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Supplemental Information

Histone H3 Lysine 9 Acetylation Obstructs ATM Activation and Promotes Ionizing Radiation Sensitivity in Normal Stem Cells

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Figure S1, related to Figure 1

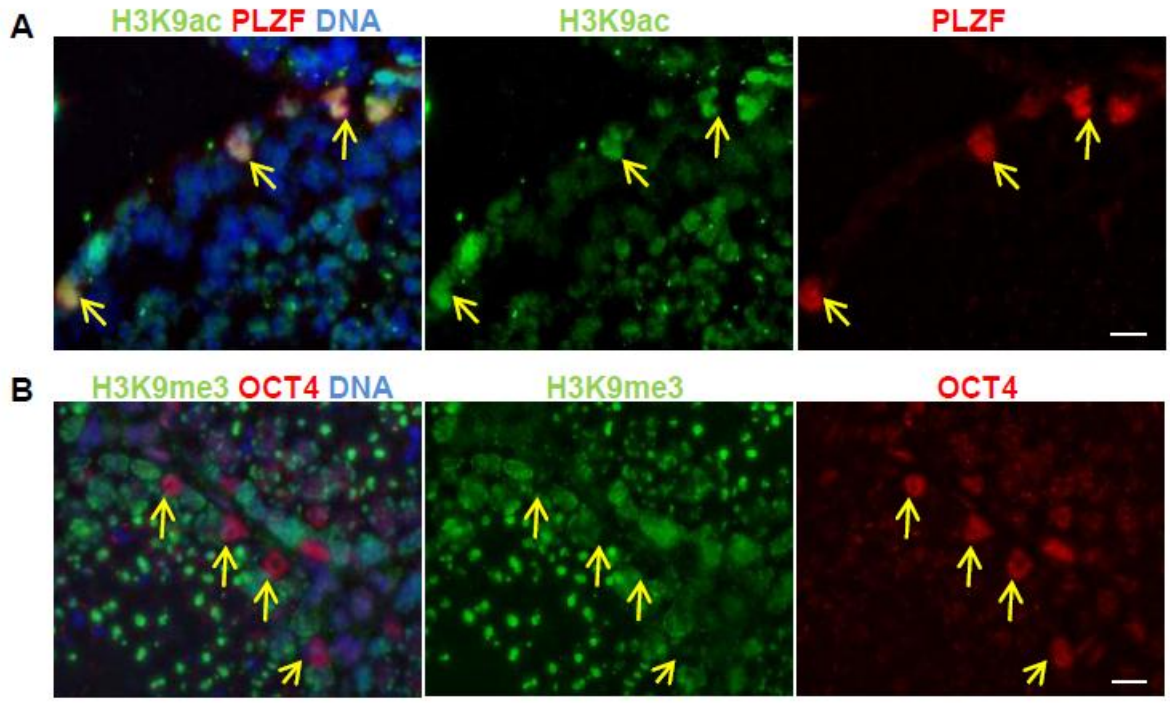


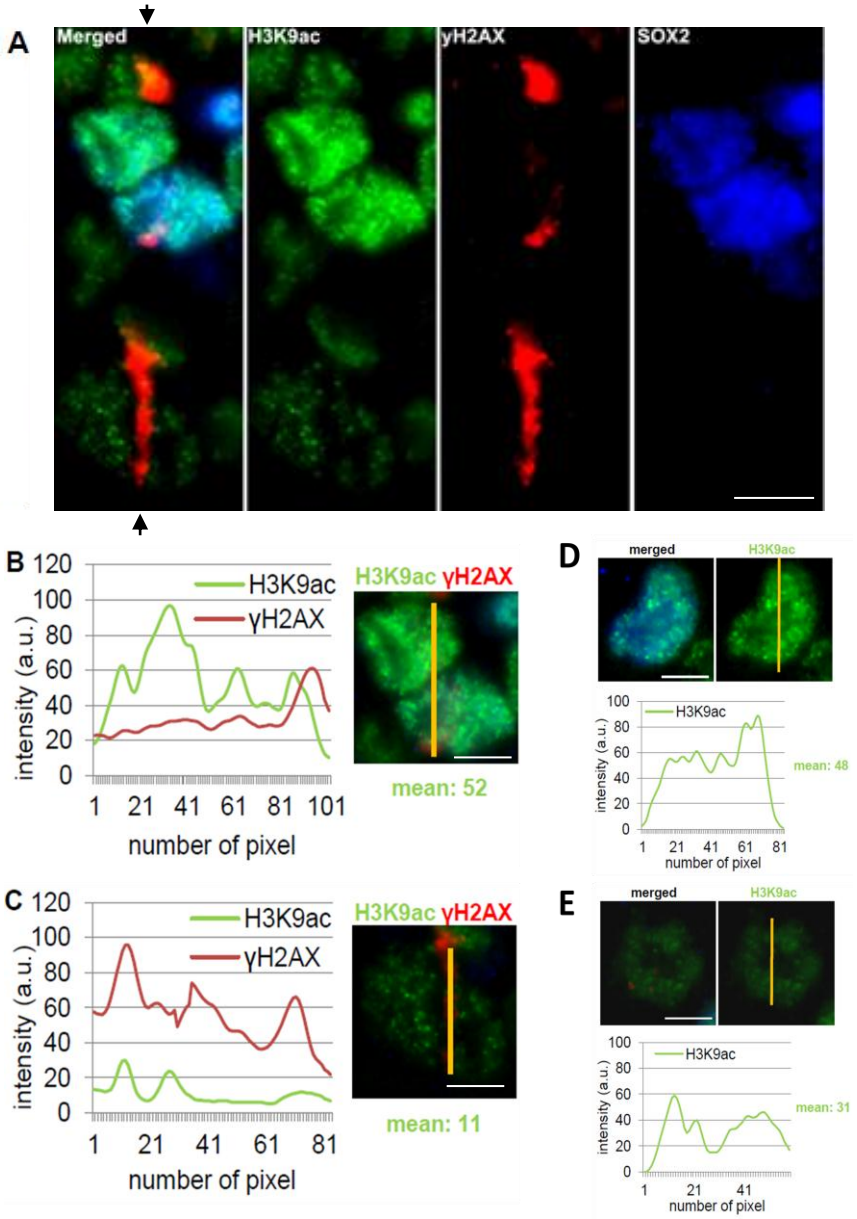
Figure S1, related to Figure 1

(A) H3K9ac, stem cell marker PLZF (promyelocytic leukemia zinc finger protein) and the DNA by DAPI staining were detected in tissue sections of adult murine testis.

(B) H3K9me3, stem cell marker OCT4 and the DNA by DAPI staining were detected in tissue sections of adult murine testis.

Arrows indicate spermatogonial stem cells. Scale bar = 10 μm.

Figure S2, related to Figure 2



F	H3K9ac					
	< 2 mins		< 5 mins		> 5 mins	
	Deacetylated	Unchanged	Deacetylated	Unchanged	Deacetylated	Unchanged
ES (n=40)	40%	60%	40%	60%	40%	60%
ED (n=45)	80%	20%	90%	10%	90%	10%
	H3K9me3					
	< 2 mins		< 5 mins		> 5 mins	
	Methylated	Unchanged	Methylated	Unchanged	Methylated	Unchanged
ES (n=30)	5%	95%	5%	95%	5%	95%
ED (n=30)	90%	10%	40%	60%	5%	95%

Figure S2, related to Figure 2

(A) Uncropped image showing stem (ES) and non-stem (ED) cells (cropped pictures shown in Figure 2). ES and ED cells were co-plated, micro-irradiated and after fixation H3K9ac, γH2AX and SOX2 detected. Black arrows on top and bottom of the merged colors image indicate the line of micro-irradiation through ES and ED cells.

(B) Intensity profile of H3K9ac and γH2AX along the yellow line of ES cells as Figure 2SA.

(C) Intensity profile of H3K9ac and γH2AX along the yellow line of ED cells as Figure 2SA.

(D) Unirradiated ES cell was fixed and H3K9ac, γH2AX and SOX2 detected. All markers are shown in the merged image (upper left) or H3K9ac alone (upper right). Lower image shows intensity profile of H3K9ac along the yellow line.

(E) Unirradiated ED cell treated as in (D). Lower image shows intensity profile of H3K9ac along the yellow line.

