Supporting Information

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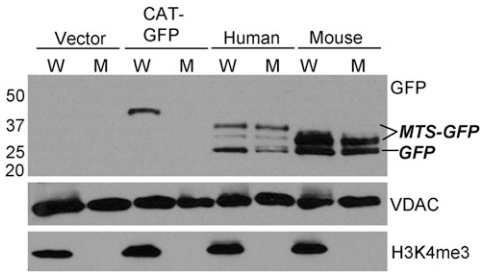

Fig. S1. The N-terminal peptides of mouse and human DNMT1 direct GFP to the mitochondria. Mitochondrial targeting sequences operate across species. Mouse and human N-terminal peptides from ATG1 to immediately upstream of ATG3 were cloned in-frame to GFP and transiently transfected into HCT116 cells. Immunoblot analysis with anti-GFP shows the presence of GFP in the mitochondrial fraction. The upper bands represents intact translation product carrying DNA methyltransferase 1 (DNMT1) leader peptides fused to GFP, whereas the lower bands are the expected molecular mass of GFP after proteolytic cleavage of the targeting peptides. MTS, mitochondrial targeting sequence. VDAC, voltage-dependent anion carrier.

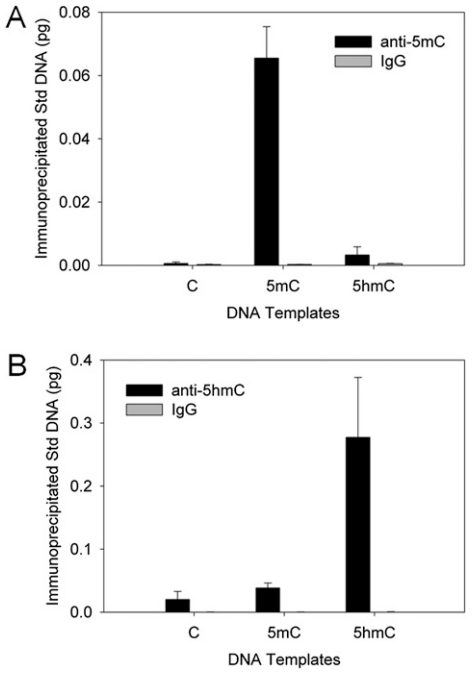

Fig. S2. Antibodies against 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) are specific for their respective modifications. Control DNA samples from the Adenomatous polyposis coli (*APC*) promoter were amplified in PCR reactions containing dCTP, 5m-dCTP, or 5hm-dCTP and tested in immunoprecipitation experiments with antibodies raised against 5mC, 5hmC, and nonspecific IgG in the presence of excess carrier phage λ DNA. Specific immunoprecipitation is shown using quantitative PCR, and data represent mean ± SD for six replicates from two independent experiments.

