SUPPLEMENTARY INFORMATION

Methylation of UHRF1 by SET7 is essential for DNA double-strand break repair

Ja Young Hahm¹, Ji-Young Kim¹, Jin Woo Park¹, Joo-Young Kang¹, Kee-Beom Kim¹, Se-Ryeon Kim¹, Hana Cho¹ and Sang-Beom Seo¹,*

¹Department of Life Science, College of Natural Sciences, Chung-Ang University, Seoul 06974, Republic of Korea

*Correspondence: sangbs@cau.ac.kr
SUPPLEMENTARY DATA

A

UHRF1 K385
RLRESKKAKMASATS
UHRF1 K408
KGMACGVRTKTECTIVP
UHRF1 K670
SPRRTSKKTKVEPYS

H3K4
ARTKQTARKS

DNMT1
SKPRTNRSKSDGEAK
p53
RAHSHHLKSQKQGSTS

B

Scintillation counting

\[^{14}C\text{CPM}\]

K385 K408 K670

\[\text{GST-only} \quad \text{GST-SET7}\]

C

K670me1: KT\text{Kme}\text{V}EPYSLT\text{AQSSLLI}\text{R}

D

UHRF1 \Delta 1 (1-100)

UHRF1 \Delta 2 (393-610)

UHRF1 \Delta 3 (393-610)

UHRF1 \Delta 4 (90-383)

UHRF1 \Delta 5 (370-685)

UHRF1 \Delta 6 (675-793)

E

SET7 FL

SET7 #1

SET7 #2

SET7 #3

SET7 #4

GST-SET7

IB: GFP (UHRF1)

CBB staining
Supplementary Figure S1  SET7-mediated UHRF1 methylation is identified at K385 and K670 in vitro and in vivo at K385

(A) Identification of a putative SET7 methylation site in UHRF1 and consensus methylation sequence in proteins methylated by SET7. (B) In vitro methylation assays were performed by incubating SET7 with peptides around K385, K408, or K670 of UHRF1. Peptide methylations were measured using a scintillation counter. (C) LC-MS/MS of full length (FL) GST-UHRF1 modified with methylation on lysine 670. (D) GST-UHRF1 FL or partial constructs were incubated with HA-SET7 overexpressed HCT116 cell lysates. Western blotting was performed to detect the interaction using anti-HA antibody. (E) The reciprocal pull-down assay between GST-SET7 or partial constructs and GFP-UHRF1. The interaction was measured by immunoblotting with anti-GFP antibody. (F) Flag-EV or Flag-UHRF1 and GFP-SET7 were
overexpressed in H1299 cells. The cell lysates were immunoprecipitated with SET7 antibody. Associated proteins were eluted, resolved by SDS-PAGE and immunoblotted using indicated antibodies. (G) The specificity of the antibodies against UHRF1 K385me1 and UHRF1 K670me1 were verified by dot blot assays. The nitrocellulose membranes were spotted with the indicated amount of unmethylated, methylated peptides at K385 or K670 of UHRF1 and immunoblotted with indicated antibodies. (H) Flag-EV or Flag-SET7 was overexpressed in HCT116 cells. The cell lysates were immunoprecipitated with UHRF1 K385me1 and UHRF1 K670me1 antibody. Associated proteins were eluted, resolved by SDS-PAGE and immunoblotted using indicated antibodies.
Supplementary Figure S2  SET7 methylates UHRF1 in response to DNA damage and cell cycle stage is important for methylation of UHRF1

(A) HCT116 cells were exposed with 50 J/m$^2$ of UV, incubated for 30 min and harvested. Cell extracts of control and damaged cells were immunoprecipitated using an anti-UHRF1 antibody and associated proteins were pulled down with A/G agarose beads. Beads were washed extensively and bound proteins were resolved by SDS-PAGE, and immunoblotted using the indicated antibodies. (B) HCT116 cells were exposed to 0 or 6 Gy of IR, incubated for 30 min and harvested. Cell extracts were immunoprecipitated using an anti-SET7 antibody. Immunoprecipitates were eluted, resolved by SDS-PAGE, and immunoblotted using the indicated antibodies. (C) HCT116 cells were exposed with 50 J/m$^2$ of UV and cell extracts were immunoprecipitated using an anti-UHRF1 K385me1 antibody. Immunoprecipitates were eluted, resolved by SDS-PAGE, and immunoblotted using the indicated antibodies. (D) HCT116 cells were exposed to 0-6 Gy of IR and cell extracts were immunoprecipitated using an anti-UHRF1 K385me1 antibody. Immunoprecipitates were eluted, resolved by SDS-PAGE, and immunoblotted using the indicated antibodies. (E and F) H1299 or DLD-1 cells treated with 0, 1 or 2 mM of H$_2$O$_2$ treatment for 30 min were immunoprecipitated using an anti-UHRF1 K385me1 antibody. Immunoprecipitates were eluted, resolved by SDS-PAGE, and
immunoblotted using the indicated antibodies. (G) Cell cycle progression in synchronized and released for 0, 3, 6, 9, 12 h HCT116 cells were detected by PI staining. Cells were fixed, stained with PI for 30 min, and analyzed by FACS. (H) HCT116 cells were arrested at G1/S boundary by double thymidine block/release and the cells were then treated with 1 mM of H2O2 for 30 min. The cell lysates were immunoprecipitated using an anti-UHRF1 K385me1 antibody. (I) Cell cycle progression in synchronized and released for 0, 1, 2, 3, 4, 5, 6 h HCT116 cells were detected by PI staining. Cells were fixed, stained with PI for 30 min, and analyzed by FACS. (J) Immunoblot data of cells used for Immunostaining in Figure 2D.
Supplementary Figure S3  UHRF1 phosphorylation is prerequisite for its methylation by SET7

