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Supplementary Materials for

DNMT1 Stability Is Regulated by Proteins Coordinating Deubiquitination and Acetylation-Driven Ubiquitination

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The PDF file includes:

Materials and Methods

Primers for somatic cell gene targeting

Primers for DNMT1 3xFlag knock-in
Left arm primers: forward 5'-GGGAAAGUCCTCAACTAATCCACCTGCCTT-3'; reverse 5'-GGAGACAUGCGTCCTTAGCAGCTTCCTCCTCCTT-3'.
Right arm primers: forward 5'-GGTCCCAUTTCTGCCCTCCCGTCACCCCTGTT-3'; reverse 5'-GGCATAGUGGAGTTTGAGACCACCCTGA-3'.
Screening primer: AGTGTCCACGGTTCTTGTGAA

Primers for DNMT3b 3xFlag knock-in
Left arm primers: forward 5'-GGGAAAGUAGAAAACCCCACAGACATGC-3'; reverse 5'-GGAGACAUGCTTCACATGCAAAGTAGTCCTTCAG-3'.
Right arm: forward 5'-GGTCCCAUTTCCAGCCAGGCCCCAAGCCCACT-3'; reverse 5'-GGCATAGUTCTCTCAGATGTGGGGAAGG-3'.
Screening primer: 5'-CGCCCTTATTTCCTGACAAA-3'

Primers for Tip60 3xFlag knock-in
Left arm primers: forward 5'-GGGAAAGUCAAGTCTTACAGCAGGTGACACTC-3'; reverse 5'-GGAGACAU GCCCACTTCCCCCTCTTGCTCCAGTCC-3'.
Right arm: forward 5'-GGTCCCAU CCAGACACTGCCCACTGCAGTGCCA-3'; reverse 5'-GGCATAGUGACTTCGTGAGCAAAGGGATAG-3'.

Primers for HAUSP knockout
Left arm: forward 5'-GGAGACAUTCGTTTAACAGATTGATTGAGCA-3'; reverse 5'-GGTCCCAU CCTGATTCTTTAAGCCGACGTAGCC -3'.
Right arm: forward 5'-GGCATAGUTTATTTTTCACGAATCAGCTACGAA -3'; reverse 5'-GGCATAGUCCGATTTCCTGTACTGCTTCATA-3'.
Screening primer: 5'-TAATCAGTAAGCCTCCTCTCTGC-3'

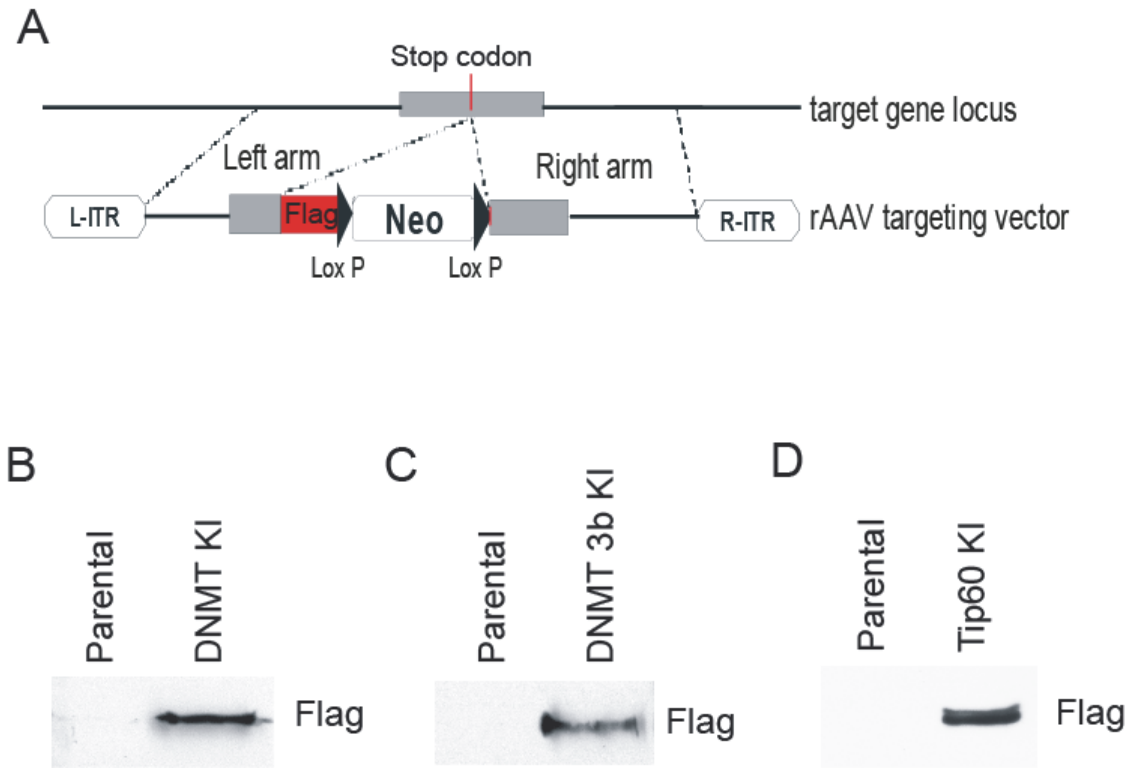

Fig. S1. Engineering of DNMT1, DNMT 3b, and Tip60 3xFlag-tagged knock-in cells. (**A**) Schematic of the KI strategy. (**B** to **D**) Cell lysates from parental and indicated 3xFlag-tagged knock-in cells were blotted with anti-Flag antibodies.

