Supporting Online Material for

UHRF1 Plays a Role in Maintaining DNA Methylation in Mammalian Cells

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Materials/Methods

Cell culture and Plasmid Constructs

COS-7, HEK293, and HeLa cells were obtained from American Type Culture Collection (ATCC). Mouse embryonic stem cells (a generous gift of Haruhiko Koseki and Masahiro Muto), E14 and *mUhrf1-/-* (19-4) were cultured on 0.1% gelatin (Sigma, St. Louis, MO) in Glasgow's Minimal Essential Medium (Invitrogen, Carlsbad, CA), supplemented with 50 units/mL mouse Leukemia Inhibitory Factor (LIF) (Chemicon, Temecula, CA), 10% Defined fetal bovine serum (Hyclone, Logan, UT), 1x non-essential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine and 55 μM beta-mercaptoethanol. The SRA domain (amino acids 393-621) was amplified from a full-length *mUhrf* cDNA construct in pcDNA3.1 (a generous gift of Paolo Di Fiore; *1*). The SRA domain (376-605) was amplified from full-length *hUHRF1* cDNA synthesized using total RNA from HeLa cells. The product was cloned into pENTR-TEV and recombined into pDEST15 (Invitrogen, Carlsbad, CA) to create the GST fusion. The full-length *mUhrf1* and *hUHRF1* were amplified and cloned into EcoRI and XhoI sites of pEGFPC2 to create each GFP fusion. DsRed-DNMT1 construct was used previously in (*2*). Chitin binding domain (CBD)-DNMT1 (amino acids 1-1465) was cloned into pcDNA4.1.

Southern blot analysis

Genomic DNA was extracted from wild-type (E14, passage 15) and *mUhrf1-/-* cells (19-4, passage 6) (*3*) using a Genomic DNA Extraction Kit (Zymo Research, Orange, CA) according to manufacturer's instructions. 750 nanograms of DNA was digested with either HpaII or MspI (New England Biolabs, Ipswich, MA) overnight and subsequently

run on a 1% agarose gel. After transfer to Hybond-N+ (Amersham, Piscataway, NJ), the blot was hybridized to radiolabelled minor satellite (a generous gift of Guoping Fan; *4*) or *IAP* probe (a generous gift of Tim Bestor; *5*) overnight, washed under high stringency conditions, and autoradiographed. Southern blots were performed at least three independent times to confirm reproducibility.

Genomic Bisulfite Sequencing

Four micrograms of genomic DNA was treated with bisulfite and desulphonated using the MethylEasy kit according to manufacturer's instructions (Human Genetic Signatures, Sydney, Australia). Two microliters of bisulfite DNA was used to amplify *IAP* and *Line-1* elements using primers and conditions in (*6*). Amplified regions were cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA) and sequenced.

Electrophoretic mobility shift assays

GST-UHRF1SRA was expressed and purified as described previously (*7*). For mUHRF1, mobility shift assays were performed with 10 ng of protein (*7*), at pH 6.8. HindIII-digested Lambda DNA (Invitrogen, Carlsbad, CA) was used as a non-specific competitor. For hUHRF1 assays were performed with 100ng of purified proteins. Since the gel shift assays were performed in the presence of a vast excess of unmethylated competitor DNA, the complexes appeared to be specific. Oligonucleotides used for binding are described in Supplemental Figure S7. For K_d analysis, at least two sets of independent experiments were performed with two different batches of GST-mUHRF1-SRA proteins. The bound fraction was plotted against the protein concentration, and data

were analyzed using non-linear regression fit using Prism version 4.0c (GraphPadSoftware, Inc., CA).

Microscopy

COS-7 cells were transfected with *DsRed-DNMT1* or *Uhrf1-GFP* (5 micrograms) plasmids with Transpass D2 reagent (New England Biolabs, Ipswich, MA) for 48 hours. Fluorescence microscopy was performed as previously described (*2*). Cells were visualized with a Zeiss 200M microscope with a 63X oil objective lens at 488 nm for GFP-UHRF1, 568 nm for DsRed-DNMT1, and 460 nm for DNA staining with Hoechst 33342.

