SETD2-dependent histone H3K36 trimethylation is required for homologous recombination repair and genome stability

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Pfister et al Supplemental Figure S1 (related to Figure 1)

A. NT siSETD2
   SETD2
   I-SceI
   Tubulin

B. qPCR-F → I-SceI site
   Exon5
   Intron5
   Exon6
   Exon7
   HPRT gene

C. I-SceI cleavage efficiency
   0h 24h
   relative PCR product
   NT siSETD2
   NT siSETD2
   n.s.

D. RT-PCR-F
   I-SceI site
   HPRT cDNA
   RT-PCR-R

E. HPRT gene transcription qRT-PCR
   Spliced
   Unspliced

F. siNT
   M a1 e I 3 4 6
   1 kb 0.5kb
   siSETD2
   M 1 2 7 9 13 18
   1 kb 0.5kb
   siRad51
   M 1 4 7 10 18 19
   1 kb 0.5kb

G. I-SceI recognition site
   Clone no.
   A. Parental TGGCAAAAACATGTTAACACCGTTATCCCTAAGTCAGGCAATATAT
      Proximal TACCCGTTAT
      Deletion TACCCGTTAT
      Distal TACCCGTTAT
      siSETD2:5(17), 3(18)
   B. Parental TGGCAAAAACATGTTAACACCGTTATCCCTAAGTCAGGCAATATAT
      Proximal GATTCAG
      Deletion GATTCAG
      Distal GATTCAG
      siNT: N1, N3, 5(19)
   C. Parental TGGCAAAAACATGTTAACACCGTTATCCCTAAGTCAGGCAATATAT
      Proximal ACCTGTTAT
      Deletion ACCTGTTAT
      Distal ACCTGTTAT
      siNT: Na1, Ne
   D. Parental TGGCAAAAACATGTTAACACCGTTATCCCTAAGTCAGGCAATATAT
      Proximal CCGGCCTGTTAT
      Deletion CCGGCCTGTTAT
      Distal CCGGCCTGTTAT
      siRAD51: R7, R10, R23
   E. Parental TGGCAAAAACATGTTAACACCGTTATCCCTAAGTCAGGCAATATAT
      Proximal TATCCCGTTAT
      Deletion TATCCCGTTAT
      Distal TATCCCGTTAT
      siSETD2: 3(9)
   F. Parental TGGCAAAAACATGTTAACACCGTTATCCCTAAGTCAGGCAATATAT
      Proximal ATACCCCCGTTAT
      Deletion ATACCCCCGTTAT
      Distal ATACCCCCGTTAT
      siSETD2: 3(1)
Figure S1. HPRT assay. Related to Figure 1.

(A) Western blot showing SETD2 knockdown and I-SceI expression in the HPRT assay. Cells were transfected with non-targeting control (NT) siRNA or SETD2 siRNA for 48 hours before I-SceI transfection. Samples were harvested 24 hours after I-SceI transfection.

(B) Schematic map showing the qPCR primers used to amplify the region flanking the I-SceI site for assessing I-SceI cutting efficiency.

(C) Quantification of the I-SceI cleavage efficiency in the NT and SETD2 depleted cells in the HPRT assay. Cells were treated with NT or SETD2 siRNA for 48 hours before I-SceI transfection. Genomic DNA samples were harvested 0 hours or 24 hours after I-SceI transfection. qPCR primers shown in (B) were used to amplify the genomic DNA. The reduction in the amount of amplified product compared to the 0 hours represents the fraction cleaved by I-SceI. Error bars show SEM from three independent experiments.

(D) Schematic map showing the qRT-PCR exonic primers used to amplify the spliced HPRT mRNA.

(E) qRT-PCR quantification of HPRT mRNA in the NT and SETD2 depleted cells. Spliced mRNA was amplified by the exonic primers shown in (D) and unspliced mRNA was amplified by the intronic/exonic primers shown in (B).