A

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   1 MAGNHRLGLE AGDTDDPPRI TQNPVINGNV ALSDGHNTAE EDMEDDTSWR
  51 SEATFQFTVE RFSRLSESVL SPPCFVRNLP WKIMVMPRFY PDRPHQKSVG
 101 FFLQCNAESD STSWSCHAQA VLKIINYRDD EKSFSRRISH LFFHKENDWG
 151 FSNFMAWSEV TDPEKGFIDD DKVTFEVFVQ ADAPHGVAWD SKKHTGYVGL
 201 KNQGATCYMN SLLQTLFFTN QLRKAVYMMP TEGDDSSKSV PLALQRVFYE
 251 LQHSDKPVGT KKLTKSFGWE TLDSFMQHDV QELCRVLLDN VENKMKGTCV
 301 EGTIPKLFRG KMVSYIQCKE VDYRSDRRED YYDIQLSIKG KKNIFESFVD
 351 YVAVEQLDGD NKYDAGEHGL QEAEKGVKFL TLPPVLHLQL MRFMYDPQTD
 401 QNIKINDRFE FPEQLPLDEF LQKTDPKDPA NYILHAVLVH SGDNHGGHYV
 451 VYLNPKGDGK WCKFDDDVVS RCTKEEAIEH NYGGHDDDLS VRHCTNAYML
 501 VYIRESKLSE VLQAVTDHDI PQQLVERLQE EKRIEAQKRK ERQEAHLYMQ
 551 VQIVAEDQFC GHQGNDMYDE EKVKYTVFKV LKNSSLAEFV QSLSQTMGFP
 601 QDQIRLWPMQ ARSNGTKRPA MLDNEADGNK TMIELSDNEN PWTIFLETVD
 651 PELAASGATL PKFDKDHDVM LFLKMYDPKT RSLNYCGHIY TPISCKIRDL
 701 LPVMCDRAGF IQDTSLILYE EVKPNLTERI QDYDVSLDKA LDELMDGDII
 751 VFQKDDPEND NSELPTAKEY FRDLYHRVDV IFCDKTIPND PGFVVTLSNR
 801 MNYFQVAKTV AQRLNTDPML LQFFKSQGYR DGPGNPLRHN YEGTLRDLLQ
 851 FFKPRQPKKL YYQQLKMKIT DFENRRSFKC IWLNSQFREE EITLYPDKHG
 901 CVRDLLEECK KAVELGEKAS GKLRLLEIVS YKIIGVHQED ELLECLSPAT
 951 SRTFRIEEIP LDQVDIDKEN EMLVTVAHFH KEVFGTFGIP FLLRIHQGEH
1001 FREVMKRIQS LLDIQEKEFE KFKFAIVMMG RHQYINEDEY EVNLKDFEPQ
1051 PGNMSHPRPW LGLDHFNKAP KRSRYTYLEK AIKIHN
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Sequence Coverage: 45%; Match to: IPI:IPI00646721.1 Score: 3978
Gene_Symbol=USP7 Ubiquitin carboxyl-terminal hydrolase

B

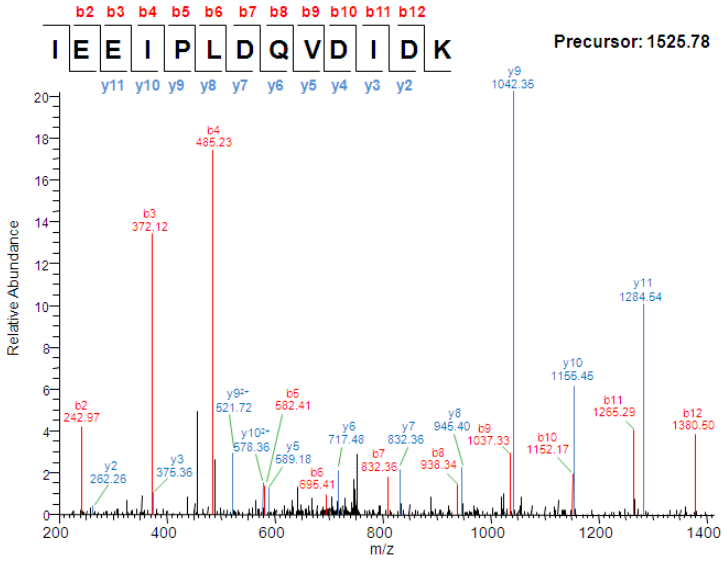

Fig. S2. Identification of HAUSP by LC-MS/MS. (**A**) Peptide sequence coverage of USP7 protein. Each of the independently identified tryptic peptides are highlighted in red. (**B**) MS/MS spectrum of a USP7 peptide (IEEIPLDQVDIDK) with the highest Mascot score. B and y ions are labeled with red and blue colors, respectively.

