Supplemental Data

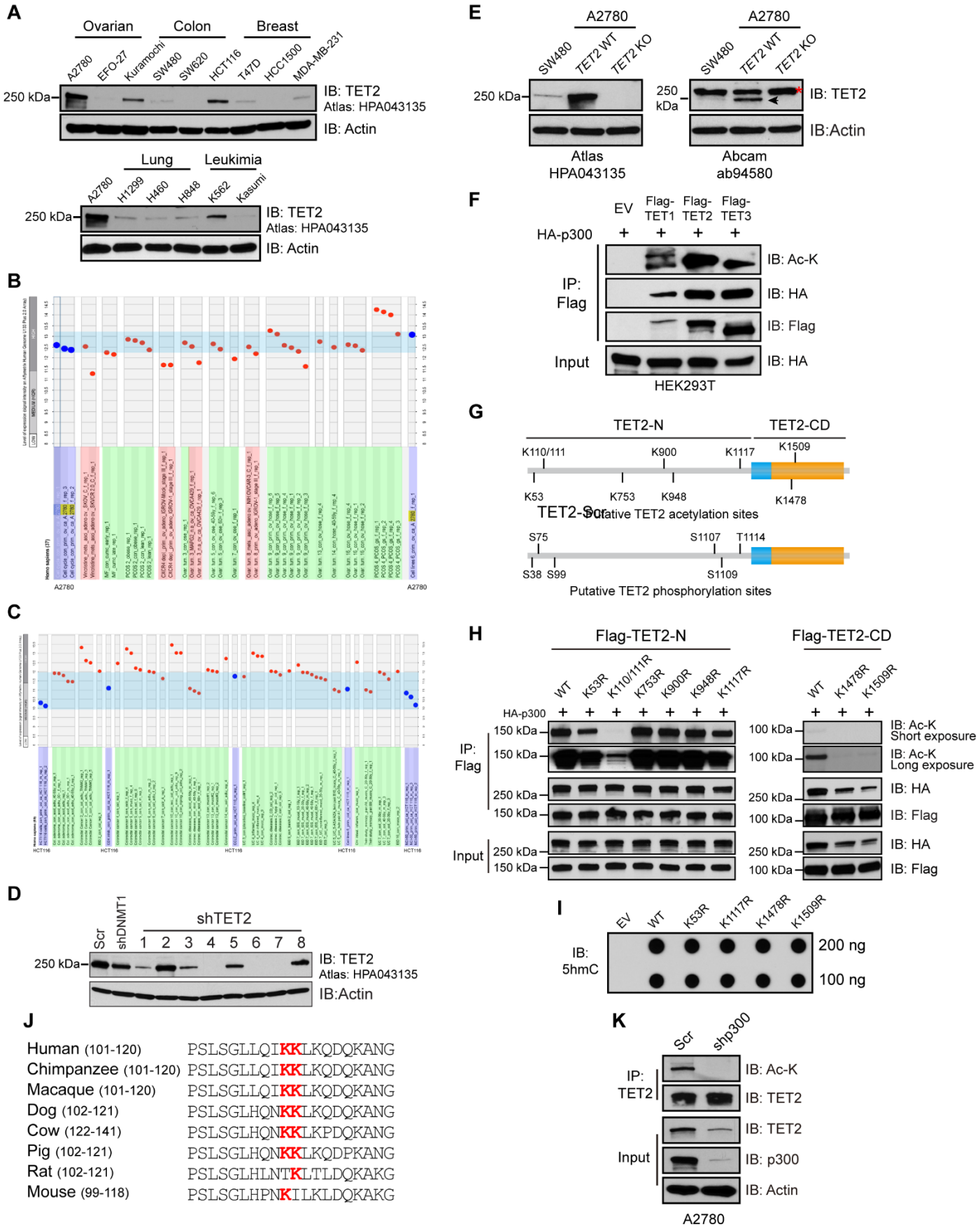

Figure S1. Related to Figure 1. TET2 is acetylated by p300

(A) Western blotting screening of cancer cell lines for endogenous TET2 proteins. TET2 antibody used: Atlas HPA043135.

(B and C) Comparison of *TET2* mRNA levels in A2780 (B) and HCT116 (C) to normal human ovarian and colon tissues. Y-axis: levels of *TET2* expressions measured by Affymetrix Human Genome U13 Plus 2.0 Array (probe set 227624_at). *TET2* levels in A2780 (B) and HCT116 (C) are highlighted in blue. X-axis: sample names. A2780 and HCT116 cell lines are highlighted in blue boxes, other ovarian cell lines (B) are highlighted in pink box (SKOV-3, SKVCR2.0, IGROV1, OVCA429, and NIH:OVCAR-3), control tissue samples are highlighted in green boxes. Graphs were generated by Genevestigator program using publicly available data.

(D and E) Validation of TET2 antibody. (D) HCT116 cells were infected with 8 individual TET2 shRNAs for 4 days and TET2 protein levels were measured by WB with anti-TET2 antibody (Atlas HPA043135). Non-targeting shRNA (Scr) was used as a negative control. (E) TET2 protein levels in A2780 *TET2* WT and KO cells were measured by WB with TET2 antibodies. A single band at ~250 kDa was detected by Atlas HPA043135 TET2 antibody in SW480 and A2780 *TET2* WT cells, but not in A2780 *TET2* KO cells. Abcam ab94580 detects double bands in the WT cells and one band in KO cells. The lower band (arrow head) represents TET2, while the upper one (asterisk) represents non-specific binding. SW480 was used as a control.

(F) TET1/2/3 are acetylated by p300. Flag-tagged TET1/2/3 were co-transfected with HA-p300 into HEK293T cells for 2 days. TET2 complexes were IPed from nuclear extract with Flag antibody and analyzed by IB.

(G) Putative acetylation (upper panel) and phosphorylation (lower panel) sites identified by MS.

(H) Validation of TET2 acetylation site(s). TET2-N or TET2-CD constructs carrying individual putative acetylation mutations were co-transfected with HA-p300 into HEK293T cells. TET2 proteins were IPed and their acetylation status were determined with anti Ac-K antibody.

(I) Full length WT, K53R, and K1117R were transfected into HEK293T cells individually and global 5hmC levels from genomic DNAs were measured by bot blot assay. Cells transfected with empty vector (EV) were used as a negative control.

(J) Sequence alignment of TET2 surrounding K110/111 from various species. Sequences used include, H. sapiens: NCBI Reference Sequence: NP_001120680.1; P. troglodytes: XP_003310448.1; M. fascicularis: XP_005555651.1; C. lupus familiaris: XP_535678.3; B. taurus: XP_010826379.1; S. scrofa: XP_003129326.3; R. norvegicus: XP_006233409.1; M. musculus: NP_001035490.2.