(F) The percentage of ES and ED cells showing a deacetylation of H3K9ac or no change in H3K9ac at DNA damage sites after laser irradiation as well as cells showing a trimethylation of H3K9 or no change was quantified at different time endopoints. N= number of analyzed cells, pooled from at least 3 independent experiments.

In the line profiles shown in (B), (C), (D) and (E) for every depicted intensity value an average of 5 adjacent pixel was calculated.

Mean = mean intensity value of H3K9ac from all values of the line profile. Scale bars = 10 µm.

Figure S3, related to Figure 3

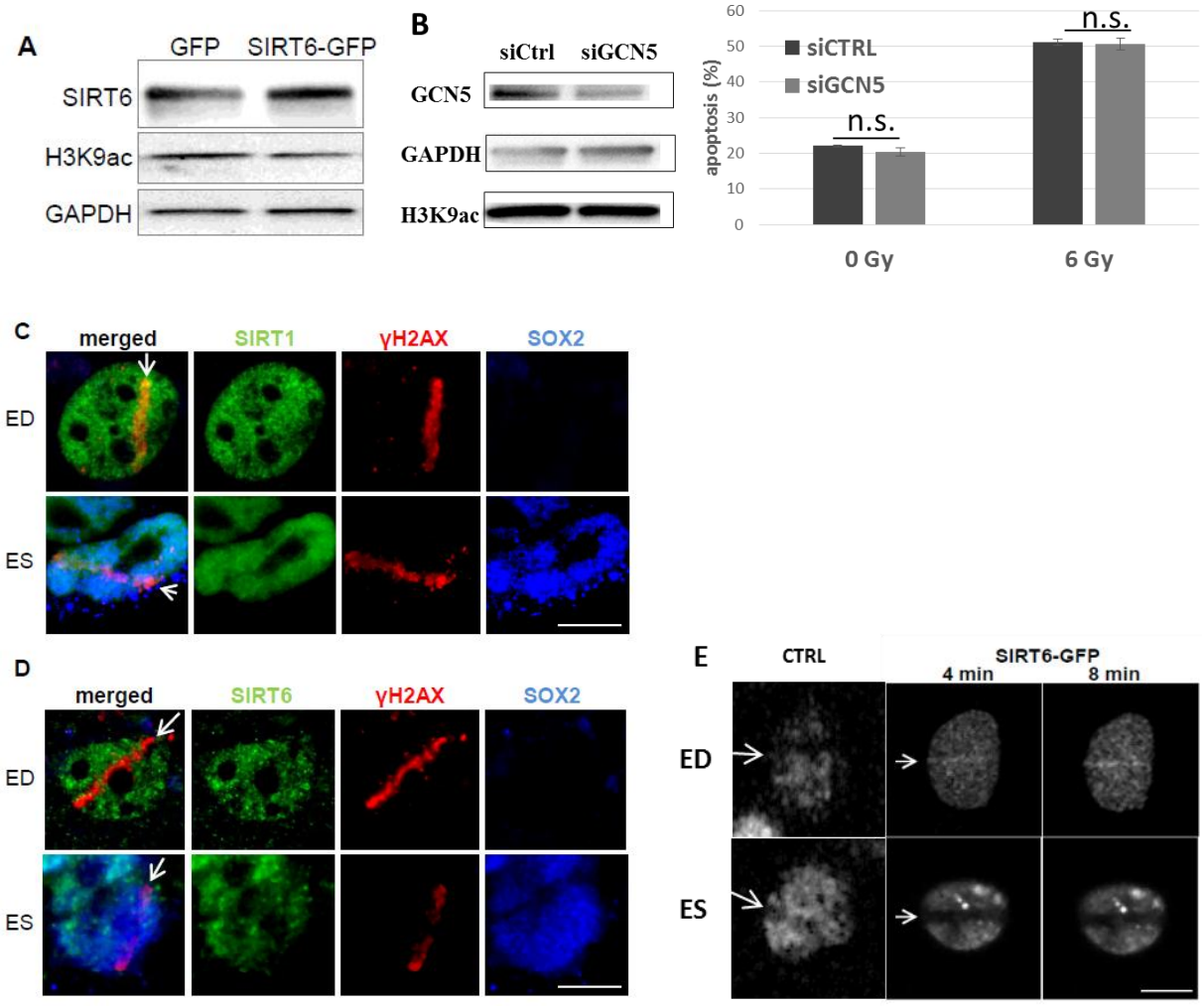


Figure S3, related to figure3

(A) Embryonic stem cells were transfected with GFP-plasmid or SIRT6-GFP-plasmid and SIRT6, H3K9ac and GADPH detected at 24h after transfection by immunoblot.

