Supplementary Information

E3-Independent Constitutive Monoubiquitination Complements Histone Methyltransferase Activity of SETDB1

Lidong Sun and Jia Fang[1]

Department of Tumor Biology, H. Lee Moffitt Cancer Center and Research Institute, Tampa, Florida 33612

[1]Correspondence and requests for materials should be addressed to:

J.F. (Jia.Fang@moffitt.org)

Phone: 813-745-6716

Fax: 813-745-7264

Sun. L, et al., Supplemental Fig.S1

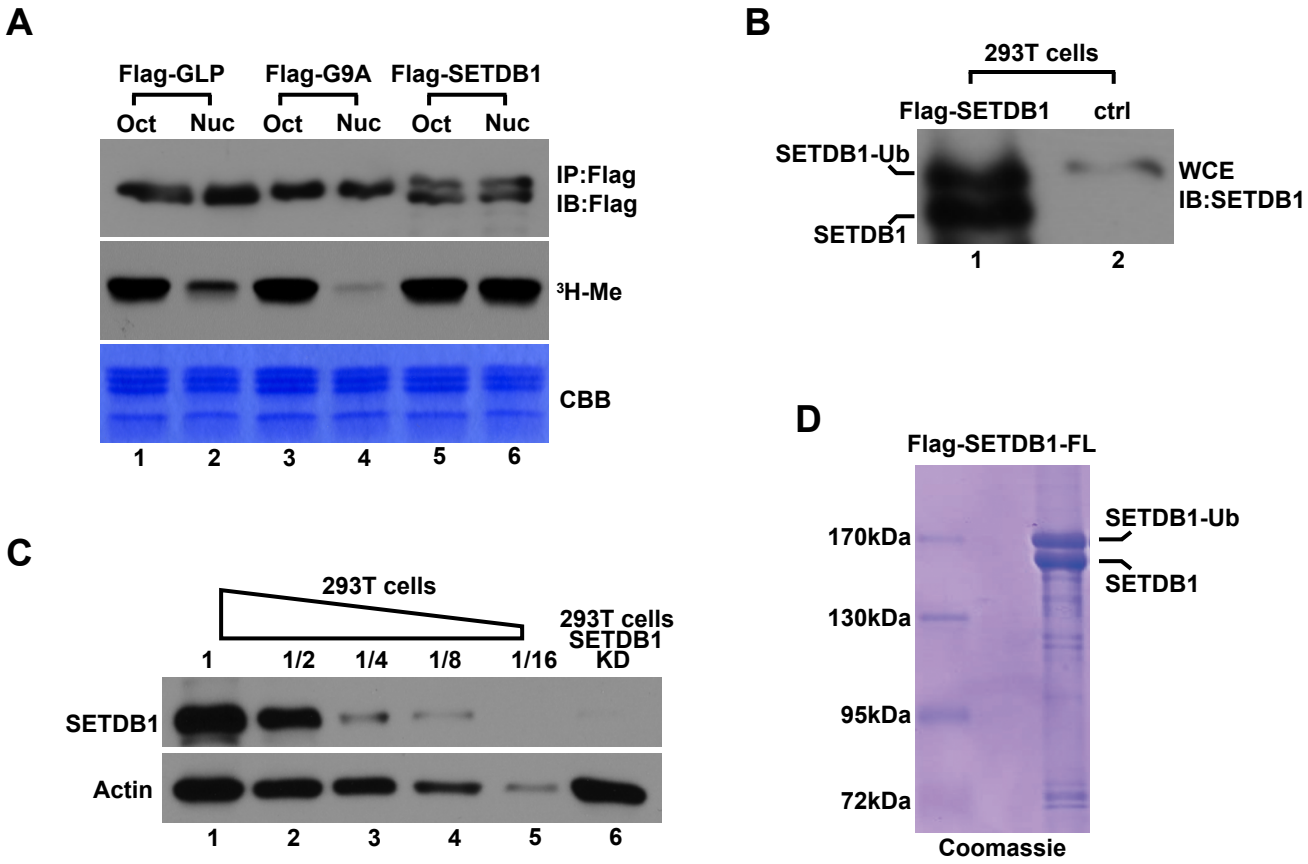

Figure S1, related to Figure 1. SETDB1 is monoubiquitinated.