(K) A2780 cells were infected with scramble or shp300 virus. Successful knockdown of p300 and its influence on TET2 were confirmed by IB. Cells were subsequently used to immunoprecipitate endogenous TET2 proteins for MS analysis shown in Figure 1G and 1H.

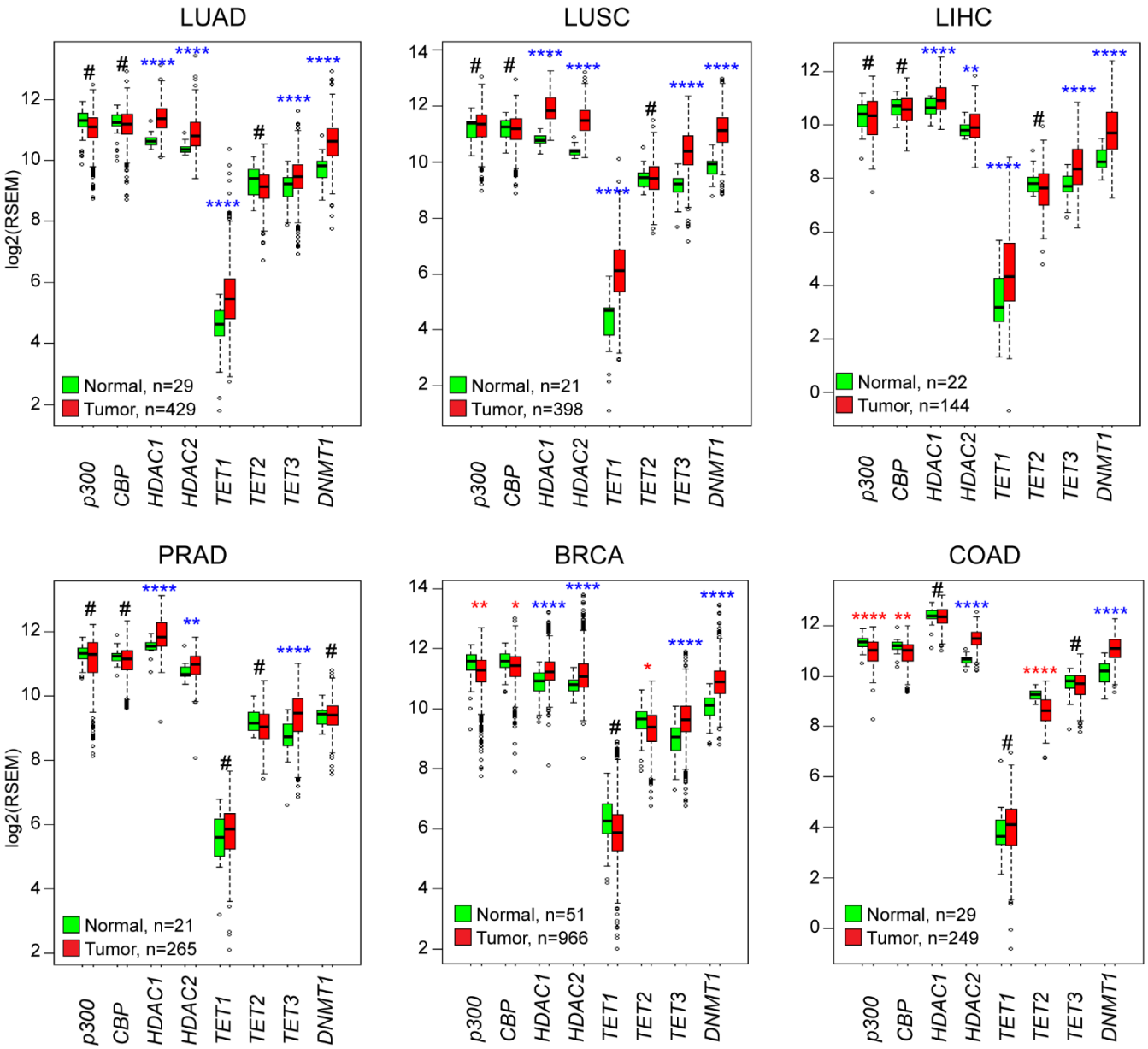

Figure S2. Related to Figure 2. mRNA levels of *p300/CBP, HDAC1/2, TET1/2/3,* and *DNMT1* in normal tissues and tumors

RNA-seq data (level 3) of various tumor types containing adequate number of normal controls (n > 20) were downloaded from the TCGA database. Transcript levels (RSEM) of indicated genes were extracted, log2 transformed, and plotted. LUAD: Lung adenocarcinoma; LUSC: lung squamous cell carcinoma; LIHC: liver hepatocellular carcinoma; PRAD: prostate adenocarcinoma; BRCA: breast invasive carcinoma; COAD: colon adenocarcinoma. *: $P < 0.05$; **: $P < 0.01$; ****: $P < 0.0001$; #: not significant. Blue asterisk (*): significantly up-regulated; red asterisk (*): significantly down-regulated; relative to normal.