(B) Embryonic stem cells were treated with siCTRL and GCN5 siRNA: H3K9ac and GADPH detected by immunoblot (left) and apoptosis analyzed 16 h after irradiation by Annexin V labeling (right). 3 independent experiments performed.

(C) ES and ED cells were micro-irradiated, fixed and SIRT1, γH2AX and SOX2 detected.

(D) ES and ED cells were micro-irradiated, fixed and SIRT6, γH2AX and SOX2 detected.

(E) ED cell (upper panel) and ES cell (lower panel) transfected with GFP-plasmid (CTRL) and SIRT6-GFP were micro-irradiated and SIRT6-GFP detected at 4 and 8 min after irradiation.

White arrows indicate direction of laser irradiation. Scale bar = 10 µm.

Error bars = SD; n.s. = p>0.05.

Figure S4, related to Figure 4

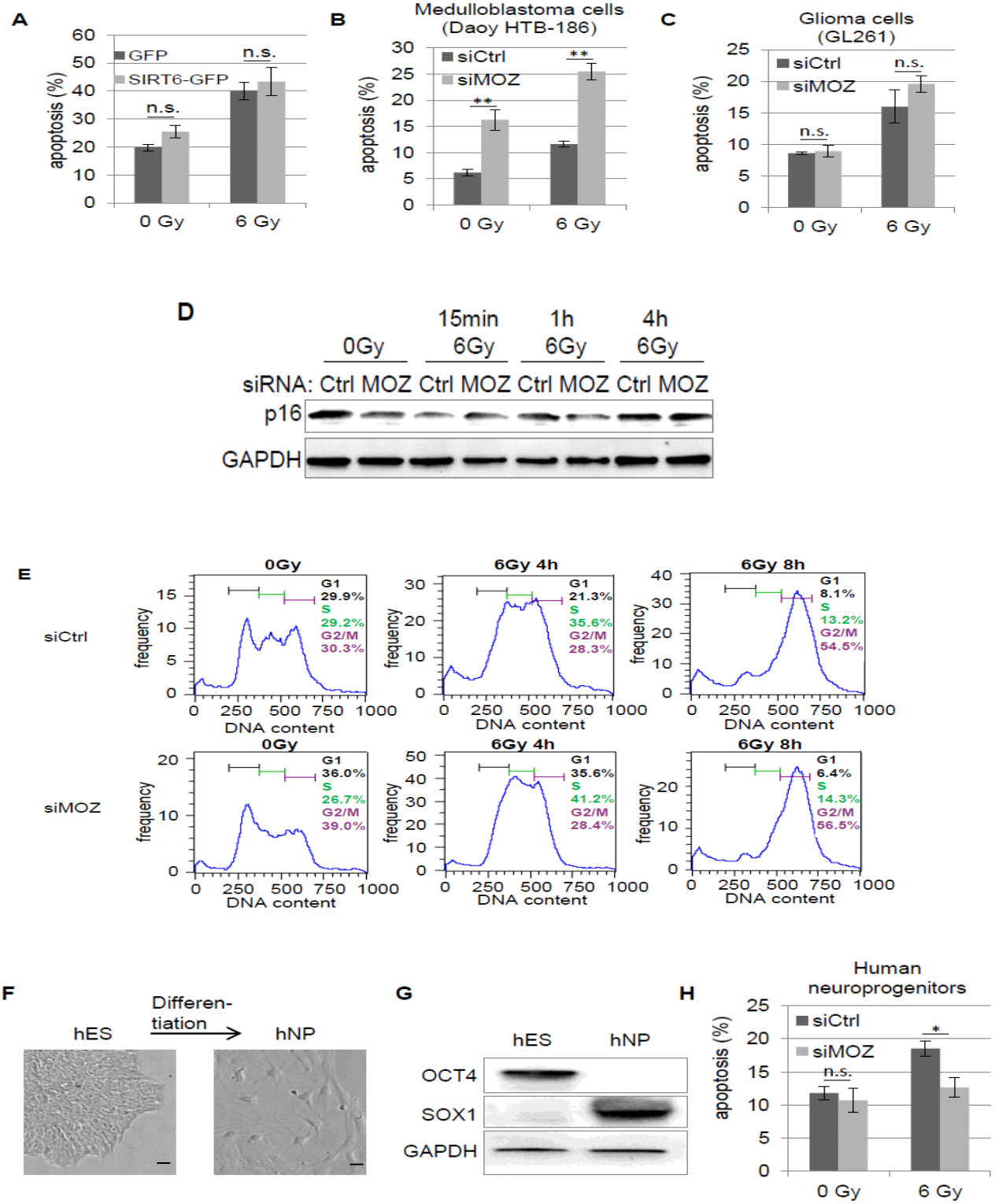


Figure S4, Related to Figure 4

(A) Embryonic stem cells were transfected with GFP-plasmid or SIRT6-GFP-plasmid, irradiated 24 h after transfection and apoptosis analyzed 16 h after irradiation by Annexin V labeling. 3 independent experiments performed.
(B) Human medulloblastoma cells (Daoy HTB-186) treated with control (siCtrl) or MOZ siRNA (siMOZ) were irradiated and apoptosis quantified at 16 h by Annexin V labeling. 3 independent experiments performed.
(C) Same assay and treatment as (B), but murine glioblastoma cells (GL261) were analyzed. 3 independent experiments performed.
(D) p16 and GAPDH were detected by immunoblot using lysates of murine ES cells treated with siRNA as in (B) and harvested at indicated timepoints.
(E) Murine ES cells were treated with siRNA as in (B) were harvested at indicated timepoints and DNA content analyzed by propidium iodide (PI) staining and flow cytometry.
(F) Micrographs of human ES cells (hES) and from these cells directly differentiated neuroprogenitors (hNP).
(G) OCT4, SOX1 and GAPDH levels of cells as in (F) were detected in the immunoblot.
(H) Human neuroprogenitors were treated with siRNA as in (B) and apoptosis analyzed 24 h after irradiation by Annexin V labeling. 3 independent experiments performed.
Error bars = SD; * = $p<0.05$; ** = $p<0.01$; n.s. = $p>0.05$. Scale bar = 10 µm.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell culture and undirected differentiation