(A) Immunoblot for control cells, UHRF1 knock down cells and cells used for IP assay in Figure 2G and 2H. (B and C) UHRF1 knock down HCT116 cells overexpressed with GFP-UHRF1 WT, GFP-UHRF1 S661A or GFP-UHRF1 S661D and Flag-SET7 were treated with 1 mM of H₂O₂ for 30 min. The lysates were immunoprecipitated using an anti-Flag antibody and immunoblotted with indicated antibodies. (D) UHRF1 knocked-down HCT116 cells
overexpressed with Flag-UHRF1 WT or Flag-UHRF1 S661A were exposed to 50 J/m² of UV for 30 min. The lysates were immunoprecipitated using an anti-UHRF1 K385me1 antibody and immunoblotted with indicated antibodies. (E) UHRF1 knocked-down H1299 cells overexpressed with GFP-UHRF1 WT or GFP-UHRF1 S661A were treated with 1 mM of H₂O₂ for 30 min. The lysates were immunoprecipitated using an anti-UHRF1 K385me1 antibody and immunoblotted with indicated antibodies.
Supplementary Figure S4  Methylation of UHRF1 promotes the interaction of UHRF1-PCNA and polyubiquitination of PCNA

(A) HCT116 shNC and shSET7 cells treated with 1 mM of H$_2$O$_2$ for 30 min were immunoprecipitated using an anti-PCNA antibody. Immunoprecipitates were eluted, resolved
by SDS-PAGE, and immunoblotted using the indicated antibodies. (B) Cell extracts of UHRF1 knocked down HCT116 cells transfected with GFP-UHRF1 WT or K385R and Flag-PCNA were treated with 1 mM of H₂O₂ for 30 min. The lysates were immunoprecipitated using an anti-Flag antibody. Immunoprecipitates were eluted, resolved by SDS-PAGE, and immunoblotted using the indicated antibodies. (C) In vitro ubiquitination assay using recombinant PCNA WT and UHRF1 WT or UHRF1 RING domain deletion mutant (UHRF1 ΔRING: 1-600 a.a). Ubiquitin, UBC13/Mms2 and UBE1 were incubated with recombinant proteins 30 °C for 3 h. (D and F) HCT116 cells were exposed to 50 J/m² of UV and H1299 cells were treated with 2 mM of H₂O₂ for 30 min. Cell extracts of control and damaged cells were immunoprecipitated using anti-PCNA antibodies and associated proteins were pulled down with A/G agarose beads. Beads were washed extensively and bound proteins were resolved by SDS-PAGE, and immunoblotted using the indicated antibodies. (E and G) HCT116 shNC and shUHRF1 transfected with Flag-PCNA were exposed to 50 J/m² of UV or H1299 shNC and shUHRF1 transfected with Flag-PCNA were treated with 2 mM of H₂O₂. The lysates were and immunoprecipitated using an anti-Flag antibody. Immunoprecipitates were eluted, resolved by SDS-PAGE, and immunoblotted using the indicated antibodies.
Supplementary Figure S5  Ubiquitination of PCNA affects HR progression

(A) Immunoblot for each set of cells used for HR assay in Figure 4D. (B) U2OS cells integrated with HR reporter were transiently knocked down with the indicated siNC or siPCNA transfection and recovered with indicated PCNA constructs for measuring HR efficiency. Results were shown as mean ± SEM, n >3; *p < 0.05, N.S: no significant difference, and immunoblot for each sample used for HR assay.
Supplementary Figure S6  LSD1 demethylases UHRF1 in H1299 cells

(A) H1299 cells treated with 500 nM GSK-LSD1 for 24 h and control cells were immunoprecipitated with anti-UHRF1 K385me1 antibody and analyzed with immunoblotting.

