Supplementary Information

Role of SUMO modification of human PCNA at stalled replication fork

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Supplementary materials and methods

Plasmid constructs

For expression in human cells, the cDNAs of human SUMO1, SUMO2, and SUMO3 were cloned in fusion with FLAG-tag in pRK2F vector resulting in plasmids pIL1759, pIL2128 and pIL2129 respectively; the cDNA of human UBC9 was cloned in fusion with His-tag in pDest26 vector resulting in pIL1779; the cDNA of wild-type human PCNA and lysine to arginine point mutant PCNAs that were generated by PCR-based method were cloned in fusion with HA-tag in pRK2H vector resulting in pIL2352 (for wt PCNA); the stop codon at the C-terminal of PCNA was removed by site-directed mutagenesis. PCNA-SUMO1 fusion was generated by PCR, and the sequence-verified PCR product was cloned in fusion with FLAG-tag in pRK2F vector resulting in pIL1602. For protein purification PCNA point mutants were cloned in fusion with GST in yeast expression vector as described for wild-type PCNA (1); PCNA-SUMO1 fusion, PIAS1, PIAS2, PIAS3, and PIAS4 were cloned in fusion with GST-tag in pGEX-6 vector resulting in plasmids pIL1725, pIL1830, pIL1831, pIL1832 and pIL1834, respectively. Expression vectors for purification of wild-type PCNA, SUMO1, SUMO2, SUMO3, UBC9, SAE1/2 and RFC were described previously (2).

Proteins

Human DNA polymerases η, κ, ι, the wild-type and point mutant human PCNA proteins were expressed in yeast as GST-fusion proteins, from which GST was removed by Prescission protease during the purification as described (3-5). SUMO1, SUMO2, SUMO3, UBC9, SAE1/2, PCNA-SUMO1, Pias1, Pias2, Pias3, and Pias4 proteins were expressed in BL21 (DE3) Codon Plus-RP bacterial strain (*Stratagene*). For their purification, total cell protein extracts were prepared by sonication in buffer A (20 mM Tris-HCl (pH 7.5), 0.01% Nonidet P-40, 10% glycerol) followed by supplementing the lysate with 1 M NaCl, 5 mM EDTA, and protease inhibitors (Mini-Complete,

Roche) before centrifugation and loading the supernatant onto a glutathione-Sepharose column (Amersham Pharmacia Biotech). Unbound proteins were washed away with buffer A+1 M NaCl followed by buffer A+100 mM NaCl. Finally, GST-fusion proteins were eluted from glutathione-sepharose in buffer A+100 mM NaCl containing 10 mM glutathione, or proteins without GST-fusion were eluted with precission protease.

In vitro SUMO and ubiquitin modification of PCNA

A standard *in vitro* SUMOylation reaction of PCNA was carried out in 10 µl of SUMOylation buffer (40 mM Tris-HCl, (pH 7.5), 8 mM $MgCl_2$, 100µg/ml BSA, 10% glycerol and 100 mM ATP) in the presence of 40 nM PCNA, 10 nM SAE1/2, 100 nM Ubc9, 500 nM SUMO1, 10 nM RFC, and 2 nM nicked PUC19 plasmid DNA at 37°C for 60 min. Samples containing unmodified and SUMOylated PCNA were separated on 10% denaturing polyacrylamide gel and visualized by Western blot using PC10 anti-PCNA antibody (Santa Cruz). The following modifications were included in some of the experiments: reactions were carried out in the presence of SUMO1, SUMO2, SUMO3, or their N-terminal GST fusion chimeras (Figure 2A); various lysine to arginine point mutant PCNAs were used (Figures 2D and S2A); reactions were incubated in the absence or presence of combinations of RFC and nicked plasmid DNA (Figure 2E); higher enzyme concentrations (20 nM SAE1/2 and 200 nM Ubc9) were used (Figure S2A); Pias1, Pias2, Pias3, or Pias4 SUMO ligases were also added to the reactions (Figure S2B). The *in vitro* ubiquitylation reactions of PCNA were carried out in the presence of Uba1, Rad6-Rad18, ubiquitin, RFC, and nicked plasmid DNA as we described (1,6). For figure S3B excess Rad6-Rad18 (150 nM) was used over PCNA (20 nM).

