Figure 3. Recruitment of MMSET to DSBs requires the ATM-H2AX-MDC1 pathway. a, ChIP analysis by PCR of indicated proteins at DSBs in HeLa-DR-GFP cells transfected with the indicated siRNA. Right panels: Western blots of H2AX and MDC1. b, Coimmunoprecipitation of MMSET and MDC1 in HeLa cells before or after IR. c, GST pull down assay of MMSET using indicated GST-fusion proteins. d, 293T cells treated and immunoprecipitated as indicated, then analyzed with anti-pSQ/TQ antibody. e, 293T cells transfected with the indicated constructs were treated as indicated, then immunoprecipitated and immunoblotted with indicated antibodies. f, the interaction between GST-MDC1-BRCT and indicated peptides were measured by BIAcore 3000.

Figure 4. Phosphorylation of MMSET is important for H4K20 methylation, 53BP1 recruitment and DNA damage response. a, HeLa DR-GFP cells were transfected with the indicated constructs, H4K20 methylation and MMSET recruitment was analyzed by PCR of ChIP samples. b-c, HCT116 cells transfected with indicated constructs were irradiated, and 10 min later, stained with indicated antibodies. d, Radiation sensitivity of cells from Figure 4c was determined by colony formation (±s.e.m., n=3). E, Model demonstrating how the MDC1-MMSET pathway regulates DNA damage-induced histone H4 Lysine 20 methylation and 53BP1 foci formation.

Full methods accompany this paper.

Methods

Plasmids and shRNAs
The MMSET-S102A mutant was generated by PCR-based site-directed mutagenesis against full length MMSET (pCEFL-MMSET-II). Wild-type MMSET or MMSET S102A mutant was cloned into a pIRES2 vector containing S- and FLAG-tag. shRNA-resistant constructs were made by introducing a silent mutation at the MMSET coding region (1666–1671; CTTCGG to CTGCGA). The MDC1 FHA and BRCT domains were cloned into the pGEX4T-1 vector for bacterial expression of GST fusion proteins. shRNAs against MMSET were provided by Ben Ho Park (Johns Hopkins University).

MMSET shRNA 1: 5’-GCACGCTACAACACCAAGTTT,
MMSET shRNA 2: 5’-GCACAGTCTTCGGAAGAGACACAATCA
Control shRNA: 5’-TTCAATAAAATTCTTGAGGT

MDC1 siRNA (MDC1 cDNA 58–76): UCCAGUGAAUCCUUGAGGUdTdT
Control siRNA: UUCAAUAAAUUCUUGAGGUdTdT.

H2AX siRNA: CAACAAGAAGACGCGAAUCdTdT
53BP1 siRNA: 5’-AAG AUA CUC CUU GCC UGA UAA-3’
RNF8 siRNA: 5’ AGA AUG AGC UCC AAU GUA UU 3’.

Antibodies and cell lines

MMSET antibodies were provided by Jonathan D. Licht, Northwestern University, Chicago, IL or purchased from Abcam. Commercial antibodies used for ChIP were obtained from Upstate biotechnology (γH2AX mouse monoclonal), Millipore (H4, H4K20me1/2/3, H2AUb), Active Motif (H4K20me2) and Novus (rabbit 53BP1). Antibodies against p53, pSQ/TQ, phospho-CHK2, CHK1 and phospho-CHK1 were
purchased from Cell Signaling. CHK2 antibody was purchased from Millipore. RNF8 antibody was purchased from Abcam. MDC1 antibodies have been previously described. 293T cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). HCT116 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS. Hela DR-GFP cell lines were cultured in DMEM supplemented with 10% FBS and 2 ng/ul puromycin. Mouse embryonic fibroblasts (MEFs) were cultured in DMEM containing 10% FBS and 5% ES.

**Immunoprecipitation, immunoblotting, and in vitro pull-down assays**

We prepared cell lysates, performed immunoprecipitation, and immunoblotting as previously described. GST fusion proteins were bound to glutathione-sepharose overnight at 4 °C. The beads were washed with PBS twice and incubated with cell lysates for 3 h at 4 °C. Beads were then washed with NETN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) three times, and proteins bound to beads were eluted by SDS sample buffer (100 °C for 12 mins) and separated by SDS-PAGE for western blot analysis.

