Supporting Information

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Hypermethylation of hepatic mitochondrial *ND6* provokes systemic insulin resistance

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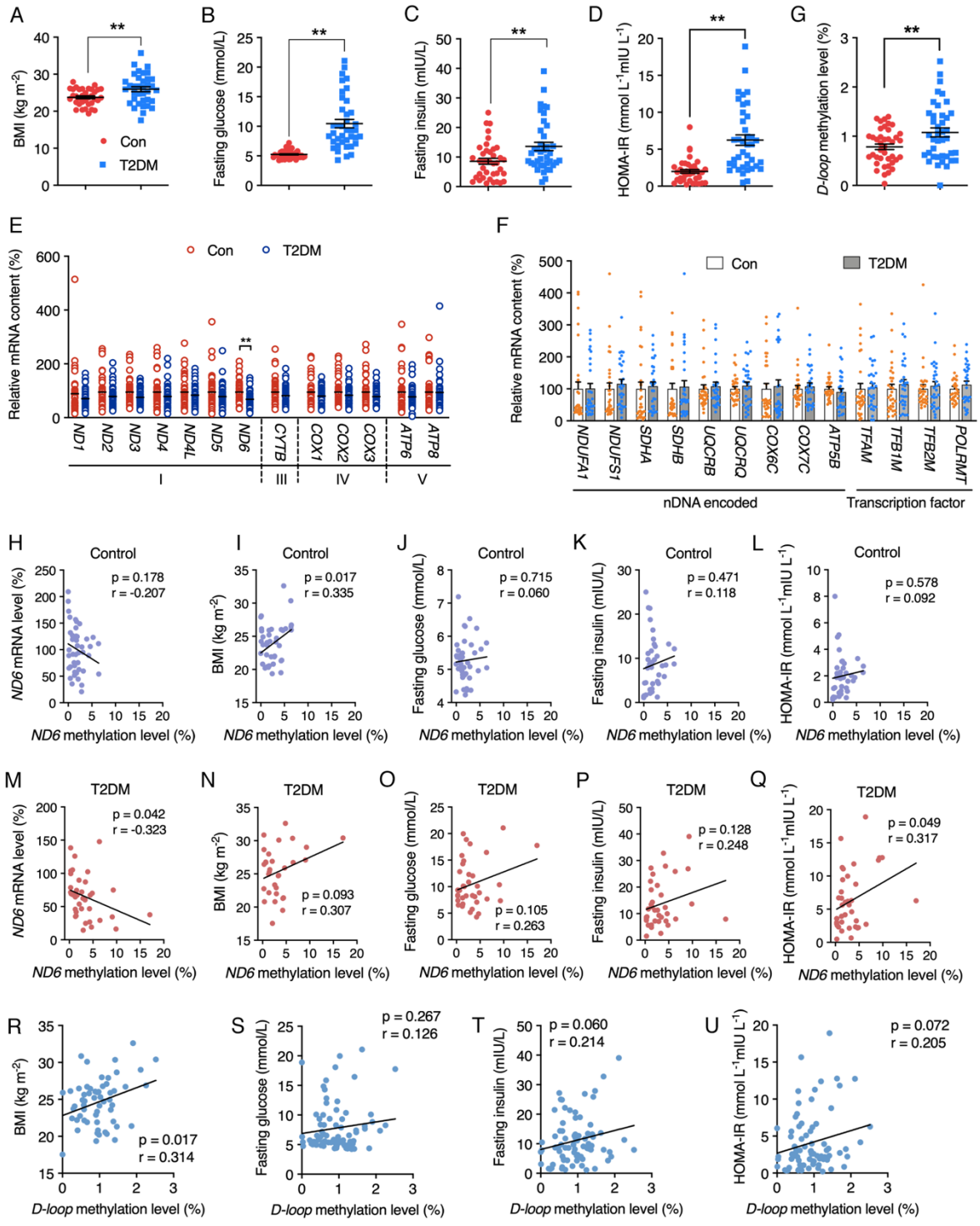

Figure S1 Linear correlations between clinical parameters and *D-loop* methylation level. **A-D,** BMI, fasting glucose level, fasting insulin level, and HOMA-IR index in T2DM and non-T2DM subjects. **E**, Relative mRNA levels of 13 mtDNA-encoded OXPHOS complex subunits; **F**, nDNA-encoded OXPHOS complex subunits and mtDNA-related transcription factors; **G**, *D-loop* methylation level in human peripheral leukocytes of T2DM and non-T2DM subjects, in **A-G**, *n*=39 for each group. **H-L,** Liner correlations between *ND6* mRNA level, BMI, fasting glucose, fasting insulin, HOMA-IR, and ND6 methylation level in human peripheral leukocytes of non-T2DM subjects, *n*=39; **M-Q,** Liner correlations between *ND6* mRNA level, BMI, fasting glucose, fasting insulin, HOMA-IR, and ND6 methylation level in human peripheral leukocytes of T2DM subjects, *n*=39; **R**, Linear correlations between BMI and *D-loop* methylation level; **S**, fasting glucose and *D-loop* methylation level; **T**, fasting insulin and *D-loop* methylation level; **U**, HOMA-IR index and *D-loop* methylation level in human peripheral leukocytes, *n*=78. Values are mean ± SEM. **p< 0.01.

