SUPPLEMENTARY METHODS

Cell lines and reagents

U2OS and U2OS-HIS-SUMO2 cell lines [1] were grown in DMEM supplemented with 10% FCS and penicillin/streptomycin (Life Technologies). SLX4$^{+/+}$ MEFs and SLX4$^{-/-}$ MEFs [2] were also supplemented with non-essential AAs. Every cell line was checked for mycoplasma contamination regularly. The result was always negative. mSLX4 was amplified by PCR from pBABE-mSLX4 [2] and cloned into pDONR207 using a BP reaction (Gateway system, Life Technologies). Subsequently, SIMs were mutated by replacing large hydrophobic residues for alanines by PCR mediated mutagenesis (see primers heading) and checked by sequencing. Later, LR reactions were performed according to the instructions of the manufacturer of the Gateway system (Life Technologies) to transfer the different mSLX4 constructs into pBABE-puro-GFP-DEST (Figure 2 and 3), pMT2-HA-DEST (immunoprecipitation Figure 4) [3] or pDEST-EGFP-C1 (for Laser microirradiation Figure 4). The NBS1-mCherry plasmid was kindly provided by Dr. Jiri Lukas and described and used in [4].

Antibodies used in this paper are the following: Mouse anti-GFP (11814460001, Roche), Rabbit anti-BTBD12/SLX4 (A302-270A, Bethyl), Mouse anti phospho-H2A.X (05-636, Milipore), Mouse anti-SUMO2/3 (ab81371, Abcam), Mouse anti-XPF (MS-1381, Thermo), Mouse-anti MUS81 (IQ285, Immuquest), Mouse anti-PARP1 (9542, Cell Signaling), Mouse anti-PML antibody was a kind gift from Dr. R. van Driel (University of Amsterdam) and is described in [5]. Sheep anti-mSLX4 antibody is described and used in [2].

Thymidine, Nocodazole and DMSO were obtained from Sigma. CDK1 inhibitor RO-3306 was from Merck-Millipore. The PARP inhibitor KU-0058948 was a kind gift from Mark O'Connor (Astrazeneca) and used at a concentration of 10 μM.

Primers

Primers to mutate the different SIMs are the following:

- SIM1:

Fw:5'-CGTGAAAAAGGAGGATGAGGCCGCCGCGGCAGCGGACTCAGATGAGGAGC-3'

Rv: 5'-GCTCCTCATCTGAGTCCGCTGCCGCGGCGGCCTCATCCTCCTTTTTCACG-3'

- SIM2:

Fw:5'-CCTCTGAACTGTTTTCAGCCGCAGATGCCGAAGAAGATCATGAACATTTCCAAAGCCC-3'

Rv:5'-GGGCTTTGGAAATGTTCATGATCTTCTTCGGCATCTGCGGCTGAAAACAGTTCAGAGG-3'

- SIM3:

Fw:5'- GCCTGCAGCTGAGATGCGGCAGAAGCCGGGGACAGTGATGATGAGG-3'

Rv:5'- CCTCATCATCACTGTCCCCGGCTTCTGCCGCATCTCAGCTGCAGGC-3'

HA-SLX4 co-immunoprecipitation to identify interactors

U2OS cells were plated in a 15 cm dish at 30% confluence. The following day, cells were transiently transfected using 2.5 µl polyethylenimine (PEI, 1 mg/ml; Alfa Aesar) per µg DNA with 24 µg of either pMT2-mSLX2-wt or ΔSIM and empty plasmid was used as negative control. Two days after transfection, cells were harvested, washed two times with icecold PBS and transferred to Eppendorf LoBind 1.5 mL tubes. From now on, all steps were performed at 4ºC. Cells were lysed in 1 ml of Lysis Buffer (20mM Tris pH 7.5, 150 mM NaCl, 1mM $MgCl_2$, Triton X-100 0.5%, Protease inhibitor cocktail minus EDTA (Roche), 200 mM Iodoacetamide) and incubated for 10 minutes in ice. 500 U of Benzonase were added and the lysate was incubated for 1 hour in a rotation wheel at 4ºC. Then, samples were centrifuged at 16,000 g for 45 minutes. The supernatant was incubated for 1 hour with 50 µL of pre-washed EZview™ Red Anti-HA Affinity Gel (Sigma). After incubation, samples were washed three times with lysis buffer and subsequently analysed by SDS-PAGE and Western blotting or by Mass Spectrometry.