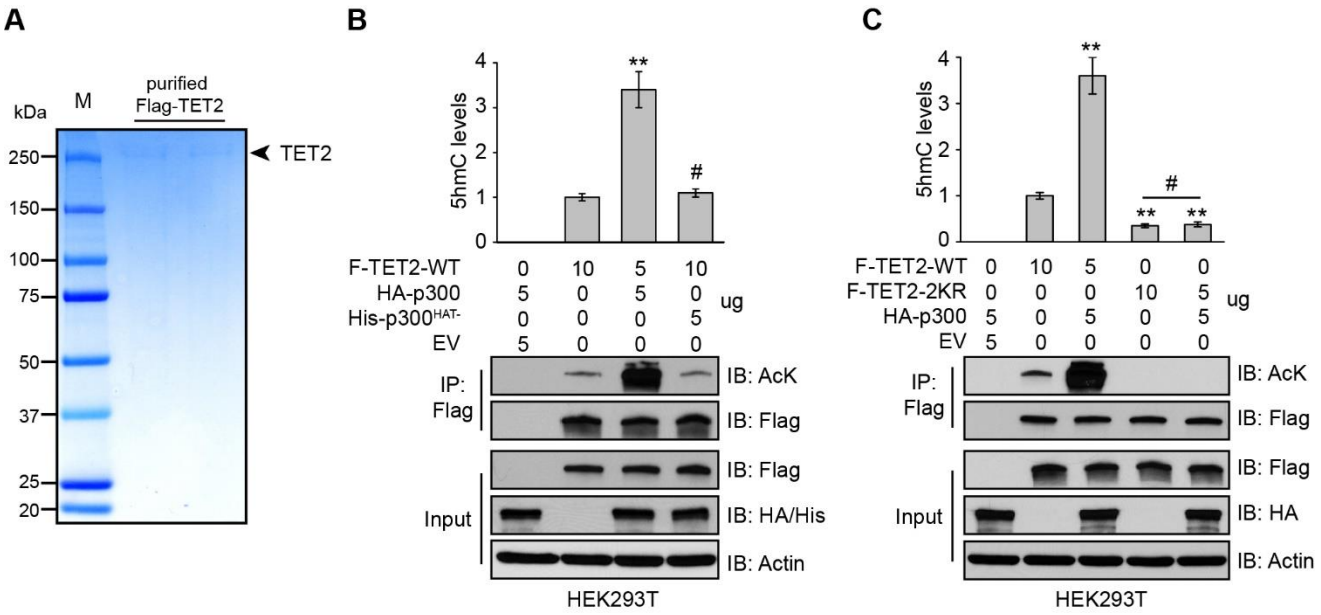

Figure S3. Related to Figure 3. Acetylation enhances TET2 enzymatic activity

(A) Purification of recombinant TET2. 293F cells grown in suspension were transiently transfected with Flag-TET2 construct using PEI for 2 days. Cells were lysed by sonication and incubated with anti-Flag affinity gel. After extensive wash, 10% beads were eluted with 3XFlag peptide and analyzed by SDS-PAGE/Coomassie staining. (B and C) Wild type TET2 with wild type p300 or a catalytically inactive mutant (His-p300^{HAT-}) (B), or wild type p300 with wild type TET2 or TET2 2KR mutant (C) were co-transfected into HEK293T cells. TET2 proteins were IPed and their acetylation status were analyzed by IB with anti-AcK antibody (lower panels). Global 5hmC levels were measured by 5hmC ELISA (upper panel). Values represent mean ± SEM (n=3). **: $P < 0.01$; #: not significant (student's t-test).

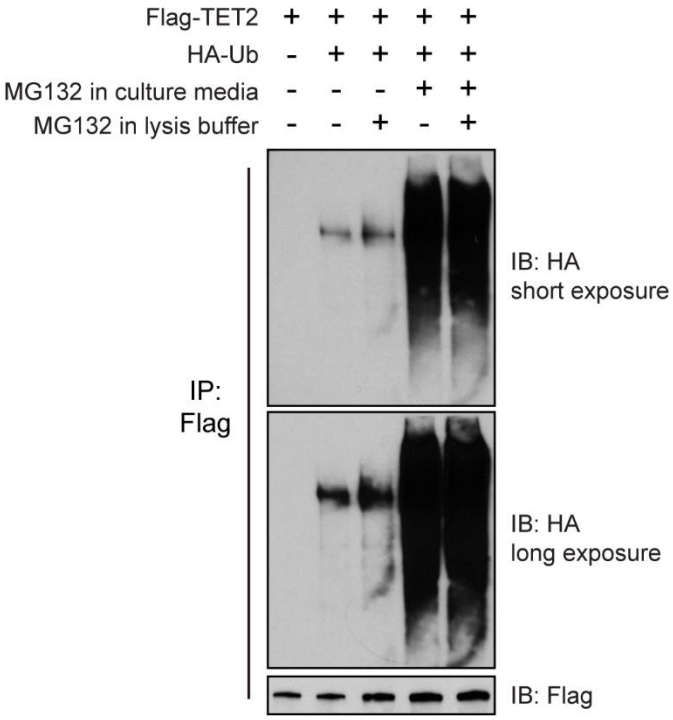

Figure S4. Related to Figure 4. TET2 ubiquitination status affected by MG132.

HEK293T cells were transfected with Flag-TET2, with or without HA-Ub for 24 hours. Cells were then treated with either DMSO or MG132 (2 uM) overnight. Buffers supplemented with or without MG132 (5 uM) were used to lyse cells from each group (DMSO or MG132) and to perform IP. Elute were analyzed by IB with HA and Flag antibodies.

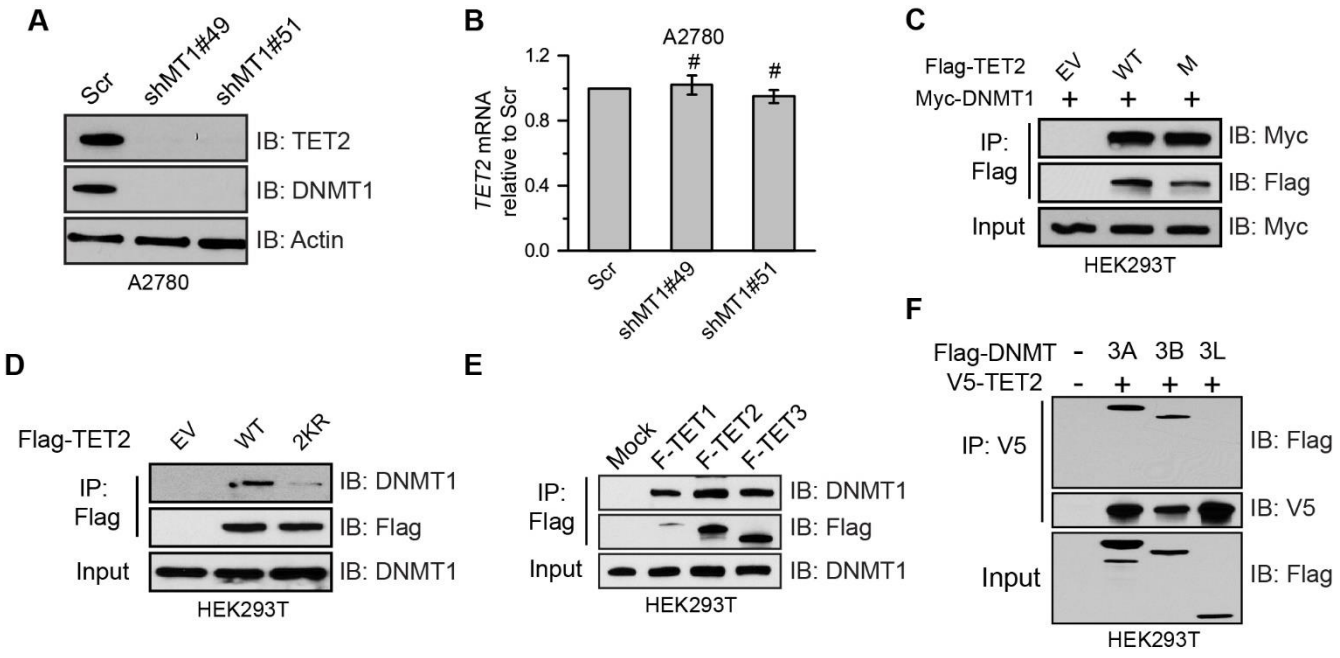

Figure S5. Related to Figure 5. TET2 acetylation enhances DNMT1 binding to promote protein stability

(A and B) Knocking down DNMT1 decreases TET2 protein. A2780 cells were infected by either Scr or DNMT1 shRNAs (shMT1#49, shMT1#51, Cai et.al., unpublished data). Endogenous TET2 protein levels (A) were measured by IB. *TET2* mRNA levels were quantified by qRT-PCR (B). Values represent mean ± SEM (n=3). #: not significant (student's t-test).