(B) H1299 cells transfected with Flag-EV or Flag-LSD1 in control or LSD1 stably knocked down cells. The lysates were immunoprecipitated using an anti-UHRF1 K385me1 antibody.
Supplementary Figure S7  UHRF1 methylation is required for proper DNA damage repair and cell viability

(A) Colony formation assay using HCT116 UHRF1 knocked down cells transfected with UHRF1 WT or UHRF1 K385R. Cells were treated with 0, 10 uM, 100 uM, 1 mM and 2 mM H2O2 for 30 min and incubated in fresh media for 7 days. Results were shown as mean ± SEM,
n = 3; *p < 0.05, **p < 0.01. (B and C) HCT116 cells were exposed with 50 J/m² of UV or H1299 cells were treated with 2 mM H_{2}O_{2} for 30 min. Flow cytometry analysis of annexin-V and propidium iodide (PI) staining of apoptotic cells transfected with UHRF1 WT, UHRF1 K385R or LSD1 in UHRF1 knocked down cells or control cells, showing % of apoptotic cells (Annexin-V positive + Annexin-V and PI double positive cells). Results were shown as mean ± SEM, n = 3; *p < 0.05, **p < 0.01, ***p < 0.001.
SUPPLEMENTARY MATERIALS AND METHODS

Plasmid constructs

The UBI domain (residues 1-100), TTD-PHD domain (residues 90-383), SRA domain (residues 370-685), RING domain (residues 675-793), UHRF1 (residues 1-793), UHRF1 K385R, UHRF1 S661A, UHRF1 K670R, UHRF K385/670R, UHRF1 RING domain deletion (residues 1-600) and other mutants or partial constructs of human UHRF1, were sub-cloned into the pGFP-C1 vector (Clontech, Mountain View, CA), pLenti-G418-vector, bacterial expression vector pGEX-4T2, and a modified pcDNA6-HA-myc-his vector (Invitrogen, Carlsbad, CA). SET7 #1 (residues 1-214), SET7 #2 (residues 52-366), SET7 #3 (residues 138-366), SET7 #4 (residues 214-366), and SET7 (residues 1-366) of human SET7 (Addgene #24084) were amplified by PCR and then sub-cloned into bacterial expression vector pGEX-4T2 and a modified pcDNA6-HA-myc-his vector (Invitrogen, Carlsbad, CA). Human SET7 H297A (Addgene #24085) was purchased from Addgene. The shRNAs against human UHRF1 and SET7 were designed using siRNA sequence designer software (Clontech). A double-stranded oligonucleotide for shRNA plasmid construction was produced using primers from the 5’ to the 3’ end. The shUHRF1 and shSET7 RNA oligonucleotide sequences were as follows: for shUHRF1: 5’- CCGGAGAT ATAACGTTAGGTTTCTCGAGAAACCCTAACGT TA TATCTTTTTTG – 3’ (top strand) and 5’– AATTCAAAAAAGATATAACGTTAGGTTTCTCGAGAAACCCTAACGT TA TATCTTTTTTG – 3’ (bottom strand), for shSET7: 5’– CCGGGCCAGGGTATTATTATAGAATCTCGAGATTCTATAATAATACCCTGGCTTTT TT - 3’ (top strand) and 5’ – AATTCAAAAAGCCAGGGTATTATTATAGAATCTCGAGATTCTATAATAATACCCTGGCTTTT TT - 3’ (top strand) and 5’ – AATTCAAAAAGCCAGGGTATTATTATAGAATCTCGAGATTCTATAATAATACCCTGGCTTTT TT - 3’ (top strand) and 5’ – AATTCAAAAAGCCAGGGTATTATTATAGAATCTCGAGATTCTATAATAATACCCTGGCTTTT TT - 3’ (top strand) and 5’ – CCGGAGGAAGGGCTCTTTCTAGCAATCTCGAGTTGCTAGAAGAGCTCCTTTTTTG – 3’ (top strand) and 5’–
AATTCAAAAAAGGAAGGCTCTTCTAGCAATACTCGAGTATTGCTAGAAGAGCCT
TCCT – 3’ (bottom strand). These oligonucleotides were inserted into the AgeI/EcoRI site of the pLKO.1 TRC vector. Small-interfering RNAs (siRNAs) that effectively inhibit PCNA was purchased from Genolution Pharmaceuticals Inc (Genolution, Seoul, Republic of Korea). siRNA sequences of negative control (siNC) and PCNA were as follows; negative control, 5’-CCUCGUGCCGUUCAUCAGGUAGUU -3’; PCNA, 5’- AAGCACAAACCAGGAGAAAGUU -3’. DR-GFP integrated U2OS cells were transfected with either 200 nmol/L of target siRNAs using lipofectamine 2000 (Invitrogen).