COS-7 cells grown on slides were transfected with 200 nM of UHRF1 siRNA (Invitrogen, Carlsbad, CA) or Litmus siRNA (New England Biolabs, Ipswich, MA) with RNAiFECT transfection reagent (Qiagen, Valencia, CA) for 24h, and then transfected with 2 micrograms of GFP-DNMT1 encoding plasmid for the next 48h. Cells were fixed in methanol, permeabilized with PBS-1% Triton X-100 and incubated with anti-PCNA (Cell Signaling Technology, Danvers, MA) for overnight after blocking the slides with PBS-5% BSA. PCNA was revealed by an anti-mouse IgG Alexa Fluor 594 (Molecular Probes, Invitrogen, Carlsbad, CA). Nuclear staining was performed by using Hoechst 33342.

mUhrf1-/- cells were fixed in methanol, permeabilized with PBS-1% Triton X-100 and incubated with anti-PCNA (Cell Signaling Technology, Danvers, MA) for overnight after blocking the slides with 1xPBS-5% BSA. PCNA was revealed by an anti-mouse IgG Alexa Fluor 594 (Molecular Probes, Invitrogen, Carlsbad, CA) and the

DNMT1 was revealed using Anti-DNMT1 polyclonal antibody overnight Santa Cruz, CA), followed by probing with anti-rabbit IgG Alexa Fluor 488 (Molecular Probes, Invitrogen, Carlsbad, CA).

Chromatin association experiments

hUHRF1 siRNAs (Invitrogen, Carlsbad, CA) or *DNMT1* siRNAs (New England Biolabs, Ipswich, MA) were introduced into HeLa cells by two transfections using RNAiFECT reagent (Qiagen, Valencia, CA), (*2*). Forty-eight hours after the second transfection, chromatin purifications were performed as described previously (*2*). Briefly, the transfected HeLa cells were subjected to crosslinking with 1% formaldehyde for 10 min at room temperature, followed by washing twice with PBS (pH 7.4) and lysis in buffer A (100 mM Tris-Cl at pH 7.5, 5 mM $MgCl_2$, 60 mM KCl, 0.5 mM DTT, 125 mM NaCl, 300 mM sucrose, 1% NP-40). After lysis on ice for 10 min, the nuclei were pelleted, resuspended in buffer B (100 mM Tris-Cl at pH 7.5, 1 mM $CaCl_2$, 60 mM KCl, 0.5 mM DTT, 125 mM NaCl, 300 mM sucrose), and added with 10 U of S7 nuclease (Roche, Indianapolis, IN) for 20 min at 37 °C. The reaction was stopped by adding 50 mM EDTA. The chromatin was pelletted and resuspended in buffer C (1% SDS, 10 mM EDTA, 50 mM Tris-Cl at pH 8.0) overnight at 4 °C. After centrifugation (13000 rpm, 2 min at room temperature), the supernatant was used for western blotting. All buffers contained PMSF and protease inhibitor cocktail (Sigma, St. Louis, MO).

Co-precipitation and Western analysis

HEK293 cells were transfected with CBD-DNMT1 by FuGENE (Roche, Indianapolis, IN) and selected with Zeocin (Invitrogen, Carlsbad, CA). Pull-down experiments were performed as described in (*2*). Nuclear extract proteins (500 μg) in PBS (pH 7.4) were incubated with chitin beads (New England Biolabs, Ipswich, MA) at 4 °C, overnight with rotation. The beads were washed with ice-cold PBS twice and the protein complex was eluted with SDS loading buffer at 95 °C for 5 min. Western blots were performed with anti-DNMT1 (New England Biolabs, Ipswich, MA), anti-hUHRF1 (BD Biosciences, Franklin Lakes, NJ, Cat.# 612264). In this way, we could assess the association of endogenous hUHRF1 with the CBD-tagged DNMT1. For the experiments shown in Figure 3B, we also utilized anti-H3 (Cell Signaling Technology, Danvers, MA) and anti-Actin antibodies (Sigma, St. Louis, MO). For protein analysis 30 micrograms of total protein extracted from *mUhrf1-/-* cells were Western blotted with anti-DNMT1, anti-DNMT3a, anti-DNMT3b and anti-PCNA antibodies (New England Biolabs, Ipswich, MA).