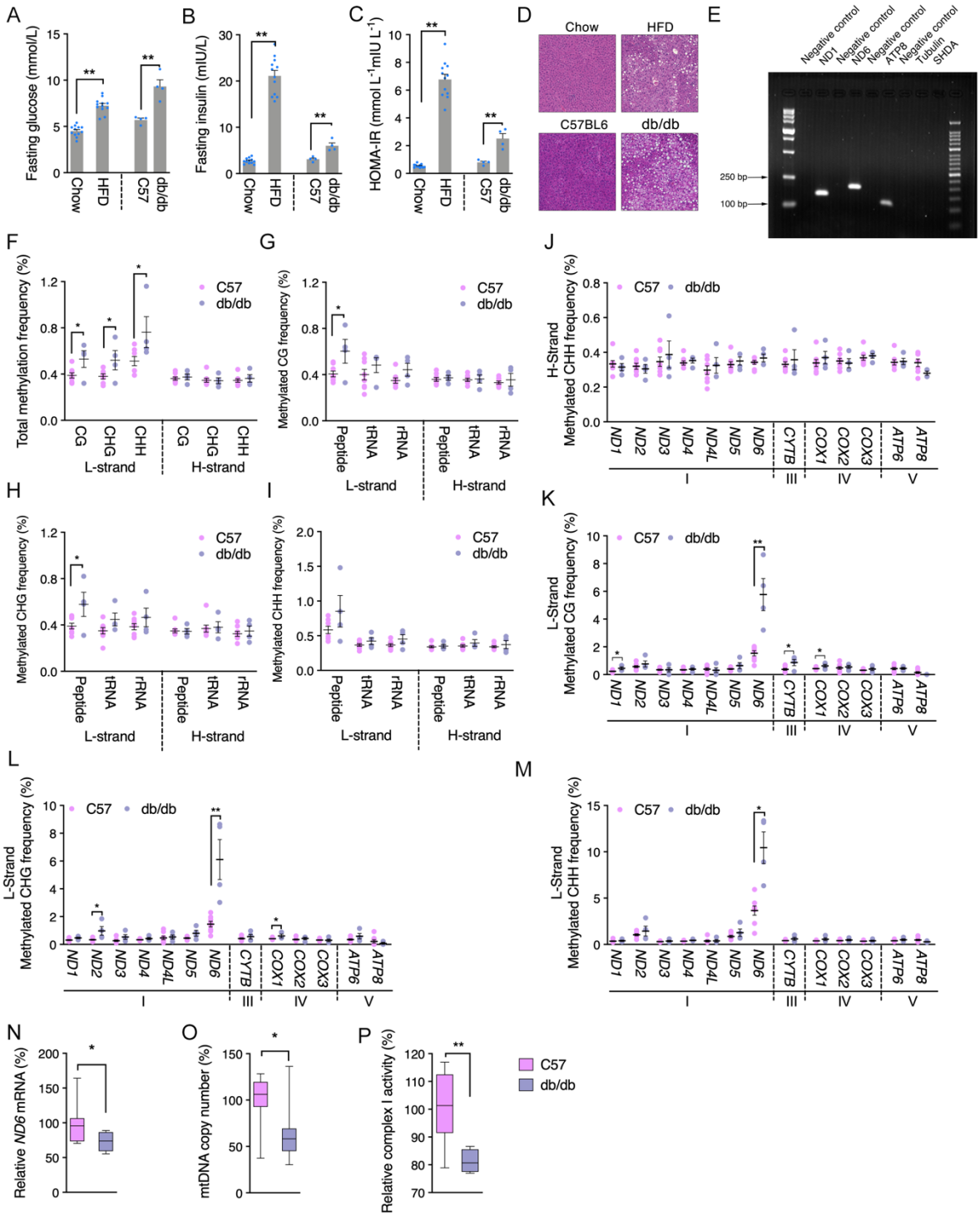

Figure S2 *ND6* is a primary target of mtDNA methylation in the liver of db/db mice. A-C, Fasting glucose, fasting insulin, and HOMA-IR index in HFD and db/db mice. **D**, HE staining of liver tissues. **E**, Confirmation the purity of extracted mtDNA via PCR with specific mitochondrial and nuclear primers. NC1-4, negative control of *ND1*, *ND6*, *ATP8* and *Tubulin*, respectively. Predicted PCR products were *ND1*, 152 bp; *ND6*, 199 bp; *ATP8*, 113 bp; *Tubulin*, 294 bp; *Sdha*, 247 bp. **F**, Total methylated CG, CHG and CHH frequencies; **G-I**, Methylated CG, CHG, and CHH frequencies of peptides, tRNAs and rRNAs on liver mtDNA. **J-M**, Methylated CHH frequencies on H-strand, methylated CHH, CHG, and CG frequencies on L-strand of 13 oxidative phosphorylation (OXPHOS) complex subunits in the liver of control and

db/db mice. **N**, Relative *ND6* mRNA level; **O**, Relative mtDNA copy number; **P,** Relative complex I activity in the liver of the control and db/db mice. Values are mean ± SEM. *n*=7 for the control and 4 for the HFD group, *p< 0.05, **p< 0.01.