(A) Immunoblot (top), autoradiogram (middle) and gel image (bottom) of in vitro histone methylation (HMT) assay with Flag-tagged full-length GLP, G9A and SETDB1 on histone octamer (Oct) or mononucleosome (Nuc). Proteins were immunoprecipitation (IP)-purified from transfected 293T cells 48 hours after transfection. **(B)** Immunoblots of whole cell extract (WCE) derived from 293T cells transfected with control or Flag-SETDB1 expressing vector. **(C)** Immunoblot of WCE derived from SETDB1 stable knockdown (SETDB1-KD) or control 293T cells using indicated antibodies. WCE of control cells was titrated to evaluate the knockdown efficiency. Actin serves as loading control. **(D)** Gel image of IP-purified Flag-SETDB1 derived from 293T cells expressing Flag-SETDB1. Gel was stained with Coomassie blue.

Sun. L, et al., Supplemental Fig.S2

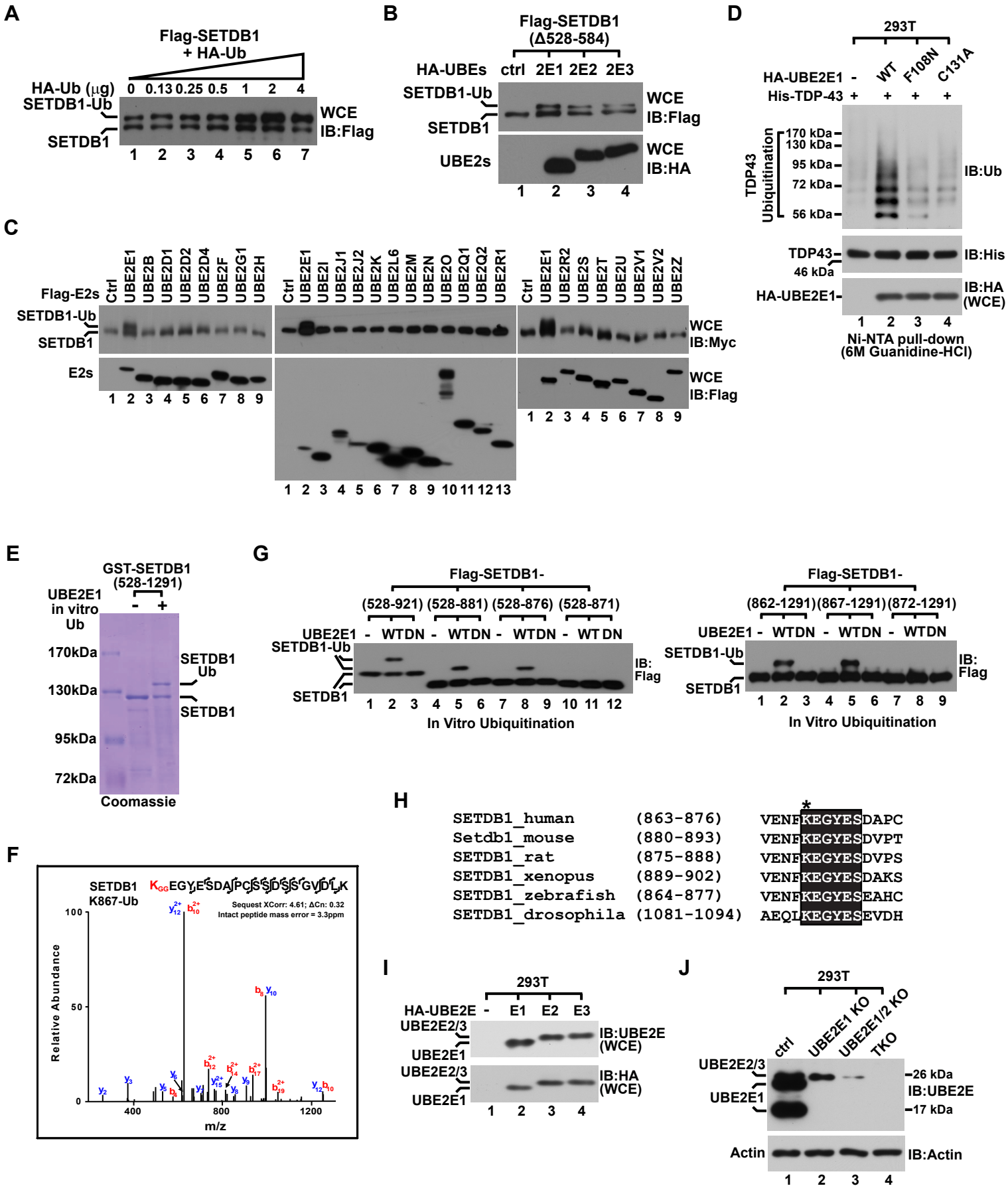

Figure S2, related to Figure 2. UBE2E family of E2s catalyze SETDB1-K867Ub1 in vitro and in vivo.

(A) Immunoblot of WCE derived from 293T cells co-expressing Flag-SETDB1 and increased amount of HA-Ubiquitin (HA-Ub). **(B)** Immunoblots of WCE derived from 293T cells co-expressing Flag-SETDB1(Δ528-584) and UBE2E family E2s including UBE2E1 (2E1), UBE2E2 (2E2) and UBE2E3 (2E3). **(C)** Immunoblots of WCE derived from 293T cells co-expressing