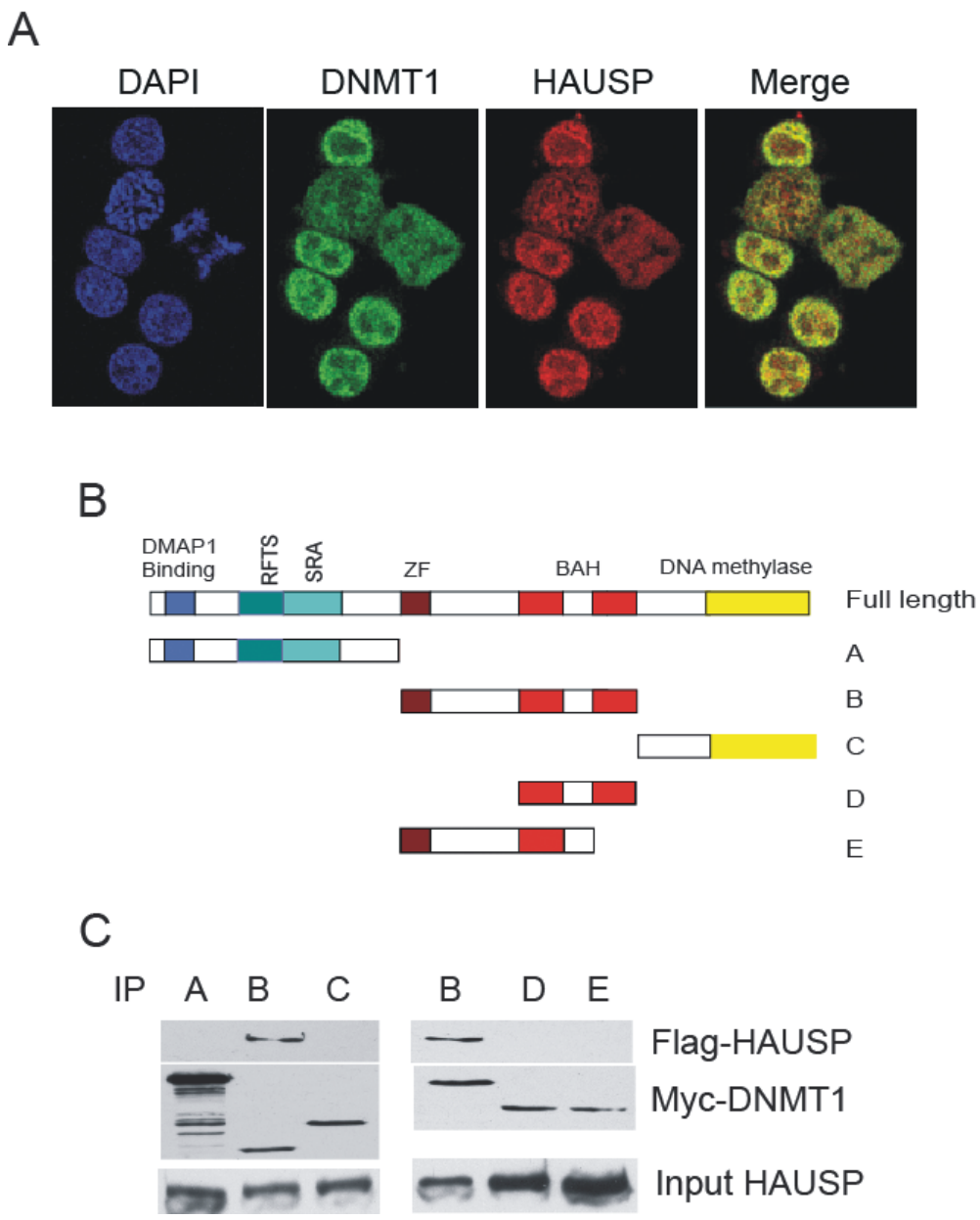

Fig. S3. Specific interaction between DNMT1 and HAUSP. (**A**) DNMT1 and HAUSP colocalize in the nucleus. DNMT1 3xFlag-tagged KI cells were stained with a mouse anti-Flag and a rabbit anti-HAUSP antibody, as well as DAPI (for DNA). (**B**) Schematic of DNMT1 deletion constructs. DMAP1 binding: DNMT1-associated protein binding; RFTS: replication foci targeting sequence; SRA: RING finger-associated; ZF: zinc finger domain; BAH: bromo-adjacent homology. (**C**) The central portion of DNMT1 interacts with HAUSP. HEK 293 cells were transfected with a plasmid expressing Flag-tagged HAUSP and the indicated Myc-tagged DNMT1 constructs. Cell lystates were immunoprecipitated with anti-Myc antibodies and blotted with anti-Flag antibodies.

