Supplementary Data

SET7–mediated TIP60 methylation is essential for DNA double-strand break repair

Author’s name: Song Hyun Kim, Junyoung Park, Jin Woo Park, Ja Young Hahm, Seobin Yoon, In Jun Hwang, Keun Pil Kim & Sang-Beom Seo

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

Running Title: TIP60 methylation by SET7 induces DNA repair.

Keywords: SET7, TIP60, methylation, DNA repair, epigenetics

Corresponding Author’s Information: sangbs@cau.ac.kr
MATERIALS AND METHODS

Plasmid constructs

TIP60 (residues 1–461), TIP60 K137R, and partial constructs of human TIP60 were sub-cloned into the pGFP-C1 vector (Clontech), bacterial expression vector pGEX-4T2 (Invitrogen). SET7 (residues 1–366) of human SET7 (#24084; Addgene) was amplified by PCR and then sub-cloned into the bacterial expression vector pGEX-4T2. A double-stranded oligonucleotide for shRNA plasmid construction was purchased using primers from the 5’ to the 3’ end. The shTIP60 and shSET7 RNA oligonucleotide sequences were as follows: shTIP60: 5’ - CCGGT CGAATTGTTTGGCAGTCGAGATCGTGCCAAAATTTCCGATTTTTG - 3’ (top strand) and 5’-AATTCAAAAATCGAATTGTTTGGCAGTCGAGATCTCGAGATCAGTGCCCAAACAATTCGATTTTTG - 3’ (bottom strand); shSET7: 5’ - CCGGGCCAGGATGTATTATAGAATCTCGAGATTCTATAATAATACCCCTGCTGTTT - 3’ (top strand) and 5’ - AATTCAAAAGCCAGGGTATTATTATAATACCCCTGCTGCTGTTT - 3’ (bottom strand). These oligonucleotides were inserted into the AgeI/EcoRI site of the pLKO.1 TRC vector. The siRNA sequence of the negative control (siNC) was as follows: negative control, 5’ - CCUCUGUGCCGUUCCAUCAGGUU - 3’.

Antibodies

Antibodies against SET7 (sc-390823), p-histone H2A.X (sc-517348), RPA (sc-56770), GFP (sc-9996), β-actin (sc-47778), TIP60 (sc-166323), and LSD1 (sc-271720) were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology). Methyl-lysine (Me-K: ab174719, ab23366) (Abcam), Flag (F3165) (Sigma-Aldrich), anti-caspase 3 (AB1899) (millipore), Rad51 (GTX70230) (Genetex) were employed.
Cell culture and transfection

HEK293T and U2OS cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco), and HCT116 cells were grown in RPMI 1640 medium (Gibco) containing 10% heat-inactivated fetal bovine serum (Gibco) and 0.05% penicillin-streptomycin (Welgene) at 37 °C in a 5% CO₂ atmosphere. HCT116 WT and U2OS cells were transfected with the indicated DNA constructs using polyethyleneimine (PEI) (Polyscience) or Lipofectamine 2000 (Invitrogen).

*In vitro* methylation assay

Methylation was 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), GST-TIP60, GST-TIP60 ∆1 (residues 1 - 104), GST-TIP60 ∆2 (residues 105 - 220), GST-TIP60 ∆3 (residues 221 - 461), and 2 µg of GST-SET7. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by autoradiography. TIP60 K137R was synthesized based on the N-terminal amino acid sequences of H3 histone (Cosmo Genetech), 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.

Immunoprecipitation (IP) assay

Cells were lysed in a lysis buffer (50 mM Tris-HCl [pH 7.5], 200 mM NaCl, 0.5% NP-40, 1× protease inhibitor cocktail) and incubated with the indicated antibodies overnight at 4 °C. Protein A/G agarose beads (GenDEPOT) were then added, and the mixture was gently rotated for 3 h at 4 °C. Bound proteins were analyzed by immunoblotting with the indicated antibodies.
LTQ-orbitrap mass spectrometry

Samples were separated by SDS-PAGE and were isolated via gel extraction. After overnight trypsin digestion at 37 °C, the eluted peptides were separated using a C18 column with a linear gradient (A: 100% H2O, 0.1% formic acid and B: 100% ACN) at a flow rate of 300 nl/min. Typically, 2 μL of the sample was injected. Mass spectrometry was performed with a dual-mass spectrometer (LTQ Orbitrap Velos; Thermo Scientific) coupled to a nano-LC system (EASY nLC; Thermo Scientific). This method consisted of a cycle combining one full MS scan (mass range: 150 - 2000 m/z). Proteins were identified from the MS/MS spectra using SEQUEST.

