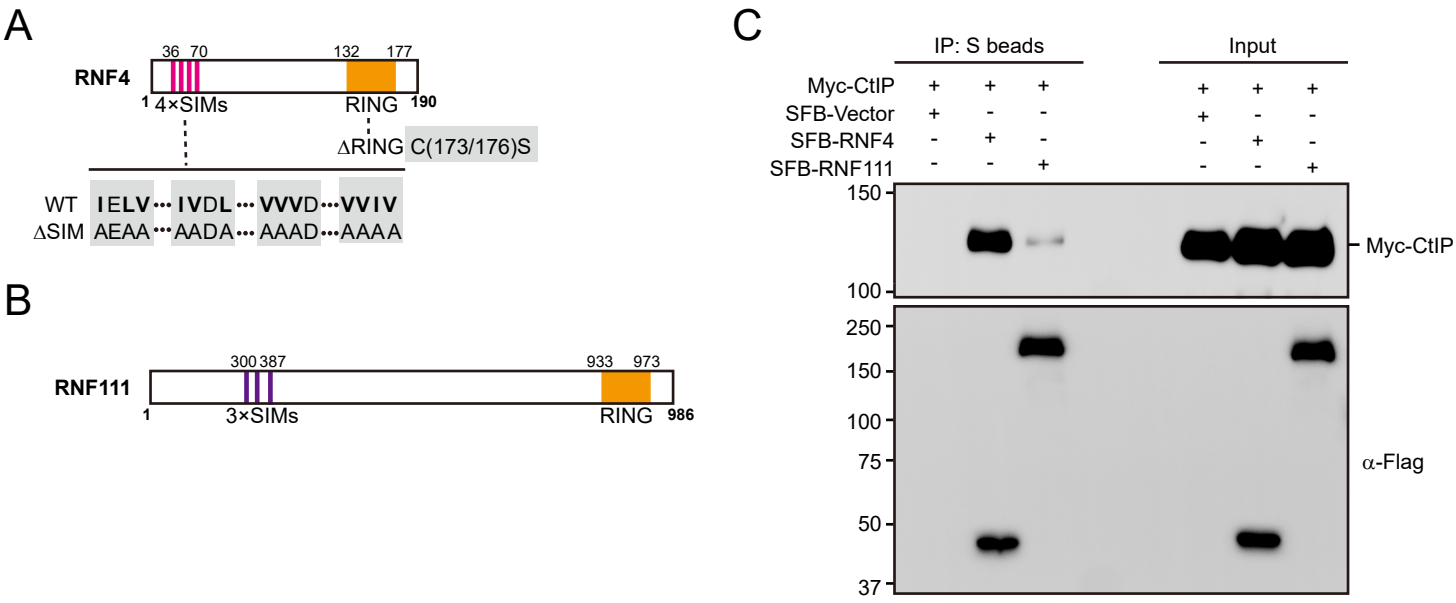
D3
D4
NLS-D5
IP
75
IgG-H
50
α-Myc
150
100
p-CtIP
CtIP
Input
75
50
α-Myc
150
100
p-CtIP
CtIP

Fig. S1. PIAS4 but not CBX4 is required for CtIP SUMOylation.