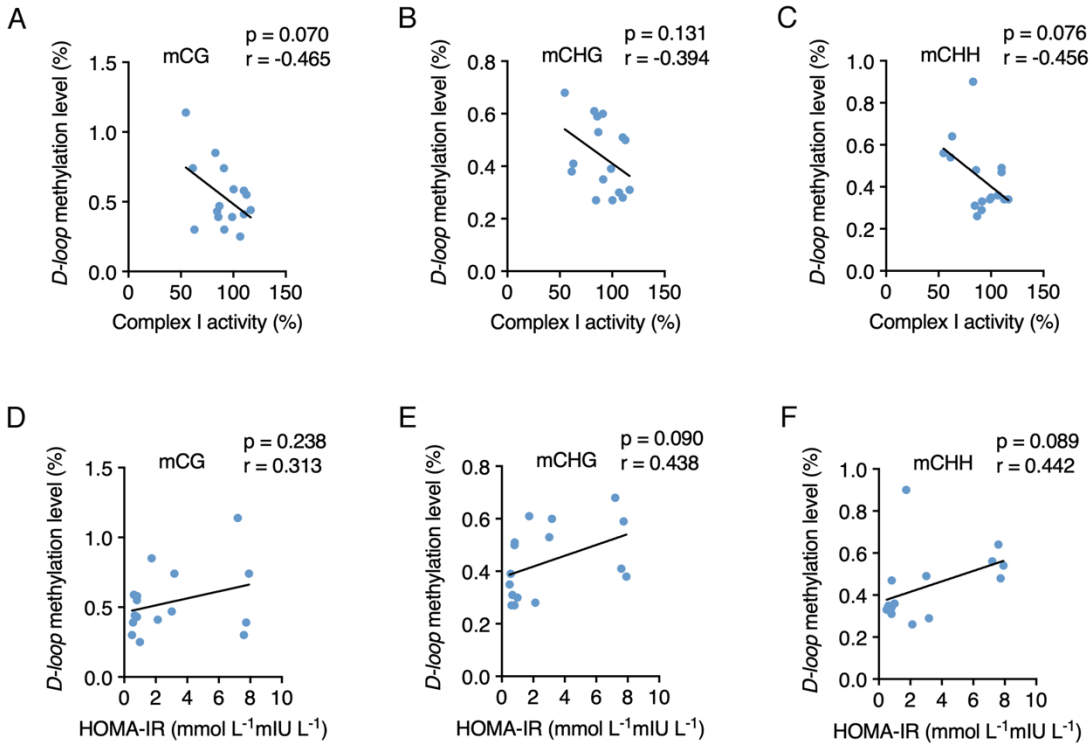

Figure S3 Corralations between D-loop methylation and complex I activity and HOMA-IR. A-C, Liner correlations between *D-Loop* methylated CG, CHG, CHH frequencies and complex I activity in the liver of control and db/db mice; **D-F**, Liner correlations between *D-Loop* methylated CG, CHG, CHH frequencies and HOMA-IR index in the liver of control and db/db mice. N=15.

