Human HDAC1 and HDAC2 function in the DNA-damage response to promote DNA non-homologous end-joining

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Supplementary Online Materials

Supplementary Methods

Supplementary References

Supplementary Table 1

Supplementary Figures 1-8 (with legends)

Supplementary Methods

Cell culture, reagents and treatments

Human U2OS and HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg ml^{-1} streptomycin and 2 mM L-glutamine. BJ cells (with or without *RAS* over-expression) were generated and grown in the medium described above supplemented with sodium pyruvate as described previously [34]. Replicative senescent BJ cells were generated in a previous study and these cells were maintained as described[62]. To create a complementation system for HDAC1, human HDAC1 was cloned into eGFP-C3. GFP-HDAC1 was then mutated by site-directed mutagenesis to obtain a siRNA resistant clone against the HDAC1_A siRNA (5'CAGCGACUGUUUGAGAACC3'). The siResA-GFP-HDAC1 clone containing the following silent amino acid substitutions (underlined) 5'CAA CGA CTG TTC GAA AAT T 3' was constructed using site-directed mutagenesis and its resistance to HDAC1_A siRNA was confirmed by Western blotting and IF analysis. For the complementation assay using Western blotting, U2OS cell lines were constructed that stably express siResA-GFP-HDAC1 (selection in 0.5 mg ml^{-1} of G418) at near endogenous levels as analyzed using an antibody against endogenous HDAC1. To test for complementation of HDAC1, these cells were transfected with HDAC2 siRNAs A and B, and either siLuc, siHDAC1_A or siHDAC1_B (which targets both endogenous and GFP-tagged HDAC1). After two rounds of siRNA transfections, cells were taken 72 h after the first siRNA transfection and were analyzed by Western blotting. To test for complementation of HDAC1 by IF analysis, U2OS cells were transfected with

empty vector or with the siResA-GFP-HDAC1 construct. These cells were then subjected to two rounds of siRNA transfection with the indicated siRNAs, treated with phleomycin for 2 h and fixed and processed as described below for IF analysis. Over 200 cells were counted for pan-nuclear γH2AX positive cells and these numbers are graphed (n=3, error bars represent S.E.M.). To analyze H3K56Ac, U2OS cells were transiently transfected with the siResA-GFP-HDAC1 construct followed by two rounds of siRNA transfections with HDAC2_A+B and HDAC1_A siRNAs. After 72 h, cells were fixed and analyzed using H3K56Ac (Epitomics) and visualizing GFP-HDAC1 using the normal protocol described below for IF analysis. To analyze NHEJ factors for live analysis, we obtained pEGFP-C1-6xHis-KU70 from Dr Philippe Frit (IPBS, Toulouse, France) and full length ARTEMIS isoform 1 cDNA was obtained by PCR from IMAGE clones 3945623 and 4272155 and cloned as a N-terminal fusion to FLAG-GFP tag into pEGFP-N1 (Clontech). Detailed sequences and cloning information can be provided if requested. U2OS cells were transfected with Lipofectamine 2000 (Invitrogen) as specified by manufacturers by pEGFP-C1-6xHis-KU70 or pEGFP-N1-ARTEMIS-FLAG. Stable cell lines expressing GFP-KU70 and GFP-ARTEMIS were established by selection with 0.5 mg ml^{-1} G418 for two weeks and used for further analysis.

Laser micro-irradiation

Laser micro-irradiation was done essentially as described before[29]. Damage was created by exposure to a UV-A laser beam in cells plated on glass-bottomed dishes (Willco Wells). Cells were pre-sensitized with 10 μM of 5-bromo-2'-

deoxyuridine (BrdU; Sigma Aldrich) in normal medium for 24 h at 37°C. Laser micro-irradiation was carried out with a FluoView 1000 confocal microscope (Olympus) and a 405 nm laser diode (6 mW, SIM scanner). Laser settings (0.40 mW output, 50 scans) were used to generate DNA damage that was restricted to the laser path in a pre-sensitization-dependent manner with minimal cellular toxicity. To analyze NHEJ factors using live analysis of GFP-tagged proteins, 75000 cells from the corresponding cell line were seeded in glass bottom dishes (Willco Wells) two days before laser micro-irradiation. The day before irradiation, medium was refreshed with phenol red-free medium supplemented with 10 μM 5-bromo-2'-deoxyuridine (BrdU, Sigma-Aldrich). Laser micro-irradiations were carried out as nuclear dots of 2.7-3 μm using a FluoView 1000 confocal microscope (Olympus) equipped with a 37°C heating stage and a 405 nm laser diode (6 mW, SIM scanner) focused through a 60x UPlanSApo/1.35 oil objective. Time of cell exposure to the laser beam was around 250 ms. Laser settings (0.40 mW output, 50 scans) were chosen as to generate a detectable damage response in a BrdU, presensitization-dependent manner. Variation of the fluorescence intensity was quantified as the difference between the average fluorescence intensity in the damaged region versus the average fluorescence intensity in an undamaged region of the same size in the same cell. Error bars represent S.E.M. Each curve corresponds to data obtained from at least 10 independently filmed cells. Data was analyzed in ImageJ.

Protein extracts and Western blotting

For whole cell extracts, cells were washed once with PBS (phosphate buffered saline), collected by adding Laemmli buffer (4% (v/v) SDS, 20% (v/v) glycerol and 120 mM Tris, pH 6.8), boiled for 5 min at 95°C, sheared through a 23-gauge needle and boiled again before loading. For chromatin fractionation, cells were processed as previously described[63]. Samples were resolved by SDS-PAGE and analyzed by standard western blotting techniques. Antigens were detected by standard chemiluminescence (ECL; Amersham) or quantified with a LI-COR Odyssey infrared imaging system (LI-COR Biosciences). Secondary antibodies used for ECL were goat anti-rabbit HRP (Perbio Science Ltd) and rabbit anti-mouse HRP (Dako Ltd). Secondary antibodies used for quantification were IRDye 680CW Donkey anti-rabbit or anti-mouse (LI-COR Biosciences).

siRNA transfection

RNA interference was performed as previously described[20]. Briefly, cells were subjected to two rounds of siRNA transfections with the indicated siRNAs using Qiagen HiPerFect (following manufacturer's protocol) and after 72 h, cells were processed and analyzed as described for each experiment. siRNA sequences used in this study are HDAC1_A (CAGCGACUGUUUGAGAACC), HDAC1_B (CUAAUGAGCUUCCAUACAA), HDAC2_A (GCGGAUAGCUUGUGAUGAA), HDAC2_B (GCAAAGAAAGCUAGAAUUG), HDAC3 (Dharmacon On-Target plus SMARTpool), SIRT1 (ACUUUGCUGUAACCCUGUA), SIRT2 (Dharmacon On-Target plus SMARTpool), CTIP (GCUAAAACAGGAACGAAUC), and Ligase IV (AGGAAGUAUUCUCAGGAAUUA). Western blot analyses with the indicated

antibodies were performed to analyze depletion efficiencies for each siRNA. For human SIRT2, siRNA depletion was tested on GFP-SIRT2 (cloned into eGFP-C1) that was transiently transfected into U2OS cells followed by two rounds of siRNA with the indicated siRNAs against SIRT2.