(F) Gels showing PCR product sizes from randomly selected HPRT-negative clones generated in cells transfected with NT siRNA (upper panel), SETD2 siRNAs (si#3 or si#5) (middle panel), or RAD51 siRNAs (bottom panel).

(G) Examples of deletion junctions of HPRT-negative clones from NT, SETD2 and RAD51 depleted cells that have undergone microhomology mediated end joining (MMEJ). For each individual junction the parental sequence of the HPRT gene loci and the intervening I-SceI site (underlined) sequence has been indicated on the top. Junctional microhomologies between the proximal and distal reference sequences are shown in gray. The examples of HPRT-negative clones relating to each type of MMEJ are indicated on the right.
Pfister et al. Supplemental Figure S2 (related to Figure 2 and 3)

A

SETD2 KD on HeLa clonogenic survival

B

U

IR

- + - +

siSETD2

SETD2

BRCA2

RAD50

CTIP

MRE11

KU80

RPA

Tubulin

C

NT

si#3

si#5

BrdU

PI

D

Rad51 foci after SETD2 KD

% cells with >5 foci

E

RPA foci after SETD2 KD

% cells with >20 foci

F

DAPI

RPA

H3K36me3

Merge

NT

si#3

si#5
Figure S2. SETD2 is required for HR. Related to Figure 2 and 3.

(A) Clonogenic survival of SETD2 knockdown (si#3 and si#5) or control (NT) in HeLa cells treated with indicated concentrations of Mitomycin C (MMC), Camptothecin (CPT) or ionising radiation (IR). Error bars show SEM from three independent experiments.

(B) Western blot analysis of the protein levels of major HR and NHEJ factors before and after SETD2 knockdown, in the presence or absence of IR damage (5Gy following 2 hours of repair).

(C) Cell cycle analysis by FACS showing BrdU incorporation in U2OS cells treated with control (NT) or SETD2 siRNA (si#3 and si#5) for 48 hours.

(D) RAD51 foci formation at indicated times of CPT treatment (10µM) in U2OS cells treated with non-targeting control siRNA (NT) or SETD2 siRNAs (si#3 and si#5). Error bars show SEM from three independent experiments. *P<0.05, **P<0.01.

(E) RPA32 foci formation at indicated times after 2-hour treatment with MMC (2µM) in U2OS cells treated with non-targeting control siRNA (NT) or SETD2 siRNAs (si#3 and si#5). Error bars show SEM from three independent experiments. ***P<0.001, **P<0.01.

(F) Loss of H3K36me3 in the nuclei after SETD2 knockdown is visualised by immunofluorescence (IF). Double staining of antibodies against RPA32 (mouse, Abcam) and H3K36me3 (rabbit, Abcam) after 1-hour treatment with CPT (10µM) in U2OS cells treated with non-targeting control siRNA (NT) or SETD2 siRNAs (si#3 and si#5).
Pfister et al. Supplemental Figure S3 (related to Figure 3)

A

![Diagram of SETD2 cleavage site and expression of SceGFP and iGFP](image)

B

| Condition | 10% IN | NS | SETD2 | h post TF |
|-----------|--------|----|--------|-----------|
| Isocitrate (2μg) | 0 8 24 48 | 0 8 24 48 | 0 8 24 48 | 0 8 24 48 |
| DNA sites | up    | lsc | down   | HA (I-SceI) |
|           |       |     |        | GAPDH     |

C

| Condition | 10% IN | NS | SETD2 | H3K36me3 | h post TF |
|-----------|--------|----|--------|----------|-----------|
| Isocitrate (2μg) | 0 8 24 48 | 0 8 24 48 | 0 8 24 48 | 0 8 24 48 | 0 8 24 48 |
| DNA sites | up    | lsc | down   | SETD2    | Tubulin   |

D

| Condition | 10% IN | NS | SETD2 | H3K36me3 | h post TF |
|-----------|--------|----|--------|----------|-----------|
| Isocitrate (2μg) | 0 8 24 48 | 0 8 24 48 | 0 8 24 48 | 0 8 24 48 | 0 8 24 48 |
| DNA sites | up    | lsc | down   | siSETD2  | Tubulin   |
Figure S3. Analysis of SETD2 and H3K36me3 at the break-site. Related to Figure 3.