(C) HEK293T cells were co-transfected with Flag-tagged TET2 WT, or catalytic mutant (M, H1382Y/D1384A), and Myc-DNMT1. TET2 complexes were IPed and analyzed by IB.

(D) TET2 acetylation affects interaction with DNMT1. HEK293T cells were transfected with Flag-TET2 WT, or 2KR mutant for 2 days. TET2 complexes were IPed and analyzed by IB.

(E) TET1/2/3 interact with DNMT1. HEK293T cells were transfected with Flag-tagged TET1/2/3 for 2 days. TET complexes were IPed and analyzed by IB.

(F) TET2 interacts with DNMT3B. HEK293T cells were co-transfected with V5-TET2 and Flag-tagged DNMT3A, DNMT3B, DNMT3L for 2 days. TET2 complexes were IPed and analyzed by IB.

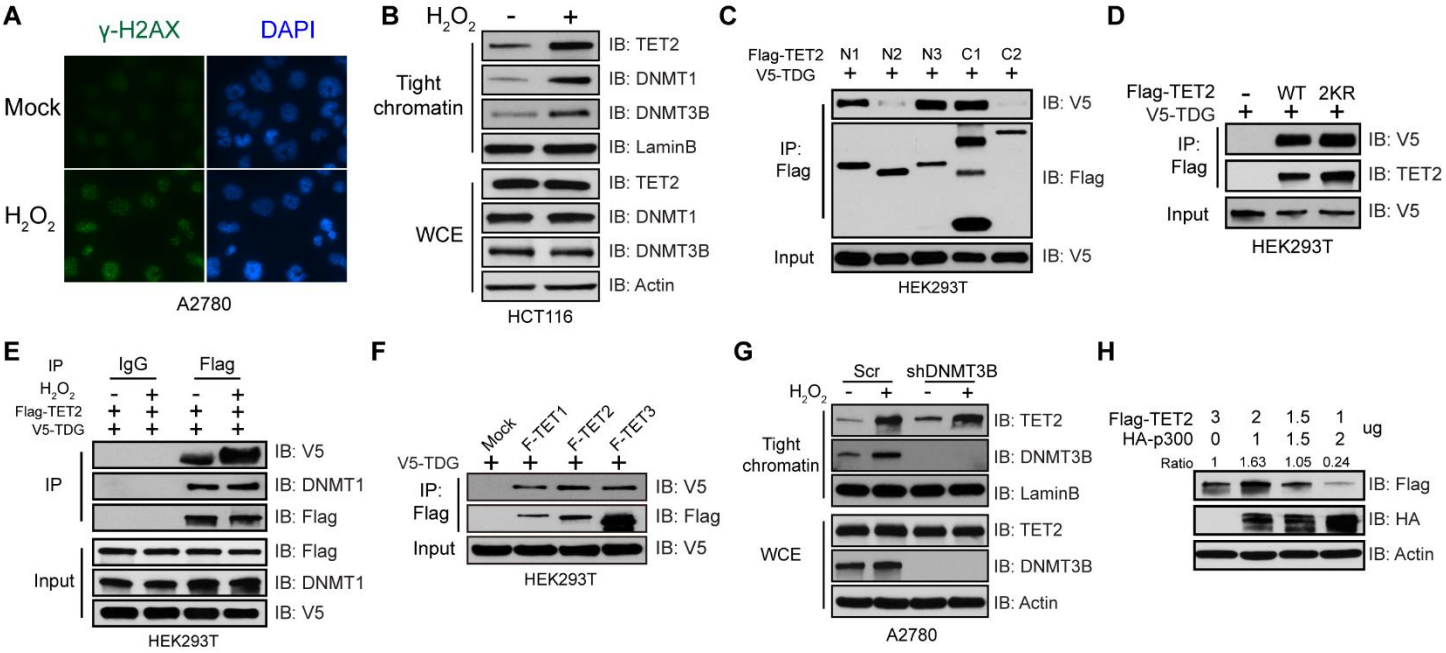

Figure S6. Related to Figure 6. Acetylation enhances targeting of TET2 to chromatin during OS

(A) H_2O_2 treatment induces DNA damage. A2780 cells were treated with or without 5 mM H_2O_2 for 30 min. Immunofluorescent staining was performed with γ-H2AX antibody. DNA was stained with DAPI.

(B) HCT116 cells were treated with or without 2 mM H_2O_2 for 30 min. Tight chromatin and WCE were analyzed by IB with indicated antibodies.

(C and D) HEK293T cells were co-transfected with V5-TDG and Flag-TET2 truncations (C), or TET2 WT and 2KR mutant (D) for 2 days. TET2 complexes were IPed and analyzed by IB with indicated antibodies.

(E) HEK293T cells were co-transfected with TDG and TET2 for 2 days and treated with or without H_2O_2 for 30 min. TET2 complexes were IPed and analyzed by IB with indicated antibodies. Spermine and Spermidine were used to extract proteins bound to chromatin.

(F) HEK293T cells were co-transfected with V5-TDG and Flag-tagged TET1/2/3 constructs. TET2 complexes were IPed and analyzed by IB with indicated antibodies.

(G) A2780 cells were infected with Scr or shDNMT3B virus for 2 days and selected for 5 days. Cells were then treated with or without H_2O_2 for 30 min and tight chromatin and WCE were analyzed by IB with indicated antibodies.

(H) HEK293T cells were co-transfected with Flag-TET2 and HA-p300 at various ratios. Total amount of DNA transfected were kept equal (3 ug). WCE were analyzed by IB.