Immunofluorescence analyses

For DNA-damage analysis with BrdU, cells were first pulsed with BrdU then treated with the indicated DNA damage. After the first round of IF, to detect BrdU, coverslips containing the first antibody already detected with the secondary antibody were re-fixed in 2% PFA for 12 min at RT. Coverslips were washed 3X in PBS and incubated in 2 M HCl for 30 min to denature double-stranded DNA. Cells were washed four times in PBS to neutralize before blocking in 5% FBS in PBS for 30 min. Cells were incubated for 1 h at RT in mouse BrdU primary antibody in PBS containing 5% FBS. Coverslips were then processed using standard IF procedures.

Chromatin Immunoprecipitation (ChIP) analysis

Cells were fixed with 1% (v/v) formaldehyde (Sigma) at room temperature for 15 min and quenched with 20 mM glycine for 5 min. Cells were rinsed two times with 10 ml of cold PBS, and collected by scraping two times with 5 ml of cold PBS followed by a centrifugation for 30 min at 13,000rpm at 4°C to pellet the cells. The pellets from two dishes were resuspended in 2 ml of cold RIPA Buffer (50 mM Tris-HCl pH8, 150 mM NaCl, 2 mM EDTA pH8, 1% (v/v) NP-40, 0.5% (v/v) sodium deoxycholate, 0.1% (v/v) SDS) supplemented with protease inhibitors

(Complete, EDTA-free from Roche). The suspension was sonicated in 15 ml conical tubes twice for 10 minutes at maximum setting (30 sec ON/OFF cycles) in a cooled Bioruptor® (Diagenode) and cleared by centrifugation for 15 min at 13,000 rpm. Chromatin was quantified and diluted with RIPA buffer to contain the equivalent of 20 ng/μl of DNA. 500 μl of chromatin corresponding to 10 μg of DNA was incubated with the appropriate antibodies overnight at 4°C, then with 25 μl of pre-washed Protein A+G Dynabeads® (Invitrogen) for 2 h at RT. Beads were washed for 5 min at RT with 1 ml of TSE-150 (0.1% (v/v) SDS, 1% (v/v) Triton X-100, 150mM NaCl, 2mM EDTA, 20mM Tris-HCl, pH 8), TSE-500 (0.1% (v/v) SDS, 1% (v/v) Triton X-100, 500mM NaCl, 2mM EDTA, 20mM Tris-HCl, pH 8), LiCl buffer (0.25M LiCl, 1% (v/v) NP-40, 1% (v/v) DOC, 1mM EDTA, 10mM Tris, pH 8) and TE (10 mM Tris-HCl, pH8, 0.1 mM EDTA). Immuno-complexes were eluted from the beads with a total of 125 μl of elution buffer (100 mM sodium bicarbonate, 1% (v/v) SDS) for 30 min at 30°C and de-crosslinked overnight at 65°C. The ChIP samples were purified with the Qiaquick® PCR purification kit (Qiagen) and DNA was eluted from the columns with 50 μl of water. From each immunoprecipitation, 2.5 μl of eluted “ChIPed” sample was used in Real-time PCR analysis on an ABI PRISM 7000 /7300 sequence detection system with the use of SYBR® Green (Applied Biosystems) for each primer pair.

The sequences of the primers used for the qPCR analysis are: DSB 1 (Forward: GATTGGCTATGGGTGTGGAC Reverse: CATCCTTGCAAACCAGTCCT), DSB 2 (Forward: CCCTGGAGGTAGGTCTGGTT Reverse:

CGCACACTCACTGGTTCCT) No DSB site (Forward: CCCATCTCAACCTCCACACT Reverse: CTTGTCCAGATTCGCTGTGA). Antibodies used were H3 (Abcam), H3K56Ac (Upstate), H3K14Ac (Upstate) and γH2AX (Epitomics). qPCR primers used for NHEJ factors were DSB site (Forward: CCCTGGAGGTAGGTCTGGTT Reverse: CGCACACTCACTGGTTCCT) No DSB site (Forward: CCCATCTCAACCTCCACACT Reverse: CTTGTCCAGATTCGCTGTGA). Antibodies used were γH2AX (Epitomics), KU (p70/80, clone 162, Thermo Scientific) and XRCC4 (Abcam).

NHEJ Assays

Assays were performed essentially as previously described[64]. Briefly, one day after siRNA-transfections, U2OS cells were transfected with BamHI-XhoI linearized pEGFP-C1 (Clontech). The next day, cells were trypsinized, counted and plated. For each siRNA, transfection efficiency was calculated one day after transfection by counting GFP-positive cells. Also, two duplicate plates, one containing G418 (0.5 mg ml^{-1}, to observe NHEJ events) and one without (for calculating plating efficiency), were incubated for 10-14 days at 37°C until colonies had formed. Colonies were stained with 0.5% (v/v) crystal violet/20% (v/v) ethanol and counted. Random-plasmid integration events were calculated using transfection and plating efficiencies normalized to siLuc. Graphs represent triplicate experiments with error bars (+/- S.D.)

Homologous recombination (HR) assays

An integrated HR reporter DR-GFP-containing U2OS cell line was as described previously[65,66]. One day after siRNA-transfections, U2OS-DR-GFP cells were co-transfected with an I-*Sce*I expressing vector (pCBA-I-*Sce*I) and a monomeric red fluorescent protein expressing vector (pCS2-mRFP). 48 h following I-*Sce*I transfection, cells were collected and subjected to FACS (fluorescence-activated cell sorting) analysis to determine HR efficiency. Only RFP positive cells were analyzed to avoid possible differences in transfection efficiencies. Samples were analyzed by FACS with Summit V4.3 software to reveal the percentage of GFP-positive cells relative to the number of RFP-transfected cells (i.e HR levels). U2OS-DR-GFP cells that were transfected with pCS2-mRFP but lacked I-SceI represented background levels of HR. Dual GFP-positive and RFP-positive cells (i.e. cells where HR had occurred) were calculated for all siRNAs minus background and results were normalized to siLuc cells.