**Immunofluorescence staining**

Cells grown on coverslips were fixed with 3% paraformaldehyde solution in 1xPBS containing 50 mM sucrose at room temperature for 15 mins. After permeabilization with 0.5% Triton X-100 buffer containing 20 mM HEPES pH 7.4, 50 mM NaCl, 3 mM MgCl₂ and 300 mM sucrose at room temperature for 5 min, cells were blocked with 5% Goat serum for 1hr at room temperature, then incubated with primary antibodies at 37 °C for
20 mins. After washing with PBS twice, cells were incubated with FITC or rhodamine-conjugated secondary antibodies at 37 °C for 20 mins. Nuclei were counterstained with 4′6-diamidino-2-phenylindole (DAPI). After a final wash with PBS, coverslips were mounted with glycerin containing paraphenylene diamine.

**ChIP**

Induction of a single DSB in HeLa-DRGFP cells was performed through transfection of the I-SceI expression plasmid. 24 hrs after transfection, about $5 \times 10^7$ cells were treated with 1% formaldehyde for 10 mins at room temperature to crosslink proteins to DNA. Glycine (0.125 M) was added and incubated at room temperature for 5 mins to stop the cross-linking. Cells were harvested and the pellets were resuspended in cell lysis buffer [5 mM Pipes (KOH), pH 8.0, 85 mM KCl, 0.5% NP-40] containing the following protease inhibitors 1 μg/ml leupeptin, 1 μg/ml aprotinin and 1 mM PMSF and incubated for 10 mins on ice. Nuclei were pelleted by centrifugation (5000 rpm for 5 mins). Nuclei were then resuspended in nuclear lysis buffer [50 mM Tris, pH 8.1, 10 mM EDTA, 1% SDS containing the same protease inhibitors as in cell lysis buffer] and sonicated to shear chromatin to an average size of 0.6 kb. Once centrifuged until clear, the lysates were precleared overnight with salmon sperm DNA/protein-A agarose slurry. 20% of each supernatant was used as input control and processed with the cross-linking reversal step. The rest of the supernatant (about 80% of the total) were incubated with 5 μg of the indicated antibody overnight at 4°C with rotation. Complexes were washed four times, once in high salt buffer (50 mM Tris–Cl, pH 8.0, 500 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% NP-40, 1 mM EDTA), once in LiCl buffer (50 mM Tris–Cl, pH 8.0,
250 mM LiCl, 1% NP-40, 0.5% deoxycholate, 1mM EDTA) and twice in TE buffer (10 mM Tris–Cl, pH 8.0, 1mM EDTA, pH 8.0). Beads were resuspended in TE containing 50 mg/ml of RNase and incubated for 30 mins. Beads were washed with water and elution buffer (1% SDS, 0.1 M NaHCO₃) was added for 15 mins. Crosslinks were reversed by adding 10 μg/ml RNase and 5M NaCl to a final concentration of 0.3 M to the elutants and incubate in a 65°C water bath for 4-5 hours. Two volumes of 100% ethanol were added to precipitate overnight at –20°C. DNA was pelleted and resuspended in 100 ul of water, 2 μl of 0.5 M EDTA, 4 μl 1 M Tris, pH 6.5 and 1 μl of 20 mg/ml Proteinase K was added and incubated for 1-2 hours at 45°C. DNA was then purified and used in PCR reactions.

The PCR primers for ChIP, about 220 bp away from the I-SceI cut site, were as follows:
Forward: 5’-TACAGCTCCTGGGCAACGTG -3’
Reverse: 5’- TCCTGCTCCTGGGCTTCTCG-3’
Amplification was performed using the following program: 95°C /5 mins, 1 cycle; 95°C /45 s, 56 °C /30 s, and 72 °C / 30 s, 30 cycles; 72 °C /10 mins, 1 cycle. A total of 12.5 μl of the PCR products were applied to a 1.2% agarose gel and visualized by ethidium bromide staining.