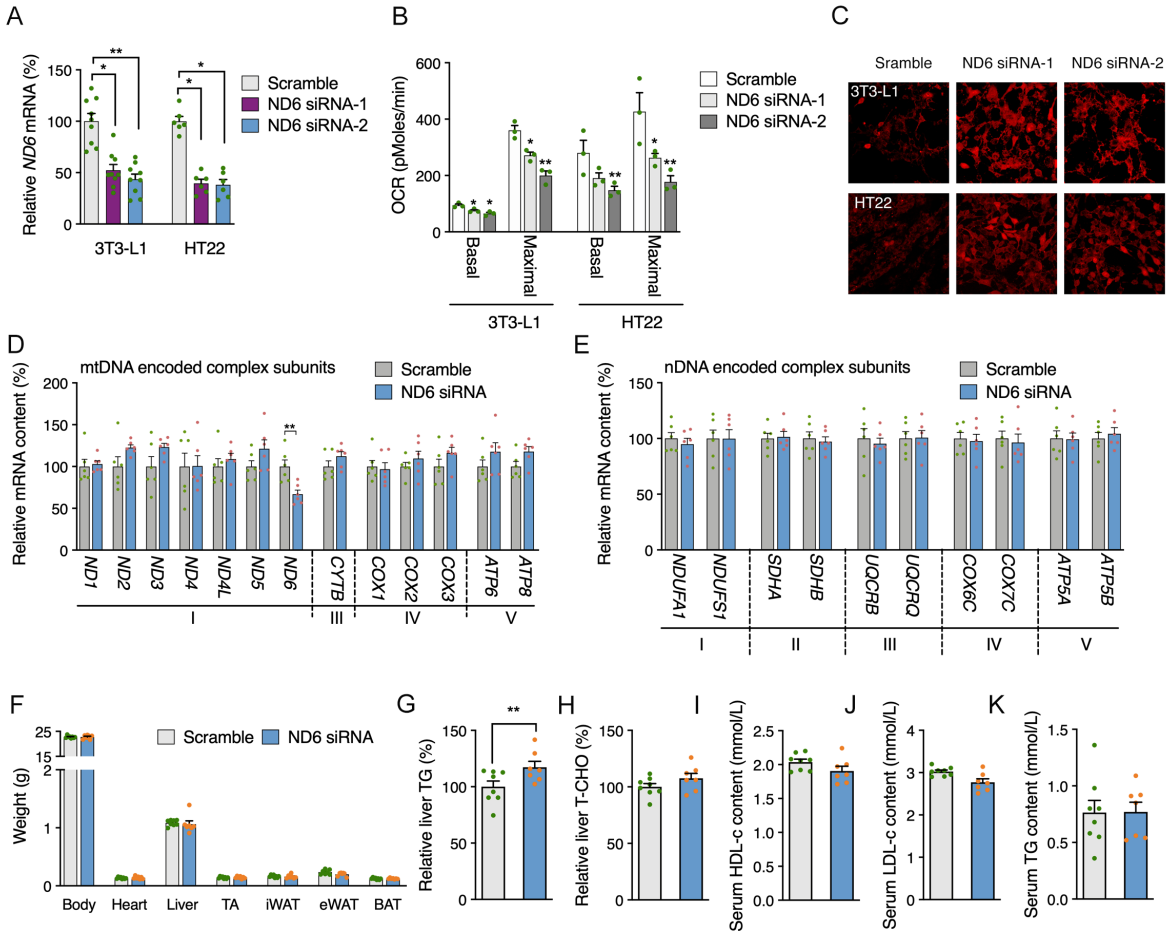

Figure S4 *ND6* knockdown promotes mitochondrial dysfunction in murine cell lines and hepatic lipid accumulation in mice. A, Relative *ND6* mRNA levels; **B**, mitochondrial OCR; **C**, fluorescence of mitochondrial superoxide in 3T3-L1 and HT22 cells transfected with *ND6* siRNA (*n*=3). **D,** Relative mRNA levels of 13 mtDNA-encoded OXPHOS complex subunits in the liver of ND6 siRNA treated mice (*n*=6). **E,** Relative mRNA levels of nDNA-encoded OXPHOS complex subunits in the liver of ND6 siRNA treated mice (*n*=6). **F**, Body and tissue weight; **G**, Relative liver TG; **H**, Relative liver T-CHO; **I**, Serum HDL-c; **J**, serum LDL-c of mice; **K**, Serum TG levels in mice treated with *ND6* siRNA for one week (*n*≥6). Values are mean ± SEM. *p< 0.05, **p< 0.01.

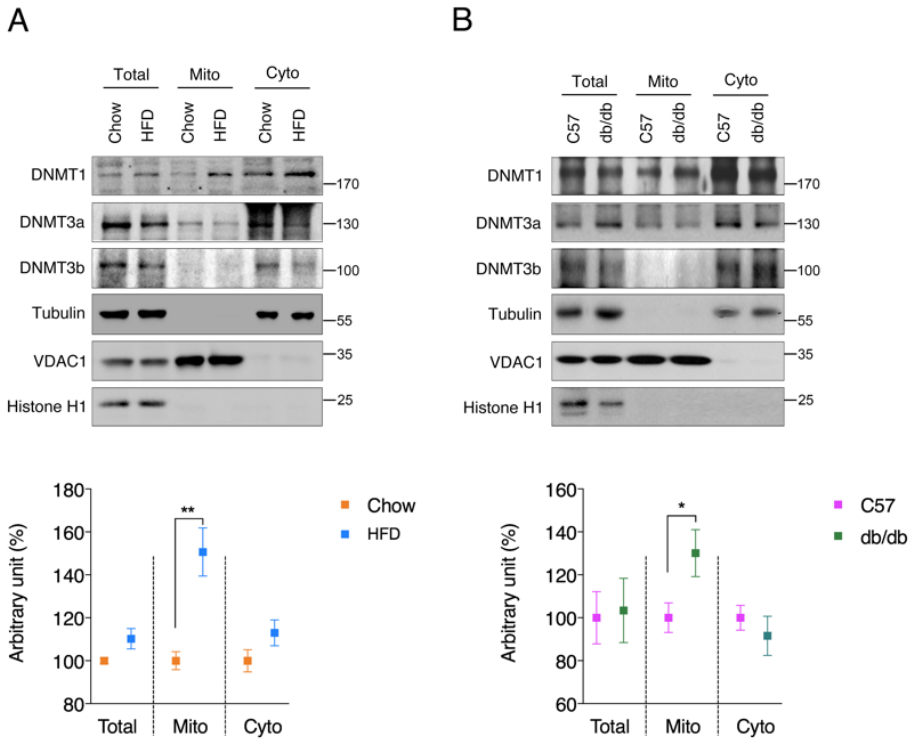

Figure S5 Increased mitochondrial DNMT1 localization in the liver of HFD mice and db/db mice. A-B, Western blot analysis of liver DNMT1, DNMT3a and DNMT3b protein levels in total, mitochondrial and cytosolic fractions of HFD mice and (b) db/db mice. Values are mean ± SEM. *n*=6, *p< 0.05, **p< 0.01.