(A) Schematic map of the DR-GFP cassette, where arrows indicate the PCR primers used to amplify genomic DNA after ChIP. The positions of primers and the amplified genomic sites (‘up’, ‘Isc’, ‘down’) are indicated.

(B) ChIP analysis on DR-GFP U2OS cells transfected with I-SceI plasmid and harvested at the indicated times post-transfection. ChIP was performed on the lysate using antibodies against SETD2 (Abcam) or H3K36me3 (Abcam) or non-specific Ig (NS); inputs (IN) are also indicated. The three PCR primer pairs (covering “up”, “Isc” and “down” sites) are shown on the schematic map. Also shown is an immunoblot (IB) of HA-I-SceI protein levels over the timecourse; GAPDH serves as a loading control.

(C) ChIP-qPCR analysis of enrichment of SETD2 or H3K36me3 at the “up” site. The data are presented as fold change over the uncut condition (0; non-Iscel induced: column 1). Fold changes were calculated from 2-ΔΔCt values; ΔΔCt=ΔCtSP- ΔCtNS, where SP stands for specific IPs, and NS stands for non-specific IPs (background). ΔCt= CtIP- corrected CtIN, where corrected CtIN=CtIN-log210 to account for the 1/10 dilution factor of the IN; IP:immunoprecipitation, IN:input chromatin. Error bars show SD from three experiments.

(D) Western blot confirming SETD2 knockdown in the cells used for ChIP. Samples were taken from the ChIP experiment shown in Figure 3D.
Pfister et al Supplemental Figure S4 (related to Figure 4)

**A**

|          | WT   | MD   |
|----------|------|------|
| siSETD2- | -    | +    |
| DOX      | -    | +    |

**B**

Rescue of siSETD2 on HR by WT

**C**

Rescue of siSETD2 on HR by MD

**D**

Doxycycline on HR

**E**

-DOX

+DOX 5µg/ml (KDM4A OE)

**F**

H3.3

K36M
Figure S4. H3K36me3 is required for HR. Related to Figure 4.

(A) Western blots showing the induction of SETD2 protein expression by Doxycycline (5µg/ml) in T-REx DR-GFP SETD2 clones 48 hours after NT or SETD2 siRNA treatment. The clone expressing wild-type (WT) SETD2 cDNA shows rescue of H3K36me3 levels after siSETD2. The clone expressing methyltransferase-dead (MD) SETD2 cDNA shows no rescue of H3K36me3 levels after siSETD2.

(B) Rescue of HR efficacy after SETD2 siRNA treatment in six independent wild-type (WT) SETD2 clones. Error bars show SEM from three technical replicates.

(C) Lack of rescue of HR efficacy after SETD2 siRNA treatment in five independent methyltransferase-dead (MD) SETD2 clones. Error bars show SEM from three technical repeats.

(D) The HR efficacy in parental T-REx DR-GFP U2OS cells (without any cDNA integration) with or without Doxycycline (5µg/ml) treatment. Error bars show SEM from three independent experiments.

(E) Cell cycle distribution of T-REx KDM4A U2OS cells with or without induction of KDM4A by Doxycycline (5µg/ml).

(F) Cell cycle distribution of U2OS cells expressing H3.3 or H3.3K36M transgene.
Figure S5. SETD2 promotes DSB end resection. Related to Figure 5.

(A) Western blots showing the single or double knockdown of CtIP (siC), SETD2 (siS) or both CtIP and SETD2 (siC+siS) in the experiment shown in Figure 5D.