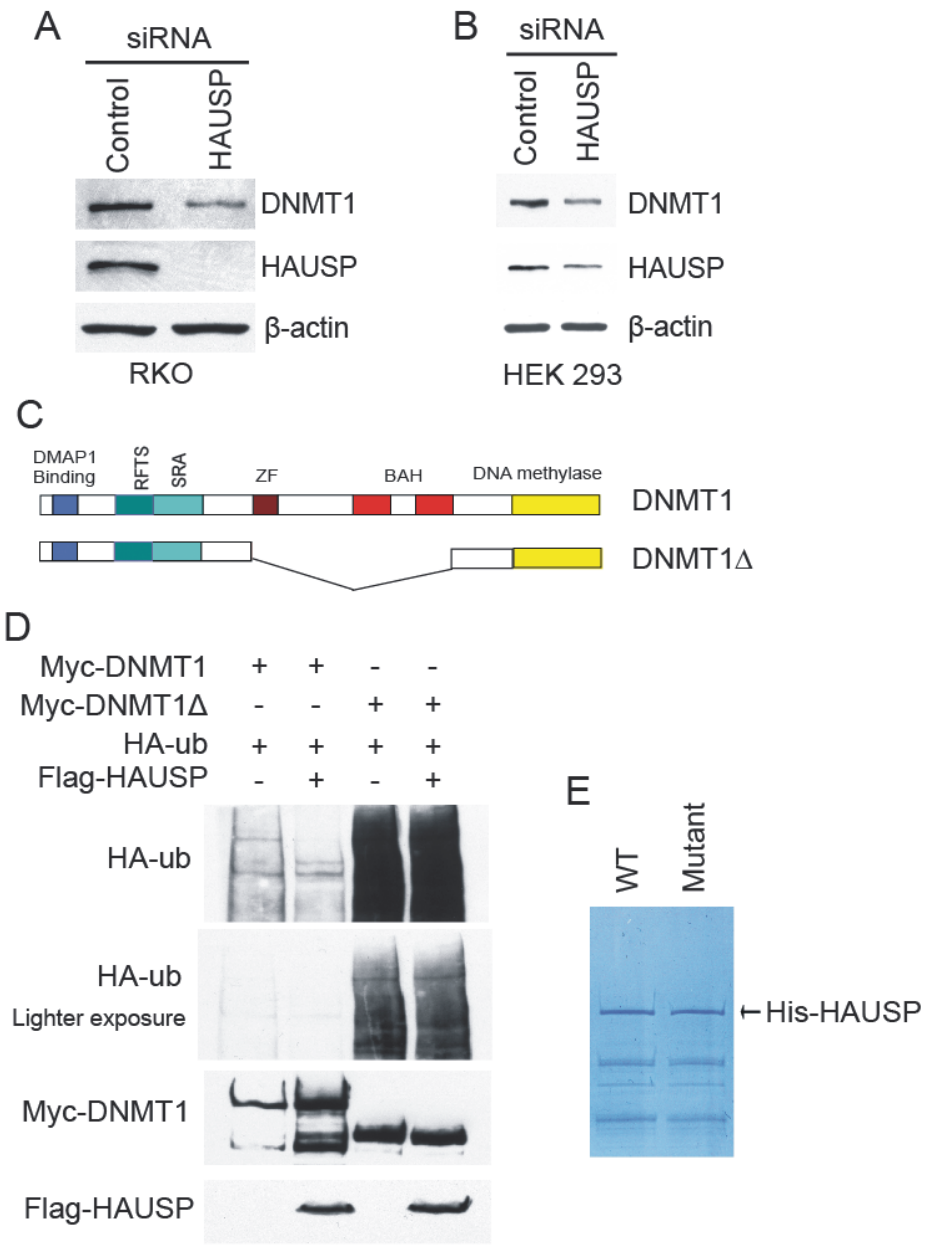

Fig. S4. Physical interaction is required for HAUSP to deubiquitinate DNMT1. (**A** and **B**) Knock-down of HAUSP destabilizes DNMT1. The indicated cells were transfected with either a control siRNA or a siRNA against HAUSP and cell lysates were blotted with the indicated antibodies. (**C**) Schematic of DNMT1 full-length and deletion constructs. DNMT1Δ: deletion of the HAUSP-interacting region, which encompasses the zinc finger domain to the C-terminus BAH domain. (**D**) Physical interaction is required for HAUSP to deubiquitinate DNMT1. HEK 293 cells were transfected with the indicated plasmids. Myc-DNMT1 proteins were immunoprecipitated and blotted with anti-HA (to detect ubiquitinated DNMT1) and anti–Myc antibodies. Cell lystates were blotted with an anti-Flag antibody to detect ectopically expressed HAUSP. (**E**) Coomassie staining of purified recombinant HAUSP or HAUSP C223S mutant proteins.

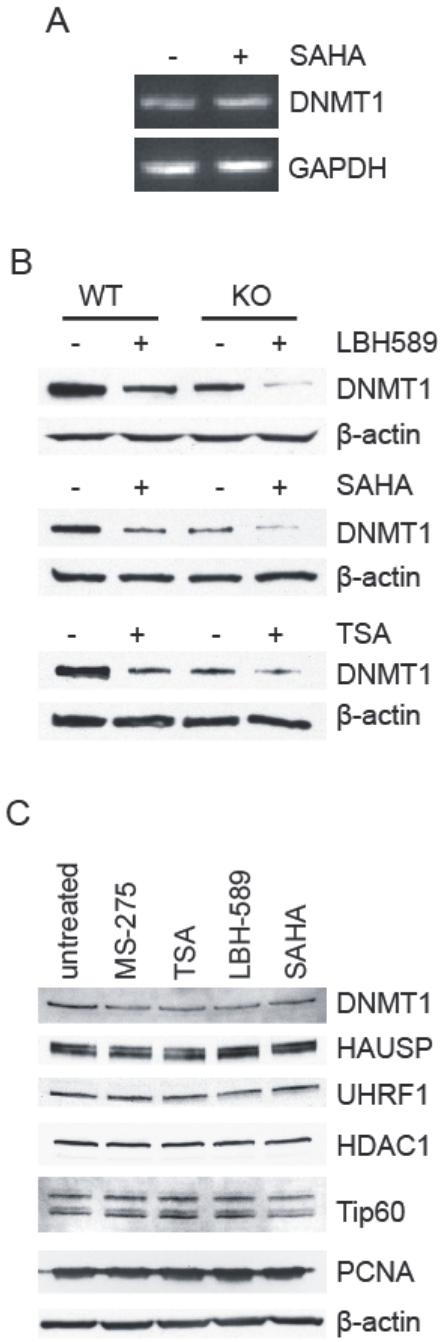

Fig. S5. HDAC inhibitors induce DNMT1 degradation. (**A**) HDAC inhibitors do not reduce DNMT1 gene transcription. DLD1 cells were treated with or without HDACi SAHA. Total RNAs were isolated and RT-PCRs were performed. (**B**) HAUSP KO potentiates HDAC inhibitor-induced DNMT1 degradation. Wild-type (WT) and HAUSP KO cells were treated with or without the indicated HDAC inhibitors. Cell lysates were blotted with anti-DNMT1 and beta-actin antibodies. (**C**) HDAC inhibitors induce degradation of DNMT1 and but not other DNMT1-associated proteins. Cells were treated with the indicated HDAC inhibitors and cells lysates were blotted with the indicated antibodies.

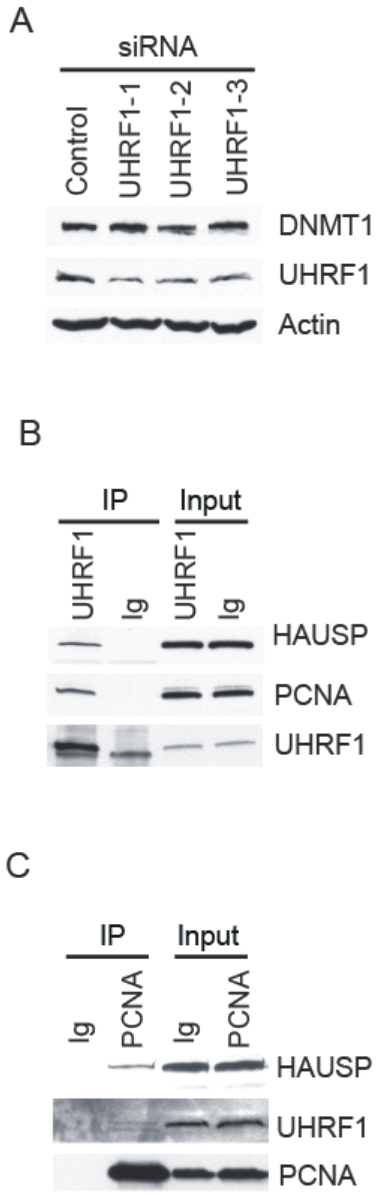

Fig. S6. DNMT1-interacting proteins associate with each other. (**A**) Knockdown of UHRF1 does not alter the abundance of DNMT1. HEK 293 cells were transfected with a control siRNA or siRNAs against UHRF1. Cell lysates were blotted with the indicated antibodies. (**B** and **C**) HAUSP, UHRF1, and PCNA interact with each other. Cell lysates were immunoprecipitated with antibodies against either UHRF1 or PCNA. The immune complexes were blotted with the indicated antibodies.