Neutral Comet assays

Comet assays were with the Single Cell Gel Electrophoresis Assay-kit (Trevigen). Briefly, U2OS cells were transfected with luciferase or HDAC1 and HDAC2 siRNAs. After 72 h post-transfection, cells were untreated or treated for 2 h with 60 mg/ml phleomycin or 20 Gy of IR followed by a 1 h recovery. Cells were then trypsinized and counted. Approximately 5 x 10^5 cells were mixed with low-melting agarose in a 1:10 ratio, of which 75 ml was transferred onto Gel Bond film and covered with a 13 mm coverslip. Samples were incubated at 4°C in the dark for 30 min to solidify. Cells were then lysed by incubation with lysis solution for 60

min. Slides were subsequently washed three times for 5 min in TBE. Slides were run for 7 min at 35 volts on a horizontal electrophoresis apparatus in TBE-buffer and subsequently fixed in 70% (v/v) ethanol for 5 min and allowed to dry overnight. Cells were visualized with SYBR-green and pictures were taken with a standard Olympus epifluorescence microscope. Analysis of comets was performed with the CometScore software (TriTek) and the average tail moment was calculated by counting around 100 cells for each sample.

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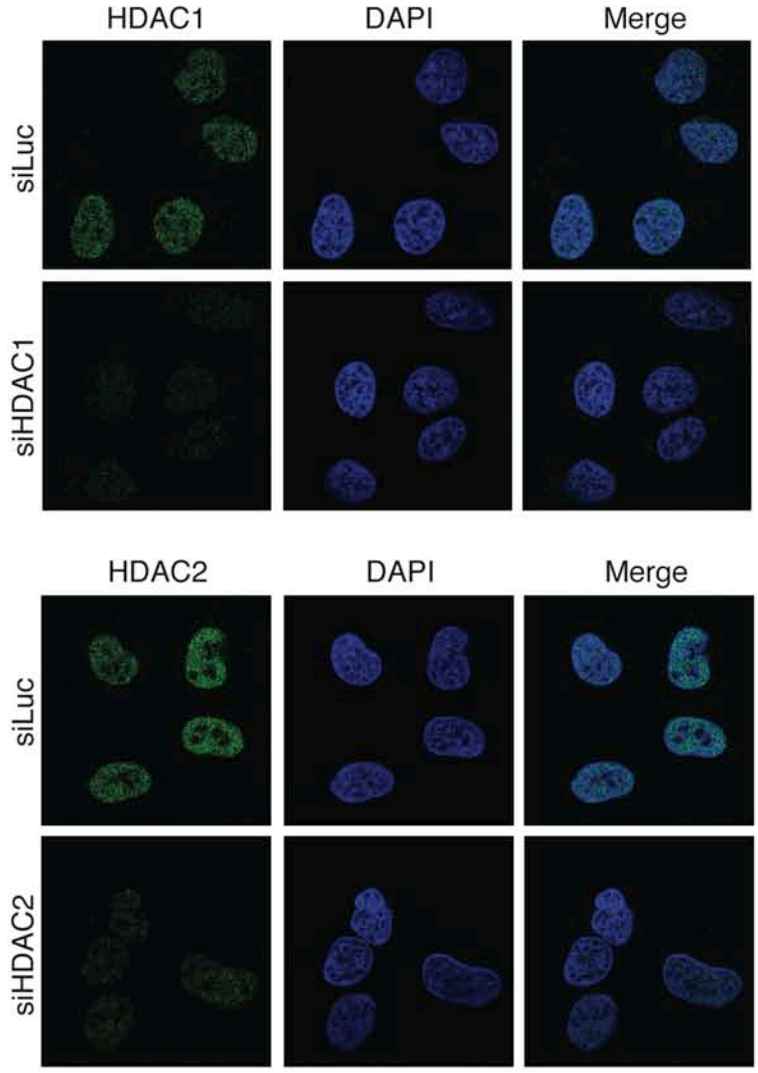
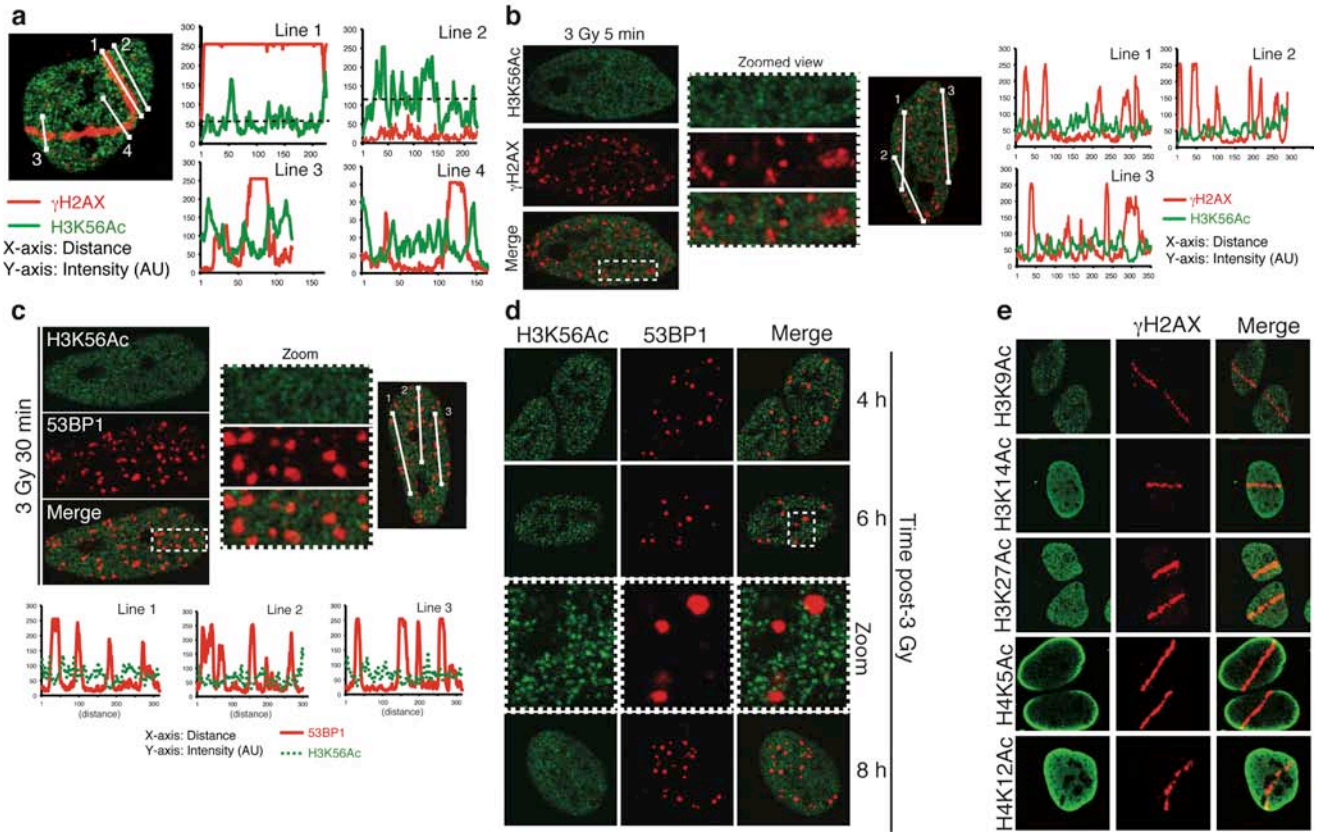
Antibody epitope	Company
H3K56Ac	Upstate
H3K56Ac	Epitomics
H3K27Ac	Upstate
H3K14Ac	Upstate
H3S10p	Abcam
H3K9Ac	Upstate
H4K5Ac	Active Motif
H4K8Ac	Active Motif
H4K12Ac	Active Motif
H4K16Ac	Active Motif
H3	Cell Signaling
H4	Abcam
H2A-ub	Upstate
SUMO1	Abcam
Pol II Ser5p	Abcam
BrdU	Amersham biosciences
Tubulin	Sigma-Aldrich
GFP	Invitrogen
HDAC1	Cell Signaling, Santa Cruz Biotechnology
HDAC2	Cell Signaling, Santa Cruz Biotechnology
HDAC3	Active Motif
HDAC8	Santa Cruz
SIRT1	Abcam (rabbit monoclonal)
53BP1 (Mouse)	[7]
53BP1 (Rabbit)	Novus Biologicals
γH2AX	Epitomics
γH2AX	Cell Signaling
γH2AX	Upstate
Chk1	Santa Cruz Biotechnology
Chk1 S345p	Cell Signaling
Chk2	Cell Signaling
Chk2 T68p	Cell Signaling
p53	Cell Signaling
p53 S15p	Cell Signaling
p53-Ac	Upstate
RPA	Abcam
RPA S4p/S8p	Bethyl Laboratories, Inc
RPA S33p	Bethyl Laboratories, Inc
CTIP	[8]
Ligase 4	Abcam
XRCC4	Abcam
XLF	Abcam
KU70	Abcam (N3H10)
DNA-PKcs	Abcam
DNA-PKcs S2056p	Abcam
DNA-PKcs T2609p	Abcam