Cell culture, transfections and stable cell lines

HEK293T and HeLa cells were cultured in DMEM supplemented with 10% FCS and antibiotics. Cells

were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. For generation of cell lines stably expressing FLAG-SUMO1 in HeLa S3 cells and FLAG-PCNA or FLAG-PCNA-SUMO1 conjugate in HeLa cells, cells were selected after 24h of transfection in medium containing 1μg/ml puromycin or 300μg/ml Hygromycin B, respectively, before single colonies were isolated and tested for stable protein expression. Human Rad18 -/- cell line was described and characterized previously (7).

Cell lysis and immunoprecipitations

Cells were lysed in a buffer containing 50 mM Tris-HCl (pH7.5), 400 mM NaCl, 1% NP-40, 0.1% SDS, 5 mM EDTA, 10% glycerol and 1 mM PMSF. Cell lysates were briefly sonicated and clarified by centrifugation at 25,000 g for 10 min at 4°C. Supernatants were diluted with equal volume of dilution buffer (lysis buffer without NaCl) and FLAG-tagged proteins were immunoprecipitated using anti-FLAG M2 affinity gel (Sigma). FLAG precipitates and initial cell lysates were analyzed by western blot using anti-FLAG M2 (Sigma F3165), anti-HA 3F10 (Roche) and anti-PCNA PC-10 (Santa Cruz) antibodies.

DNA substrate and polymerase assay

The DNA substrate for DNA polymerase assays was generated by annealing of the 75-nt oligonucleotide template containing biotin at each end (5'- AGC TAC CAT GCC TGC CTC AAG AAT TCG TAA CAT GCC TAC ACT GGA GTA CCG GAG CAT CGT CGT GAC TGG GAA AAC-3') to a 27-nt 5′ 32P-labeled oligomer, (5'- CGA CGA TGC TCC GGT ACT CCA GTG TAG -3'). Before adding to the reactions these partial-heteroduplex DNAs were preincubated with streptavidin which protects against PCNA sliding off. A standard DNA polymerase reaction (10 μl) was carried out in P150 buffer (40 mM Tris-HCl (pH 7.5), 8 mM MgCl2, 150 mM NaCl, 100 μg/ml BSA, 10% glycerol,

100 μM ATP) supplemented with 100 μM dGTP, dATP, dTTP, and dCTP. As indicated in the figure legends, Polη, Polκ or Polι (2 nM each) in the presence of RFC (5 nM), and PCNA (10 nM) or PCNA-SUMO1 fusion protein (10 nM) were incubated with the DNA substrate (10 nM) at 37°C for 10 min before adding the loading buffer (40 μl) (95% formamide, 20 mM EDTA, 0.25% bromophenol blue, and 0.25% cyanol blue). The reaction products were resolved on 10% polyacrylamide gels containing 8 M urea and visualized using a Molecular Dynamics STORM PhosphoImager and ImageQuant software.

Flow cytometric analysis

Exponentially growing stable cell lines expressing FLAG-control, FLAG-PCNA or FLAG-PCNA-SUMO1 were harvested, washed with PBS and fixed in 70 % ethanol. Cells were RNAseA (0.2μg/ml) treated for 15 min at 37^0C and stained with propidium iodide (50 μg/ml). Flow cytometric profiles were determined using a Becton-Dickinson FACScan and analysed with ModFitLT software.

Recombination assay

A GFP based recombination reporter assay in HeLa cells was adapted, which measures the recombination frequency between an integrated DNA fragment encoding a C-terminally truncated GFP and a transiently transfected DNA fragment encoding N-terminally truncated GFP proteins(8). Briefly, reporter cells were co-transfected with DNA fragment encoding N-terminally truncated GFP proteins and either a control empty vector, or PCNA, or PCNA-SUMO1 fusion protein expressing vectors, and, for Figure 4A, also with pCMV3nls-I-SceI expression vector using Lipofectamine 2000 (Invitrogen). After 2 weeks the frequency of GFP+ recombinants were analyzed.

Cell viability assay

Cells stably expressing FLAG, FLAG-PCNA or FLAG-PCNA-SUMO1 were plated with 5000 cells/well in a 96-well plate. Twenty four hours after plating cells were treated with different doses of UV, and for 3h with MMS or cisplatin followed by washing three times with 1xPBS and further culturing for 72 h in regular growth media. Cell survival was determined using the Vialight Plus cell proliferation/cytotoxicity Kit assay (Lonza) according to the manufacturer's instructions.