Electrophoresis and immunoblotting

Samples were separated on Novex 4-12% Bis-Tris gradient gels (Invitrogen) using MOPS buffer. Subsequently, separated proteins were transferred onto a Hybond-C extra membrane (Amersham, BioSciences). Total protein levels were visualized by staining membranes with Ponceau S (Sigma). After washing in PBS containing 0.1% Tween-20 (PBS/T, MERCK), membranes were blocked in PBS/T containing 8% milk. Membranes were incubated with specific primary antibodies overnight. After washing membranes with PBS/T, they were incubated with secondary antibody, either donkey anti-rabbit or goat anti-mouse coupled to HRP (Thermo Scientific). After extensive washing with PBS/T, Pierce ECL 2 western blotting solution (Thermo Scientific) or Amersham ECL Prime (GE Healthcare) were used to visualize signals on FUJI medical X-ray films (FUJIFILM).

Mass Spectrometry sample preparation

After immunoprecipitation, EZview™ Red Anti-HA Affinity Gel beads were washed three times with ammonium bicarbonate 50 mM pH 8. Then the beads were resuspended in 250 µL ammonium bicarbonate 50 mM pH 8 and 0.25 µg of sequencing grade Trypsin (Promega).

Samples were incubated overnight at 37ºC while shaking. Then the supernatant was separated from the beads and passed through a pre-washed 0.45 µm filter (Millipore) and samples were transferred to Epperdorf LoBind 1.5 mL microcentrifuge tubes. Samples were acidified with 2% Trifluoroacetic acid (Sigma). Then samples were desalted with C-18 stage tips as described in [1].

Mass Spectrometry

Mass Spectrometry samples were analysed by nanoscale LC-MS/MS on an Orbitrap Q-Exactive instrument (Thermo) and raw data were analysed using MaxQuant Software version 1.5.1.2 and Perseus Software version 1.5.1.6. The mass spectrometry data have been deposited to the ProteomeXchange Consortium [6] via the PRIDE partner repository with the dataset identifier PXD001681.

SIM tetra-SUMO2 interaction assay

U2OS cells were plated in a 15 cm dish at 30% confluence. The following day, cells were transiently transfected using 2.5 µl polyethylenimine (PEI, 1 mg/ml; Alfa Aesar) per µg DNA with 24 µg of either pMT2-mSLX2-wt or ΔSIM; empty plasmid was used as negative control. Two days after transfection, cells were harvested, washed two times with icecold PBS and transferred to Eppendorf LoBind 1.5 mL tubes. From now on, all steps were performed at 4ºC. Cells were lysed in 1 ml of Lysis Buffer (20 mM TRIS-HCl pH 7.5, 150 mM NaCl, 1mM $MgCl_2$, NP-40 0,5%, Protease inhibitor cocktail minus EDTA (Roche)) and incubated for 10 minutes on ice. Samples were then sonicated for 5 seconds to fragment DNA and centrifuged at 16,000 g for 45 minutes. Supernatants were transferred to a new tube containing 50 µL of pre-washed EZview™ Red Anti-HA Affinity Gel (Sigma) and incubated for 1 hour at 4ºC while rotating. Then, samples were washes 3 times with washing buffer (20 mM TRIS-HCl pH 7.5, 750 mM NaCl, 1mM $MgCl_2$, NP-40 0,5%, Protease inhibitor cocktail minus EDTA (Roche)), and incubated for 1 more hour in 1 mL of Lysis buffer plus 10 µg Bovine Serum Albumin (New England Biolabs) and 100 ng of recombinant 6HIS-tetra-SUMO2 produced in *E.coli* [7]. Samples were washed 3 times with washing buffer and then eluted for 30 minutes in 50 µL of washing buffer plus 100 ng/µL HA peptide (Sigma).

Survival assay

MEFs were plated in 10 cm plates and allowed to attach overnight before treatment. MMC was added to cells in DMEM for 24 hours at different concentrations. Then cells were allowed to

grow for 10 days and fixed for 5 minutes in paraformaldehyde 4% in PBS. Cells were stained with Cristal Violet 0.05% for 30 minutes and washed 3 times with water. Afterwards, Cristal Violet was re-solubilized in Methanol and $O.D._{545}$ was measured. The value of untreated cells was set at 100%.