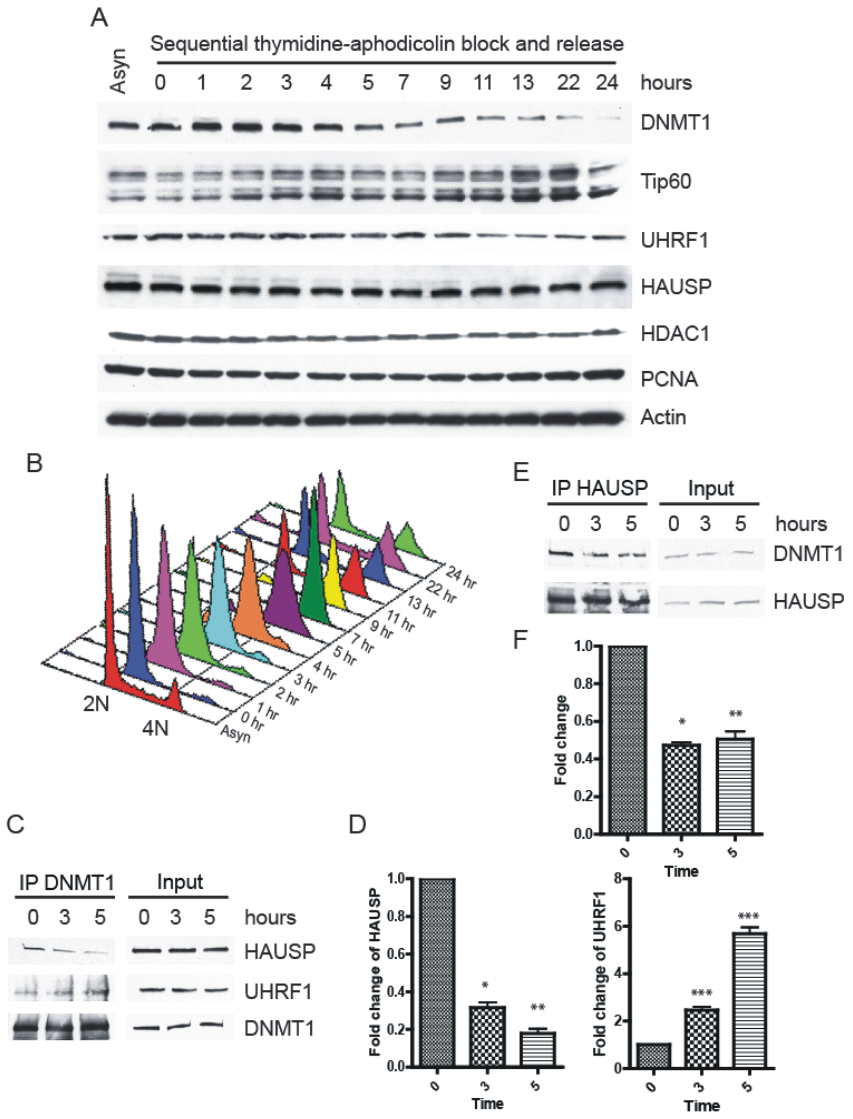

Fig. S7. The abundance of DNMT1 changes during the cell cycle. (**A**) The abundance of DNMT1 is inversely correlated with that of Tip60 during the cell cycle. Cells were synchronized at early S phase by sequential thymidine-aphodicolin block and then released. Samples were taken at the indicated time points after release. Western blots were performed with the indicated antibodies. Asyn: asynchronized cells. (**B**) Cell cycle profiles of cells at the indicated time points. FACS analysis of DNA content was plotted. (**C** to **F**) HAUSP dissociates from DNMT1 during late S phase. Cells were synchronized as in (A) and cell lysates were made at the indicated time points after release, immunoprecipitated with anti-DNMT1, and blotted with the indicated antibodies (C). (**D**) Immunoprecipitated HAUSP or UHRF1 protein amounts (in C) were normalized to DNMT1 and plotted (N=4, Wilcoxon rank sum test, * $p = 0.1$; ** $p = 0.014$; *** $p = 0.029$). (**E**) Immunoprecipitation was performed with anti-HAUSP antibodies. (**F**) DNMT1 abundance was normalized to HAUSP and plotted (N=4, Wilcoxon rank sum test, * $p = 0.014$; ** $p = 0.014$).