Supplementary Table S1 List of antibodies used in this study.

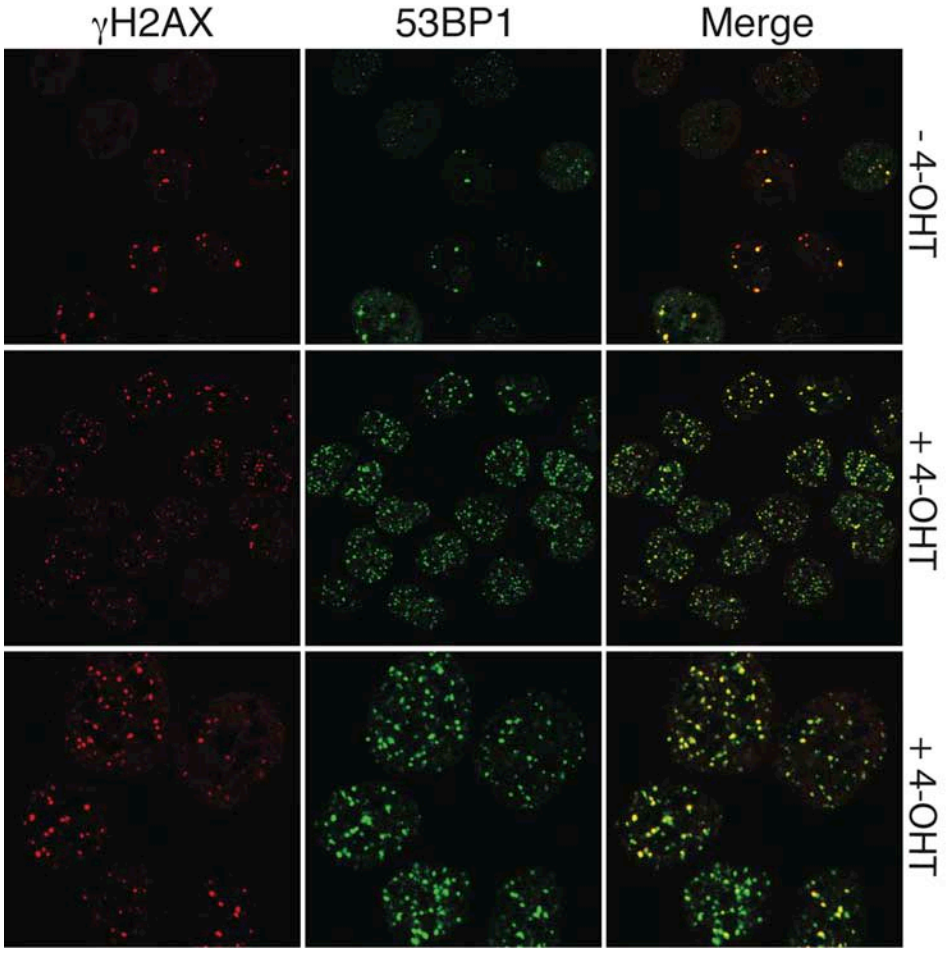
Miller_Figure S1



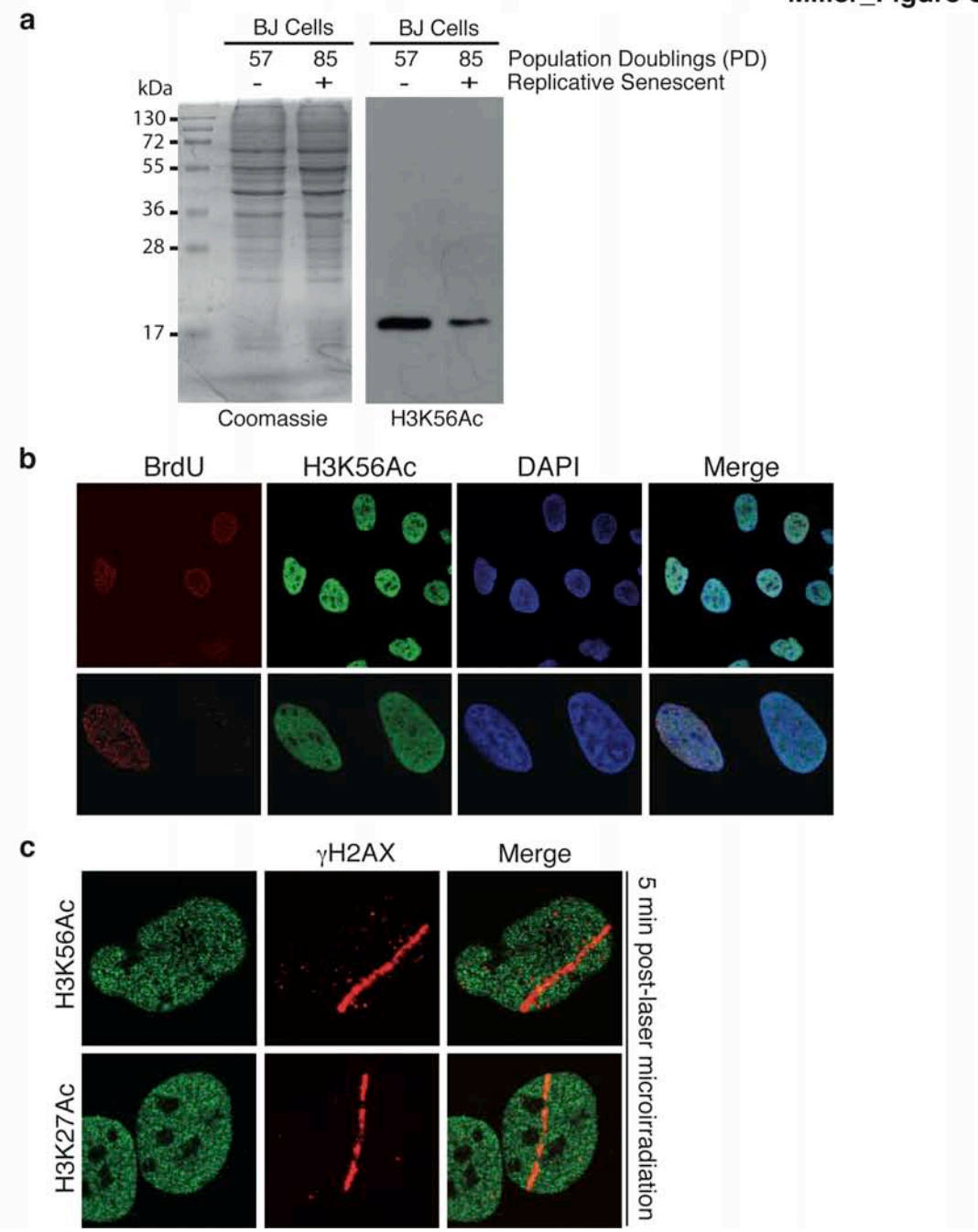
Supplementary Fig. S1 HDAC1 and HDAC2 antibodies are specific in IF analysis. U2OS cells were treated with siLuc, siHDAC1 or siHDAC2 and analyzed by IF. Specific decreases in signal intensities were detected by IF for HDAC1 and HDAC2 when their corresponding siRNAs were used as compared to siLuc control cells.



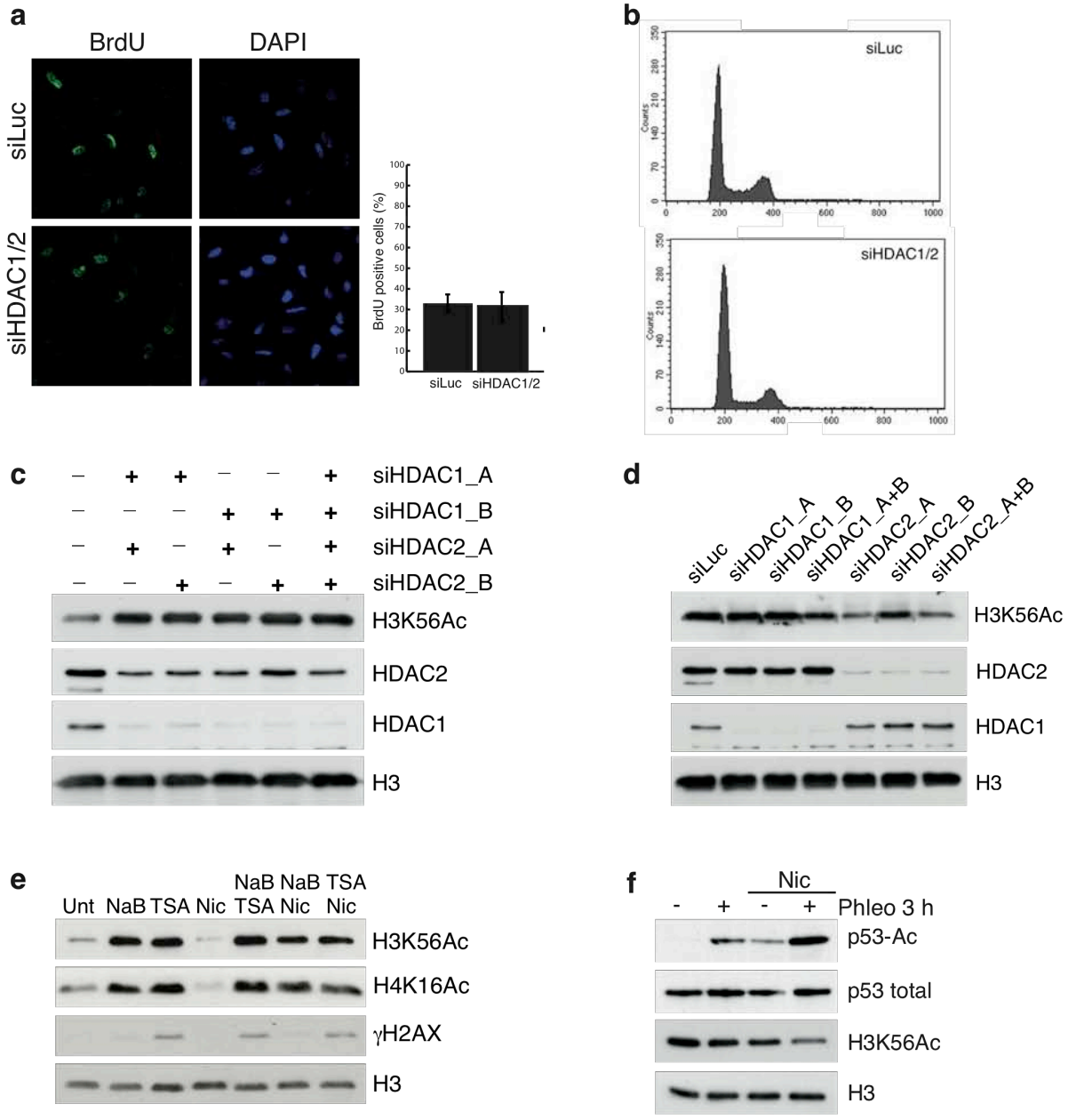
Supplementary Fig. S2 Histone H3 lysine 56 acetylation (H3K56Ac) is diminished in damaged versus undamaged regions. **(a)** Quantification of H3K56Ac and γH2AX intensities from a cell in Fig. 1c. Intensities and distances are shown in arbitrary units. Line 1 represents damaged area and Line 2 represents non-damaged area. Dotted lines show the average intensity of H3K56Ac. Lines 3 and 4 contain both damaged and undamaged regions. Quantitative analyses of intensities versus distance of H3K56Ac and γH2AX were obtained with the line-intensity function of Volocity software. **(b)** IRIF analysis of H3K56Ac and γH2AX demonstrates that H3K56Ac does not co-localize with DNA damage foci. HeLa cells were IR-treated and analyzed by IF for H3K56Ac (Epitomics) and γH2AX. Box denotes magnified portion of image. Right panel: Quantification of H3K56Ac and γH2AX intensities of cell in left panel as performed in a **(c)** IRIF analysis