Flag-SETDB1(Δ528-584) and different E2s. **(D)** His-TDP43 and HA-UBE2E1- (wt, C131A or F108N mutant) were co-expressed in 293T cells followed by Ni-NTA pull-down under the denaturing condition. WCE and purified proteins were then analyzed by immunoblots using indicated antibodies. **(E)** Gel image of purified recombinant GST-SETDB1(528-1291) before and after in vitro ubiquitination by recombinant UBE2E1. **(F)** LC Mass Spectrometry analysis of the top ubiquitinated protein band of purified GST-SETDB1(528-1291) from **(A)**. Arrows are the fragment ions that confirm K867 as the ubiquitination site. **(G)** Immunoblots of different IP-purified Flag-SETDB1 deletions after they were incubated with UBE2E1-wt or C131A (DN) for in vitro ubiquitination. **(H)** Sequence alignment of human SETDB1 sequence encompassing 867-872aa with different counterparts from various organisms. **(I)** Immunoblots of WCE derived from 293T cells transfected with expression vector for HA- UBE2E1, UBE2E2 or UBE2E3 using indicated antibodies. Anti- UBE2E and HA antibodies showed similar results, indicating a comparable affinity of UBE2E antibody for all three UBE2E enzymes. **(J)** Immunoblots of WCE derived from control, UBE2E1-KO, UBE2E1/2-double knockout and UBE2E1/2/3 triple knockout (TKO) 293T cells using indicated antibodies.