In vitro interaction analysis

GST fusions of mUHRF1 and DNMT1 were created in pGEX5.1 (Amersham Biosciences, Piscataway, NJ), and expressed and purified by using glutathione sepharose beads (Amersham Biosciences, Piscataway, NJ). DNMT1 was expressed and purified from baculovirus-infected Sf9 cells. Purified mUHRF1 was obtained by PCR cloning into NdeI/XhoI sites of pET-28a (Novagen, San Diego, CA) and Ni-Sepharose chromatography. GST pull-down assays were performed by incubating the GST or GST fusion protein beads with purified DNMT1 or mUHRF1 in binding buffer (50 mM Tris

pH 7.5, 28 μM $ZnCl_2$, 0.4% Triton X-100, 150 mM NaCl, 10 % glycerol) at 4 °C for 2 h. The beads were washed 3 times with the binding buffer containing 500 mM NaCl and the protein complex was eluted with SDS sample buffer at 95°C for 5 min. Interacting proteins were visualized with anti-DNMT1 (New England Biolabs, Ipswich, MA) or anti-mUHRF1 antibodies (a generous gift of Haruhiko Koseki and Masahiro Muto).

Methylation Specific PCR

hUHRF1 siRNAs (Invitrogen, Carlsbad, CA) were introduce into HeLa cells by three transfections using RNAiFECT reagent (Qiagen, Valencia, CA) (*2*). Two micrograms of genomic DNA was treated with bisulfite and desulfonated using EpiTect Bisulfite kit (Qiagen, Valencia, CA). Two to four microliters of bisulfite DNA was used to amplify, IGS-*rDNA* (*2*), *Line-1* (Methylated: 5'-AAGATGGTCGAA-TAGGAATAGTTTC-3' and 5'-CACTCCCTAATAAAATAAACCCGAT-3', Unmethylated: 5'-AGATGGTTG-AATAGGAATAGTTTTG-3' and 5'-CACTCCCTAATAAAATAAACCCAAT-3') and *RAR-β2* (*8*) using primers specific for methylated or unmethylated sequences and conditions in (*9*).

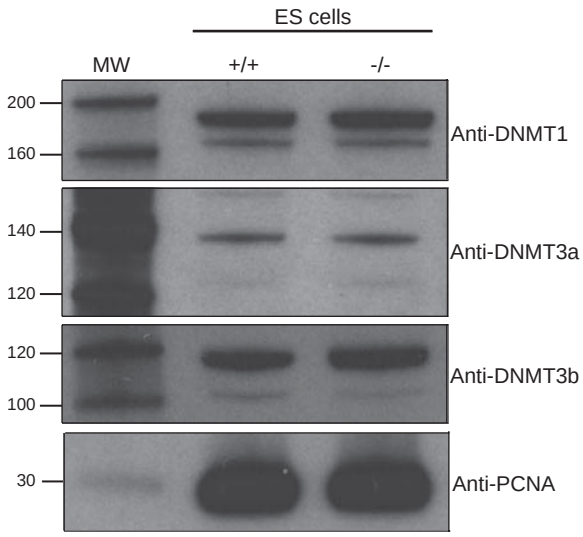

Figure S1. *mUHRF1-/-* cells have wild-type levels of DNA methyltransferases. Western blot of DNMT1, DNMT3a, DNMT3b, and PCNA with cell extracts from wild-type (+/+) or *mUHRF1-/-* (-/-) embryonic stem (ES) cells. MW denotes the lane with protein molecular weight markers, with sizes on left of gel in kDa.