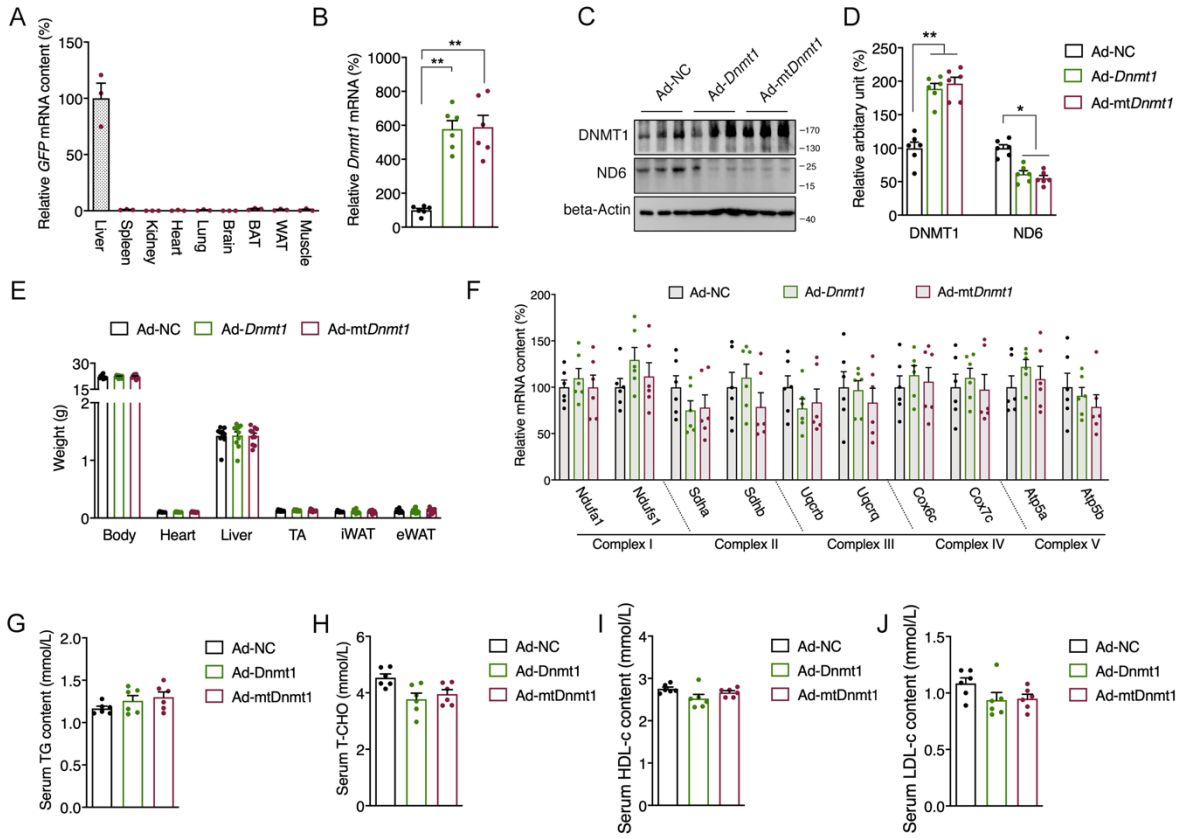

Figure S6 Characterization of mouse adenovirus infection through tail vein injection. A, *GFP* mRNA expression in the tissues of mice infected with pAd/CMV/V5-*GFP* adenovirus via the tail vein injection (*n*=3). **B,** Relative *Dnmt1* mRNA level in the liver of mice infected with pAd/CMV/V5-*Dnmt1*(Ad-*Dnmt1*), pAd/CMV/V5-mt*Dnmt1*(Ad-mt*Dnmt1*) or pAd/CMV/V5-NC (Ad-NC) adenovirus (*n*=6). **C-D,** Protein levels of DNMT1 and ND6 in the liver of mice infected with adenovirus (*n*=6). **E,** Body and tissue weight of mice after *Dnmt1* overexpression (*n*=8). **F,** Relative mRNA level of nDNA-encoded OXPHOS complex subunits (*n*=6). **G,** Serum TG; **H,** Serum T-CHO; **I**, Serum HDL-c; **J**, Serum LDL-c of mice infected with pAd/CMV/V5-*GFP* adenovirus via the tail vein injection (*n*=6). Values are mean ± SEM. *p< 0.05, **p< 0.01.