Sun. L, et al., Supplemental Fig.S3

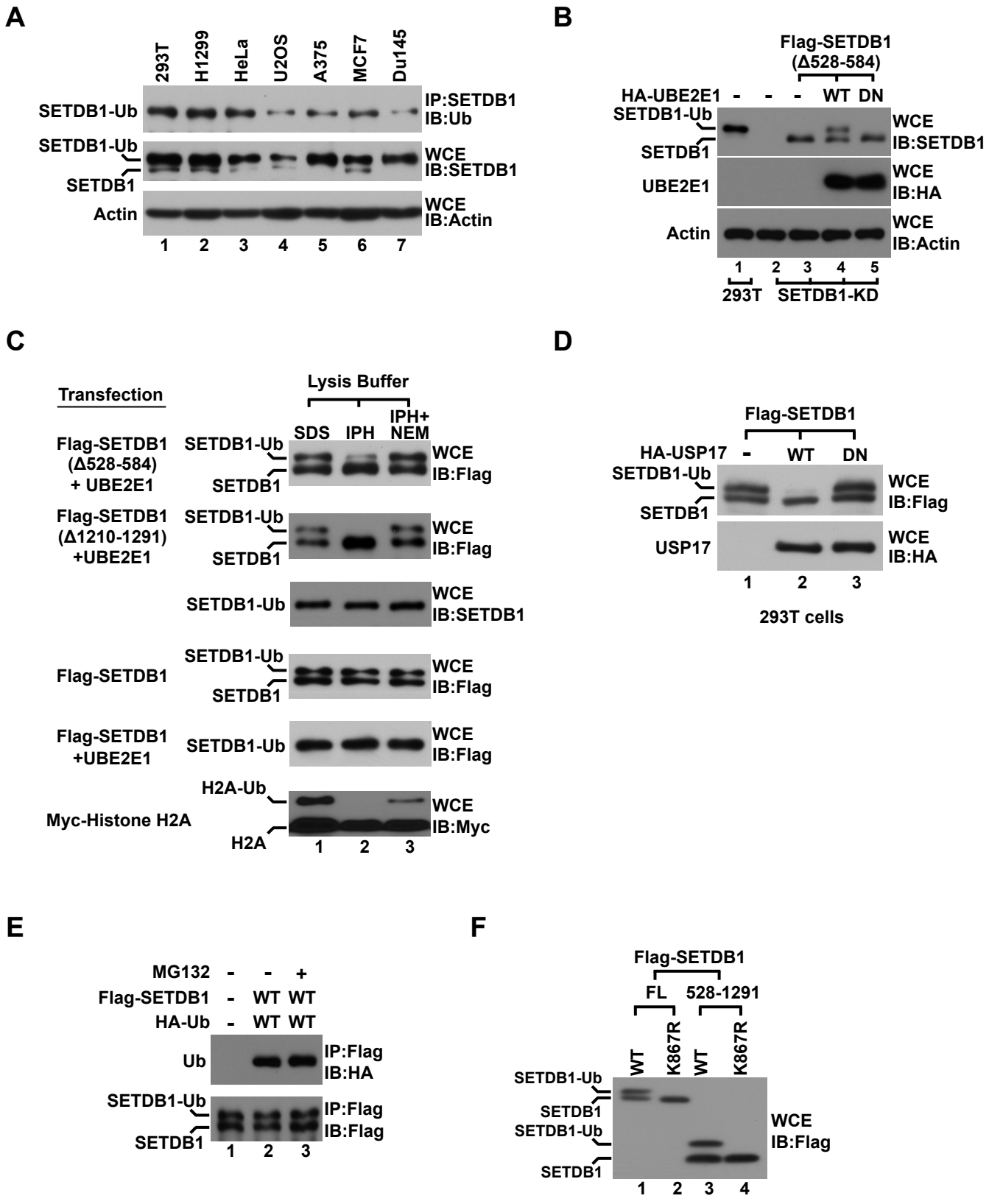

Figure S3, related to Figure 3. Conjugated-K867Ub1 is protected from deubiquitination on full-length SETDB1.

(A) Immunoblots of IPed endogenous SETDB1 and WCE derived from seven different cell lines using indicated antibodies. **(B)** Immunoblots of WCE derived from control or SETDB1-KD 293T cells in which rescue SETDB1(Δ528-584) was co-expressed with UBE2E1-wt or C131S (DN). **(C)** Immunoblots of WCE derived from 293T cells that were transfected with expression vectors for indicated proteins. Cells were lysed with 2% SDS containing buffer (SDS), IPH buffer (IPH) or IPH buffer supplemented with 10mM N-Ethylmaleimide (IPH+NEM) 48 hours after transfection. **(D)** Immunoblots of WCE derived from 293T cells expressing Flag-SETDB1 and USP17-wt or its C89S inactive mutant (DN). **(E)** IP-western analysis of SETDB1 co-expressed with HA-Ub in 293T cells. 42 hours after transfection, cells were treated with 10μg/ml MG132 or DMSO for additional 6 hours before lysis. **(F)** Immunoblots of WCE derived from 293T cells over-expressing Flag-SETDB1-FL or (528-1291) deletion (wt or K867R).