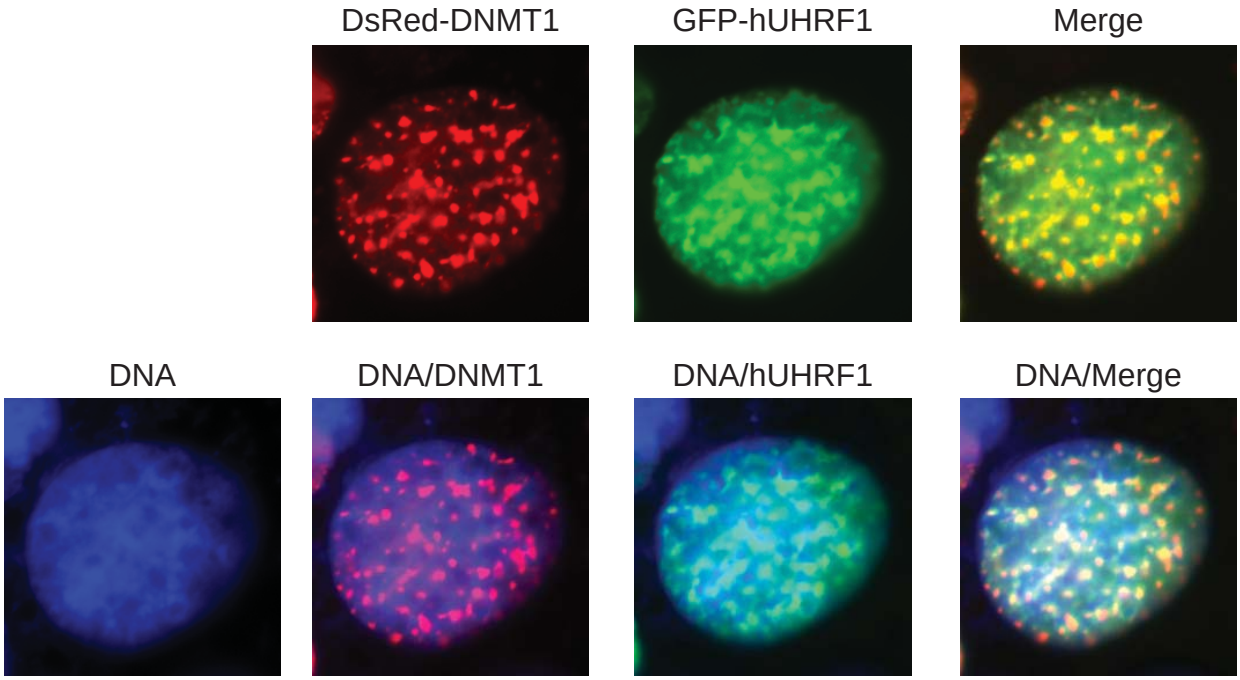

Figure S2. Colocalization of hUHRF1 and DNMT1. Detection of DsRed-DNMT1 and GFP-hUHRF1 transiently expressed in synchronized COS-7 cells. Cells were released from G1 arrest and visualized in late S Phase. DNA in the nucleus (DNA) was visualized with Hoechst stain.

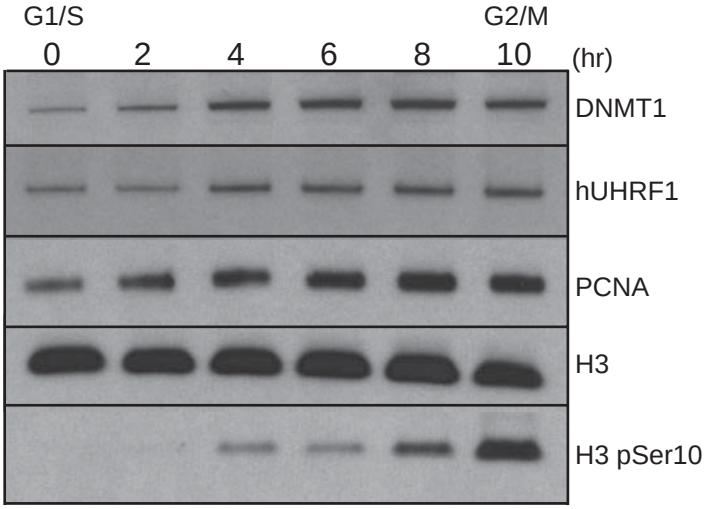

Figure S3. Cell cycle-dependent chromatin association of DNMT1, hUHRF1, and PCNA. HeLa cells were synchronized by thymidine/ aphidicolin block and released into regular medium. Time points in hours (hr) after the release are indicated at the top of the panels. Western blots for DNMT1, hUHRF, and PCNA to detect association with chromatin. H3 and H3 phospho-Ser10 (H3 pSer10) antibodies were used for a loading control and mitosis marker, respectively.