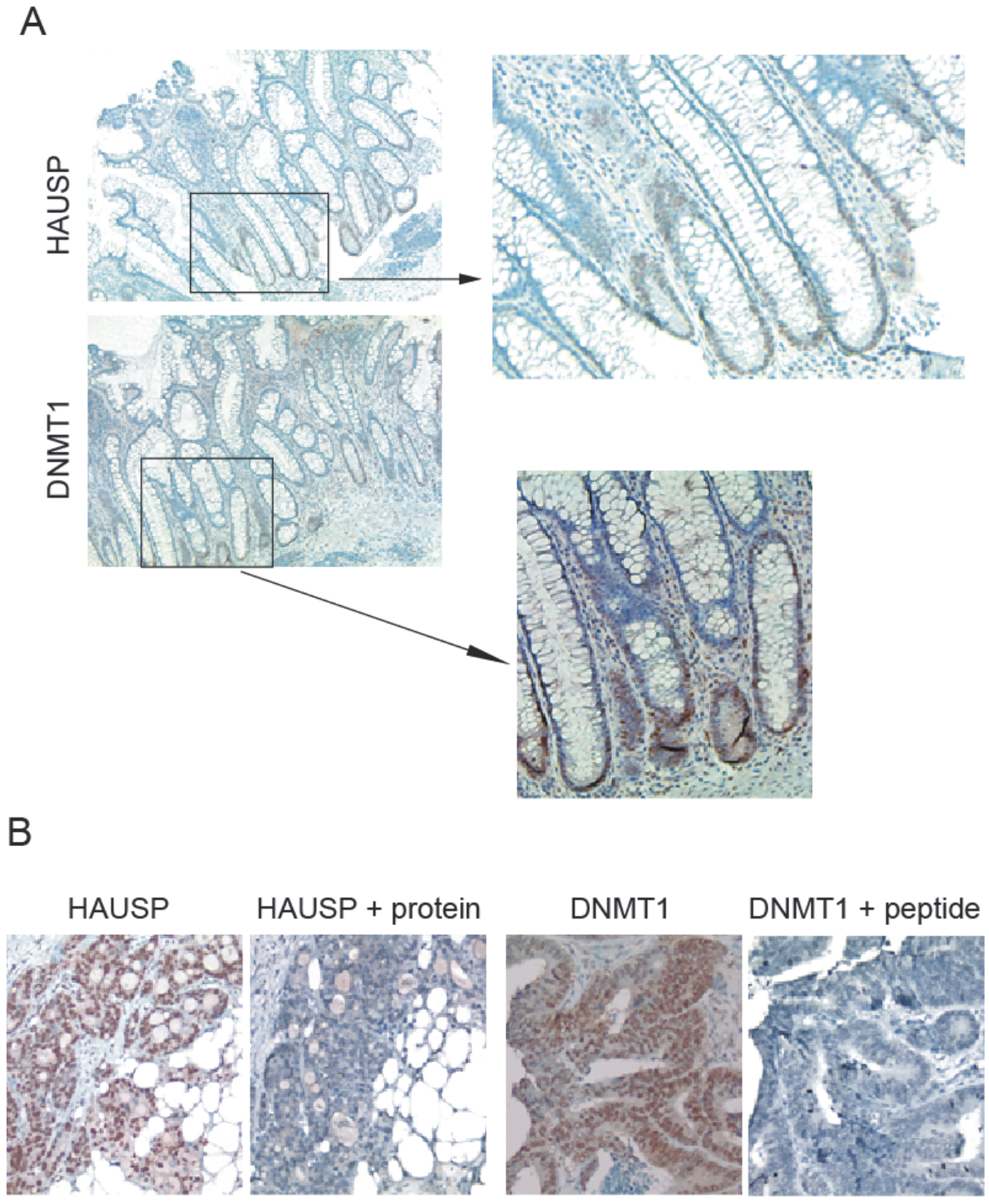

Fig. S8. IHC staining of HAUSP and DNMT1 in normal colon tissues. (**A**) HAUSP and DNMT1 are found in proliferative cells in normal colon. IHC was performed on normal colon tissue with the indicated antibodies. (**B**) Antibody competition assays. Colon tumors were stained with anti-HAUSP antibody, anti-HAUSP antibody mixed with recombinant HAUSP protein, anti-DNMT1 antibody, or anti-DNMT1 antibody mixed with its peptide antigens respectively.