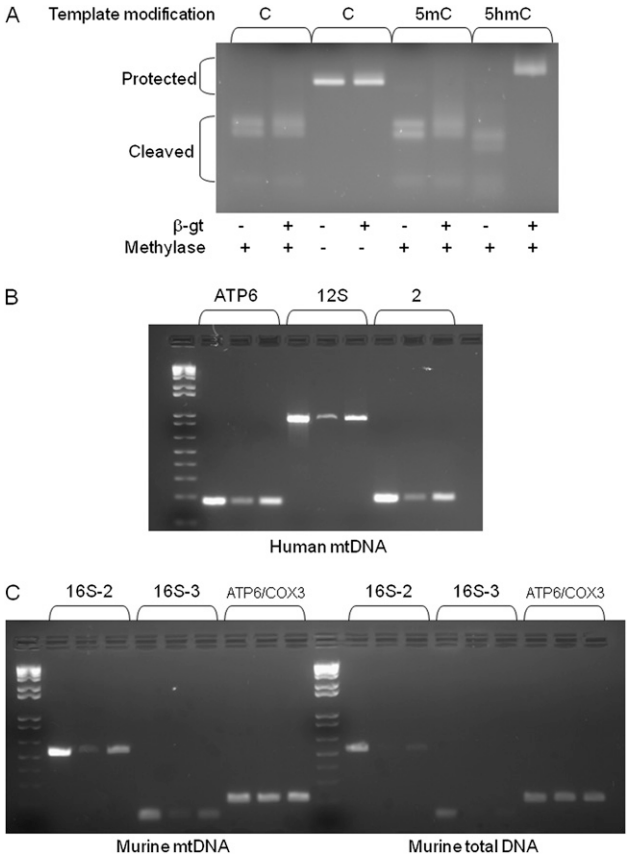

Fig. S3. Site-specific detection of 5hmC at Gla1 restriction sites. (*A*) Control DNAs (described in Fig. S2) were used to validate the specificity of the Quest 5hmC detection assay (Zymo Research). DNA (150 ng) was incubated in the presence or absence of β-glucosyltransferase (β-gt) and methylase mixture (*M.Sss*1 and *M. CviP*1) as indicated, followed by Gla1 cleavage. Products of the reaction were resolved on a 3% NuSieve 3:1 agarose gel (Lonza). Gla1 cleavage was specific for methylated or hydroxymethylated DNA and was prevented by glucosylation of 5hmC (lanes 7 and 8). Complete methylation by the methylase mixture resulted in complete cleavage (lanes 3 and 4). (*B* and *C*) Endpoint PCR demonstrates protection from Gla1 cleavage of 80 ng human mitochondrial DNA (mtDNA) (*B*) and 80 ng mouse mtDNA or total genomic DNA (*C*) following incubation with β-gt and methylase mixture. For each primer set, lane 1 represents input DNA, lane 2 represents minus β-gt control, and lane 3 represents + β-gt protection. Mouse amplicon ATP6/COX3 was expected to contain a single Gla1 site, but showed protection both in the absence and presence of β-gt. DNA sequence analysis verified the presence of an SNP deleting the restriction site (ACGT to ATGT). The data demonstrate that the assay is applicable to both purified mtDNA and total cellular DNA.

Table S1. Conservation of the mitochondrial targeting sequence for DNMT1 and binding sites for p53 and NRF1 in several mammalian species

Species	ATG number	Position upstream of ATG (aa)*	Probability of export to mitochondria	Consensus p53 binding site	Consensus NRF1 binding site
Mouse	ATG1	53	0.92	Y	Y
	ATG2	45	0.96		
	ATG3*	–	0.36		
Human	ATG1	101	0.84	Y	Y
	ATG2	62	0.72		
	ATG3*	–	0.48		
Chimp	ATG1	100	0.92	Y	Y
	ATG2	62	0.70		
	ATG3*	–	0.47		
Rat	ATG1	53	0.90	Y	Y
	ATG2*	–	0.33		
Cow	ATG1	97	0.43	Y	Y
	ATG2	86	0.14		
	ATG3	67	0.14		
	ATG4	33	1.00		
	ATG5*	–	0.97		

The mammalian genome databases were examined for 5′ flanking sequences upstream of *DNMT1*. To date, sequences for this region are only available for mouse, human, rat, chimpanzee, and cow. Y, Yes.
*Published translational start site.