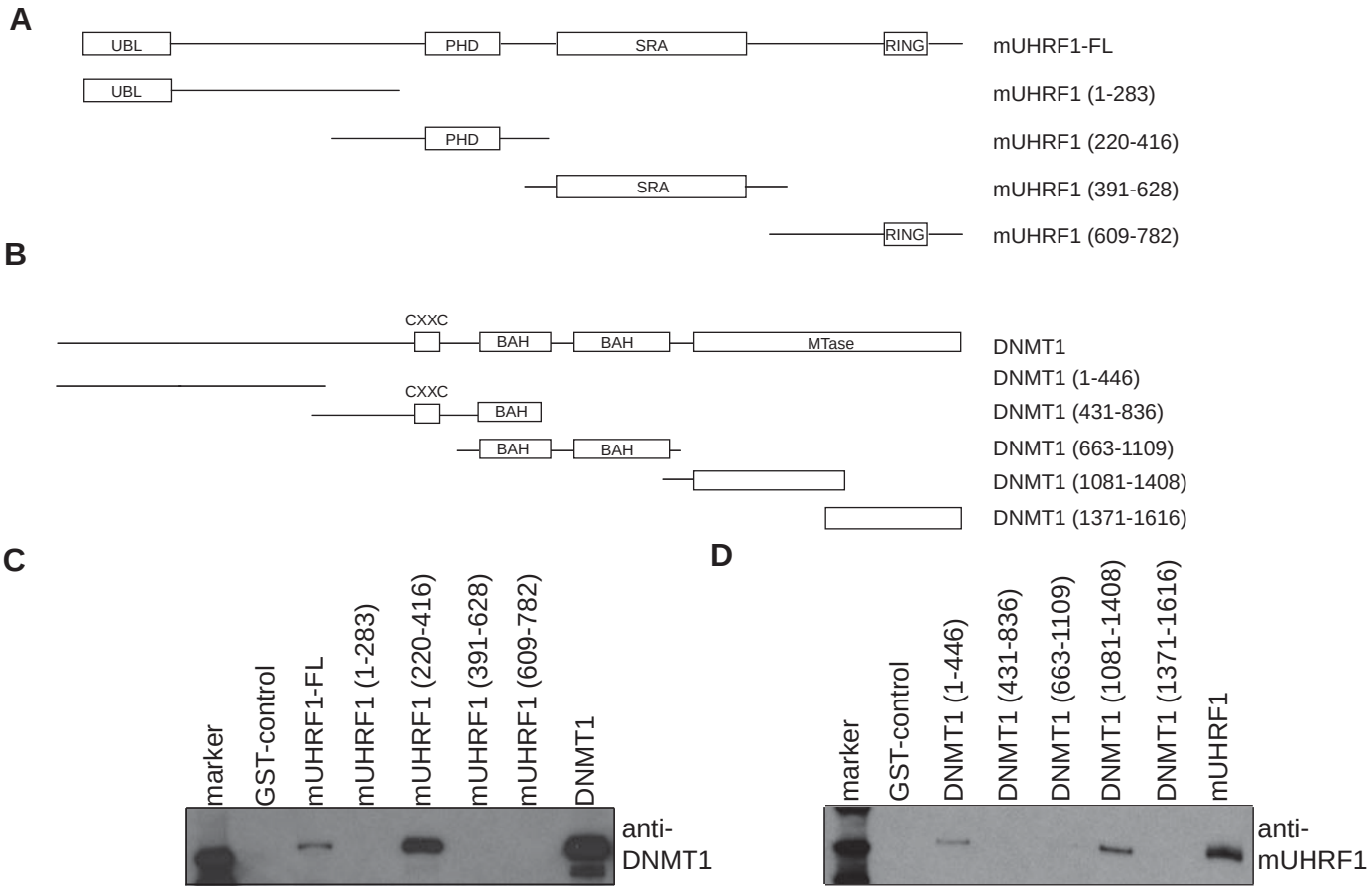

Figure S4. Mapping DNMT1 and mUHRF1 interaction domains. (**A**) Various domains of mUHRF1 are indicated by a schematic presentation of the GST fusion constructs shown with amino acid numbers. UBL, ubiquitin-like; PHD, plant homeodomain; SRA, SET and RING associated; RING, Really Interesting New Gene. (**B**) Various domains of DNMT1 are indicated by schematic diagram of the GST fusion fragments shown with amino acid numbers. CXXC, Zinc-finger; BAH, Bromo adjacent homology; MTase, methyltransferase. (**C**) GST pull-down assay demonstrating interacting regions between purified GST-fused UHRF1 fragments and baculovirus expressed full-length DNMT1. The input DNMT1 is included as control. All GST fusions were validated by western blot analysis with anti-GST antibodies (data not shown). After the transfer of the proteins, the blots