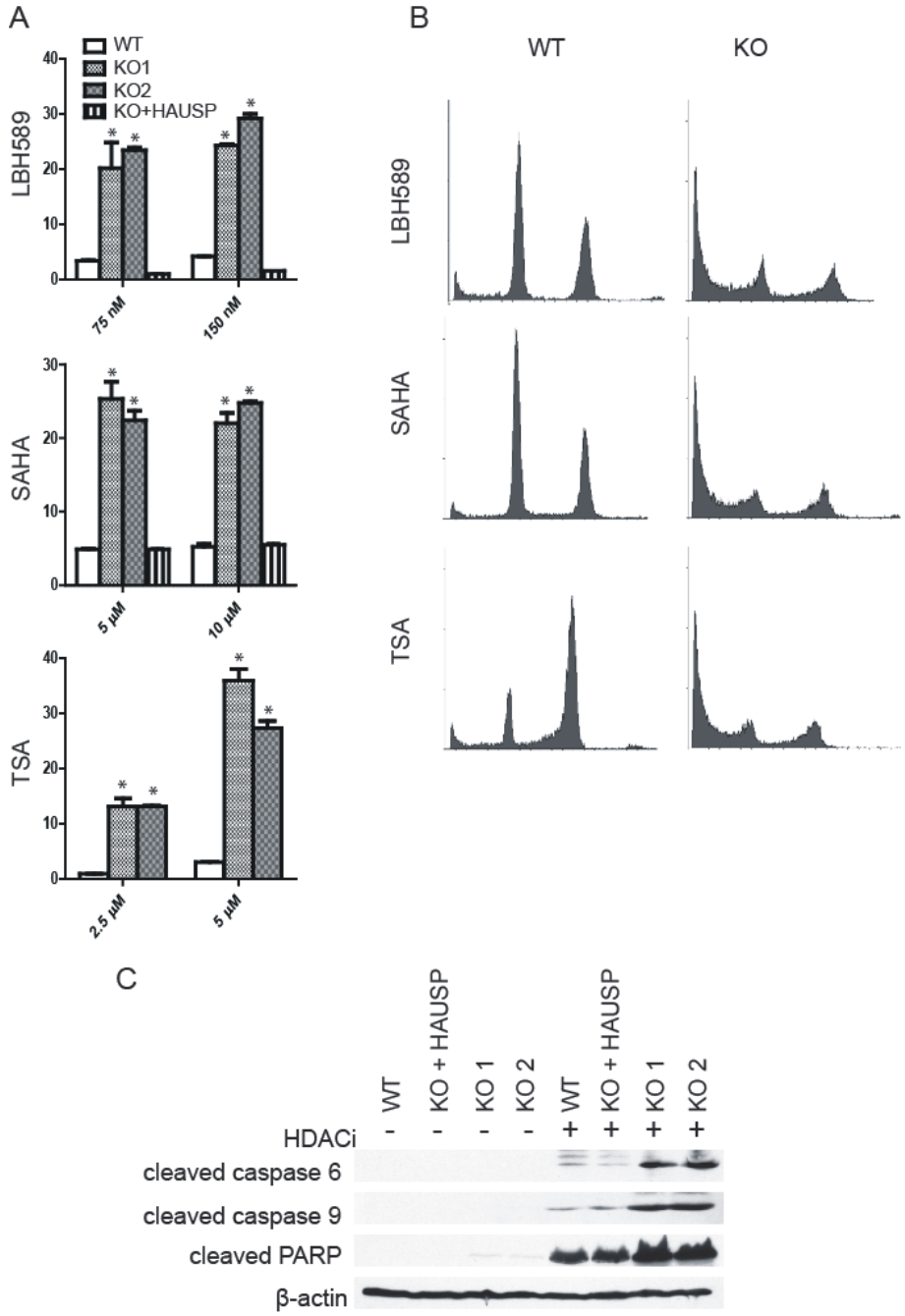

Fig. S9. HDAC inhibitors induce apoptosis in HAUSP knockout cells. (**A** and **B**) HDAC inhibitors induce apoptosis in HAUSP knockout cells. WT and HAUSP knockout cells with or without treatment by the indicated HDAC inhibitors. FACS analyses were performed to profile apoptotic sub G1 cells. Quantification of three independent experiments is shown in (A) and representative FACS profiles are shown in (B). * $p < 0.001$. (**C**) HDAC inhibition increases the abundance of apoptotic cell markers. Wild-type, two independent HAUSP knockout clones, and a HAUSP knockout clone ectopically expressing HAUSP were treated or not with HDAC inhibitor. Cell lysates were blotted with the indicated antibodies.

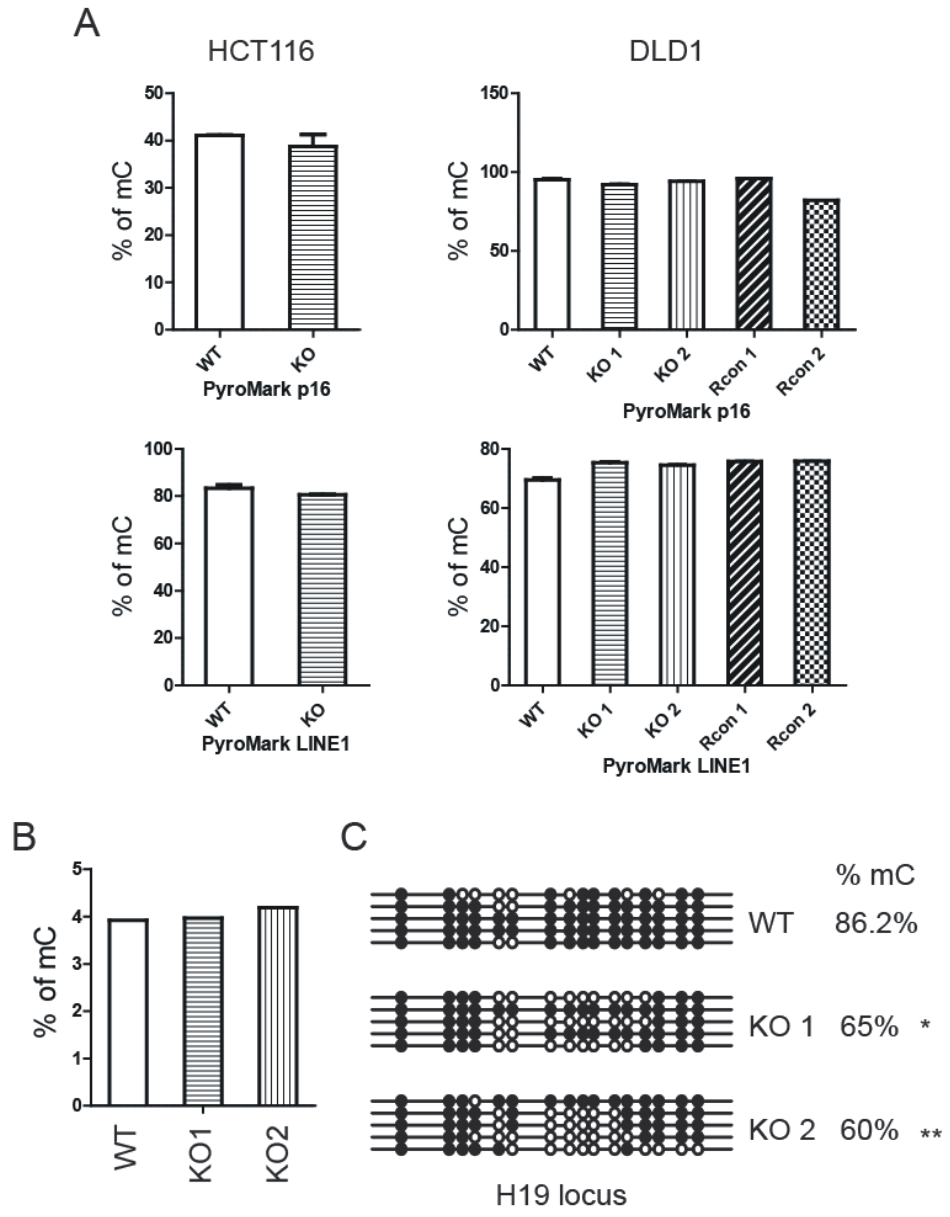

Fig. S10. DNA methylation status of wild-type and HAUSP knockout cells. (**A**) No change of CpG methylation was observed in the p16 promoter and LINE1 regions. Genomic DNA was isolated from the indicated clones and bisulfate treated. Pyro-sequencing was used to quantify DNA methylation status of the p16 locus and the LINE1 elements. (**B**) Global DNA methylation is not altered in HAUSP KO cell. The percentages of methylated cytosine in wild-type and two knockout DLD1 clones were measured by LC-MS/MS analyses. (**C**) Methylated CpG sites are decreased in HAUSP KO cells at the H19 locus. Genomic DNAs from wild-type and two HAUSP knockout DLD1 clones were bisulfate treated. The CpG island of H19 locus was PCR amplified, cloned, and sequenced with the Sanger sequencing method. * $p = 0.015$; ** $p = 0.0012$.