Immunofluorescence and detection of γH2AX

For the detection of γH2AX foci, cells stably expressing FLAG, FLAG-PCNA or FLAG PCNA-SUMO1 (as shown in Figure S4B) were grown on coverslips and mock- or MMS (0.01%)-treated for 1h followed by washing thoroughly with PBS before treating with 0.4% Triton X-100 and fixing in 3% PFA for 10 min at room temperature. Coverslips were blocked in PBS+1% BSA for 20 min before immunostaining with anti-γH2AX antibody. For the localization study shown in Figure 3C cells stably expressing FLAG-PCNA or FLAG-PCNA-SUMO1 were grown on coverslips and pulse labelled with 20 μM BrdU for 1h before rinsing in PBS and fixing in cold methanol followed by immunostaining with anti-FLAG and anti-BrdU antibodies. Finally, cover slips were mounted with mounting solution containing glycerol and DAPI followed by visualization with an Olympus confocal laser scanning microscope. For immunostaining an anti-FLAg mAb 1:400 M2 (Sigma F3165), anti-FLAG 1:400 (Sigma F7425), anti-BrdU 1:500 (Ab-direct Serotech), anti- γH2Ax 1:5000 (Upstate 05-636), anti-mouse Cy3 1:1000 (Sigma C2181), AlexaFluor 488-labeled goat anti-rat antibody 1;1000 (Molecular Probes, Inc.) and anti-rabbit FITC 1:1000 (Sigma F0382) antibodies were used.

Single cell gel electrophoresis (neutral comet assay)

The comet assay was performed under neutral conditions with modifications of previously described protocol(9,10). Briefly, cells stably expressing FLAG, FLAG-PCNA, or FLAG-PCNA-SUMO1 were treated with 0.01 % MMS or mock for 1h followed by washing thoroughly with PBS. Next, cells were allowed to recover before collecting them at various time points as indicated in figure legends. Collected cells were suspended in 0.75 % low-melting-point agarose (Sigma), dissolved in PBS and spread onto microscopic slides pre-coated with 1 % normal-melting agarose (Sigma). Cells were then lysed in buffer containing 2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl (pH 10), 1% Triton X-100 for 90 min at 4^{o}C. After lysis slides were washed in 0.4M Tris-HCl (pH 7.4) and placed in an alkaline buffer containing 0.3 M NaOH, 1 mM EDTA (pH 13) for 20 min to allow complete degradation of RNA. Slides were neutralized in 0.4 M (Tris-HCl) pH7.4 and placed in an ice-cold electrophoresis chamber containing 1XTBE buffer for 20 min to equilibrate. Electrophoresis was subsequently conducted in the same TBE buffer at 4^{o}C for 20 min at an electric field strength of 25 V and 15 mA. Finally, slides were stained with 20 μg/ml EtBr in PBS, photographed and analyzed with Komet 5.0 video image analysis software.