Wild-type murine embryonic stem cells (LK-1 used throughout this study, EDJ22 were used only as comparison to LK-1 cells in Figure 4B) were originally isolated from mouse blastocysts and obtained from the Murine Embryonic Stem Cell Core at Washington University in Saint Louis. Cells were cultured as previously described for EDJ22 and RW.4 cells (Jacobs et al., 2016). Cells were karyotypical normal and cultured for not more than 20 passages and tested bi-monthly for mycoplasma. Undirected differentiation of cells was achieved by depletion of LIF (leukemia inhibitory factor) and beta-mercaptoethanol for at least 4 days as described earlier (Jacobs et al., 2016). Human medulloblastoma cells (Daoy HTB-186, obtained from ATCC) were cultured in MEM containing 10 % serum and murine glioma cells GL261 were obtained from the National Cancer Institute and cultured in DMEM/F12 containing 10 % serum. Neural stem cells were isolated from the dentate gyrus of P0-P2 newborn mice and cultured or differentiated. After dissection of the hippocampal region, cells were dissociated by pipetting the tissue up and down and neural stem cells were cultured in suspension in dishes non-treated for tissue culture. Neural stem cells were cultured in AB2 Basal Neural Medium (Aruna Biomedical) containing 20 ng/ml EGF (epidermal growth factor), 10 ng/ml bFGF (basic fibroblast growth factor), 1 % N2-Supplement and 2 % B27 (Invitrogen). Differentiation of neural stem cells was induced by culturing cells for at least 5 days with 10 % serum (Hyclone) and without EGF/FGF on tissue culture-treated dishes for attachment.
Human neuroprogenitor cells were cultured at the Human Embryonic Stem Cell core facility of Washington University. We directly derived the cells from H9 cells grown on Matrigel covered plates in mTeSRTM1 with supplement (Stem Cell Technologies) and passaged using ReLeSRTM (Stem Cell Technologies) and 5 μM Rock inhibitor (Calbiochem). They were differentiated into the neural lineage using the Stem Cell Technologies monolayer protocol. For this, cells were grown on poly-L-ornithine and laminin coated plates for several days in STEMdiffTM Neural Induction Medium and several days in STEMdiffTM Neural Progenitor Medium containing supplement A and B, using accutase for passages.