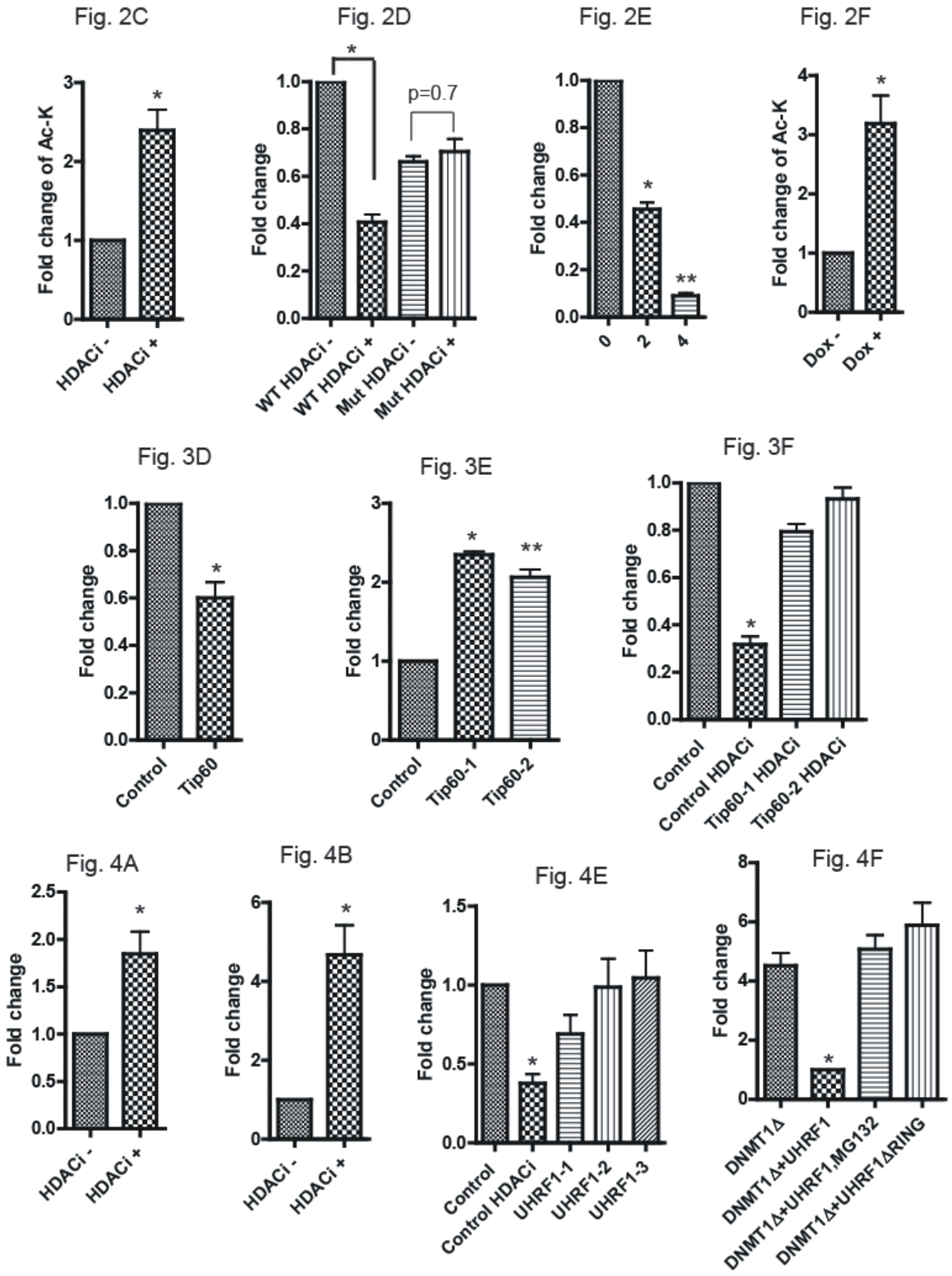

Fig. S11. Quantification and statistical analysis of Western blots. The Wilcoxon rank sum test was used to determine whether difference in any given two groups of data was statistically significant.

(Fig. 2C). The intensities of Ac-K were normalized to that of DNMT1 from two independent experiments with two blots each. N=4; *p = 0.014.
(Fig. 2D). The intensities of Myc-DNMT1 were normalized to that of beta-actin from two independent experiments with two blots each. N=4; *p = 0.014.
(Fig. 2E). The intensities of DNMT1 were normalized to that of beta-actin from three independent experiments. N=3; *p = 0.05, **p = 0.05.
(Fig. 2F). The intensities of Ac-K were normalized to that of DNMT1 from two independent experiments with two blots each. N=4; *p = 0.014.
(Fig. 3D). The intensities of DNMT1 were normalized to that of beta-actin from four independent experiments. N=4; *p = 0.014.
(Fig. 3E). The intensities of DNMT1 were normalized to that of beta-actin from three independent experiments. N=3; *p = 0.05, **p = 0.05.
(Fig. 3F). The intensities of DNMT1 were normalized to that of beta-actin from four independent experiments. N=4; *p = 0.014.
(Fig. 4A). The intensities of Flag-UHRF1 were normalized to that of Myc-DNMT1 from two independent experiments with two blots each. N=4; *p = 0.014.
(Fig. 4B). The intensities of DNMT1 were normalized to that of UHRF1 from two independent experiments with two blots each. N=4; *p = 0.014.
(Fig. 4E). The intensities of DNMT1 were normalized to that of beta-actin from three independent experiments. N=3; *p = 0.05.
(Fig. 4F). The intensities of Myc-DNMT1Δ were normalized to that of beta-actin from two independent experiments with two blots each. N=4; *p = 0.014.