Antibodies

Antibodies against SET7 (ABE1001), phospho-H2A.X (05-636), RPA (NA-19L) (Millipore, Billerica, MA), phospho-H2A.X (Abcam, ab2893), Flag (F3165) (Sigma, St. Louis, MO), Rad51 (Genetex, GTX70230), β-actin (sc-47778), GFP (sc-9996), HA (sc-805), SET7 (sc-390823), normal rabbit IgG (sc-2027), UHRF1 (sc-373750), PCNA (sc-56), and LSD1 (sc-271720) (all from Santa Cruz Biotechnology, Dallas, TX) were employed. UHRF1 K385me1 and UHRF1 K670me1 antibodies were generated by Abfrontier (South Korea)

In vitro methylation assay

In vitro methylation assays performed at 30 °C for 3 h in 30 µL volumes containing 50 mM Tris-HCl [pH 8.5], 20 mM KCl, 10 mM MgCl₂, 10 mM β-mercaptoethanol, 1.25 M sucrose, 100 nCi of [14C]-SAM (Perkin Elmer, Waltham, MA) and GST-UHRF1, GST-UHRF1 #1 (residues 1-100), GST-UHRF1 #2 (residues 90-383), GST-UHRF1 #3 (residues 370-685), or GST-UHRF1 #4 (675-793) and 2 µg of GST-SET7. Proteins were separated via 12% sodium
dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by autoradiography. Peptides (UHRF1 K385, UHRF1 K408, UHRF1 K670) were synthesized based on the N-terminal amino acid sequences of H3 histone (Cosmogenetech). The peptides were filtered using p81 filter paper (Upstate) and washed three times with cold 10% trichloroacetic acid (TCA) and 95% ethanol for 5 min at room temperature. The filters were allowed to air dry, after which 2 ml of Ultima Gold (Perkin Elmer) was added, and the [14C]-SAM was quantified using a scintillation counter.

**Cell culture and synchronization**

HEK293T and U2OS cells were grown in Dulbecco’s modified Eagle’s medium (DMEM), and HCT116 WT, HCT116 SET7 or UHRF1 knocked-down stable cells in RPMI 1640, each containing 10% heat-inactivated fetal bovine serum and 0.05% penicillin-streptomycin, at 37 °C in a 5 % CO2 atmosphere. HCT116 WT, H1299 and U2OS cells were transfected with the indicated DNA constructs using polyethyleneimine (PEI) (Polyscience, Warrington, PA) or Lipofectamine 2000 (Invitrogen, Carlsbad, CA). For inhibition of HCT116 or H1299 cells were treated with 500 nM GSK-LSD1. After incubation for 24 h, cells were harvested and used in experiments. G1/S synchronization was achieved by a double thymidine block. In brief, cells were cultured in the presence of 2.5 mM thymidine for 19 h, and then released to grow for 10 h. Cells were then treated for another 15 h with 2.5 mM thymidine, causing the cells to arrest at the G1/S boundary. The arrested cells were allowed to enter the S phase by washing the thymidine away with phosphate buffered saline (PBS).
**FACS analysis**

To measure the cell-cycle profile, HCT116 cells were trypsinized, washed and fixed in ice-cold 70% ethanol for 30 min. Immediately before flow cytometric analysis, the cells were treated with RNase A (0.4 mg/ml) and stained with propidium iodide (PI, Sigma) for 30 min, then subjected to fluorescence-activated cell sorting (FACS) analysis using a BD Accuri C6 cytometer (BD Biosciences). Data were analyzed using BD Accuri C6 software (BD Biosciences). Apoptosis rate was measured with FITC Annexin V Apoptosis Detection Kit using the manual procedure according to manufacturer’s instructions.

**MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay**

UHRF1 stable knocked-down HCT116 cells and the control cells were seeded in 48-well plates (5 × 10³ cells/well) and transfected with pLenti-UHRF1 WT and pLenti-UHRF1 K385R. After 24 h, cells were treated with 1 mM H₂O₂ for 30 min and then changed with fresh media. At 24, 48, and 72 h after H₂O₂ treatment, MTT was added to the cells (final concentration 1 mg/mL), and the cells were incubated for a further 2 h at 37 °C. The medium was then removed by aspiration, and DMSO was added (200 µL). The OD at 575 nm was determined using a spectrophotometer.

**Colony formation assay**

UHRF1 stable knockdown and control cells were plated at 5 × 10³ cells/ plate in a six-well culture dish and transfected with pLenti-UHRF1 WT and pLenti-UHRF1 K385R. After 48 h, cells were treated with 0 - 2 mM H₂O₂ for 30 min and then changed with fresh media and incubated for 7 days. Surviving colonies were stained with 0.005 % crystal violet and visible
colonies were counted.