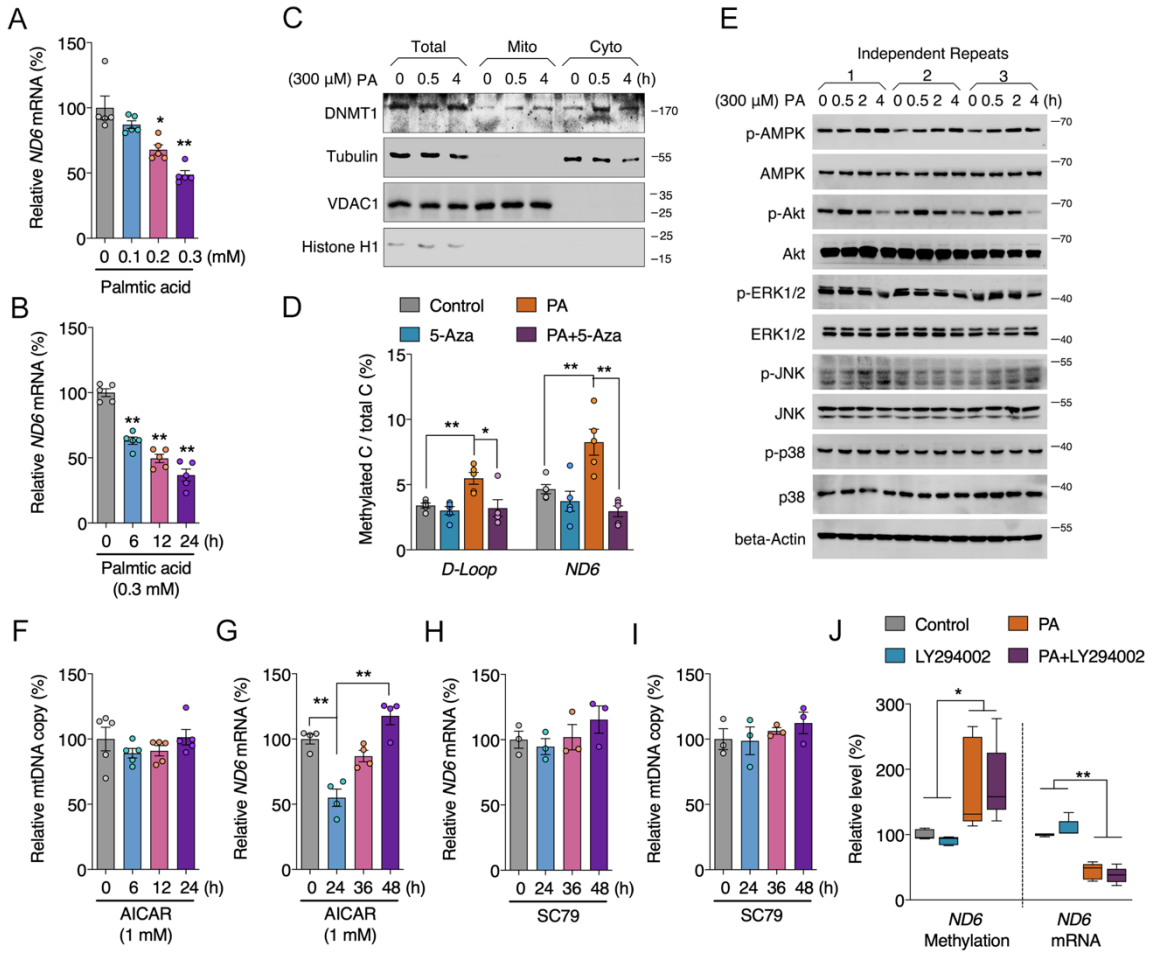

Figure S7 AMPK activation is associated with PA induced *ND6* hypermethylation by DNMT1. A, Relative *ND6* mRNA level in HepG2 cells under dose dependent PA treatment for 24 h (*n*=5). **B,** Relative *ND6* mRNA level in HepG2 cells under time dependent PA treatment at 300 μM (*n*=5). **C,** Western blot analysis of DNMT1 protein subcellular localization in mouse primary hepatocytes under 300 μM PA treatment. **D,** Methylation levels of *D-loop* and *ND6* in PA treated cells with or without 2 μM 5-Aza for 24 h (*n*=5). **E,** Western blot of p-AMPK, AMPK, p-Akt, Akt, p-ERK1/2, ERK1/2, p-JNK, JNK, p-p38 and p38 in HepG2 cells under time dependent PA treatment at 300 μM. **F,** mtDNA copy number in 1 mM AICAR treated cells (*n*=4). **G,** Relative *ND6* mRNA level in cells after prolonged AICAR treatment (*n*=4). **H,** Relative *ND6* mRNA level in HepG2 cells under time dependent 2 μM SC79 treatment (*n*=3). **I,** Relative mtDNA copy number in HepG2 cells under time dependent 2 μM SC79 treatment (*n*=3). **J,** *ND6* methylation and mRNA levels in PA treated cells with or without 10 μM LY294002 for 24 h (*n*=5). Values are mean ± SEM. *p< 0.05, **p< 0.01.

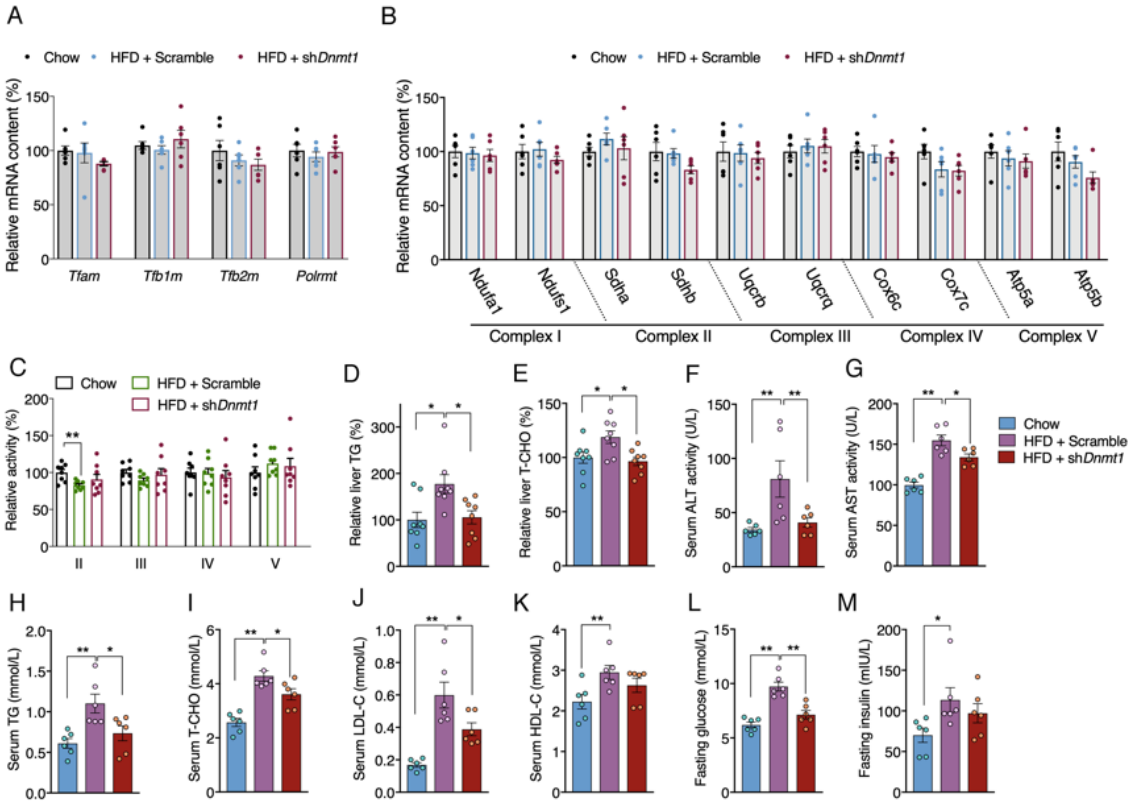

Figure S8 Hepatic knockdown of *Dnmt1* ameliorates metabolic abnormality induced by HFD. A-B, Relative mRNA levels of mtDNA-related transcription factors and nDNA-encoded OXPHOS complex subunits in the liver of mice under diets and AAV intervention (*n*=6). **C,** Relative complex II-V activities in liver (*n*=8). **D-E,** Relative TG and T-CHO level in the liver (*n*=8). **F-K,** Serum ALT activity, AST activity, TG level, T-CHO level, LDL-c level, and HDL-c level in mice (*n*=6). **L-M,** Fasting glucose and fasting insulin level in mice after diets and AAV intervention (*n*=6). Values are mean ± SEM. *p< 0.05, **p< 0.01.