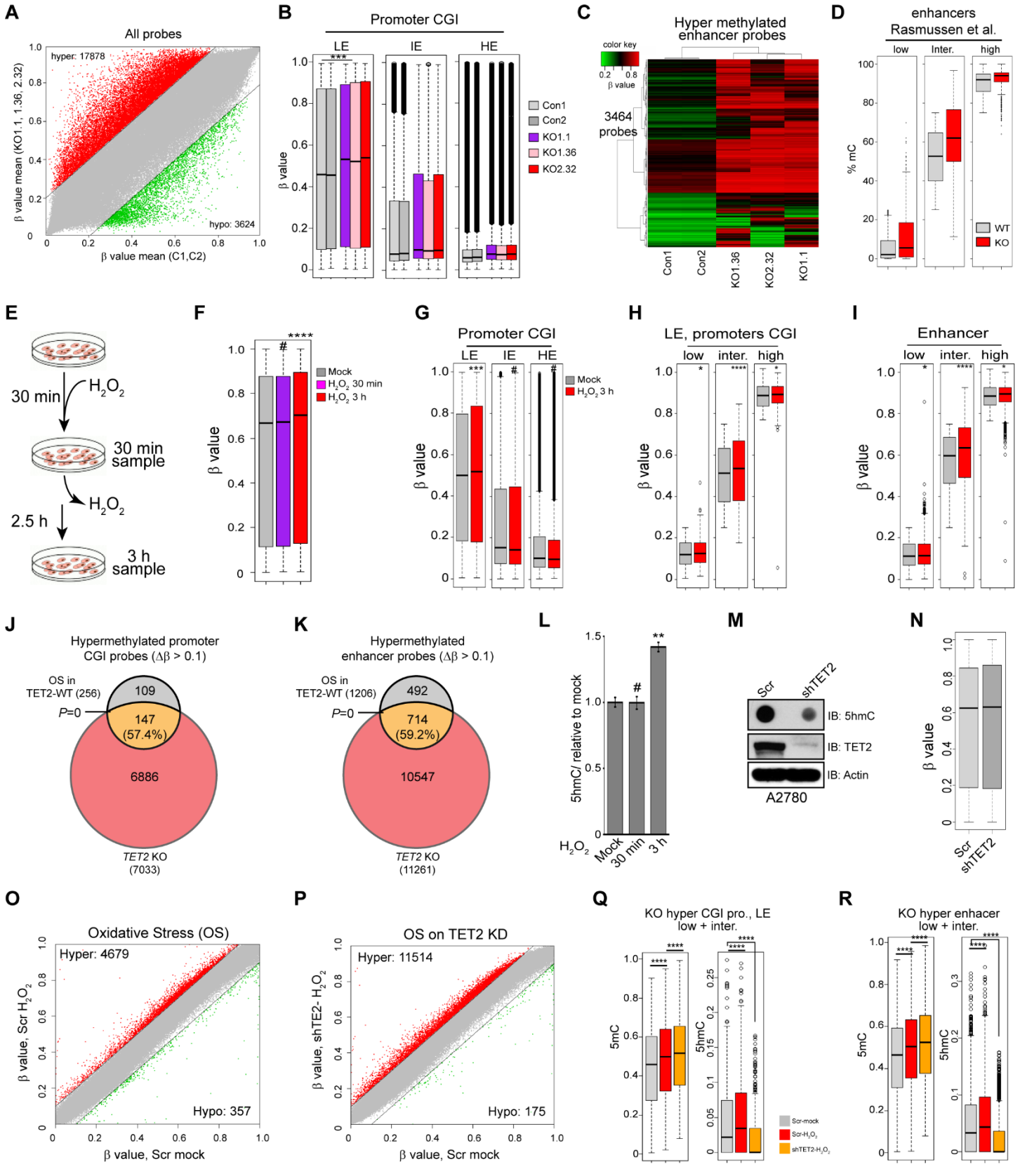
A
All probes
hyper: 17878
hypo: 3624
β value mean (KO1.1, 1.36, 2.32)
β value mean (C1,C2)
B
Promoter CGI
LE
IE
HE
β value
Con1
Con2
KO1.1
KO1.36
KO2.32
C
Hyper methylated enhancer probes
color key
β value
3464 probes
Con1
Con2
KO1.36
KO2.32
KO1.1
D
enhancers
Rasmussen et al.
low
Inter.
high
% mC
WT
KO
E
30 min
H2O2
30 min sample
2.5 h
3 h sample
F
Mock
H2O2 30 min
H2O2 3 h
β value
G
Promoter CGI
LE
IE
HE
Mock
H2O2 3 h
β value
H
LE, promoters CGI
low
inter.
high
β value
I
Enhancer
low
inter.
high
β value
J
Hypermethylated promoter CGI probes (Δβ > 0.1)
OS in TET2-WT (256)
109
P=0
147 (57.4%)
6886
TET2 KO (7033)
K
Hypermethylated enhancer probes (Δβ > 0.1)
OS in TET2-WT (1206)
492
P=0
714 (59.2%)
10547
TET2 KO (11261)
L
5hmC/ relative to mock
H2O2
Mock
30 min
3 h
M
Scr
shTET2
IB: 5hmC
IB: TET2
IB: Actin
A2780
N
β value
Scr
shTET2
O
Oxidative Stress (OS)
Hyper: 4679
Hypo: 357
β value, Scr H2O2
β value, Scr mock
P
OS on TET2 KD
Hyper: 11514
Hypo: 175
β value, shTE2- H2O2
β value, Scr mock
Q
KO hyper CGI pro., LE low + inter.
5mC
5hmC
Scr-mock
Scr-H2O2
shTET2-H2O2
R
KO hyper enhacer low + inter.
5mC
5hmC

Figure S7. Related to Figure 7 and Table S1. TET2 protects against abnormal DNAm

(A) Loss of *TET2* leads to DNA hypermethylation. Scatter plot showing correlation of DNA methylation levels between control and *TET2* KO clones. Mean of two control clones (x-axis) and three KO clones (y-axis) were used.

(B) Box plot showing overall DNA methylation changes for promoter CGI probes associated with low expression (LE), intermediate expression (IE), and high expression (HE) genes between Con and *TET2* KO clones. ***: $P < 0.001$ (student's t-test).

(C) Heatmap showing hypermethylated enhancer probes ($\Delta\beta$ (KO - WT) > 0.2) in *TET2* KO clones.

(D) Enhancer probes identified by Rasmussen et al. (Rasmussen et al., 2015) were classified according to Figure 7B. DNA methylation levels of each group in *TET2* WT and KO samples were shown by box plot.

(E and F) (E) Schematic diagram of H_2O_2 treatment. Cells treated with H_2O_2 for 30 min were either assayed immediately (30 min), or rested in fresh medium for an additional 2.5 hours (3h) and assayed. Global DNA methylation (F) were assayed by Infinium 450K. #: not significant; ****: $P < 0.0001$ (student's t-test).

(G-I) Box plot showing DNA methylation changes induced by OS on promoter CGI probes associated with low, intermediate, and high expression (LE, IE, and HE) genes (G). LE, promoter CGI (H) or enhancer (I) probes were divided into low ($\beta < 0.25$), intermediate ($0.25 < \beta < 0.75$) and high methylation ($\beta > 0.75$) groups according to their basal DNA methylation levels and changes of DNA methylation in each group were shown by box plot. #: not significant; *: $P < 0.05$; ***: $P < 0.001$; ****: $P < 0.0001$ (student's t-test).