(B) XRCC4 ChIP were performed in 4OHT treated DIvA cells and analysed close to (<100bp) selected DSBs. ChIP efficiencies (as % of input) are shown for DSB-II, a DSB reported as RAD51-bound in (Aymard et al., 2014). The mean and SEM (n=4, technical replicate) of a representative experiment are presented. Note that SETD2 siRNA does not drastically affect XRCC4 binding at DSB, indicative of an equivalent cleavage in SETD2 depleted and control cells.
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell Culture

U2OS (human osteosarcoma) cells were cultured at 37°C with 5% CO₂ in Dulbecco’s modified Eagle’s medium supplemented with 10% v/v foetal bovine serum and penicillin (100 units/ml) and streptomycin (0.1 mg/ml). HR reporter UGFP#1 cells (U2OS with integrated pDR-GFP reporter construct (Pierce et al., 1999) were maintained in 7µg/ml Puromycin. NHEJ reporter cells (H1299 non-small cell lung carcinoma cells with integrated NHEJ reporter) were a kind gift from Atsushi Shibata and Takashi Kohno and were maintained in 2µg/ml Puromycin. U2OS-CtIP cells (U2OS cells with integrated GFP-tagged CtIP) were kind gift from Annika Baude and Jiri Lukas and were maintained in 5µg/ml Puromycin. HT1080 cells were supplemented with 1% Sodium Pyruvate (100mM), 1% L-Glutamine (200 mM), 25% MEM (amino acids) and HAT medium before the I-SceI transfection, and 6-TG medium 5 days after the I-SceI transfections.

I-SceI inducible HPRT Mutation Assay

Construction of cells with a functional but I-SceI-cleavable HPRT gene (clone 5.2.1) from human fibrosarcoma (HT1080) cells will be described in detail elsewhere. Briefly, an 18 bp I-SceI recognition site was inserted into exon 6 of the hypoxanthine phosphoribosyltransferase (HPRT) gene using a gene targeting procedure while retaining HPRT function. To assay for I-SceI induced loss of HPRT function, clone 5.2.1 was transfected with an I-SceI or a
GFP expression plasmid in 6-well plates 48h after the siRNA knockdowns. The transfection efficiencies were determined after 12h by flow cytometry. The cells were expanded to 100 mm petri dishes after 24h and were kept in non-selective medium. After 5 days, $10^4$ cells were seeded in triplicate into 100 mm Petri dishes for 12 h and fed with complete medium containing 15µM freshly prepared 6-TG. The plating efficiency was determined similarly, by culturing $10^3$ cells in the absence of 6-TG. After 10-14 days of culturing, HPRT negative cell colonies were visualised by staining with Brilliant Blue R Concentrate (Sigma).

The mutation frequency was determined by dividing the number of 6-TG-resistant (HPRT negative) colonies by the total number of cells plated after being corrected for the colony-forming ability. To determine the mutation sequence, independent single cell colonies (30 colonies from each background) were isolated in 24-well plates, genomic DNA was extracted and the I-SceI site within the exon 6 was PCR amplified. The PCR products were sequenced (Sanger Sequencing) using the primers flanking the I-SceI site. Sequence alignment was conducted using DNA Data Bank of Japan (DDBJ) ClustalW program (version 2.1). High fidelity Phusion master mix (x2) (New England BioLabs) was used for PCR amplification across the I-SceI site using forward (GATCCTGCACCTACAAAATCC) and reverse (GCATAGGTAAGGTGAGGAGGTG) primers (Sigma). PCR settings were as follows: 98 °C for 30 s, 30 cycles of 98°C for 10 s, 67°C for 1 min, and 72°C for 1min. Products were visualised on 2 % agarose gel with 0.5 µg/ml ethidium bromide.
**qPCR for HPRT I-SceI cleavage efficiency**

Genomic DNA was isolated using the Qiagen kit at 0h and 24h after the I-SceI transfection of the HT1080 (clone 5.2.1) cells. Quantitative PCR was performed using 7500 Fast Real-Time PCR detection system (Applied Biosystems). Reactions (25µl each) were prepared in triplicate in a 96-well reaction plate. Each reaction contained 20ng genomic DNA, 200nM of each primer, 10µl water and 12.5µl Absolute Blue QPCR SYBR low ROX Mix (Thermo Scientific). DNA levels were normalized to the GAPDH calculated using a 2-ΔΔCt method. QPCR settings were as follows: Initialisation at 95°C for 15 min, denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds and repeat for 40 cycles.