were stained with ponceau stain (data not shown) to determine the quality of protein transfer. (**D**) GST pull-down assay demonstrating interacting regions between purified GST-fused DNMT1 fragments and *E.coli* expressed full-length 6xHis tagged UHRF1. The input mUHRF1 is included as control. All GST fusions were validated by western blot analysis with anti-GST antibodies (data not shown). After the transfer of the proteins, the blots were stained with ponceau stain (data not shown) to determine the quality of protein transfer.

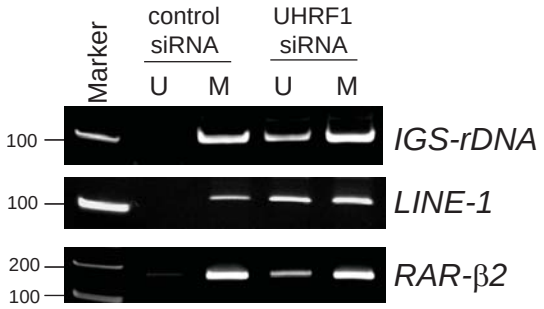

Figure S5. DNA Methylation levels are decreased by UHRF1 knockdown in HeLa cells. Methylation-specific PCR for *IGS-rDNA*, *LINE-1*, and *RAR-β2* after siRNA-mediated knockdown of hUHRF1 in HeLa cells. Unmethylated (U) and methylated (M) gene-specific products are shown. Markers are in basepairs.