Supplementary references

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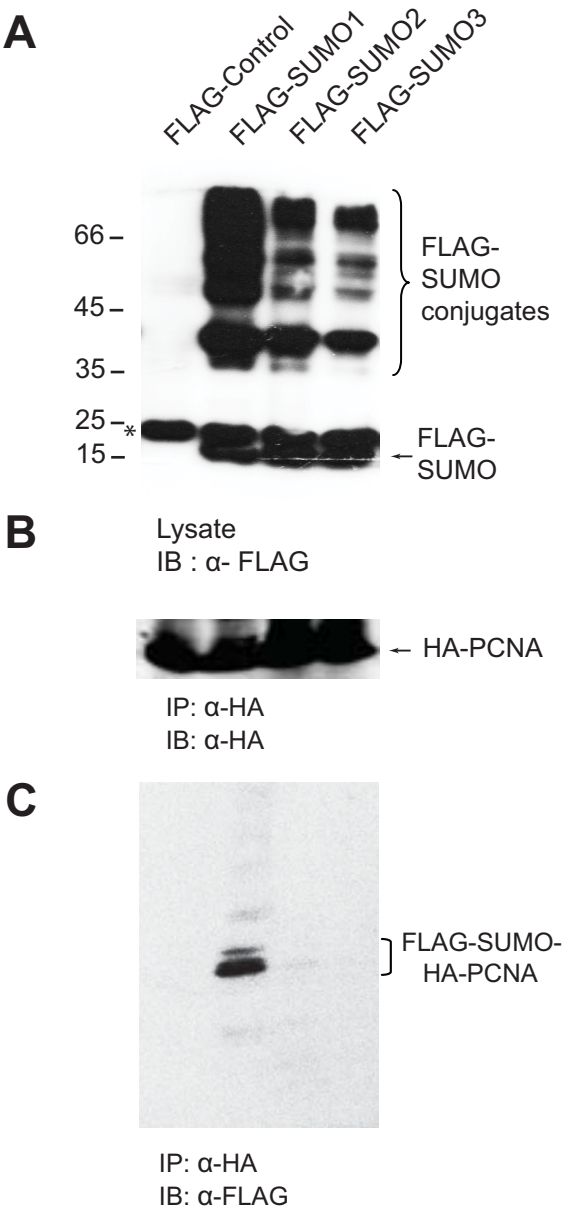
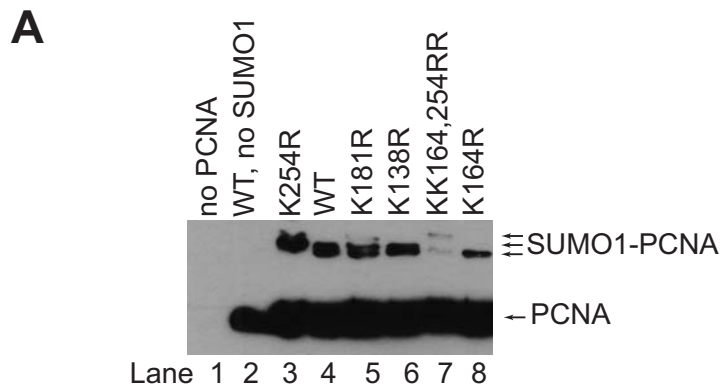
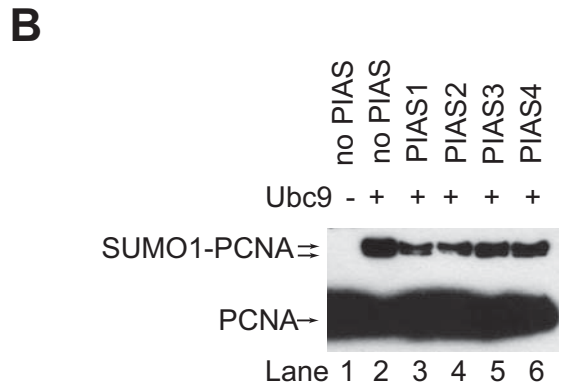
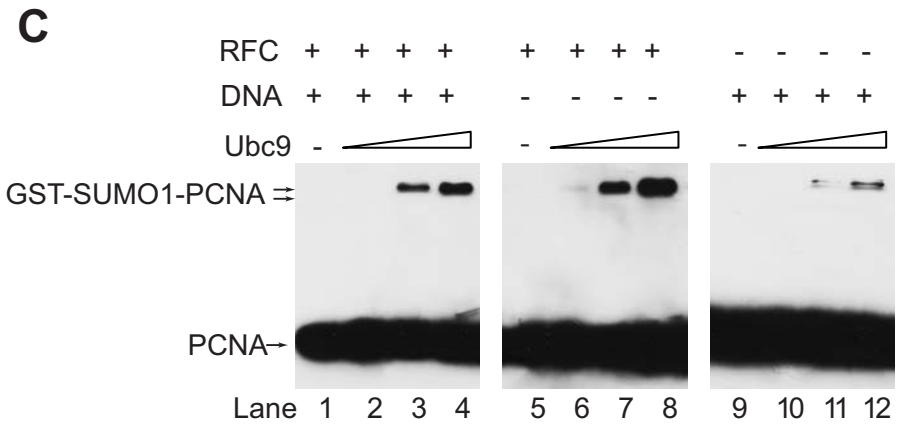