Sun. L, et al., Supplemental Fig.S4

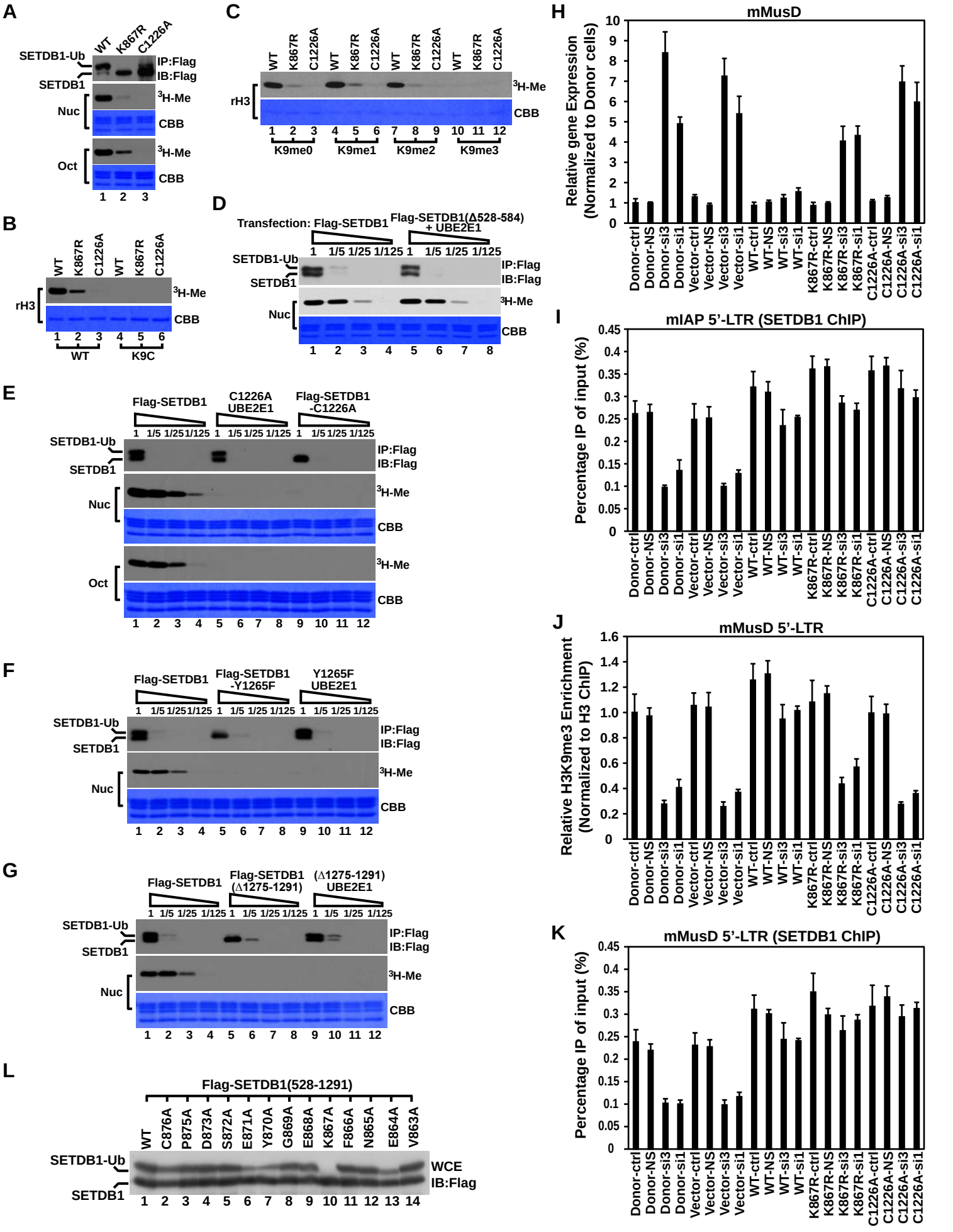

Figure S4, related to Figure 4. K867Ub1 augments the enzymatic activity of SETDB1 and is necessary to maintain SETDB1-mediated ERV silencing in mES cells.