(J and K) Venn diagram showing overlaps and corresponding *P* values (Chi-square test, Yates corrected) of probes hypermethylated in *TET2* KO clones and those gain abnormal DNAm in OS system. $\Delta\beta$ (KO - WT) > 0.1 was used as cut-off.

(L) Global 5hmC levels in mock, H_2O_2-30 min and H_2O_2-3h samples were measured by 5hmC ELISA. Values represent mean ± SEM (n=3). #: not significant, **: $P < 0.01$; (student's t-test).

(M and N) A2780 cells were infected with Scr or shTET2 shRNA for 4 days. TET2 protein and global 5hmC levels were measured by IB and 5hmC dot blot assay, respectively (M). Global DNA methylation levels were assayed by Infinium 450K and compared by box plot (N).

(O and P) A2780 cells were infected with Scr or shTET2 shRNA for 4 days, followed with or without H_2O_2 treatment and DNA methylation levels were assayed by Infinium 450K. DNAm changes induced by OS alone (O) or double treatment (P, OS upon TET2 KD) were shown by scatter plot.

(Q and R) LE promoter CGI (Q) and enhancer (R) probes that are hypermethylated in *TET2* KO clones tend to gain abnormal DNAm in OS system. Such DNAm gains tend to increase further under double treatment condition (OS upon TET2 KD), coincide with a decrease of 5hmC. ****: $P < 0.0001$ (student's t-test).

Table S1. Related to Figure 7 and Figure S7. Hyper methylated low expression, promoter CGI probes in *TET2* KO clones

Low expression, promoter CGI and enhancer probes gained DNA methylation in TET2 KO clones (mean(Δβ) >0.2) are listed.

Hypermethylated genes in cancer, bivalent genes in human ESCs used in this study are also listed.

Supplemental Experimental Procedures

DNA constructs, antibodies and chemicals

A human TET2 cDNA clone encoding transcript variant 1 (2002 aa) was purchased from OriGene (RC226438). 3xFlag or V5 epitope tags were inserted at the N-terminus of TET2 by PCR and cloned into the pcDNA4/TO vector (Life Technologies) using KpnI and NotI. All TET2 mutants were generated by QuikChange Mutagenesis (Stratagene). All TET2 fragments were PCR amplified with an N-terminal 3xFlag epitope tag and cloned into the pcDNA4/TO vector. V5-tagged TDG was PCR amplified from pGEX-4T2-TDG (a gift from Dr. A-Lien Lu-Chang, University of Maryland) and cloned into the pcDNA4/TO vector. HA-Ubiquitin was a gift from Edward Yeh (Addgene plasmid # 18712). pcDNA3/Myc-DNMT1 was a gift from Arthur Riggs (Addgene plasmid # 36939). pcDNA3β-FLAG-CBP-LD-HA was a gift from Tso-Pang Yao (Addgene plasmid # 32906). pcDNA3.1-300(HAT-) was a gift from Warner Greene (Addgene plasmid # 23254). DNMT1 fragments were PCR amplified and cloned into the pcDNA/Myc vector via ECoRI and KpnI. LentiCRISPR v2 was a gift from Feng Zhang (Addgene plasmid # 52961). sgRNAs were cloned into the LentiCRISPR vector according to instruction (Shalem et al., 2014). CBP, p300, MOF, and Tip60 plasmids were kindly provided by Dr. Jianyuan Luo (University of Maryland), SBP-TET1, TET2, and TET3 were provided by Dr. Xiaochun Yu (University of Michigan), Flag-HDAC1/3 by Dr. Alan Friedman (the Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins), and Flag-HDAC2 by Dr. Ed Seto (Moffitt Cancer Center).

Rabbit anti-TET2 antibody was provided by Atlas Antibodies (HPA043135). Anti-Flag, anti-DNMT1, anti-TDG, and anti-β-actin antibodies were purchased from Sigma; anti-HA, anti-p300 from Santa Cruz; anti-HDAC1, anti-HDAC2, and anti-V5 from Abcam; anti-AcK from Cell Signaling; anti-γ-H2AX from Millipore; anti-5hmC from Active motif; and anti-5mC from Eurogentec.

TSA was purchased from Selleckchem and dissolved in DMSO. MG132, NAM, Calpeptin, Z-VAD-FMK Cycloheximide, and 3XFlag peptide were purchased from Sigma; recombinant p300 catalytic domain from Enzo and recombinant DNMT1 from Active Motif. 5mC substrate was purchased from Zymo research.

Immunoprecipitation, pull down, and Western blotting

Immunoprecipitation was performed as described previously (O'Hagan et al., 2011). Cell pellets were washed in CEBN buffer (10 mM HEPES (pH 7.8), 10 mM KCl, 1.5 mM MgCl2, 0.34 M sucrose, 10% glycerol, 0.2 % NP-40), followed by CEB buffer (CEBN buffer without NP-40), resuspended in modified RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS), sonicated for 12 pulses, rotated at 4°C for 30 minutes, and cleared by high-speed centrifugation (14,000 rpm, 30 min, 4°C). All solutions were supplemented with 1X protease inhibitor cocktail (Invitrogen), 1X phosphatase Inhibitor cocktail, N-ethylmaleimide (Sigma) and Pefabloc SC AEBSF (Roche Applied Science). Nuclear extracts were incubated with primary antibodies overnight at 4 °C with rotating, followed by additional 3 hours of incubation with magnetic protein G beads (Life Technologies). After extensive washing with TNEN buffer (50 mM Tris-HCl pH7.5, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.5% NP40, 0.5% TritonX-100)**,** the beads were boiled in 2x laemmli buffer. Proteins were resolved on 4-15% SDS-PAGE (Bio-Rad), transferred to PVDF membrane, and probed with indicated antibodies. For TET2/DNMT1/DNMT3B Co-IP, spermine (30 mM) spermidine (10 mM) were added in RIPA buffer, and additional 10 pulse of sonication were conducted after 30 min incubation.

For pull down assay, Flag-TET2 was transiently transfected into HEK293T cells for 48 hour using PEI "Max" and immunoprecipitated using Flag magnetic beads under high salt conditions (400 mM NaCl). After extensive washing with TNEN buffer**,** the immunoprecipitated TET2 was then mixed with purified recombinant human DNMT1 protein (Active Motif), or TDG, in Modified RIPA buffer overnight at 4 °C. Beads were washed 5x with TNEN buffer for 10 minutes at 4°C, boiled in 2x Laemmli buffer and analyzed by Western blot.