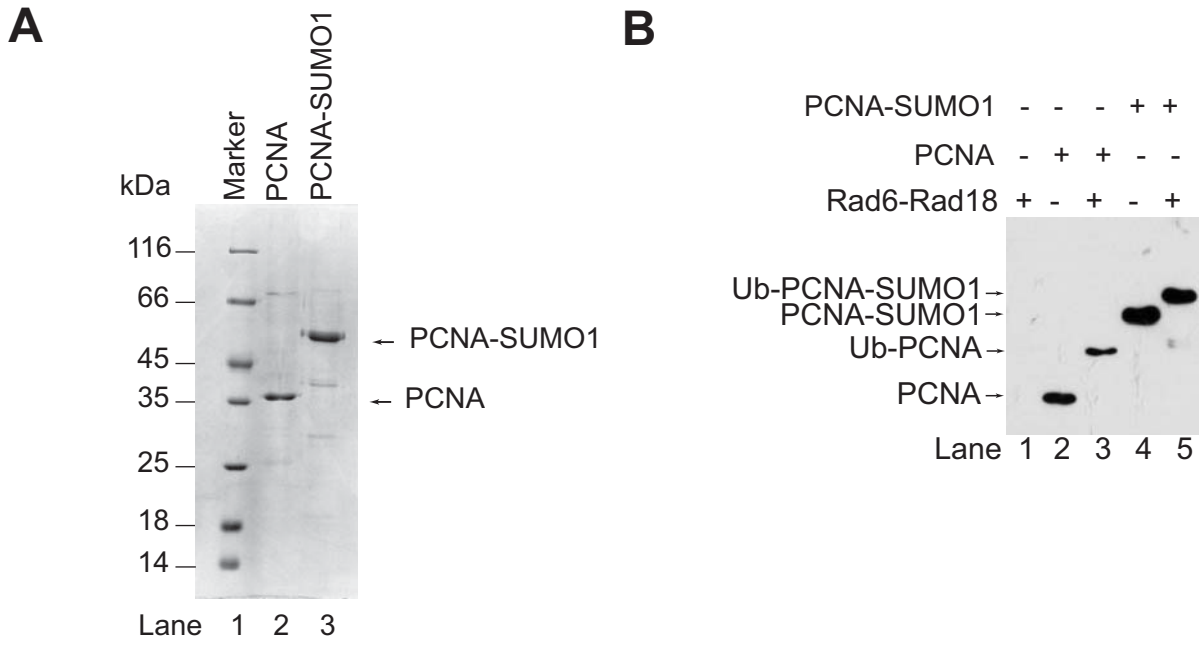
Supplementary Fig.1







Supplementary Fig.2



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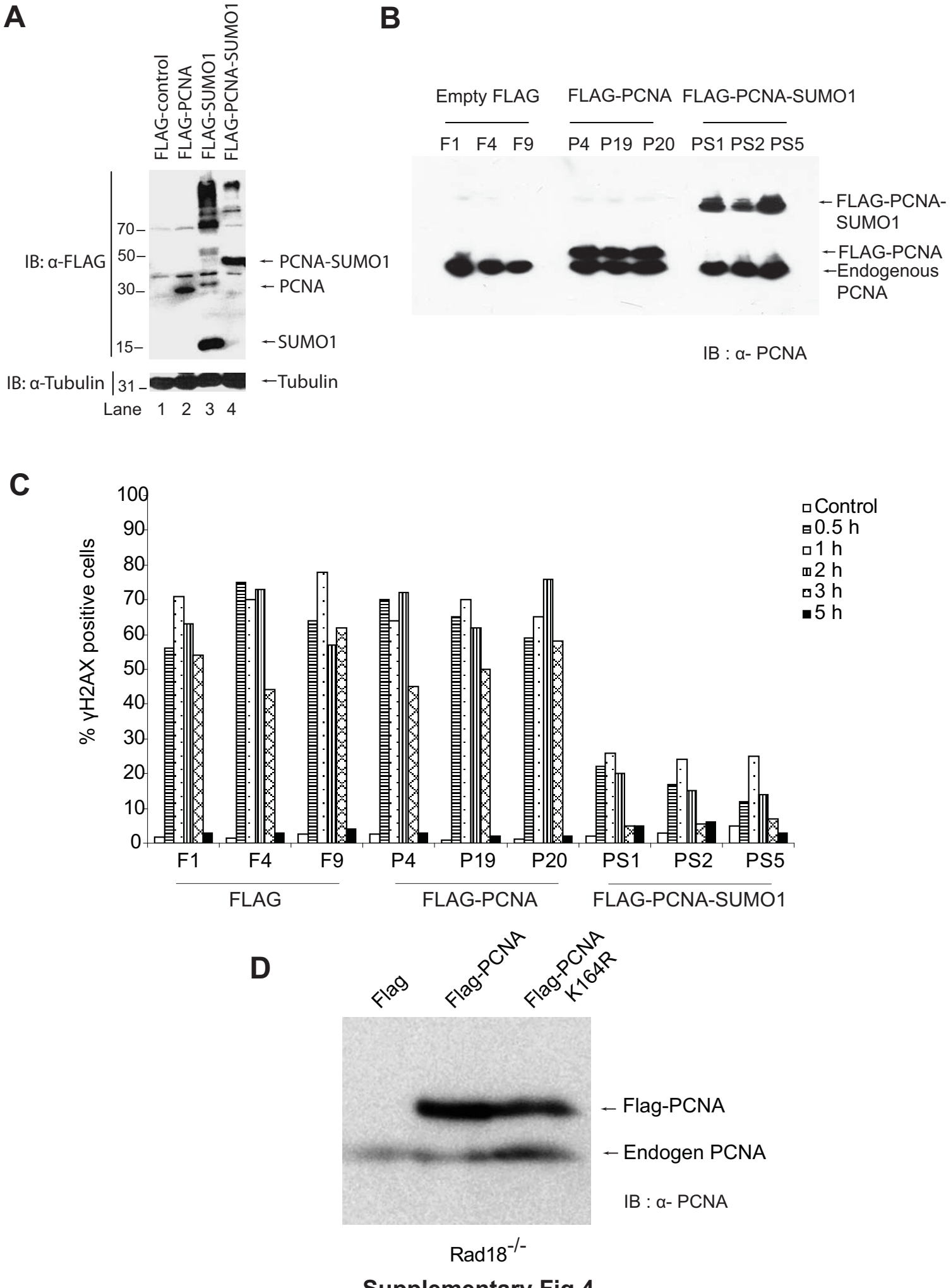