(A) Immunoblot, autoradiogram (^{3}H-Me) and gel image (CBB) of in vitro HMT assay with Flag-SETDB1 (wt, K867R or C1226A mutant) on histone octamer (Oct) or mononucleosome (Nuc). **(B)** Autoradiogram (^{3}H-Me) and gel image (CBB) of the similar in vitro HMT assay with Flag-SETDB1, K867R or C1226A on purified recombinant histone H3 (wt or K9C). **(C)** Autoradiogram (^{3}H-Me) and gel image (CBB) of the similar in vitro HMT assay with Flag-SETDB1, K867R or C1226A on purified recombinant histone H3 which were chemically installed with different methyl-lysine analogues at K9. **(D)** Immunoblot, autoradiogram (^{3}H-Me) and gel image (CBB) of in vitro HMT assay with titrated Flag-SETDB1 or Flag-SETDB1(Δ528-584) on mononucleosome (Nuc). Flag-SETDB1(Δ528-584) was co-expressed with UBE2E1 in 293T cells and then IP-purified. **(E-G)** Immunoblot, autoradiogram (^{3}H-Me) and gel image (CBB) of in vitro HMT assay with Flag-tagged three catalytic inactive SETDB1 mutants or the same mutant that was ubiquitinated by co-expressed UBE2E1. Similar to Flag-SETDB1, Flag-SETDB1-C1226A **(E)**, Flag-SETDB1-Y1265F **(F)** or Flag-SETDB1(Δ1275-1291) **(G)** was IP-purified from transfected 293T cells or cells co-expressing the mutant with UBE2E1. **(H-K)** Control E14 mES cells or cells stably expressing SETDB1-WT, K867R or C1226A mutant were treated with siRNA targeting endogenous Setdb1 (si3 and si1) or non-sense control (NS) for 96 hours followed by quantitative analyses. **(H)** mRNA level of mMusD element was analyzed by real-time RT-PCR (normalized to GAPDH expression). mRNA level in control cells was normalized as 1. **(I)** ChIP-qPCR analysis using antibody for SETDB1 and primers for mIAP 5'-LTR. **(J)** ChIP-qPCR analysis using antibodies for H3 and H3K9me3 and primers for mMusD 5'-LTR. ChIP enrichment of H3K9me3 was normalized to enrichment of H3. **(K)** ChIP-qPCR

analysis using antibody for SETDB1 and primers for mMusD 5'-LTR. **(L)** Immunoblot of WCE derived from 293T cells expressing Flag-SETDB1-wt or different alanine mutations.

SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Recombinant Proteins

Purified His-E1 (human ubiquitin activating enzyme) and ubiquitin-wt were purchased from Enzo. ubiquitin-I44A was obtained from Boston Biochem. Other His- or GST- tagged recombinant proteins were purified from *E coli* and listed below. Briefly, cDNA was cloned into pET-28 or pGEX-KG vector which was transformed into *E. coli* BL21 (DE3) Codon-plus RIL (Stratagene). Expression was induced with 0.3 mM isopropyl β-D-thiogalactoside (IPTG, RPI) for 16 h at 16 °C in 2xYT medium. Proteins were then purified with Ni-NTA or Glutathione agarose using standard protocols as we described (Chang et al., 2011; Kokura et al., 2010). Recombinant xenopus histone H3 and H3-K9C were generated and purified according to previously reported protocols (Dyer et al., 2004). The installation of different methyl-lysine analogs at H3K9 was carried out using previous published methods (Kokura et al., 2015; Simon, 2010; Simon et al., 2007).

Protein	**Protein**
GST-SETDB1(528-1291)	His-Ubiquitin-WT
His-SETDB1(528-1291)	His-Ubiquitin-I44A
GST-SETDB1-528-1291-(Δ877-1191)	His-Ubiquitin-K0(lysine-less)
His-SETDB1(867-872)	His-UBE2E1-WT
His-SETDB1(867-872)-K867R	His-UBE2E1-C131A
His-USP17-Full length	His-UBE2E1-C131S
His-USP17-Full length-C89S	GST-UBE2E1-WT
GST-USP2A-Full length	GST-UBE2E1-C131A
GST-USP2A- Full length-C276A	GST-UBE2E1-C131S

Transient and stable knockdown and CRISPR/Cas9 Knockout

To knockdown mouse Setdb1 in E14 mES cells, three siRNAs were tested individually for their efficiency by western blot. Two sequences with better efficiency were used for experiments.