DNA repair assay (HR reporter assay)

An integrated DNA repair reporter system was used to determine the HR efficiency. U2OS cells integrated with an HR reporter were transfected with the indicated constructs and the I-SceI plasmid, which induces DSB. Cells were harvested 48 h after transfection, and the percentage of GFP-positive cells was determined by fluorescence-activated cell sorting (FACS) analysis using a BD Accuri C6 cytometer (BD Biosciences). Data were analyzed using BD Accuri C6 software (BD Biosciences). Repair frequencies represent the mean of at least three independent experiments.

Immunofluorescence staining

HCT116 TIP60 knockdown cells were cultured on coverslips and transfected with Flag-TIP60 WT or methylation-deficient TIP60 (Flag-TIP60 K137R) using Lipofectamine 2000 Reagent (Thermo Fisher Scientific). After treatment with 5 mM HU for 4 h, the cells were fixed in 4% paraformaldehyde for 1 h and permeabilized in 0.2% Triton X-100 solution for 10 min at room temperature. Cells were blocked with 1% bovine serum albumin in PBS and incubated with a primary antibody for 2 h. Cells were washed with PBS and incubated with a secondary antibody for 1 h. The
coverslips were mounted onto glass slides and visualized using an ECLIPSE 80i fluorescence microscope (Nikon, Tokyo, Japan).

Comet assay

After treatment of cells with HU for 4 h, the media were replaced with fresh media, and the cells were incubated at 37 °C. After harvesting the next day, neutral comet assay was performed. The cells were mixed with 1% UltraKem LE Agarose (Young Science: 1:10 [v/v]), and upon agarose solidification, the cells were lysed with a lysis buffer (2% sarkosyl, 0.5 M EDTA–Na2, and 0.5 mg/mL proteinase K, pH 8.0) at 37 °C overnight, followed by three 20 min washes with an electrophoresis buffer (90 mM Tris buffer, 90 mM boric acid, and 2 mM EDTA–Na2, pH 8.5). The proteins in the washed gels were subjected to electrophoresis at 20 V for 25 min. The slides were stained with propidium iodide (Sigma–Aldrich), and fluorescence images were captured using an Olympus BX53 epifluorescence microscope (Olympus). The tail moment was quantified using CASP version 1.2.3 beta2 (CaspLab).

MTT assay

HCT116 cells (shTIP60) and control cells were transfected with FLAG–TIP60 WT or FLAG–TIP60 K137R and seeded in 48–well plates at a density of 5 × 10^3 cells per well. After 24 h, the cells were treated with 5 mM HU for 4 h, and the medium was replaced with a fresh medium. At 24, 48, and 72 h after HU treatment, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to the cells (final concentration 0.5 mg/mL), after which the cells were incubated further for 2h at 37 °C. The medium was then removed, and dimethyl sulfoxide (DMSO) was added (200 µL/well). Finally, the OD was determined using a microplate spectrophotometer (BioTek) at a wavelength of 575 nm.
Colony formation assay

Cells transfected with FLAG-TIP60 WT or FLAG-TIP60 K137R were seeded in 35 mm culture dishes at a density of $5 \times 10^3$ cells per well. After 24 h, cells were treated with 5 mM HU for 4 h, and the medium was replaced with a fresh medium. After incubation for 6 days, surviving colonies were stained with 0.005 % crystal violet.

Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM) of three independent experiments. Data were analyzed using GraphPad Prism (version 9; GraphPad Software, USA). Differences between the groups were evaluated by Student’s t-test. $P < 0.05$ was considered statistically significant.
Supplementary Fig. 1. SET7 methylates TIP60 at Lys 137, and the region is conserved in various species. (A) HCT116 cells were transfected with the indicated plasmids and immunoprecipitated with anti-methyl lysine antibodies. Associated proteins were eluted, resolved by SDS–PAGE, and immunoblotted with indicated antibodies. Methyl lysine levels were normalized by input of TIP60. (B) Conserved region in various species. The arrow indicates TIP60 at K137.
Supplementary Fig. 2. SET7 doesn’t affect TIP60-mediated acetylation and localization.

(A, B) pcDNA3.1–SET7, pcDNA3.1–SET7 H297A transfected 293T cells and HCT116 cells were lysed and immunoblotted using the indicated antibodies. (C) HCT116 cells, transfected with pcDNA3.1–SET7, pcDNA3.1–SET7 H297A or empty vector, were separated into nuclear and cytoplasmic fractions. H3 and β-tubulin were used as loading controls.
Supplementary Fig. 3. SET7 interacts with TIP60 *in vitro* and *in vivo*. (A) Extracts of HCT116 cells transfected with SET7 were incubated with purified GST or GST–TIP60. Associated proteins were eluted, resolved by SDS–PAGE, and immunoblotted (top). The amount of TIP60 in the cell extract was determined by Coomassie staining (bottom). (B) Flag–EV or Flag–TIP60 and pcDNA3.1–SET7 were overexpressed in HCT116 cells. The cell lysates were immunoprecipitated with an anti–SET7 antibody. Associated proteins were eluted, resolved by SDS–PAGE, and immunoblotted using the indicated antibodies.
Supplementary Fig. 4. Hydroxyurea (HU)-mediated DNA damage induces SET7-dependent methylation of TIP60 and induces homologous recombination (HR). (A) HCT116 cells were treated with 5 mM hydroxyurea (HU) for 4 h, 8 h. Apoptotic cells were measured by fluorescence-activated cell sorting (FACS) analysis. (B) HCT116 shNC and shSET7 cells treated with 5 mM HU for 4 h were immunoprecipitated using anti-methyl lysine antibodies. Immunoprecipitates were eluted, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotted using indicated antibodies. Methyl lysine levels were normalized by input of TIP60. (C) HCT116 cells were transfected with the indicated plasmids and treated with 5 mM HU for 4 h. Cell extracts of control and damaged cells were immunoprecipitated using anti-SET7 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. (D) PcDNA3.1–SET7, PcDNA3.1–SET7 H297A, or Flag–TIP60 was subjected to HR assay. Results were shown as the mean ± SEM; n = 3, **P < 0.01, N.S: no significant difference. (E) HCT116 cells with TIP60 knockdown were transfected with TIP60 WT or TIP60 K137R. Rad51 foci was examined following 5 mM HU treatment for 4 h.
Supplementary Fig. 5. LSD1 demethylates TIP60 methylation. HCT116 cells were transfected with Flag–EV or Flag–LSD1 in control or LSD1 stably knocked down cells. The lysates were immunoprecipitated using anti–methyl lysine antibodies. Methyl lysine levels were normalized by input of TIP60.
Supplementary Fig. 6. HU-dependently increased apoptosis in TIP60 K137R compared to TIP60 WT. (A) HCT116 cells were transfected with the indicated plasmids and treated with 5 mM hydroxyurea (HU) for 4 h. Apoptotic cells were measured by fluorescence-activated cell sorting (FACS) analysis. (B) HCT116 cells were transfected with the indicated plasmids and treated with 5 mM HU for 4 h. Apoptotic cells were measured by fluorescence-activated cell sorting (FACS) analysis. Results were shown as the mean ± SEM; n = 3, *P < 0.05, N.S: no significant difference. (C) Cell viability was determined using the MTT assay. HCT116 cells were transfected with Flag-empty vector (EV) or Flag–TIP60. Cells were treated with 500 nM GSK–LSD1 for 24 h and incubated in fresh media for 0 - 72 h. Results were shown as the mean ± SEM; n = 3, **P < 0.01, *P < 0.05, N.S: no significant difference.