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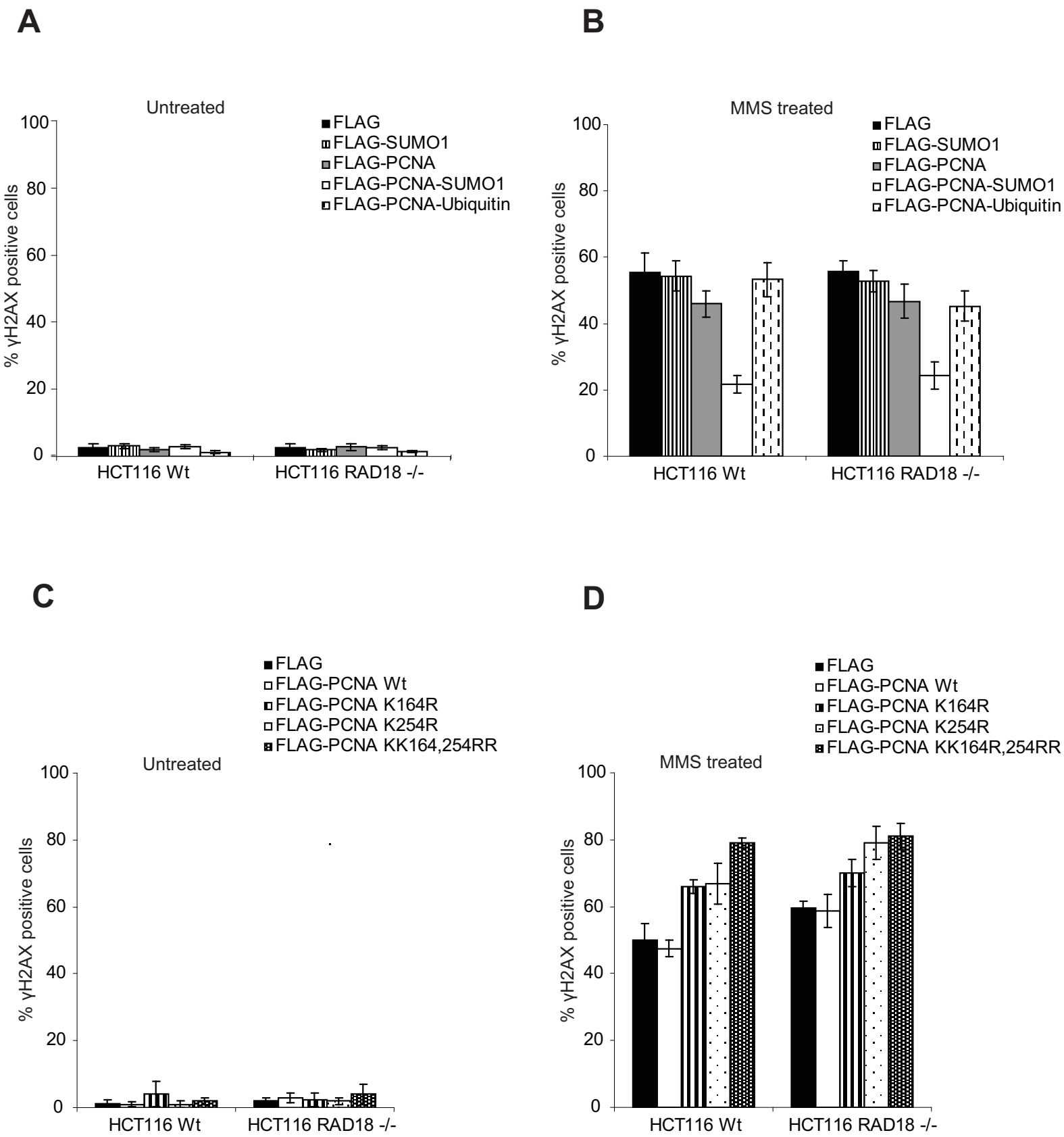
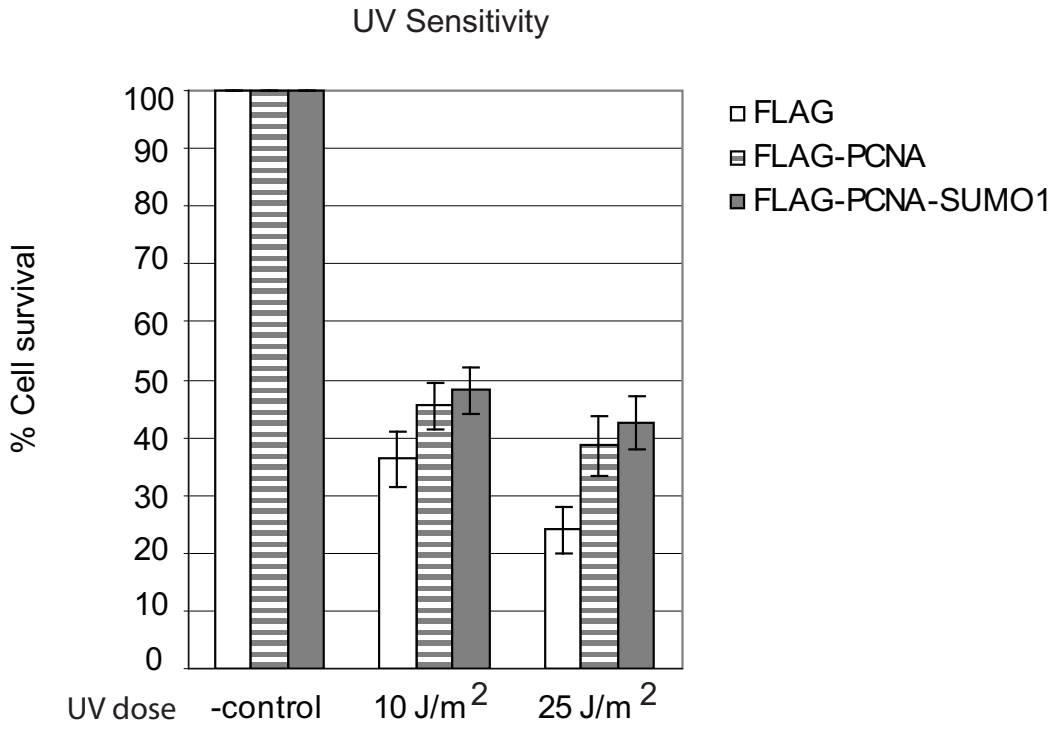


Supplementary Fig.5



Supplementary Fig.6

Supplementary figure legends

Supplementary Figure 1. (**A**) Comparison of the expression of FLAG-tagged SUMO1, SUMO2 and SUMO3 in HEK293 cell lysates used for the experiment shown in Figure 1A by Western blot analysis using anti-FLAG antibody. The nonspecific band present in all samples is labeled by asterisk. (**B**) The above samples precipitated on HA-beads followed by Western-blot analysis by anti-HA antibody. (**C**) HA-PCNA precipitates were immunoblotted with anti-FLAG antibody to detect the SUMO-modified forms of PCNA .