revealed that H3K56Ac is excluded from 53BP1-containing DNA-damage foci. U2OS cells were treated with 3 Gy of IR and samples were taken for IF analysis at 30 min. For IF analysis for H3K56Ac, a rabbit anti-H3K56Ac antibody and a mouse anti-53BP1 or mouse anti-γH2AX antibody were used for co-localization to avoid antibody cross-reactivity artefacts. Inset shows a zoomed view from the region of the cell marked by a white dotted box. Right panel: Quantitative analysis of the cell in left panel shows that H3K56Ac is depleted in γH2AX-containing regions. Analysis was as in a. **(d)** H3K56Ac is not coincident from 53BP1-containing DNA-damage sites. Cells were analyzed as in c at the indicated time points. We note that previous work by Das et al. [1] used rabbit antibodies directed against both H3K56Ac and γH2AX, while for our analysis we used the same rabbit antibody for H3K56Ac but used mouse antibodies against γH2AX or 53BP1. Given the potential for cross-reactivity of secondary antibodies with two antibodies raised in the same species and our results presented here, we conclude that H3K56Ac does not accumulate at DNA-damage sites, but is reduced or excluded from sites of DNA damage. **(e)** Multiple histone acetylations are unaffected by laser micro-irradiation. IF analysis of acetylations of H3 on K9, K14 and K27 and H4 acetylations on K5 or K12 by laser-induced DNA damage. Experiments were performed as described in Fig. 1c.