Primers used for qPCR are listed below:

- **qPCR-F:** TGGTGAGAATTACTGTGCTGAA
- **qPCR-R:** TGCGACCTTGACCATCTTTG

**qRT-PCR**

RNA was extracted using RNeasy mini kit (Qiagen). SuperScript® VILO™ (Invitrogen/Life Technologies) cDNA synthesis kit was used to reverse transcribe cDNA from total RNA according to manufacturer's instructions. Quantitative real time PCR was carried out as described in the qPCR section.

Primers used for the qRT-PCR are listed below:

For spliced HPRT mRNA:

- **RT-PCR-F:** CCCTGGCGTCGTGATTAGT
- **RT-PCR-R:** TTCATCACATCTCGAGCAAGAC

For un-spliced HPRT mRNA:
qPCR-F: TGATGAGAATTACTGTGCTGAA
qPCR-R: TGCGACCTTGACCACATCTTTG

For GFP mRNA:
GFP-F: TATATCATGGCCGACA,
GFP-R: ACATGTCCTGCTGGAGTTC.

Primers are 360-560bp downstream of the I-SceI site and only amplify the SceGFP locus and not the iGFP.

**Generation of cell lines expressing wild-type or methyltransferase dead SETD2**

Site-directed mutagenesis primers were designed so that the SETD2 cDNA was mutated from the original sequence (AGAAACGTCTCCAGTCTGTT) to the sequence AGGAATCGGCTGCAATCCGTG without changing any amino acid. The new cDNA is resistant to SETD2 siRNA#3 because it is refractory to siRNA hybridisation. The refractory cDNA was then mutated to abolish the methyltransferase activity at amino acid 1625 (R to G) and 1631 (C to A) by a two-step mutagenesis. Primers for site-directed mutagenesis are listed below:

SS1/SS2 for R1625G, and SS3/SS4 for C1631A.

SS1(CTCAAAAAGGAAATTGCTCTGGTTTCATGAATCACAGCTGT)
SS2(ACAGCTGTGATTCATGAAACCAGAGCAATTTCCTTTTTGAG)
SS3(CTCGTTTCATGAATCACAGCGCTGAACCAAATTGTGAAAC)
SS4(GTTTCACAATTTGGTTCAGCGCTGATTCATGAAACGAG)

U2OS-Flp-In/T-Rex/DRGFP cells and pDESTfrtto plasmid as described later were used to generate stable cell lines expressing SETD2 cDNA. U2OS-Flp-In/T-Rex/DRGFP cells were co-transfected with the resulting SETD2
expression plasmid and pOG44 (Invitrogen) in a ratio of 1:9 using Fugene transfection reagent (Promega). 48 hours after transfection, cells were split and stable integrants were selected for by 100µg/ml Hygromycin and 15µg/ml Blasticidin. About 20 days after selection, colonies were isolated and expanded individually. Individual colonies were tested for SETD2 expression in response to Doxycyline by Western blotting. Cells expressing SETD2 under the control of the T-REx system were subsequently subjected to treatments.

**Generation of inducible KDM4A over-expression cell lines**

The U2OS-Flp-In/T-Rex cell line was originally generated in the laboratory of Jeffrey D. Parvin (Ohio State University) and was a kind gift of Catherine Millar (University of Manchester). These cells express the tet repressor and contain a single integrated copy of the pFRT/IacZeo plasmid (Invitrogen) (Gordon et al., 2009). U2OS-Flp-In/T-Rex/DRGFP cells were generated by transfecting the pDR-GFP expression plasmid (Pierce et al., 1999) into the U2OS-Flp-In/T-Rex cells. Stable integrants were selected with 1 µg/ml puromycin (Invivogen), 15 µg/ml Blasticidin S (Invivogen) and 100 µg/ml Zeocin (Invitrogen). Clones with best inducible expression were chosen for further studies.