(**A**) CBX4 depletion did not affect CPT-induced endogenous CtIP SUMOylation. HEK293T cells stably expressing His-SUMO2 were transfected with the indicated siRNAs. At 48 h after transfection, the cells were either left untreated or treated with 1 μM CPT for 1 h, lysed under denaturing conditions, and subjected to cobalt pulldown. The resulting isolated proteins were assessed by western blotting using anti-CtIP antibody.
(**B**) The sequence of the region containing the lysine 896 site of CtIP was aligned with those from various species.
(**C**) knockdown of PIAS4 with a mixture of two different siRNAs (a 1:1 mixture of two PIAS4 specific siRNAs) largely reduced CPT-induced CtIP SUMOylation. HEK293T cells stably expressing His-SUMO2 were transfected with the indicated siRNAs. 48 h after transfection, the cells were either left untreated or treated with 1 μM CPT for 1 h, lysed under denaturing conditions, and subjected to cobalt pulldown. The resulting isolated proteins were assessed by western blotting using anti-CtIP antibody.
(**D**) HEK293T cells stably expressing His-SUMO2 were transfected with the indicated siRNAs. 48 h after transfection, the cells were either left untreated or treated with 1 μM CPT for 1 h, lysed under denaturing conditions, and subjected to cobalt pulldown. The resulting isolated proteins were assessed by western blotting using anti-CtIP antibody. The asterisk indicates a non-specific band.
(**E**) Schematic representation of PIAS4 mutants used in this study.
(**F**) HeLa cells were transfected with Myc-tagged wild-type PIAS4 or the indicated mutants for 24 h. The cells were then fixed, permeabilized, and stained with anti-Myc antibody. NLS indicates nuclear localization sequence.
(**G**) The PINIT motif of PIAS4 was required for its interaction with CtIP. HEK293T cells transfected with the indicated plasmids were treated with 1 μM CPT for 1 h. The cells were then lysed with NETN buffer in the presence of Benzonase. The cell lysates were incubated with protein A agarose beads conjugated to anti-CtIP antibody and then subjected to Western blotting.
(**H**) HEK293T cells stably expressing His-SUMO2 were transfected with the indicated plasmids for 24 h. The cells were either left untreated or treated with 1 μM CPT for 1 h, lysed under denaturing conditions, and subjected to cobalt pulldown. The resulting isolated proteins were assessed by western blotting using anti-CtIP antibody. SUMO-modified hyperphosphorylated CtIP levels and ratios of phosphorylated to unphosphorylated CtIP were quantified using ImageJ and are indicated beneath the corresponding lanes.

Figure S2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RING
190
ΔRING C(173/176)S
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B
RNF111
300 387
933 973
1
3×SIMs
RING
986
C
IP: S beads
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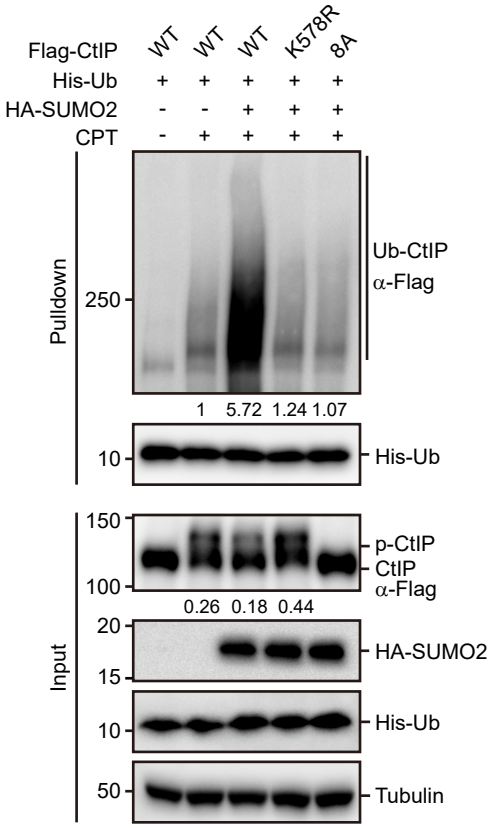
SFB-Vector + - - + - -
SFB-RNF4 - + - - + -
SFB-RNF111 - - + - - +
150
100
250
150
100
75
50
37
Myc-CtIP
α-Flag

Fig. S2. RNF4, but not RNF111, interacts with CtIP.

(**A, B**) Schematic representation of RNF4 and RNF111 constructs used in this study.
(**C**) HEK293T cells were transfected with the indicated plasmids for 24 h. Cell lysates were then prepared and subjected to immunoprecipitation with S beads and Western blot analysis was carried out as indicated.