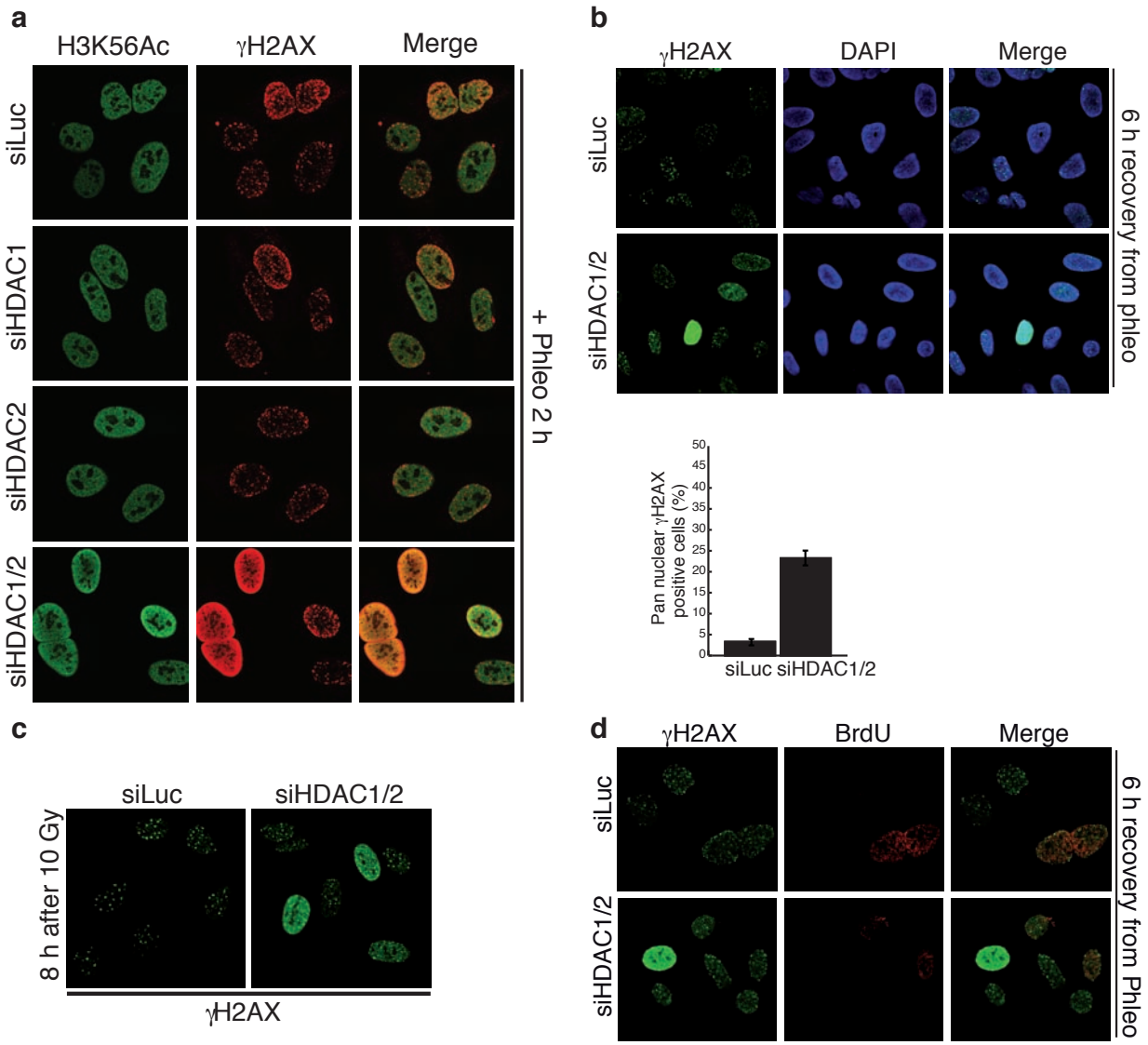
Supplementary Fig. S3 U2OS cells containing *Asi*SI-ER display increased γH2AX and 53BP1 foci upon addition of 4-OHT. U2OS-*Asi*SI-ER cells were untreated or treated with 300 nM of 4-OHT for 4 h followed by IF analysis with mouse anti-γH2AX and rabbit anti-53BP1 antibodies.