**Quantitative analysis of ChIP samples**
Quantitative PCR (Q-PCR) was performed on a 7500 RT-PCR System (Applied
Biosystems) using the SYBR Green-detection system with the following program: 95 °C /5 min, 1 cycle; 95 °C /45 s and 62 °C /45 s, 40 cycles. As an internal control for the normalization of the specific fragments amplified, a locus outside the region of the DSB was amplified, in this case *FKBP5*, using the input control sample as template. The internal control (*FKBP5*) primers were as follows:

Forward: 5’-CAGTCAAGCAATGGAAGAAG-3’

Reverse: 5’- CCCGTGCCACCCCTCAGTGA-3’

After Q-PCR amplification, the *FKBP5* input controls for untransfected (no DSB) and I-SceI transfected (DSB) were used to normalize the untransfected and transfected samples respectively. After normalization, the relative levels of the indicated proteins on a DSB were calculated by comparison of untransfected and I-SceI transfected samples to their respective IgG controls. All Q-PCR reactions were performed in triplicate, with the s.e.m. values calculated from at least three independent experiments.

**BiaCore analysis**

Binding was analysed in a BiaCore 3000 system. The relevant biotinylated peptides (MMSET peptide sequences: biotin-AKLRFESQEMKG; pMMSET peptide sequences: biotin-AKLRF(p)SQEMKG; H2AX peptide sequences: KKATQASQEY; γH2AX peptide sequences: biotin-KKATQApSQEY) were bound to an SA sensor chip (GE Healthcare). The indicated concentrations of bacterially expressed GST–MDC1-BRCT in HBS-EP [HEPES-buffered saline with EDTA and polysorbate 20; 10 mM HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA and 0.005% (v/v) polysorbate 20] were injected over the immobilized peptides at a flow rate of 80 μl/min. Interactions between each peptide and
GST–MDC1-BRCT were analysed and steady-state binding was determined at each concentration. Regeneration of the sensor chip surface between each injection was performed with three consecutive 5 µl injections of a solution containing 50 mM NaOH and 1 M NaCl.

**In vitro histone methyltransferase assay**

HA-MMSET and HA-53BP1 were expressed and purified from 293T cells with hemagglutinin (HA) tag antibody and subsequent HA peptide elusion. Recombinant Histone 4 protein was from Upstate, Charlottesville, VA. In vitro histone methyltransferase assay was carried out according to the manufacturer's instructions (SAM510: SAM Methyltransferase Assay kit, G-Biosciences, St Louis, MO, USA). In brief, all proteins were dialyzed against 0.1M Tris-HCl, pH8.0. 20 µM HA-MMSET (or HA-MMSETS102A mutant) and 20 µM H4 (or HA-53BP1) were used for every reaction. Absorbances at 510nm were measured every 10-30 seconds at 37°C until the increasing absorbances reached a plateau or the reactions were stopped by boiling in SDS buffer, their contents separated by 15% SDS-PAGE, and the methylation of H4 was visualized by immunoblotting with anti-H4K20Me2 antibodies (Upstate).

**Laser irradiation and immunofluorescence staining**

A partially customized “laser-scissors” microirradiation system with an inverted microscope (Nikon, Ti-E), a laser ablation unit (Photonic Instruments, MicroPoint) and microscope automation and imaging software (Molecular Devices, MetaMorph) were used to introduce DNA damage in cultured cells. A 337 nm nitrogen laser (with 1-20 Hz
repetition rate, 2-6 ns pulse duration and 120 µj/pulse energy) transmits radiation through an optical fiber and a dye cell containing a solution that produces a 551 nm dye laser. The laser microbeam is then focused by a 63x (NA 1.4) oil immersion microscope objective. The total laser energy delivered to each focused spot was set by an attenuator plate (50% transmission) and the number of pulses. Cells were cultured on 35 mm glass-bottomed dishes (MatTek Cultureware, P35G-15-14-C) prior to laser irradiation.

Following laser irradiation, cells were fixed with 4% paraformaldehye (Electron Microscopy Sciences) for 10 min at room temperature. Immunofluorescence staining was performed as previously described\textsuperscript{30}. Cells were then imaged using the Nikon microscope and the MetaMorph software described above.