X-ray irradiation and microirradiation

Cells were irradiated with 160 keV X-rays with indicated doses at a dose rate of 1.7 Gy/min in an RS-2000 Biological Research Irradiatior (Rad-Source). For microirradiation co-plated embryonic and differentiated cells were cultured for 2 days in 70 μM BrdU and irradiated with a 405 nm and 633 nm laser using a LSM 510 Confocal Microscope (Zeiss, Plan-APOCHROMAT 63x/1.4 oil objective) with incubation chamber as previously described (Jacobs et al., 2016). Stem cells were identified directly before irradiation by CDy1 (Active Motif) according to manufacturer's instructions in order to target stem cells growing adjacent to differentiated cells. ZEN software was used to select cells and target irradiation. Fixation of cells for immunofluorescence staining was performed immediately after irradiation.

RNA interference

24 h after plating cells were transfected using RNAiMAX Lipofectamine (Invitrogen) according to the manufacturer's instructions. siRNA against murine or human MOZ (Santa Cruz, sc-149523 mouse, sc-37959 human), GCN5 (Santa Cruiz, sc-37947) and Suv39h1 (Santa Cruz, sc-38464 mouse) was used at an end concentration of 50 nM and incubated 24 h before irradiation. According to manufacturer, siRNA products consist of pools of three to five target-specific 19-25 nt siRNAs designed to specifically knockdown gene expression. As control cells were transfected with the same concentration of non-targeted control siRNA (Dharmacon).

Plasmid transfection

SIRT6-GFP-plasmid (Liszt et al., 2005) was obtained from Addgene (#20275) and to generate control GFP-plasmid the SIRT6 insert was cut out using BamHI and BglII (New England Biolabs). Efficient restriction digest was tested by gel electrophoresis. For transfection of plasmids Lipofectamine LTX with Plus Reagent (Invitrogen) was used according to manufacturer's instructions. Transfection efficiency (80 % for GFP-plasmid, 55% for Sirt6-GFP-plasmid) was evaluated 24 h after transfection by measuring GFP positivity using flow cytometry. Only GFP-positive cells were analyzed for apoptosis induction.

Inhibitors

ATM inhibitor (Sigma Aldrich, KU-55933) was dissolved in DMSO and the cells incubated with 10 µM inhibitor for 1h prior irradiation.

Immunoblot

Trypsinized cells were lysed in RIPA buffer (Thermo Scientific) containing protease inhibitor cocktail, 1 mM PMSF and phosphatase inhibitors (Sigma). Lysates were sonicated in 5 times for 30 seconds, centrifuged for 15 min at 8000 g and protein concentration determined using the BCA protein assay (Thermo Scientific). 15 µg of lysate were loaded on a 4 - 15 % tris glycine PAA-Gel (BioRad) using tris glycine running buffer (novex). Protein size was determined with the Kaleidoscope prestained marker (BioRad). Transfer to a PVDF membrane was performed in a wet chamber with tris glycine transfer buffer (novex). For blocking the membrane was incubated in 5 % milk or BSA in TBS-T for 30 min. Primary antibodies against phospho-S1981-ATM (Cell Signaling, 10H11.E12, mouse, 1:1000 in milk), MOZ (Acris, AP00341PU-N, rabbit, 1:200 in BSA), Suv39h1 (Abgent, rabbit, 1:500 in milk), SIRT6 (Proteintech Group, 13572-1-AP, rabbit, 1:1000 in BSA) GAPDH (Sigma Aldrich, G8795, mouse, 1:100,000 in milk), H3K9ac (Millipore, 06-942, rabbit, 1:1000 in BSA), H3K9me3 (Millipore, CMA308, mouse, 1:25,000 in BSA), SOX2 (Abcam, ab79351, mouse, 1:1000 in BSA), SOX1 (R&D Systems, AF3369 , goat, 1:1000 in BSA), OCT4 (Abcam, ab19857, rabbit, 1:1000 in BSA), p16 (Thermo Scientific, MA5-17142, mouse, 1:5000 in BSA) were incubated over night at 4 ◦C and secondary peroxidase conjugated antibodies (anti mouse or anti rabbit, Sigma, 1:5000; anti goat Santa Cruz 1:5000) for 2 h at RT. The chemoluminescence signal was detected with ECL substrate (GE/Pierce) in a ChemiDoc MP digital system (BioRad).