The tetracycline inducible Flp-In gateway destination vector pDESTfrrto was generated by replacing the CMV promoter of the pcDNA5/FRT plasmid backbone (Invitrogen) with the CMV/TO promoter of pcDNA4/TO/myc-His A (Invitrogen) on an MluI/HindIII fragment. The Gateway conversion cassette RfA was then inserted into the EcoRV site of the resulting plasmid construct.
The KDM4A cDNA was purchased from Genecopoeia and was transferred into the pDESTfrito plasmid with a Gateway LR reaction. U2OS-Flp-In/T-Rex and U2OS-Flp-In/T-Rex/DRGFP cells were co-transfected with the resulting KDM4A expression plasmid and pOG44 (Invitrogen) in a ratio of 1:9 using Fugene transfection reagent (Promega). 48 hours after transfection, cells were split and stable integrants were selected for by 100 µg/ml Hygromycin and 15 µg/ml Blasticidin. About 20 days after selection, colonies were isolated and expanded individually. Individual colonies were tested for KDM4A expression in response to Doxycycline by Western blotting. Cells expressing KDM4A under the control of the T-Rex system were subsequently subjected to treatments.

**Generation of cell lines expressing H3.3K36M**

The H3.3 and H3.3-K36M lentiviral plasmids were generous gifts from the laboratory of Dr David Allis (Lewis et al., 2013). Briefly, the lentiviral plasmids and viral packaging mix (System Biosciences) were co-transfected into 293T cells using lipofectamine transfection reagent (Invitrogen). Viral particles produced from 293T cells were collected and used to infect U2OS cells. Five days after viral infection, U2OS cells were selected for stable integration of the gene using Puromycin (5µg/ml). Stable cell lines were tested for H3.3 or H3.3-K36M expression by Western blotting and subsequently subjected to treatments.

**siRNA Transfections**

The sequences of the siRNAs used in this project are: Non-targeting (NT) proprietary sequence of supplier (Qiagen). SETD2#3 (si#3) (Dharmacon):
GAAACCGUCUCCAGUCUGU; SETD2#5 (si#5) (Dharmacon): UAAAGGAGGUAAUCGAAU. RAD51 (Qiagen): AAGGGAAUUAGUGAAGCCAAA, CAGGAUAAGCUUCCGGGAAA, CACUUCUAUUAAUUGGUA, CACGCUUAGGAGCAGUGUGGCA. CtIP (Dharmacon): GAGGUUAUAUUAAGGA, GGAGCUACCUCUAGUAU, GAACAGAAUAGGACUGA, GCACGUUGCCCAAAGAU,

All siRNAs (10nM final concentration) were delivered to the cells by RNAiMAX (Invitrogen) according to manufacturer's instructions.

**Cell Survival assay**

400 U2OS or 200 HeLa cells were seeded per well of 6-well plates 48 hours post siRNA treatment. Cells were then treated with desired concentrations of DNA damaging reagents 6 hours after seeding. Mitomycin C (MMC) and Camptothecin (CPT) were purchased from Sigma. Ionising radiation (IR) was carried out by a caesium-137 irradiator at dose rate of 1.87Gy/min. Colonies were allowed to form for up to 14 days. Cells were then fixed and stained with Brilliant Blue (Sigma) and numbers of colonies were counted. Plating efficiency (PE) and surviving fraction (SF) were calculated according to (Franken et al., 2006), where PE=number of colonies formed without treatment/number of cell seeded, and SF=number of colonies formed after treatment/(number of cell seeded×PE).