Table S1 Probabilities of DNMT1 isoforms to mitochondria predicted by MitoProt Ⅱ.

Species	Isoform	Length (amino acid)	Probability to mitochondria	Mitochondrial leader peptide
Human	NM_001130823.3 NP_001124295.1 DNMT1 isoform a	1632	47.94%	MPARTAPARV
Human	NM_001318730.2 NP_001305659.1 DNMT1 isoform c	1619	48.33%	MPARTAPARV
Human	NM_001318731.2 NP_001305660.1 DNMT1 isoform d	1511	38.96%	MADANSPPKPLSKPRTPRRS
Human	NM_001379.4 NP_001370.1 DNMT1 isoform b	1616	47.33%	MPARTAPARV
Human	DNMT1 isoform b supplemented with an additional MTS reported by Shock et al. (mtDNMT1)	1717	85.54%	MNECLGHRTHLPANRGAWTS PLLRVGGVCARLAHACSLGM AGSVPSFCTGYRLSPFGTSPPP PRPDWGGRRRLRSSPLPIGFRA KSRG
Human	NM_003201.3 NP_003192.1 TFAM isoform 1 precursor	246	92.26%	MAFLRSMWGVLSALGRSGAE LCTGCGSRLRSPFSFVYLPRWF SSVLASC
Mouse	NM_001199431.1 NP_001186360.2 Dnmt1 isoform 1	1620	36.13%	MPARTAPARV
Mouse	NM_001199432.1 NP_001186361.1 Dnmt1 isoform 3	1501	72.45%	MADSNRSPRSRPKPRGPRRS
Mouse	NM_001199433.1 NP_001186362.1 Dnmt1 isoform 4	1502	72.45%	MADSNRSPRSRPKPRGPRRS
Mouse	NM_001314011.1 NP_001300940.1 Dnmt1 isoform 5	1627	36.42%	MPARTAPARV
Mouse	NM_010066.4 NP_034196.5 Dnmt1 isoform 2	1619	36.13%	MPARTAPARV
Mouse	Dnmt1 isoform 1 supplemented with an additional MTS reported by Shock et al. (mtDnmt1)	1673	91.70%	MRTPFGHSMVFPHSLALCGT CCFRLRRPLPIGFRAREKAGV SFRAVLSSATCKMPARTAPARV

Table S2 Primers used in methylation-specific PCR.

Species	Region	Primer (5'-3')
Human	*D-loop-M*	Forward: CGTTTTTTTTAAATAAGATATTACGA
		Reverse: AAAAATCAAAAACAAATACTACGAC
Human	*D-loop-U*	Forward: TGTTTTTTTTAAATAAGATATTATGA
		Reverse: AAAAATCAAAAACAAATACTACAAC

Human	*ND6-M*	Forward: TTTCGTATTAATAGGATTTTTTCGA Reverse: AATTATCTTTAAATATACTACAACGAT
Human	*ND6-U*	Forward: TTTTGTATTAATAGGATTTTTTTGA Reverse: ATAATTATCTTTAAATATACTACAACAAT
Mouse	*D-loop-M*	Forward: GTTTTTTTTAAATAAGATATTTCGA Reverse: TACTATCCTTTCATACCTTAACGAC
Mouse	*D-loop-U*	Forward: GTTTTTTTTAAATAAGATATTTTGA Reverse: TACTATCCTTTCATACCTTAACAAC
Mouse	*ND6-M*	Forward: GATATTTTTTAGTAGTTATAGTAGTCGT Reverse: TAATTTTAAAATTTAATAAATCGTT
Mouse	*ND6-U*	Forward: TGGATATTTTTTAGTAGTTATAGTAGTTGT Reverse: TAATAATTTTAAAATTTAATAAATCATT

Table S3 Primers used in mtDIP.

Species	Region	Primer (5'-3')
Human	*D-loop*	Forward: TGCTTGTAAGCATGGGGAGG Reverse: ACATTACTGCCAGCCACCAT
Human	*ND6*	Forward: GGGGTTTGTGGGGTTTTCTTC Reverse: ACCCATATAACCTCCCCCAA

Table S4 Primers used in real-time PCR.

Species	Gene	Primer (5'-3')
Mouse	*ND6*	Forward: CTACCCCAATCCCTCCTT Reverse: GGTTTGGTGGATCGTTTT
Mouse	*Dnmt1*	Forward: GAGGCTGATGATGATGTGTC Reverse: GGAATCATCTGGAATGACCG
Mouse	*Ndufa1*	Forward: ATGTGGTTCGAGATTCTCCC Reverse: GCAACTCGTTTTTCCTTGC
Mouse	*Ndufs1*	Forward: ATCCCATAGGTCAGACAAGC Reverse: CAGTTGTGCGAACATATCCT
Mouse	*Sdha*	Forward: AAGATTACAAAGTGCGGGTC Reverse: TTTCATCAGTAGGAGCGGAT
Mouse	*Sdhb*	Forward: GTTCTCGGCAGAGTCGG