Retroviral mediated expression of GFP-mSLX4 constructs

GFP-mSLX4 retroviruses were made in 293GP cells using pBABE-puro-GFP-mSLX4 constructs. U2OS and SLX4-/- MEFs were infected at a MOI of 2. Three days after infection, GFP-mSLX4 constructs expressing cells were selected with puromycin at 3 μg/mL.

Cell cycle synchronization

U2OS-HIS-SUMO2 cells were treated with 4mM Thymidine for 16 hours to arrest them in G1/S transition. Cells were released in fresh medium and harvested after 0, 2.5, 5.5, 9 or 12 hours, being G1/S, early S, mid S, late S and S/G2 time points respectively. 6 hours after the Thymidine block release, either RO-3306 10μM (G2/M) or Nocodazole 0.33μM (prometaphase) were added to the cultures and cells were harvested 6 hours later. For the G1 time point, cells were harvested after releasing the cultures from G2/M arrest in fresh medium for 12 hours.

Microscopy and multiphoton laser micro-irradiation.

Co-localization images were taken with a Leica TCS SP8 confocal microscope equipped with 405, 488, 552 and 638 lasers. Images were analysed using ImageJ. For SUMO2/3 co-localization 423 foci in 53 cells were analysed. For PML co-localization 435 foci in 59 cells were analysed.

Laser track experiments were performed using a Leica SP5 confocal microscope with an environmental chamber set to 37 °C. Briefly, U2OS cells were grown on 18 mm coverslips and transiently co-transfected with NBS1-mCherry and GFP-mSLX4 constructs. Two days after transfection, media was replaced by CO2-independent Leibovitz's L15 medium supplemented with 10% FCS and pen/strep. Laser micro-irradiation was carried out on a Leica SP5 confocal microscope equipped with an environmental chamber set to 37°C. DNA damage tracks (1 μm width) were generated with a Mira modelocked titanium-sapphire (Ti:Sapphire) laser (l = 800 nm, pulse length = 200 fs, repetition rate = 76 MHz, output power = 80 mW) using a UV-transmitting 63× 1.4 NA oil immersion objective (HCX PL APO; Leica). Confocal images were recorded before and after laser irradiation at 10- or 20-s time intervals over a period of 5-10 min. Images were analysed using Leica LAS AF software.

Immunostaining

Cells were grown on 9 mm coverslips and fixed with 4% paraformaldehyde for 15 minutes at 37°C in PBS and permeabilized with 0.1% Triton X-100 in PBS for 15 minutes. Next, cells were washed twice with PBS and once with PBS with 0.1% Tween-20 (PBS-T). Cells were then blocked for 10 minutes with 0.5% blocking reagent (Roche) in 0.1 M Tris, pH 7.5 and 0.15 M NaCl (TNB), and treated with primary antibody in TNB for one hour. Coverslips were washed five times with PBS-T and incubated with the secondary antibody (Goat anti-mouse coupled to Alexa-594) in TNB for one hour. Next, coverslips were washed five times with PBS-T and dehydrated by washing once with 70% ethanol, once with 90% ethanol, and once with 100% ethanol. After drying the cells, coverslips were mounted onto a microscopy slide using citifluor/DAPI solution (500 ng/mL).

Statistics

Previous to t-tests, all the samples were checked for normality by Kolmogorov-Smirnov test and Homoscedasticity was checked using the F-test. Adequate t-tests were performed.

p-values from figure 4E are the following:

TTEST		wt DMSO	wt PARPi	ΔSIM DMSO	ΔSIM PARPi
SLX4	wt DMSO	1			
	wt PARPi	2.92741E-06	1		
	ΔSIM DMSO	1.18625E-09	0.354098899	1	
	ΔSIM PARPi	2.23693E-26	6.20418E-08	5.40895E-08	1

TTEST		wt DMSO	wt PARPi	ΔSIM DMSO	ΔSIM PARPi
NBS1	wt DMSO	1			
	wt PARPi	0.109500366	1		
	ΔSIM DMSO	0.254286952	0.664815316	1	
	ΔSIM PARPi	0.202538335	0.793403829	0.874200269	1

SUPPLEMENTARY REFERENCES

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