**Western blotting**

For histone western blots, proteins from whole cell lysates were separated by NuPAGE Bis-Tris gel (Invitrogen) and transferred to a nitrocellulose
membrane (0.2µm pore size) (Invitrogen). For western blot analysis of other proteins, whole cell lysate were separated by NuPAGE Tris-Acetate or Bis-Tris gel (Invitrogen) and transferred to a PVDF membrane (0.45µm pore size) (Invitrogen). After blocking, the membranes were incubated with appropriate primary antibodies at 4°C overnight followed by secondary antibodies at room temperature for 1 hour. Imaging and quantification of the amount of protein was performed by the Odyssey Infrared imaging system (LI-COR).

Primary antibodies used for western blotting are listed below: SETD2 (Abcam), H3K36me3 (Abcam), RAD51 (Santa Cruz), H3 (Abcam), LEDGF (Bethyl), CtIP (GeneTex), RPA32 (Abcam) and KDM4A (Cell Signaling). Secondary Alexa Fluor antibodies were purchased from Invitrogen.

**Co-Immunoprecipitation**

U2OS cells were undamaged or damaged with either 15 uM camptothecin or 6 Gy IR, and allowed to repair for 4 and 2 hours respectively. Cells were lysed and pre-cleared with anti-rabbit serum and incubated overnight with 2-3µg of indicated antibody for immunoprecipitations at 4°C overnight. Antibodies used for co-IP were: H3K36me3 (Abcam ab 9050) and LEDGF (Bethyl A300-847A). Protein A beads were added to and incubated for further 2 hours. Beads were washed with 150mM salt buffers and the resulting eluate was subjected to electrophoresis and analysed by immunoblotting. Western blots were incubated with indicated antibodies at 1:1000 dilution and secondary HRP-conjugated antibodies at 1:3000 dilution.
HR and NHEJ Reporter assays

Transfection of the plasmids encoding I-SceI was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Cells were left to repair the DSBs for 48 hours before analysing GFP positive cells by FACS (FACSCalibur). For FACS analysis, non-overlapping gates were defined by three populations of cells from the same cell line: normal cells, cells transfected with EGFP or DsRed plasmids. 20000 cells were analysed for each sample.

Immunofluorescence

For repair foci analysis, U2OS cells were treated with DNA damaging reagent (10µM CPT for 2 hours or 5Gy IR) and left to recover for time indicated. Cells were fixed with 4% paraformaldehyde for 10 minutes and permeablised with 0.5% Triton-X. After blocking in 3% BSA, cells were incubated at 4°C overnight with antibodies diluted in 3% BSA as following: RAD51 (Santa Cruz, sc-8349) 1:1000, RPA32 (Abcam, ab2175) 1:200 or γH2AX (Upstate, 05-636) 1:1000. After washing with PBS, cells were incubated with secondary antibodies (Alexa Fluor dyes, Invitrogen) 1:1000 at room temperature for 1 hour. After washing and DAPI staining, fluorescence signals were visualised by Incell Analyzer 3.0 (GE healthcare) and number of foci per cell were analysed using Incell Analysis 3.5 software (GE healthcare).

ChIP analysis at I-SceI resection sites.

Chromatin immunoprecipitations were performed as previously described (Zalmas et al., 2013). Briefly, U2OS DR-GFP cells were transfected with 2µg
of HA-I-SceI plasmid and cells were harvested for ChIP at various timepoints post-transfection, as indicated. For siRNA DR-GFP ChIPs, cells were treated with non-targeting (NT) or SETD2 siRNA for 72 h, trypsinised and allowed to re-plate before transfection with DNA for a further 18 hours. ChIPs were performed using species-matched non-specific immunoglobulins (NS) or specific antibodies against H3K36me3 (Abcam), SETD2 (Abcam), RPA (Millipore), RAD51 (Santa Cruz) and LEDGF (Bethyl). DNA was amplified with Paq5000 polymerase (Agilent) with specific primers pairs for promoter regions of DR-GFP primers (up, Isc, down) designed to amplify regions the DR-GFP cassette, as indicated. Primer sequences are available upon request. PCR settings were as follows: 95 °C for 3 min, 33 cycles of 95 °C for 30 s, 61 °C for 1 min, and 72°C for 1 min, and 72 °C for 5 min. Products were visualised on 2 % agarose gel with 0.5 µg/ml ethidium bromide. QPCR on ChIP samples was carried out on the Agilent MX3005P real-time PCR instrument. Brilliant III SYBR Green QPCP Mastermix was used according to manufacturer’s instructions. IP data are shown as fold change of ΔΔCt over control treatment (non-Iscel induced; 0µg IsceI), after subtracting values from non-specific signals and normalising to input levels.