Cells were transfected twice with siRNA every 48 h and collected 96 h after first transfection.

siRNA-1 (si1): GCGCAGAGUUAACCGCAAA

siRNA-3 (si3): CAUCCAGACUGUUGGGCUA

Nonsense control siRNA (NS): UGGUUUACAUGUCGACUAA

Stable knockdown of human SETDB1 in 293T cells was carried out using lentiviral vector as we previously described (Sun et al., 2015). Human SETDB1 shRNA targeting sequence is "GAAGCAGCTAGCAGAGTTA". For rescue expression, this sequence was replaced by "GAAGCAGCTGGCCGAACTG" in shRNA resistant Flag-SETDB1 in a lentiviral expression vector which harbors EF1α promoter and IRES-Blasticidin cassette. The same lentiviral vector was also used to stably express SETDB1-WT or mutants in E14 mES cells.

For CRISPR/Cas9 knockout of human UBE2Es in 293T cells, the following sgRNAs were used.

sgUBE2E1: CATTGTAATATTAACAGTCA

sgUBE2E2: CACTGTAATATTAACAGCCA and TCAACTATATTGGGACCCCC

sgUBE2E3: CCTTCATATACAGAACCCGG and AAGGAGATAACATTTATGAA

Two individual human UBE2Es triple-knockout 293T cell lines were derived from different combination of UBE2E2 and UBE2E3 sgRNAs in a validated UBE2E1 knockout clone.

In vitro Histone Methyltransferase (HMT) assay

Reaction was performed as we described previously (Kokura and Fang, 2009; Kokura et al., 2015). Briefly, 4μg mononucleosome or histone octamer purified from HeLa cell (Kokura and Fang, 2009; Kokura et al., 2015) or 1μg recombinant histone H3 was mixed with SETDB1 in a 20μl reaction containing 0.55μCi ^{3}H-Adenosyl-L-Methionine (Perkin Elmer) at 30^{o}C for 30min with rotation. Reaction was stopped by adding SDS-PAGE sample buffer, resolved by SDS-

PAGE (15%) and analyzed by autoradiography.

Chromatin Immunoprecipitation (ChIP), real-time quantitative PCR and RT-PCR

The ChIP assay was carried out as we described previously (Kokura et al., 2010; Sun et al., 2015). Quantitative PCR was carried out using SYBR green (Quanta) and a C1000 thermal cycler (Bio-Rad). PCR primers for 5'LTR of mIAP and mMusD and mGAPDH promoter were described previously (Matsui et al., 2010).

For RT-PCR, RNA was isolated with RNeasy Mini Kit (Qiagen), treated with DNase-I (Sigma) and reverse transcribed with ImProm-II^{TM} Reverse Transcription System (Promega). PCR was carried out under the same condition using published primers for mGAPDH (Polo et al., 2010), and mIAP & mMusD (Oliveri et al., 2007). All other primers were reported (Matsui et al., 2010).

LC-Mass spectrometry

To identify the ubiquitination site on SETDB1, IP-purified Flag-SETDB1 or in vitro ubiquitinated recombinant SETDB1 was resolved by SDS-PAGE. After coomassie blue staining, ubiquitinated protein band was excised, soaked in water for 2 hours, treated with TCEP and iodoacetamide and then digested with trypsin and chymotrypsin. After peptides were extracted from gel and concentrated under vacuum centrifugation, a nanoflow liquid chromatography (U3000, Dionex) coupled to an electrospray ion trap mass spectrometer (LTQ-Orbitrap, Thermo) was used for tandem mass spectrometry peptide sequencing experiments. The sample was first loaded onto a pre-column (5mm x 300 μm ID packed with C18 reversed-phase resin, 5μm, 100Å) and washed for 8 minutes with aqueous 2% acetonitrile and 0.04% trifluoroacetic acid. The trapped peptides were eluted onto the analytical column (C18, 75 μm ID x 15 cm, Pepmap