For whole cell lysate analysis, cell pellets were re-suspended in 10 ul 1x PBS, lysed directly with 4% SDS and passed through QIAshredder (Qiagen). Protein concentration was measured by BCA assay. Samples were mixed with 5x Laemmli buffer, and subjected to SDS-PAGE and Western blotting analysis with indicated antibodies.

Protein purification

For recombinant TET2 purification, 1L HEK293F cells were transfected with 1 mg Flag-TET2 using PEI "Max" at ratio of 1:3 for 2 days. Cells were resuspended in Lysis Buffer (50 mM HEPES, pH 8.0, 150 mM NaCl, 1 mM DTT, 5% glycerol, supplemented with protease inhibitor cocktail) and lysed by sonication. After centrifuge, cell lysate was adjusted with 5M NaCl to reach a final concentration of 500 mM NaCl, and incubated with anti-Flag affinity gel for 2 hours at 4 ˚C. After extensive wash (twice with high

salt wash buffer (50 mM HEPES, pH 8.0, 500 mM NaCl, 1 mM DTT, 5% glycerol), twice with lysis buffer), a fraction of beads (~10%) were eluted with 200 ug/ml 3XFlag peptide in Lysis buffer. The remaining TET2 immobilized on beads were used immediately for in vitro acetylation and enzymic assay.

Endogenous TET2 proteins were purified similarly with TET2 antibody. 10-20x 15cm plates of A2780 cells were resuspended in Lysis Buffer (50 mM HEPES, pH 8.0, 150 mM NaCl, 1 mM DTT, 5% glycerol, supplemented with protease inhibitor cocktail and 5 uM TSA) and lysed by sonication. After centrifuge, cell lysate was adjusted with 5M NaCl to reach final concentration of 500 mM NaCl, and incubated with TET2 antibody at 4 ˚C overnight, followed by incubation with magnetic beads for 3 hours at 4 ˚C. After extensive wash (twice with high salt wash buffer (50 mM HEPES, pH 8.0, 500 mM NaCl, 1 mM DTT, 5% glycerol), twice with lysis buffer), TET2 proteins were eluted with 1% SDS and submitted for mass spectrometry analysis.

For purification of TDG, pGEX-4T2-TDG was transformed into in BL21 CodonPlus *E. coli* cells. Cells were induced with 500 uM IPTG when OD_{600} reached 0.6 and incubated for 16 h at 16 ˚C. Cells were centrifuged at 5,000rpm for 10 min, resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% NP-40, with protease inhibitor), and passed through a French Press Cell three times. Crude cell lysates were centrifuged at 20,000 rpm for 30 min and clarified supernatant was incubated with glutathione resin (GE) for 4 hours at 4°C. TDG protein was cleaved from GST beads using Thrombin (RT overnight).

Mass spectrometry

In-solution proteolysis: Proteins in solution were reduced with 2x DTT, alkylated with 2x Iodoacetomide and TCA/acetone precipitated. Protein pellet were re-constituted in 10uL 500mM TEAB+40uL water and in-solution proteolyzed with 9.4ng/uL trypsin (lyophilized, Promega) in 100mM TTEAB, total volume 50uL, at 37C overnight according to Shevchenko et al., 1996 Analytical Chemistry 68(5); 850-858. Tryptic peptides were desalted on HLB u-Oasis plate (Waters), eluted with 60%ACN/0.1%TFA, and dried.

LC/MSMS analysis: Peptides were re-constituted in 8uL 2%acetonitrile/0.1%Formic acid and 2uL were analyzed by liquid chromatography/tandem mass spectrometry (LCMS/MS) on nano-LC-QExative HF in FTFT (Thermo Fisher Scientific) interfaced with a nano-Acquity (Waters), using reverse-phase chromatography (2%-90% acetonitrile/0.1% FA gradient over 78min at 300nl/min) on 75 um x 150 mm ProntoSIL-120-5-C18 H column 5µm, 120Å (BISCHOFF). Eluting peptides were sprayed into an QExactive Plus mass spectrometer through 1 µm emitter tip (New Objective) at 2.0 kV. Survey scans (full ms) were acquired on Orbi-trap within 350-1800Da m/z using Data Dependent Top 15 method with dynamic exclusion of 15 s. Precursor ions were individually isolated with 1.9Da, fragmented (MS/MS) using HCD activation collision energy 28. Precursor and the fragment ions were analyzed at resolution 120,000/30,000 respectively, AGC target 3xe6, max IT 60ms and AGC target 1xe5, mx IT150ms.

Data Analysis: Tandem MS2 mass spectra were processed by Proteome Discoverer (v1.4 ThermoFisher Scientific) in three ways, using 3Nodes: common, Xtract (spectra are extracted, charge state deconvoluted, and deisotoped using Xtract option, at resolution 50K at 400Da), MS2 Processor. MS/MS spectra from 3Nodes were analyzed with Mascot v.2.5.1 (Matrix Science, London, UK) using 2015_RefSeq_72r_human_database (fall of 2015) with added enzymes sequences, with concatenated decoy database. Search criteria included; all species for each database, trypsin as the enzyme, allowing two missed cleavages; precursor mass tolerance 8ppm, fragment mass tolerance 0.018Da, cysteine carbamidomethylation as a fixed modification, methionine oxidation, lysine acetylation, asparagine and glutamine deamidation as variable modifications. Mascot ".dat" files from the search against the certain database complied in Scaffold.

In vitro acetylation of TET2 and TET2 enzymatic assay

Immunoprecipitated and purified TET2 proteins (immobilized on beads) were incubated with or without 1 ug recombinant p300 catalytic domain with 50 uM acyl-CoA, or with p300 catalytic domain in the presence or absence of Acetyl-CoA, in HAT buffer (50 mM Tris–HCl, pH 8.0, 0.1 mM EDTA, 1 mM DTT) at 30 °C for 30 min. Beads were washed and incubated with 5mC substrate (500 ng) in buffer containing 50 mM HEPES (pH 8.0), 100 mM NaCl, 100 µM Fe(NH4)2(SO4)2, 2 mM ascorbate, 1 mM DTT, and 1 mM ATP at 37°C for 1 hour. DNA was purified and 5hmC levels were quantified by dot blot and ELISA using 5hmC antibody. Beads (TET2) were washed, boiled and subjected to SDS-PAGE analysis.

Protein stability assay

Cells were seeded in a 6-well plate one day before experiments. After 24 hours, fresh medium with 100 ug/ml cycloheximide (Sigma) was added to cells which were incubated and harvested at indicated time points. Cell pellets were stored at -80 °C until all samples were collected. One well of cells immediately before adding cycloheximide were harvested as control (t=0 h). Cell pellets were lysed in 4% SDS, and subjected to standard SDS-PAGE and western blot analysis with indicated antibodies.

shRNA knockdown and CRISPR/Cas9 knockout

shRNAs against *TET2*, *DNMT1*, *HDAC1/2* were ordered from Sigma. Lentivirus was produced by co-transfecting each shRNA plasmid with psPAX2 and pMD2.G using Lipofectamine 2000 (16 ug shRNA plasmid, 12 ug psPAX2 and 4 ug pMD2.G per 150 mm dish) overnight. Fresh media was added to cells the next morning and then collected thereafter at 24 and 48 hours. Media was pooled, filtered through a 0.45 um filter, and concentrated using Amicon Ultra-15 Centrifugal Filter Units, aliquoted and stored at -80 °C.