**UV laser micro-irradiation**

Performed as indicated in (Suzuki et al., 2011). Briefly, 2×10^5 cells were plated on a 22mm×22mm cover slip. After incubating in 10µM BrdU for 48 hours cells were covered with 5µm micropore membrane and exposed to 30Jm^{-2} UV light at a dose rate of 1Jm^{-2}s^{-1}. Irradiated cells were fixed 15 minutes after irradiation and stained with mouse anti-γH2AX (Upstate). Image acquisition was performed on a Zeiss LSM780 confocal microscope. Images
at excitation wavelength 488nm and 543nm were acquired individually. Image analysis was carried out on ImageJ.

**In vivo DNA end resection assay**

DIVA cells (DSB Inducible via AsiSI, (Aymard et al., 2014)) were transfected with siRNA using the Cell Line Nucleofactor kit V (Amaxa) according to the manufacturer’s instructions. The following siRNA were purchased from Eurogentec: SETD2 siRNA GUGAAGGAGUAUGCACGAAtt; Control siRNA: CAUGUCAUGUGUCACAUUtt. 48 hours after transfection, cells were treated or not with 300nM of 4-hydroxytamoxifen (Sigma; H7904) for 4h. DNA was next purified using Qiagen DNeasy kit according to manufacturer instruction (the Rnase A incubation step was added as recommended by the manufacturer). Single strand DNA generated at an AsiSI induced DSB was analyzed using the procedure described in (Zhou et al., 2014), with the following modifications. Briefly, for each sample, 100ng of extracted DNA was subjected to an RNAseH treatment for 15 min, and digested in 30 µL with 16 Units of BanI, or mock digested (no enzyme) at 37°C overnight. Samples were heat inactivated at 65°C, and analyzed by qPCR. qPCR was performed on Biorad Device using 2 µL of digested or mock digested samples, with the Takara Bio SYBR Premix Ex Taq (Tli RNase H Plus), and the following oligonucleotides:

DSBII_231_FW ACCATGAACGTGTTCCGAAT;
DSBII_231_REV GAGCTCCGCAAAGTTTCAAG;
DSBII_918_FW ACAGATCCAGAGCCACGAAA;
DSBII_918_REV CCCACTCTCAGCCTTTCAG;
DSBII_1656_FW CCCTGGTGAGGGGAGAATC;
The percent of Single Strand DNA was calculated with the following equation:

\[ \text{ssDNA\%} = \frac{1}{(2^{\Delta \text{Ct}} - 1) + 0.5} \times 100 \]

where \( \Delta \text{Ct} \) is calculated by subtracting the Ct obtained from mock digested sample from the BanI digested sample.

**ChIP analysis at AsiSI restriction sites**

ChIP assays were carried out according to the protocol described in (Iacovoni et al, 2010). Briefly, 200 µg of chromatin was immunoprecipitated with an antibody against XRCC4 (Abcam, ab145) or without antibody (mock). Immunoprecipitated DNA and input DNA were analyzed by Real time quantitative PCR, using primer pair located at the vicinity of an AsiSI-induced DSB (DSB II in Aymard et al, 2014). XRCC4 ChIP was analyzed closer to the DSB (less than 100bp, FW: CCGCCAGAAAGTTTCCTAGA; REV CTCACCCTTGCAGCACTTG). ChIP efficiencies were calculated as percent of input DNA immunoprecipitated.
SUPPLEMENTAL REFERENCES

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