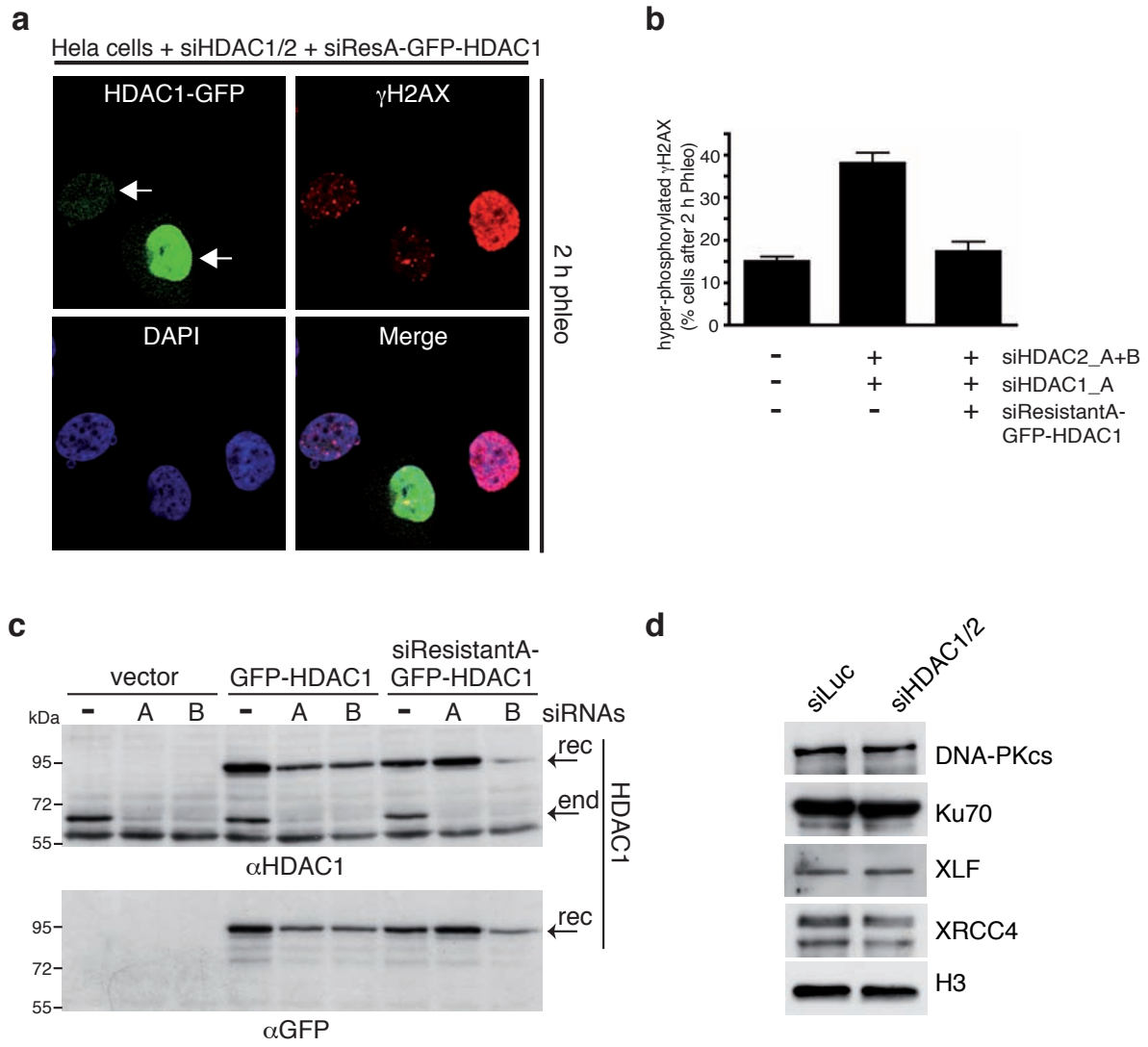
Supplementary Fig. S4. Western blotting, cell cycle and laser micro-irradiation analyses for H3K56Ac. **(a)** Control gels for the H3K56Ac antibody and loading for the H3K56Ac gel in Fig. 2d. Left: Coomassie-stained gel for samples used in Fig. 2d confirms equal loading of the two samples. Right: full Western blot for H3K56Ac shows that only one band, migrating at the size expected for histone H3, is detected with the Epitomics antibody against H3K56Ac, which is consistent with a previous reports that have verified this antibody towards H3K56Ac [1, 2]. **(b)** H3K56Ac levels are not markedly altered in S-phase. U2OS cells were pulsed with BrdU to detect S-phase cells and analyzed by IF for BrdU and H3K56Ac. **(c)** H3K56Ac, like H4K16Ac, but unlike H3K27Ac, are rapidly reduced at sites of DNA damage. U2OS cells were micro-irradiated and analyzed 5 min afterwards. Cells were processed as in Fig. 1c.