For shRNA acute knockdown, cells were seeded overnight and virus were added to fresh medium containing 8ug/ml polybrene. Cells were infected for 2 days and selected with 1 ug/ml puromycin for an additional 3 days. For CRISPR knockout, cells were infected with virus for 2 days and selected with 1 ug/ml puromycin for an additional 10 days. Pooled cells were used to measure TET2 half-life showed in Figure 5I and 5J. For single cell clone isolation, cells were diluted to 1 cell/100 ul and seeded to 96-well plates. Single clones were expanded and validated by WB and genotyping.

Cell culture and H_2O_2 treatment

A2780, HCT116, and HEK293T cells were maintained in RPMI-1640, MoCoy's 5A and DMEM media supplied with 10% FBS, respectively, and incubated at 37°C with 5% CO_2. For H_2O_2 exposure, 30% H_2O_2 (Sigma) was diluted in fresh medium and added to cells immediately at final concentration of 5 mM, protected from light. After 30 min, cells were trypsinized and equal amount of cells were harvested and analyzed as indicated. For DNAm analysis, cells were washed with 1xPBS after 30 min exposure and maintained in fresh medium for additional 2.5 hours.

Tight chromatin fractionation

A tight chromatin fractionation was prepared as described previously (O'Hagan et al., 2011). Briefly, cell pellets treated with or without 5 mM H_2O_2 were washed sequentially in CEBN buffer, CEB buffer, soluble nuclear buffer (3 mM EDTA, 0.2 mM EGTA), and 0.45 M NaCl buffer (50 mM Tris pH 8.0, 0.05% NP40, 0.45 M NaCl) (2M NaCl buffer for DNMT3A and DNMT3B). Pellets were then resuspended in modified RIPA buffer, sonicated for 12 pulses, rotated for 30 minutes with 30 mM spermine and 10 mM spermidine at 4°C, sonicated for 10 more pulses, and cleared by high-speed centrifugation (14,000 rpm, 30 min, 4°C). The supernatant was transferred to new tube and subjected to SDS-PAGE and Western Blot analysis.

γ-H2AX immunofluorescence

Cells were seeded on chamber slides (Nunc Lab-Tek II) overnight, fixed with 4% Formaldehyde, permeabilized with 0.25% Triton X-100 in PBS, blocked, incubated with γ-H2AX antibody (1:500), and Alexa Fluor 488 goat anti-mouse secondary antibody. DNA was stained with DAPI.

Dot-blot assay

Genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega). DNA samples were diluted in TE buffer, denatured in 0.4 M NaOH/10 mM EDTA for 10 min at 95 °C, neutralized with equal volume of 2 M NH_4OAc (pH 7.0), and spotted on a nitrocellulose membrane (pre-wetted in 1 M NH_4OAc,pH 7.0) in two-fold serial dilutions using a Bio-Dot Apparatus Assembly (Bio-Rad). The blotted membrane was rinsed briefly in 2x SSC, air-dried, baked at 80 °C for two hours, blocked in 5% non-fat milk for 1 hour at room temperature, and incubated with anti-5hmC (Active motif, 1:5,000) or anti-5mC (Eurogentec, 1:1,000) antibodies at 4 °C overnight. After 3x 5 min washing, the membrane was incubated with HRP-conjugated anti-rabbit or anti-mouse IgG secondary antibody (GE, 1:5,000), treated with ECL substrate and developed using film. Membrane were stained with 0.02% methylene blue in 0.3 M sodium acetate (pH 5.2) to ensure equal loading of input DNA.

5hmC ELISA

Genomic DNA was diluted in DNA coating solution (Thermo Scientific) to 1ng/ul, boiled for 10 min., cooled down on ice, added to 96-well microplates (100 ng per well), and incubated at room temperature (RT) overnight. Unbound DNA was washed off with PBST (0.1% Tween 20), and plates were blocked with 2% BSA in PBST for 2 hours at RT, incubated with primary antibody (anti-5hmC, 1:2000, in 2% BSA/PBST) for 2 hours at RT, washed, and incubated with secondary antibody (1:6000 in 2% BSA/PBST) for 2 hours at RT. After wash, TMB substrate was added and absorbance was measured at 450 nm.

Gene expression microarray

Total RNA were extracted using RNeasy Mini kit (Qiagen), and hybridized to Agilent 44K expression array, according to manufacturer's instructions. Expression data were analyzed in R using Limma.

Bioinformatics

All data were analyzed in R. For genome-wide DNAm analysis, bisulfite converted genomic DNA (EZ DNA methylation Kit, Zymo Research) was analyzed by the Illumina Infinium Human Methylation450 (HM450) BeadChip. Raw data (IDAT files) were processed using the minfi Bioconductor package (Aryee et al., 2014), and normalized by SWAN (Maksimovic et al., 2012). β-Values were computed as the signal of the methylation-specific probe over the sum of the signals of the methylation- and unmethylated-specific probes. Probes with poor signals (detection *P*-value > 0.01) were not considered. CEGX Bisulfite (BS) and oxidative-bisulfite (oxBS) treatments were performed according to manufacturer's instructions, and 5hmC values were obtained by subtracting β-Values generated by oxBS from those generated by BS. Negative 5hmC values were considered as 0. The list of promoter CpG islands probes, cancer specific hypermethylated genes (Easwaran et al., 2012), and bivalent chromatin genes in hESCs (Ku et al., 2008) were described previously. Distal probes, greater than ± 2 kb from a known TSS from the HM450 array and overlap with known enhancer regions, identified recently (Yao et al., 2015) were designated as "enhancer probes" and used in the present study. Enhancer-gene pair used in Figure 7H were derived from the above report (Yao et al., 2015). Probes with basal $\beta < 0.25$ were considered as unmethylated, $0.25 < \beta < 0.75$ as intermediately methylated, and $\beta > 0.75$ as methylated.

To identify low, intermediate, and high expressing genes in A2780, microarray data from A2780 (Li et al., 2014) were normalized using the Limma package and mock channels (A2780 mock) were extracted. Gene expressing levels (log2 (intensity)) were ranked, and the 0-25, 25-75, and 75-100 percentile genes were considered low, intermediate, and high expressing genes.

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