Figure S3

A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CPT - + + + +
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Ub-CtIP
α-Flag
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His-Ub
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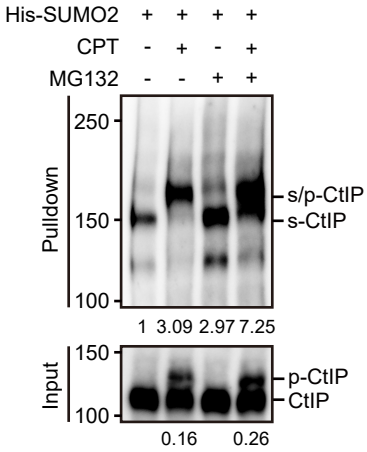
B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MG132 - - + +
Pulldown
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150
100
s/p-CtIP
s-CtIP
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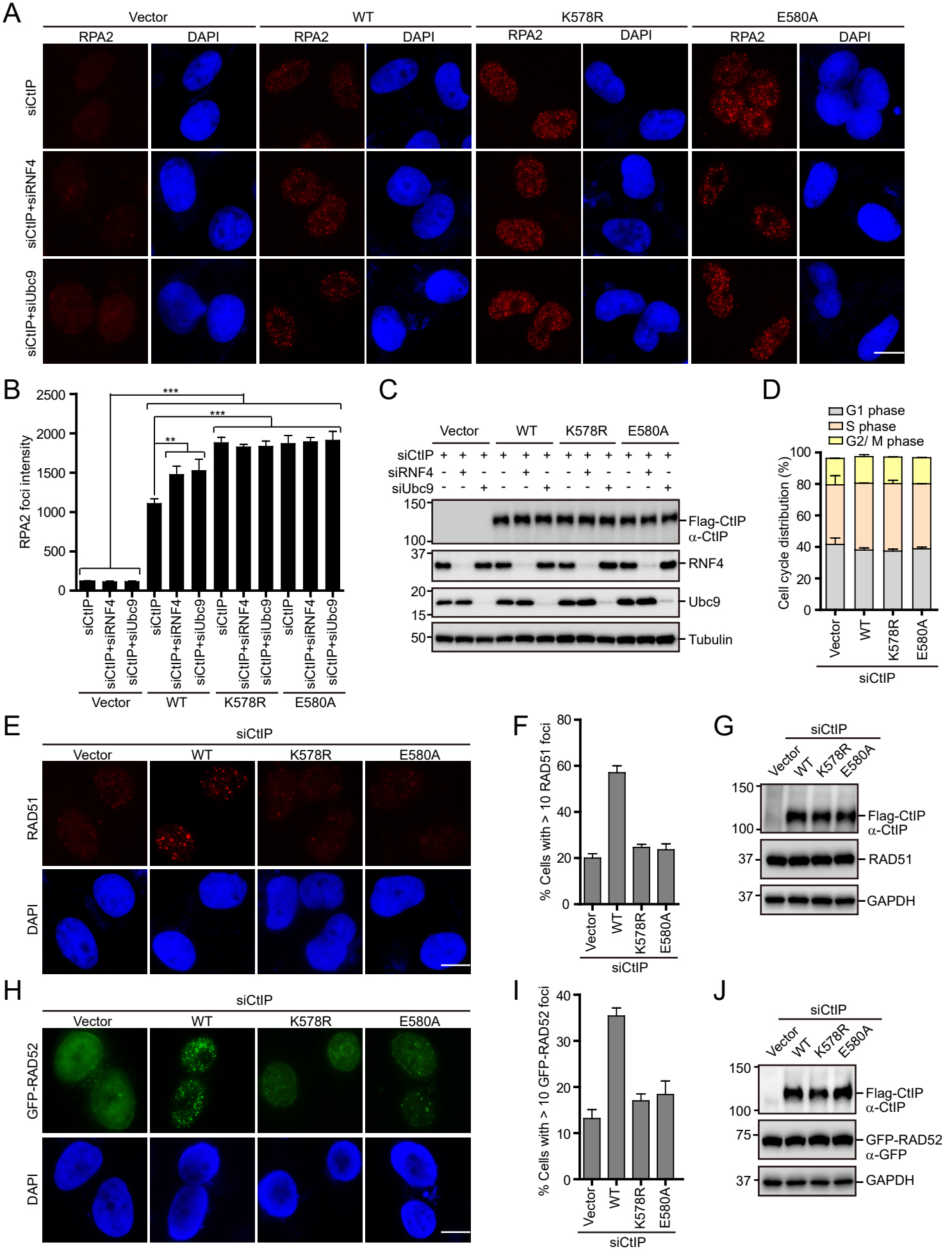
Input
150
100
p-CtIP
CtIP
0.16 0.26

Fig. S3. DSB-induced hyperphosphorylation-dependent SUMOylation of CtIP stimulates its ubiquitination.