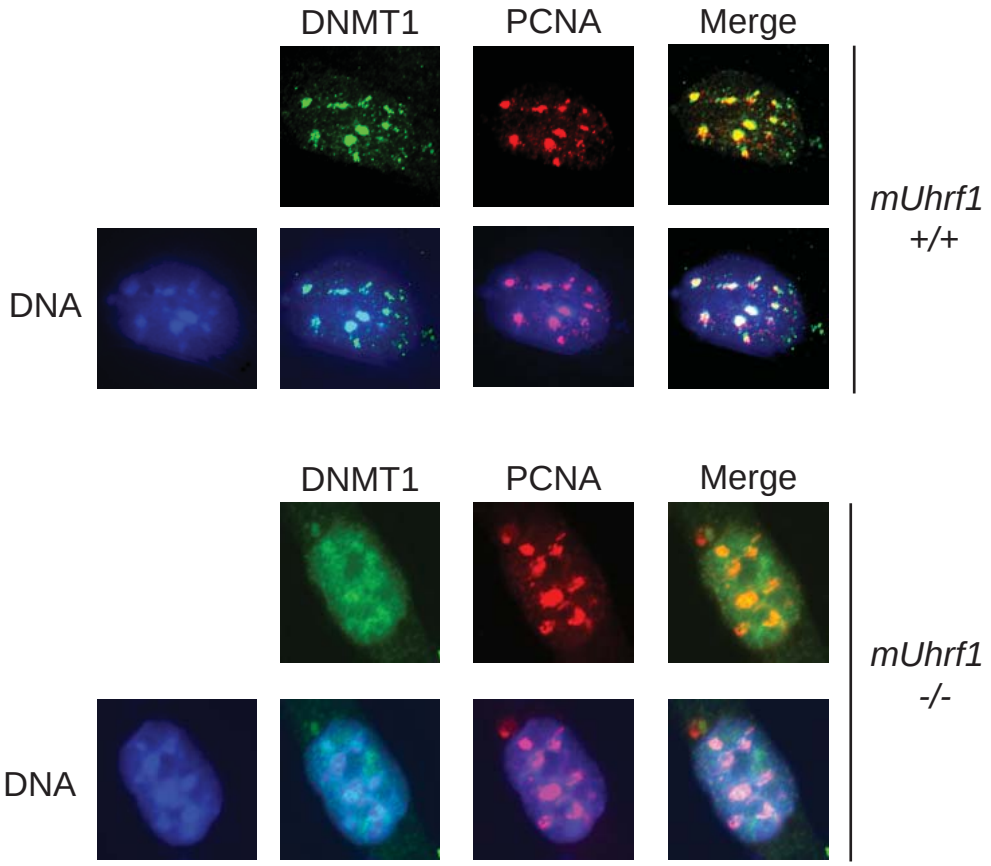

Figure S6. DNMT1 localization is disrupted in *mUHRF1-/-* cells. Immunofluorecence images of wild-type (top panel) or *mUhrf1-/-* (bottom panel) cells. DNMT1 localization was visualized with rabbit anti-DNMT1 antibodies and anti-rabbit AlexaFluor 488. PCNA was visualized with mouse anti-PCNA antibodies and anti-mouse AlexaFluor 594. The nuclear DNA was stained with Hoechst 33342 for visualization.

CG	U	cgcgacgacgcaccacgacgcacgacgcgaacgcgccaa gccgtgctgcgtggtgctcggtgctgcgcttcgcgggtt
	M	mgmgamgamgcamgamgamgcamgamgmgaamgmgmgaa gmgmtgmtgmgtgmtgmtgmgtgmtgmgmttgmgmgmtt
	Hs	mgmgamgamgcamgamgamgcamgamgmgaamgmgmgaa gccgtgctgcgtggtgctcggtgctgcgcttcgcgggtt
	Ha	cgcgacgacgcaccacgacgcacgacgcgaacgcgccaa gmgmtgmtgmgtgmtgmtgmgtgmtgmgmttgmgmgmtt
CHG	U	cagcagacagtcagcagttcagcagacagcagccagcag gtcgtctgtcactcgtcaagtcgtctgtcgtcggtcgtc
	M	magmagamagtmagmagttmagmagamagmagcmagmag gtmgtmtgtmagtmgtmaagtmgtmtgtmgtmggtmgtm
	Hs	cagcagacagtcagcagttcagcagacagcagccagcag gtmgtmtgtmagtmgtmaagtmgtmtgtmgtmggtmgtm
CHH	U	cactcccccactctcccacccactcactccctcccactt gtgaggggggtgagagggtgggtgagtgagggagggtgaa
	M	mamtmcmcmamtmtmcmamcmamtmamtmcmtmcmamtt gtgaggggggtgagagggtgggtgagtgagggagggtgaa

Figure S7. Oligonucleotides used in mobility shift assays. Double-stranded oligonucleotides containing unmethylated (U), fully methylated (M), or hemimethylated DNA with the methyl-cytosine on sense (Hs) or antisense (Ha) strands, in either a CG , CHG, or CHH sequence context (H = A, T, or C). c, cytosine; g, guanosine; a, adenosine; t, thymidine; m, 5-methyl cytosine.