Supplementary Figure 2. (**A**) *in vitro* SUMOylation reaction of wild-type and mutant human PCNA (40 nM) was carried out in the presence of purified SAE1/2 (20 nM), Ubc9 (200 nM), RFC (10 nM), nicked PUC19 plasmid DNA (2 nM) and SUMO1 (500 nM) at 37°C for 60 min. Samples containing unmodified and SUMOylated PCNA proteins were separated on 10% denaturing polyacrylamide gel and visualized by Western blot using anti-PCNA antibody. (**B**) PCNA was subjected to *in vitro* SUMOylation reaction in the absence or presence of PIAS1, PIAS2, PIAS3 or PIAS4 (100 nM). (**C**) *in vitro* SUMOylation reactions of PCNA were compared in the absence or presence of combinations of RFC and nicked plasmid DNA and increased concentration of Ubc9 (10 nM, 50 nM, and 200 nM) as indicated.

Supplementary Figure 3. Qualitative analysis of PCNA-SUMO1 fusion protein. (**A**) Purified human PCNA and human PCNA-SUMO1 fusion proteins were analyzed on 10% polyacrylamide gel by Coomassie staining. (**B**) PCNA-SUMO1 fusion protein is proficient for PCNA ubiquitylation. PCNA and PCNA-SUMO1 were assayed for ubiquitylation *in vitro* in the presence of purified ubiquitin, Uba1, Rad6-Rad18, RFC, and DNA followed by Western blotting using anti PCNA antibody.

Supplementary Figure 4. Effect of SUMO modification of PCNA on the accumulation of γH2AX foci. (**A**) Immunoblot with anti-PCNA antibody showing the transient expression of FLAG-PCNA, FLAG-SUMO1, and FLAG-PCNA-SUMO1 in recombination reporter HeLa cell line used for *Figures 4A and B*. (**B**) Immunoblot with anti-PCNA antibody showing the expression of endogenous PCNA and the respective PCNA forms in three different HeLa cell clones each stably expressing FLAG (cell lines: F1, F4, F9), FLAG-PCNA (cell lines: P4, P19, P20) or FLAG-PCNA-SUMO1 (cell lines: PS1, PS2, PS5). (**C**) Percentage of cell populations that showed more than two foci for γH2AX at 0.5, 1, 2, 3, and 5 h after 0.01% MMS treatment was calculated using three independent stable cell lines for each of control FLAG (F1,F4,F9), FLAG-PCNA (P4, P19, P20) and FLAG-PCNA-SUMO1 (PS1, PS2, PS5). (**D**) Control for the experiment shown in *Figure 5D* using immunoblot analysis with anti-PCNA antibody for the expression of FLAG-tagged wt and K164R human PCNA proteins as compared to the endogenous PCNA in Rad18$^{-/-}$ HCT16 cells.

Supplementary Figure 5. (**A,B**) Effect of the overexpression of FLAG, FLAG-SUMO1, FLAG-PCNA, FLAG-PCNA-SUMO1 or FLAG-PCNA-Ubiquitin in HCT116 Wt and HCT116 RAD18$^{-/-}$ cells was revealed by γH2AX staining 3h after mock (A) or MMS treatment (B) and the percentage of cell populations that showed more than two foci for γH2AX was calculated from three independent experiments; error bars show standard deviations. (**C,D**) Effect of the expression of K164R, K254R and K164R K254R PCNA in HCT116 Wt and HCT116 RAD18$^{-/-}$ cells on γH2AX foci formation was calculated as above 3h after mock (C) or MMS treatment (D).

Supplementary Figure 6. HeLa cells stably expressing FLAG, FLAG-PCNA or FLAG-PCNA-SUMO1 were assayed for cell survival after treatment with 10 and 25 J/m^2 UV as compared to the untreated cells. Error bars show standard deviation from the results of three independent experiments.