(**A**) SUMOylation of CtIP promoted its ubiquitination. HeLa cells stably expressing Flag-tagged wild-type CtIP or the indicated mutants were transfected with HA-tagged SUMO2 and His-tagged ubiquitin. After 24 h, the cells were pre-treated with 10 μM MG132 for 1 h prior to CPT (1 μM) treatment. After 1 h, the cells were lysed under denaturing conditions and subjected to cobalt pulldown. The resulting isolated proteins were analyzed by western blotting using the indicated antibodies. Ubiquitinated CtIP levels and ratios of phosphorylated to unphosphorylated CtIP were quantified using ImageJ and are indicated beneath the corresponding lanes.
(**B**) HEK293T cells stably expressing His-tagged SUMO2 were mock treated or pre-treated with 10 μM MG132 for 1 h followed by treatment with 1 μM CPT for 1 h. The cells were then lysed under denaturing conditions and subjected to cobalt pulldown. The resulting isolated proteins were analyzed by western blotting using the indicated antibodies. SUMO-modified hyperphosphorylated CtIP levels and ratios of phosphorylated to unphosphorylated CtIP were quantified using ImageJ and are indicated beneath the corresponding lanes.

Figure S4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RPA2 foci intensity
2500
2000
1500
1000
500
0
***
***
**
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siCtIP+siRNF4
siCtIP+siUbc9
Vector
WT
K578R
E580A
C
Vector
WT
K578R
E580A
siCtIP
siRNF4
siUbc9
150
100
37
20
15
50
Flag-CtIP
α-CtIP
RNF4
Ubc9
Tubulin
D
G1 phase
S phase
G2/ M phase
Cell cycle distribution (%)
100
80
60
40
20
0
Vector
WT
K578R
E580A
siCtIP
E
siCtIP
Vector
WT
K578R
E580A
RAD51
DAPI
F
% Cells with > 10 RAD51 foci
80
60
40
20
0
Vector
WT
K578R
E580A
siCtIP
G
siCtIP
Vector
WT
K578R
E580A
150
100
37
37
Flag-CtIP
α-CtIP
RAD51
GAPDH
H
siCtIP
Vector
WT
K578R
E580A
GFP-RAD52
DAPI
I
% Cells with > 10 GFP-RAD52 foci
40
30
20
10
0
Vector
WT
K578R
E580A
siCtIP
J
siCtIP
Vector
WT
K578R
E580A
150
100
75
37
Flag-CtIP
α-CtIP
GFP-RAD52
α-GFP
GAPDH

Fig. S4. Disruption of CtIP SUMOylation results in defective HR.

(**A–C**) Disruption of CtIP SUMOylation resulted in excessive end resection. HeLa cells stably expressing an empty vector (Vector), siRNA-resistant Flag-tagged wild-type CtIP, or its SUMOylation-defective mutants were transfected with CtIP and RNF4 or UBC9 siRNAs and then treated with 1 μM CPT. After 1 h, the cells were processed for RPA2 immunofluorescence. Representative RPA2 foci are shown (**A**). Quantification results represent the means ± SEM of three independent experiments (**B**). More than 100 cells were counted in each experiment. ***p<0.001, **p<0.01, one-way ANOVA test. Western blot analysis of the expression of wild-type CtIP or its mutants was shown (**C**). Scale bar: 10 μM.

(**D**) Disruption of CtIP SUMOylation did not affect cell cycle phase distribution. BrdU incorporation assays were performed as described in the Materials and Methods section.

(**E–G**) CtIP SUMOylation is required for efficient RAD51 foci formation. HeLa cells stably expressing an empty vector (Vector), siRNA-resistant Flag-tagged wild-type CtIP, or its SUMOylation-defective mutants were transfected with CtIP siRNAs and then treated with 1 μM CPT. After 4 h, the cells were processed for RAD51 immunofluorescence. Representative RAD51 foci are shown (**E**). Quantification results represent the means ± SEM of three independent experiments (**F**). More than 100 cells were counted in each experiment. Western blot analysis of the expression of wild-type CtIP or its mutants was shown (**G**). Scale bar: 10 μM.

(**H–J**) CtIP SUMOylation is required for efficient RAD52 foci formation. HeLa cells stably expressing an empty vector (Vector), siRNA-resistant Flag-tagged wild-type CtIP, or its SUMOylation-defective mutants were transfected with GFP-tagged RAD52. After 24 h, cells were transfected with CtIP siRNAs and then treated with 1 μM CPT. After 4 h, the cells were processed for GFP-RAD52 immunofluorescence. Representative GFP-RAD52 foci are shown (**H**). Quantification results represent the means ± SEM of three independent experiments (**I**). More than 100 cells expressing GFP-RAD52 were counted in each experiment. Western blot analysis of the expression of wild-type CtIP or its mutants was shown (**J**). Scale bar: 10 μM.