Cell cycle measurement

Cells were harvested using trypsin, washed with PBS and resuspended in 500 µl cold PBS. 2 ml of cold 100% EtOH were added drop-wise while vortexing cells slowly. Samples were stored at -20◦C until staining. After centrifugation for 8 min at 1800 g, PBS wash and centrifugation at 500 for 8 min, cells were incubated in 100 µl 0.1 µg/µl RNase (Cell Signaling) in PBS for 1h 15 min at 37◦C. 200 µl 1 µg/µl propidium iodide (BD Pharmingen) in PBS were added and samples incubated 10 min at RT until measurement with flow cytometer.

Immunofluorescence staining

For analysis with flow cytometry cells were trypsinized, washed with PBS, fixed 5 min with 4 % formaldehyde in PBS, permeabilized 2 min in 0.2 % Triton X-100 in PBS, washed with PBS and blocked in 2 % BSA in PBS over night at 4 ◦C. Centrifugation steps were carried out at 300 g before fixation and 800 g after fixation for at least 5 min. Cells were incubated with antibodies against phospho-S1981-ATM (Cell Signaling, 10H11.E12, mouse, 1:200) diluted in 2% BSA/PBS for 1 h at RT. After washing in PBS cells were incubated with anti-mouse FITC antibody (Vectashield, 1:100) for 1 h at RT. After washing in PBS signal intensities of 1000 cells were measured using a Miltenyi flow cytometer.

For microscopy cells were grown on cover slips and fixed for 5 min with 4 % formaldehyde in PBS and permeabilized for 4 min in 0.2 % Triton X-100 in PBS. After several washes in PBS, samples were incubated at least 20 min with 2 % BSA in PBS. Incubation with primary or secondary antibody was conducted for 1 h at 37◦C. For antibody staining of microscopy samples standard protocols were used as described earlier (Jacobs et al., 2016). The following primary antibodies were used: H3K9ac (Millipore, 06-942, rabbit 1:200), H3K9me3 (Millipore, CMA308, mouse, 1:200), γH2AX (Millipore, 05-636, mouse, 1:1000), SIRT1 (Millipore, 07-131, rabbit, 1:200), SIRT6 (Proteintech, 13572-1-AP, rabbit, 1:100), SOX2 (Abcam, ab79351, mouse, 1:200) and OCT4 (Abcam, ab19857, rabbit, 1:200). Secondary anti-rabbit and anti-mouse antibodies conjugated to fluorescein, Texas Red or AMCA (Vector) were used at a dilution of 1:100. Samples were mounted in Vectashield mounting medium containing DAPI (Vector).

Three-color staining of micro-irradiation samples required a sequential staining as described previously (Jacobs et al., 2016). For this, samples were incubated with anti-H3K9ac (rabbit), anti-SOX2 (mouse) and secondary antibody dilutions (anti-mouse-AMCA and anti-rabbit-fluorescein) as described above. This was followed by a 5 min incubation with γH2AX antibody dilution and subsequent 5 min incubation with anti-mouse Texas Red-conjugated antibody. Short 5 min incubation times were chosen to avoid cross reactivity between anti-mouse antibody stainings.

Samples showing nuclear-wide binding of anti-mouse-Texas Red antibody to Sox2 antibody were excluded from evaluation.