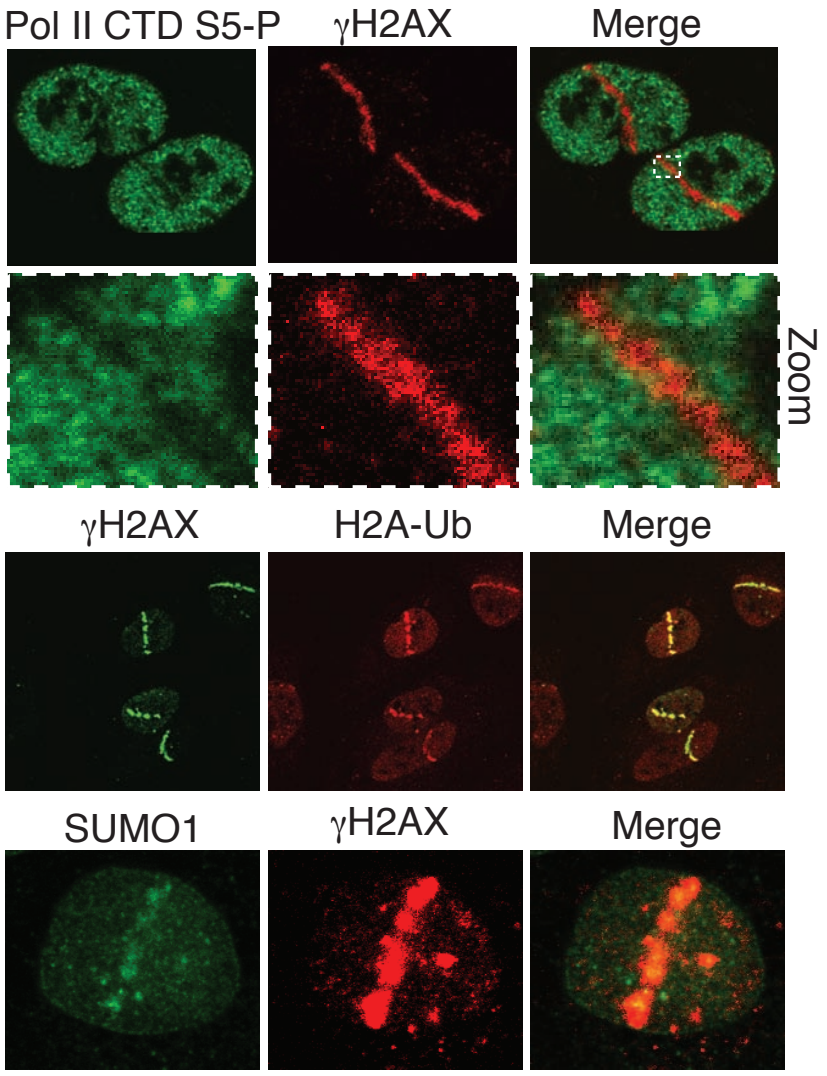
Supplementary Fig. S5 HDAC1 and HDAC2 regulate H3K56 and H4K16 acetylation and HDAC1/2 depletion does not produce detectable changes in cell-cycle distributions. **(a)** BrdU staining of cells treated with siLuc or siHDAC1/2. IF analysis and quantification of BrdU-positive cells showed no major differences in cells in S-phase between siLuc and cells depleted for HDAC1 and HDAC2. **(b)** FACS profiles of U2OS cells from siLuc or siHDAC1/2 cells. FACS analysis confirmed that depletion of siHDAC1/2 did not result in detectable changes in cell-cycle distribution compared to siLuc control cells. **(c)** Combinations of multiple siRNAs targeting HDAC1 and HDAC2 all result in the hyper-acetylation of H3K56. Two different siRNAs (A and B) targeting either HDAC1 or HDAC2 were used in pairwise combinations, as well as all 4 siRNAs together and analyzed by WB with the indicated antibodies. **(d)** U2OS cells were transfected with siRNAs targeting Luciferase, HDAC1 or HDAC2, and whole cell extracts were probed with the indicated antibodies. We observe that cells depleted of HDAC2 often display decreased levels of H3K56Ac compared to siLuc or siHDAC1 cells. **(e)** Class I/II HDAC inhibition (16 h) by sodium butyrate (NaB, 5 mM) or Trichostatin A (TSA, 1.3 mM), but not Class III HDACs, inhibition by nicotinamide (Nic, 20 mM), increases H3K56Ac and H4K16Ac levels. Inhibition of Sirtuins does not have a major effect on H3K56Ac and H4K16Ac levels. **(f)** Nicotinamide treatment did not result in an increase in H3K56Ac levels but did increase the acetylation of the SIRT1 target p53 [3, 4]. U2OS cells were untreated or treated with nicotinamide for 16 h (25 mM) and then either untreated or treated with phleomycin (60 μg ml^{-1}) for 3 h.



Supplementary Fig. S6 HDAC1/2 deficient cells exhibit increased amounts of, and exhibit pan nuclear, γH2AX. **(a)** siHDAC1/2 cells display increased H3K56Ac levels and γH2AX formation, which is dependent on DNA damage. After siRNA transfections, cells were treated with phleomycin and analyzed by IF with the indicated antibodies. **(b)** HDAC1/2-deficient cells exhibit pan nuclear γH2AX staining 6 h after phleomycin treatment. Graph represents quantification of pan-nuclear γH2AX cells from three independent experiments. Error bars represent +/- S.D. **(c)** HDAC1/2 deficient cells exhibit pan-nuclear γH2AX staining following IR treatment. Cells were treated with 10 Gy of IR, allowed to recover for 8 h and then analyzed by IF. **(d)** Pan-nuclear γH2AX staining in HDAC1/2 depleted cells after DNA damage is not restricted to S-phase. Cells were pulsed with BrdU to detect S-phase cells, damaged with phleomycin and allowed to recover for 6 h before processing for IF. Quantification of pan-nuclear γH2AX-positive cells showed that only 31% of these cells were also BrdU-positive. We conclude that HDAC1/2 depleted cells can suffer hyper-activation of the DDR, as seen by pan-nuclear γH2AX staining, in cell cycle phases including, but not limited to, S-phase.



Supplementary Fig. S7 siRNA-resistant HDAC1 rescues the DNA-damage dependent defect in γH2AX in HDAC1/2 depleted cells and HDAC1/2 depletion does not affect levels of NHEJ factors. **(a)** HeLa cells depleted for HDAC1/2 and lacking siResA-GFP-HDAC1 display increased phosphorylated γH2AX upon DNA damage compared to siResA-GFP-HDAC1 positive cells. HeLa cells were transiently transfected with siResA-GFP-HDAC1 followed by two rounds of siRNA transfections with siRNAs against HDAC2_A+B and HDAC1_A. Cells were then treated with phleomycin for 2 h and then processed and analyzed by IF. White arrows depict siResA-GFP-HDAC1 positive cells. **(b)** Expression of siResA-GFP-HDAC1 reverses the hyper-phosphorylation of γH2AX in siHDAC1/2 cells following DNA damage. Cells were untreated, treated with siRNAs against HDAC1_A and HDAC2_A+B, or treated as in a. After 72 h, cells were damaged with phleomycin for 2 h and then fixed and processed by IF. Over 200 cells were scored for hyper-phosphorylation of γH2AX for each condition and the results are shown here in graphed form. (n=3, error bars represent S.E.M). **(c)** siResA-GFP-HDAC1 is resistant to HDAC1_A siRNA. Cells were transfected with empty vector, or with vectors containing GFP-HDAC1 or siResA-GFP-HDAC1 followed by siRNA transfections with the indicated siRNAs that target HDAC1. **(d)** Levels of core NHEJ proteins are not influenced by the absence of HDAC1 and HDAC2. Extracts used in Fig. 3a were used in WB analyses to analyze proteins involved in NHEJ.



Supplementary Fig. S8 Laser micro-irradiation reveals the presence of several transcription repressive marks at sites of DNA damage. U2OS cells were micro-irradiated and analyzed with the indicated antibodies 2 h post-laser induced damage. Pol II S5p marks the elongating form of Pol II and its hypo-phosphorylated form is indicative of transcriptional repression. There is an exclusion of Pol II S5p from DNA damage sites, suggesting that these sites are transcriptionally inactive. Using our laser settings, we also detect ubiquitylated histone H2A (H2A-Ub) and SUMO1, two modifications that are also associated with transcriptional repression (note that these marks are used as controls as they have already been previously shown to mark sites of DNA damage [5, 6]).