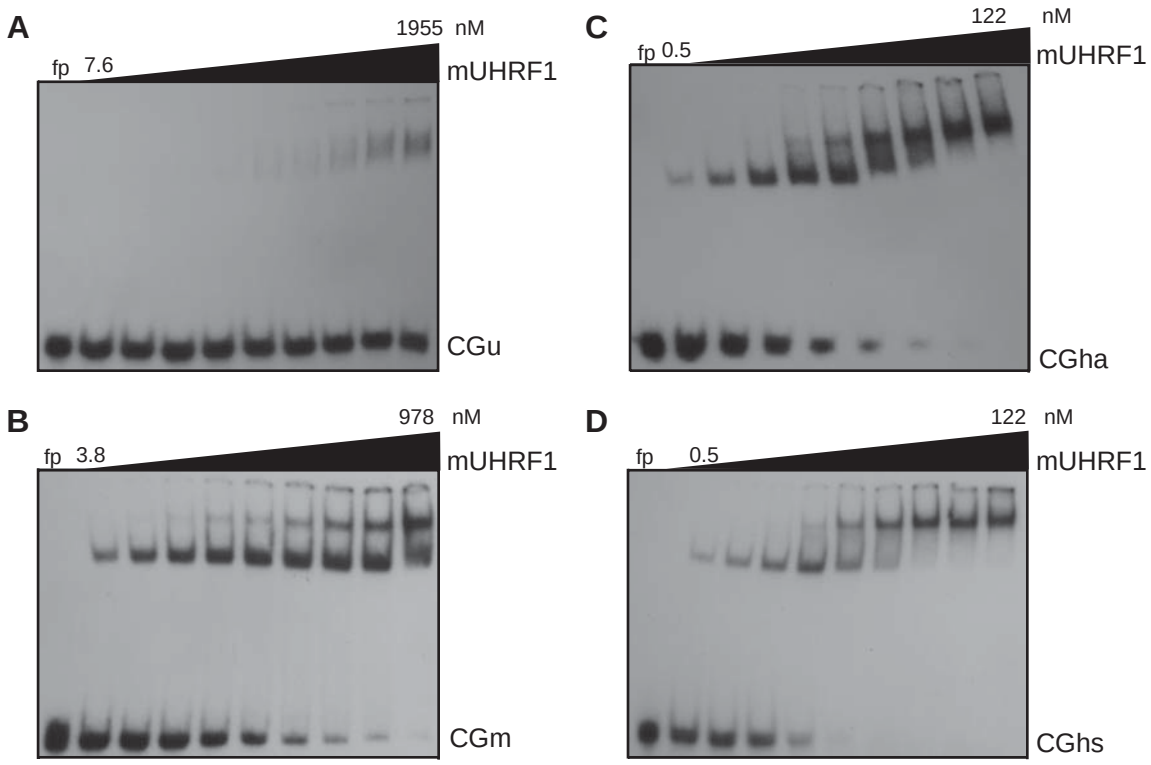

Figure S8. Binding Assays of mUHRF1 utilized for Figure 4B. Electrophoretic mobility shift assays with increasing amounts of GST-mUHRF1 binding unmethylated (CGu, **A**), fully methylated (CGm, **B**), or hemi-methylated (CGha, **C**; CGhs, **D**) double-stranded oligonucleotide, as representative of the data presented in Fig. 4B. Protein concentrations are doubled in each lane, starting at the left, with the lowest and highest amount indicated above the second and last lanes, respectively. fp is free probe.

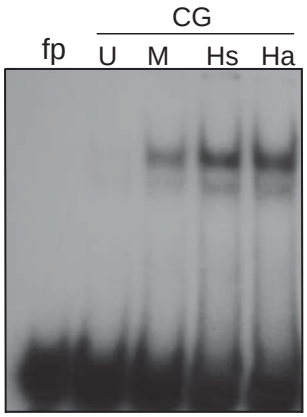

Figure S9. The SRA domain of hUHRF1 prefers hemimethylated DNA. Electrophoretic mobility shift assays with 100 ng of GST-hUHRF1 SRA domain binding to double-stranded oligonucleotides with CG dinucleotides either unmethylated (U), fully methylated (M), or hemimethylated on the sense (Hs) or antisense (Ha) strand. fp is free probe.

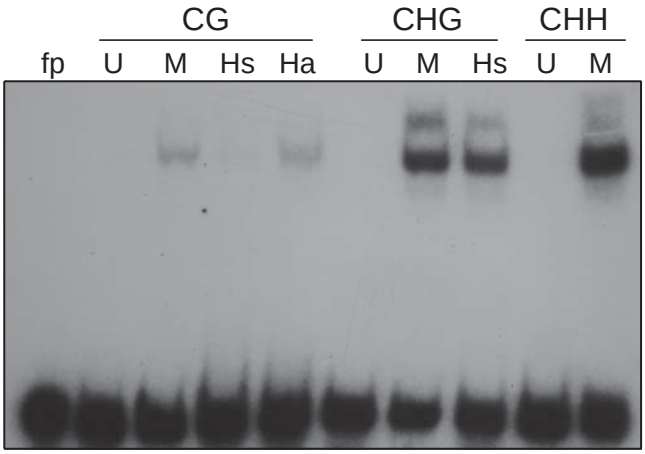

Figure S10. SuvH6 SRA binding specificity is different than mUHRF. Electrophoretic mobility shift assays with GST-SuvH6SRA binding to double-stranded oligonucleotides containing unmethylated (U), fully methylated (M), or hemi-methylated with the methyl-cytosine on the sense (Hs) or antisense (Ha) strand containing CG dinucleotides, CHG or CHH trinucleotides, where H = A, T, or C. fp is free probe.

Supplementary References

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