Immunhistochemistry

6-8 weeks old male C57BL/6 mice were sacrificed and tissues frozen in OCT media. Cryosections of 10 µm thickness were obtained from the histology core at Washington University. Frozen sections were thawed in cold PBS, fixed for 15 min in 4 % formaldehyde in PBS and permeabilized in 0.2 % Triton-X-100 in PBS for 15 min. After several short PBS washes sections were blocked in 2 % BSA in PBS for 1 h. Antibodies against H3K9ac (Millipore, 06-942, rabbit, 1:200), H3K9me3 (Millipore, CMA308, mouse, 1:200), H3K9me3 (Abcam, ab8898, mouse, 1:1000) together with SOX2 (Abcam, ab79351, mouse, 1:100), PLZF (Santa Cruz, sc-28319, mouse, 1:50), OCT4 (Abcam, ab19857, rabbit, 1:200) or H3 (Cell Signaling, D1H2, rabbit, 1:250) were used for 2 h in a wet chamber at 37 ◦C. After PBS washes secondary antibody (1:100, Vector) incubation was carried out for 45 min. Slides were washed, dried and mounted in Vectashield mounting medium containing DAPI (Vector).

Microscopy and image processing

Imaging was performed using a Zeiss Axioplan 2 microscope with 20x, 63x or 100x objectives (Plan-NEOFLUAR 20x/0.5, Plan-APOCHROMAT 63x/1.4 Oil, Plan-NEOFLUAR 100x/1.3 Oil) and Meta Systems ISIS imaging software. ImageJ was used to process micrographs, which included cropping of images and minimal adjustment of signal intensity. All images of one experiment were processed in the same way.

Neutral Comet Assay

At indicated timepoints after irradiation cells were trypsinized, centrifuged for 5 min at 300 g and resuspended in media to a final concentration of $0.5*10^6$ cells/ml. 5 µl of this cell suspension were resuspended in 50 µl prewarmed (37◦C) agarose (Trevigen) and distributed on a glass slide (CometSlide, Trevigen). Slides were cooled at 4◦C for 15 min and incubated in lysis solution (Trevigen) at 4◦C for 1h. After lysis slides were shortly washed in water and stored in cold TAE. After collection of all samples electrophoresis was conducted in a cooled chamber (Hoefer Scientific instruments) with TAE at 25 V for 35 min. Slides were washed in water, dried and stained with SybrGreen (Trevigen) for 90 min. After additional washes in water slides were dried. Images were taken using a Zeiss Axioplan 2 microscope using a 20x objective. Evaluation of olive moments was performed with CometScore software (TriTek).

Clonogenic assay

LK-1 murine embryonic stem cells with 24 h of MOZ siRNA treatment were irradiated with indicated doses and incubated 6 h after irradiation to allow for DNA repair to occur. Cells were then trypsinized and counted using a Vi-Cell cell counter. The same number of cells for control and MOZ siRNA treated cells were plated and grown for 7 days. Fixation was performed by PBS wash and incubation in cold methanol on ice for 10 min. For staining cells were incubated for 10 min with crystal violet (Sigma) at RT. Cells were washed twice with water, dried and counted. Plating efficiency for LK-1 cells was 15 %.

Cytogenetic analysis

24 h after MOZ or control siRNA treatment cells were irradiated. The dose of 2 Gy was chosen to still allow cells to enter mitosis after irradiation. 5 h after irradiation cells were treated with 100 ng/ml colcemid (Gibco) for additional 1 h and 45 min. Mitotic shake-off in cold trypsin was performed, harvested cells were centrifuged for 8 min at 250 g and treated with hypotonic buffer (0.56 % KCl) for 8 min. Samples were fixed in acetic acid/methanol (1:3) for at least 45 min and fluorescence-*in-situ*-hybridization of telomeres performed as previously described (Jacobs et al., 2016). Stained chromosomes were mounted in Vectashield mounting medium containing DAPI (Vector). Imaging of chromosomes was performed using Zeiss Axioplan 2 microscope with a 20 x objective. Chromosome aberrations were defined as chromosome or chromatid breaks, translocations or radials. At least 40 chromosome plates were scored.

SUPPLEMENTAL REFERENCE

Liszt, G., Ford, E., Kurtev, M., and Guarente, L. (2005). Mouse Sir2 homolog SIRT6 is a nuclear ADP-ribosyltransferase. J. Biol. Chem. *280*, 21313–21320.