100, Dionex). The 120-minute gradient was programmed as: 95% solvent A (2% acetonitrile + 0.1% formic acid) for 8 minutes, solvent B (90% acetonitrile + 0.1% formic acid) from 5% to 50% in 90 minutes, then solvent B from 50% to 90% in 7 minutes and held at 90% for 5 minutes, followed by solvent B from 90% to 5% in 1 minute and re-equilibrate for 10 minutes. The flow rate on analytical column was 300 nL/min. Five tandem mass spectra were collected in a data-dependent manner following each survey scan. The MS scans were performed in Orbitrap to obtain accurate peptide mass measurement and the MS/MS scans were performed in linear ion trap using 60 second exclusion for previously sampled peptide peaks. Sequest and Mascot searches were performed against the Swiss-Prot human database. Two trypsin missed cleavages were allowed, the precursor mass tolerance was 1.08 Da. MS/MS mass tolerance was 0.8 Da. Dynamic modifications included carbamidomethylation (Cys), oxidation (Met), deamidation (Asn, Gln) and ubiquitination. Both MASCOT and SEQUEST search results were summarized in Scaffold 2.0.

Reference

Chang, Y., Sun, L., Kokura, K., Horton, J.R., Fukuda, M., Espejo, A., Izumi, V., Koomen, J.M., Bedford, M.T., Zhang, X., *et al.* (2011). MPP8 mediates the interactions between DNA methyltransferase Dnmt3a and H3K9 methyltransferase GLP/G9a. Nat Commun *2*, 533.

Dyer, P.N., Edayathumangalam, R.S., White, C.L., Bao, Y., Chakravarthy, S., Muthurajan, U.M., and Luger, K. (2004). Reconstitution of nucleosome core particles from recombinant histones and DNA. Methods in enzymology *375*, 23-44.

Kokura, K., and Fang, J. (2009). In vitro histone demethylase assays. Methods in molecular biology (Clifton, N.J *523*, 249-261.

Kokura, K., Sun, L., Bedford, M.T., and Fang, J. (2010). Methyl-H3K9-binding protein MPP8 mediates E-cadherin gene silencing and promotes tumour cell motility and invasion. The EMBO journal *29*, 3673-3687.

Kokura, K., Sun, L., and Fang, J. (2015). In vitro histone demethylase assays. Methods in molecular biology (Clifton, N.J *1288*, 109-122.

Matsui, T., Leung, D., Miyashita, H., Maksakova, I.A., Miyachi, H., Kimura, H., Tachibana, M., Lorincz, M.C., and Shinkai, Y. (2010). Proviral silencing in embryonic stem cells requires the histone methyltransferase ESET. Nature *464*, 927-931.

Oliveri, R.S., Kalisz, M., Schjerling, C.K., Andersen, C.Y., Borup, R., and Byskov, A.G. (2007). Evaluation in mammalian oocytes of gene transcripts linked to epigenetic reprogramming. Reproduction *134*, 549-558.

Polo, J.M., Liu, S., Figueroa, M.E., Kulalert, W., Eminli, S., Tan, K.Y., Apostolou, E., Stadtfeld, M., Li, Y., Shioda, T., *et al.* (2010). Cell type of origin influences the molecular and functional properties of mouse induced pluripotent stem cells. Nature biotechnology *28*, 848-855.

Simon, M.D. (2010). Installation of site-specific methylation into histones using methyl lysine analogs. Curr Protoc Mol Biol *Chapter 21*, Unit 21 18 21-10.

Simon, M.D., Chu, F., Racki, L.R., de la Cruz, C.C., Burlingame, A.L., Panning, B., Narlikar, G.J., and Shokat, K.M. (2007). The site-specific installation of methyl-lysine analogs into recombinant histones. Cell *128*, 1003-1012.

Sun, L., Kokura, K., Izumi, V., Koomen, J.M., Seto, E., Chen, J., and Fang, J. (2015). MPP8 and SIRT1 crosstalk in E-cadherin gene silencing and epithelial-mesenchymal transition. EMBO reports *16*, 689-699.