		Reverse: TCCGCACTTATTCAGATCCA
Mouse	*Uqcrb*	Forward: CACTCTCAGGTCAAAATGGC
		Reverse: ATCTCGCATTAACCCCAGT
Mouse	*Uqcrq*	Forward: GCGTCTATCTTCTGTCCCA
		Reverse: ACCACTACAAACGGCGG
Mouse	*Cox6c*	Forward: CATTGGCTACCATGAGTTCC
		Reverse: CAGCCACGCCAAACTTATAG
Mouse	*Cox7c*	Forward: GAGCAAGGTCGTGTAGAAAG
		Reverse: GAAAATGGCAAATTCTTCCCC
Mouse	*Atp5a1*	Forward: CATTTTGTGCCAGTCGTCC
		Reverse: ATTTTTGGAGACCAGTCCCG
Mouse	*Atp5b1*	Forward: AATTCCTGTTGGTCCTGAGA
		Reverse: CCAAAGAGTCCGATTTTCCC
Mouse	*Tfam*	Forward: AGACTACACTGGGAAACCAC
		Reverse: GTTAGGCTTATAGGGACCCA
Mouse	*Tfb1m*	Forward: CCCAAACCAAAAGTTGATGT
		Reverse: TTTCCTTCGAAACTGAAACG
Mouse	*Tfb2m*	Forward: GGCATTACTTAAAGCTGGTG
		Reverse: TTTATAGGAACACCTGCTGAC
Mouse	*Polrmt*	Forward: TTCCCTCAGGAGTTTGTCTG
		Reverse: TGATAGGGCTGTATGATGGG
Mouse	*18S rRNA*	Forward: GTAACCCGTTGAACCCCATT
		Reverse: CCATCCAATCGGTAGTAGCG
Mouse	*D-loop* (for DNA)	Forward: AGGCATGAAAGGACAGCA
		Reverse: TTGGCATTAAGAGGAGGG
Mouse	*18S rRNA* (for DNA)	Forward: GAGAAACGGCTACCACATCC
		Reverse: CACCAGACTTGCCCTCCA
Human	*ND1*	Forward: TTCGCCCTATTCTTCATAGC
		Reverse: GGAGGTTAGAAGTAGGGTCT
Human	*ND2*	Forward: ACACCCTTAATTCCATCCAC
		Reverse: GAGATAGGTAGGAGTAGCGT
Human	*ND3*	Forward: AGAAAAATCCACCCCTTACG
		Reverse: CATGGTAGGGGTAAAAGGAG
Human	*ND4*	Forward: CTCTCTGTGCTAGTAACCAC

		Reverse: ATGGGGGATAGGTGTATGAA
Human	*ND4L*	Forward: CACACCTCATATCCTCCCTA
		Reverse: GCATTGGAGTAGGTTTAGGT
Human	*ND5*	Forward: CCTATTCGCAGGATTTCTCA
		Reverse: AATGTGCATAGTGGGGATTT
Human	*ND6*	Forward: CAACCATCATTCCCCCTAAA
		Reverse: GTGGGTTTAGTAATGGGGTT
Human	*CYTB*	Forward: AAACTTACTATCCGCCATCC
		Reverse: GTAAGGGTGGAAGGTGATTT
Human	*COX1*	Forward: CCTAATCACAGCAGTCCTAC
		Reverse: ATGGGAGATTATTCCGAAGC
Human	*COX2*	Forward: AAACCGTCTGAACTATCCTG
		Reverse: AATGGGGGAAGTATGTAGGA
Human	*COX3*	Forward: CGCTAAATCCCCTAGAAGTC
		Reverse: AGCCAATAATGACGTGAAGT
Human	*ATP6*	Forward: GATCCCCACCTCCAAATATC
		Reverse: TAAGAGATCAGGTTCGTCCT
Human	*ATP8*	Forward: AACACAAACTACCACCTACC
		Reverse: CAATGAATGAAGCGAACAGA
Human	*NDUFA1*	Forward: ACCCAGAAGTAGGGTTTTGG
		Reverse: TTCTCCAAACCCTTTGACAC
Human	*NDUFS1*	Forward: CAAGGATTCTAGTCCCTCCG
		Reverse: CTTAACATATTGCTTCTCCCCG
Human	*SDHA*	Forward: AACATGGAGGAGGACAACTG
		Reverse: CATATCGCAGAGACCTTCCA
Human	*SDHB*	Forward: TTGTTCCCGATTTGAGCAAC
		Reverse: TCACGCTCTTCTATGGACTG
Human	*UQCRB*	Forward: TGGTCAAAATGGCTGGTAAG
		Reverse: CTCGCATTAACCCCAGTTTA
Human	*UQCRQ*	Forward: TCAGCTACAGCTTGTCACC
		Reverse: AAACACTACAAACTGCGGC
Human	*COX6C*	Forward: GCTTTGTATAAGTTTCGTGTGG
		Reverse: AACTGAGAAAGACTTACTGTCC
Human	*COX7C*	Forward: GATCTGCATTTGCTACACCC

		Reverse: GGCTGCACCTCTTAAAATGC
Human	*ATP5B1*	Forward: CCTGTTGGTCCTGAGACTTT
		Reverse: CCAAAAAGCCCAATTTTGCC
Human	*TFAM*	Forward: CCAAGAAGCTAAGGGTGATT
		Reverse: TGTTTCTTTATTGTGCGACG
Human	*TFB1M*	Forward: AAGTTGTTAAGACTGCAAGC
		Reverse: TTTCAACCACCAGAAGTTCA
Human	*TFB2M*	Forward: TGGATGGAAAACTACGAGTG
		Reverse: GGAACTGCTTCTATTCCCAA
Human	*POLRMT*	Forward: AAGGTCAAGCAAATAGGAGG
		Reverse: GTCAGGCCCTTCCTGTA
Human	*18S rRNA*	Forward: GTAACCCGTTGAACCCCATT
		Reverse: CCATCCAATCGGTAGTAGCG
Human	*D-loop* (for DNA)	Forward: CAGTACCTAACAAACCCACA
		Reverse: GAGGTCGTAAACCCTATTGT
Human	*18S rRNA* (for DNA)	Forward: TGGTGAGCTGCGAGAATAG
		Reverse: TTTTATGGTAATAACGCGGC