Table S2. Primers used in endpoint and quantitative PCR

Species	Primer set	Forward	Reverse
Amplification of mitochondrial targeting sequences			
Mouse	mMTS	5′-ATGCGCACTCCCTTCGGGCATAG-3′	5′-CTTGCAGGTTGCAGACGACAG-3′
Human	hMTS	5′-ATGAATGAATGCCTCGGGCAC-3′	5′-CTCGGAGGCTTCAGCAGACGC-3′
Quantitation of DNMT1 abundance			
Mouse	mtDNMT1	5′-ACTCTCTTGCCCTGTGTGGTACATG-3′	5′-TCTTTCCAAGTCTTTGAGCCGCC-3′
	Total DNMT1	5′-TCAGAGCTGTTCTGTCGTCTGCAA-3′	5′-TCTTTCCAAGTCTTTGAGCCGCC-3′
	β-Actin	5′-GACCCAGATCATGTTTGAGACC-3′	5′-ATCAGAATGCCTGTGGTACGAC-3′
Human	mtDNMT1	5′-TCCCTGGGCATGGCCGGCTC-3′	5′-CTCTTTCCAAATCTTGAGCCGCC-3′
	Total DNMT1	5′-TCCGAGATGCCGGCGCGTACC-3′	5′-CTCTTTCCAAATCTTGAGCCGCC-3′
	β-Actin	5′-CCACGAAACTACCTTCAACTCC-3′	5′-TCATACTCCTGCTTGCTGATCC-3′
Mitochondrial gene expression			
Mouse	ND1	5′-CAGGATGAGCCTCAAACTCCA-3′	5′-CGGCTCGTAAAGCTCCGA-3′
	ATP6	5′-ATTCCCATCCTCAAAACGCC-3′	5′-TGTTGGAAAGAATGGAGACGGT-3′
	ND6	5′-AGTTATGTTGGAAGGAGGGATTGG-3′	5′-TACCCGCAAACAAAGATCACCCAG-3′
	COX1	5′-TCGCAATTCCTACCGGTGTC-3′	5′-CGTGTAGGGTTGCAAGTCAGC-3′
	18S rRNA	5′-GTCTGTGATGCCCTTAGATG-3′	5′-AGCTTATGACCCGCACTTAC-3′
Mitochondrial immunoprecipitation			
Human	ND1	5′-TGCGAGCAGTAGCCAAACAAT-3′	5′-TGATGGCAGGAGTAATCAGAGG-3′
	ND6	5′-AAACACTCACCAAGACCTCAACCC-3′	5′-ATTGATTGTTAGCGGTGTGGTCGG-3′
	No CpG	5′-CTGGTGATAGCTGGTTGTCCAAGA-3′	5′-CCTAGTGTCTAAAGAGCTGTTCCT-3′
Mitochondrial DNA immunoprecipitation (DIP) (anti-5mC and anti-5hmC)			
Human	ATP6	5′-ATTCAACCAATAGCCCTGGCCG-3′	5′-ACGTAGGCTTGGATTAAGGCGAC-3′
	COX2	5′-ACAGATGCAATTCCCGGACGTC-3′	5′-TGGGCATGAAACTGTGGTTTGCTC-3′
	12S	5′-AGTTCACCCTCTAAATCACCACG-3′	5′-TGACTTGGGTTAATCGTGTGACC-3′
	16S-1	5′-ACCTTACTACCAGACAACCTTAGCC-3′	5′-TAGCTGTTCTTAGGTAGCTCGTCTGG-3′
Quantitative PCR quantitation of 5hmC			
Mouse	16S-2	5′-AAAAGAGGGACAGCTCTTCTGGAACG-3′	5′-TCGTTTAGCCGTTCATGCTAGTCC-3′
	16S-3	5′-CGGTTTCTATCTATTTACGATTTCTCCC-3′	5′-GCCACCCTAATAACCTTCTCTAGG-3′
	ATP6/COX3	5′-CCCACCAACAGCTACCATTAC-3′	5′-CTAGACCTGATGTTAGAAGGAGGG-3′
Human	ATP6	5′-ATTCAACCAATAGCCCTGGCCG-3′	5′-ACGTAGGCTTGGATTAAGGCGAC-3′
	12S	5′-AGTTCACCCTCTAAATCACCACG-3′	5′-TGACTTGGGTTAATCGTGTGACC-3′
	2	5′-GAGACAAGTCGTAACATGGTAAG-3′	5′-GGGTAAGGTTTGCCGAGTTCCT-3′