Supplemental Figure. 1. Human TRMT1 and TRMT1L contain subcellular localization signals. (A) Prediction of bipartite nuclear localization signals in TRMT1 and TRMT1L using cNLS Mapper (<u>http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi</u>). (B) Prediction of a mitochondrial targeting signal and cleavage sites in TRMT1 using (B) MitoFates (<u>http://mitf.cbrc.jp/MitoFates/cgi-bin/top.cgi</u>) or (C) TPpred2 (<u>http://tppred2.biocomp.unibo.it/tppred2</u>). Proteolytic cleavage sites in TRMT1 for mitochondria processing peptidase (MPP) and intermediate cleavage peptidase 55 (Icp55) were predicted using MitoFates. For TPpred2, two mitochondrial cleavage sites are predicted as defined: R3a = RX [FLY]j[SA] and R3b = RX[FLY]jX. (D) TRMT1 tagged with GFP was transiently expressed in HeLa cells with mitochondrial staining using Chromeo live cell stain and nuclear staining with DAPI.

A TRMT1

MQGSSLWLSLTFRSARVLSRARFFEWQSPGLPNTAAMENGTGPYGEERPREVQETTVTEGAAKIAFPSAN EVFVNPVQEFNBDLTCAVITBFARIQLGAKGIQIKVPGEKDTQKVVVDLSEQEEKVELKESSNLASGDQ PRTAAVGEICEEGLHVLEGLAASGLRSIRFALEVPGLRSVVANDASTRAVDLIRRNVQLNDVAHLVQPSQ ADARMLMYQHQRVSERFDVIDLDPYGSPATFLDAAVQAVSEGGLLCVTCTDMAVLAGNSGETCYSKYGAM ALKSRACHEMALRIVLHSLDLRANCYQRPVVPLLSISADFYVRVFTQQAKVKASASKQALVFQCVG CGAFHLQRLGKASGVPSGRAKFSAACGPPVTPECEHCGQRHQLGGPMWAEPIHDLDFVGRVLEAVSANPG RFHTSERIRGVLSVITTELPDVPLYTLDQLSSTIHCNTPSLLQLRSALHADFRVSLSHACKNAVKTDA PASALWDIMRCWEKECPVKRERLSETSPAFRILSVEPRLQANFTIREDANPSSRQRGLKRFQANPEANWG PRPRARPGGKAADEAMEERRLLQNKKKEPPEDVAQRAARLKTFPCKRFKEGTCQRGDQCCYSHSPPTPR VSADAAPDCPETSNQTPFGFGAAAGFGID

| Predicted bipartite NLS | Score |
|-------------------------------------|-------|
| RRRLLQNKRKEPPEDVAQRAARLKTFPCKRFKE | 5.5 |
| RRRLLQNKRKEPPEDVAQRAARLKTFPCKRFKEGT | 5.3 |
| RLKTFPCKRFKEGTCQRGDQCCYSHSPPTPRVSAD | 5.2 |

TRMT1L

MENMAEEELLPLEKEEVEVAQVQVPTPARDSAGVPAPAPDSALDSAPTPASAPAPAPALAQAPALSPSL ASAPEEAKSKRHISIQRQLADLENLAFVTDGNFDSASSLNSDNLDAGNRQACPLCPKEKFRACNSHKLR RHLQNLHWKVSVEFEGYRMCICHLPCRPVKPNIIGEQITSKMGAHYHCIICSATITRRTDMLGHVRHM NKGETKSSYIAASTAKPPKEILKEADTDVQVCPNYSIPQKTDSYFNFKMKLNRQLIFCTLAALAEERKP LECLDAFGATGIMGLQWAKHLGNAVKVTINDLNENSVTLIQENCHLNKLKVVVDSKEKEKSDDILEEGE KNLGNIKVTKMDANVLMHLRSFDFIHLDPFGTSVNYLDSAFRNIRNLGIVSVTSTDISSLYAKAQHVAR RHYGCNIVRTEYYKELAARIVVAAVARAAARCNKGIEVLFAVALEHFVLVVVRVLRGPTSADETAKKIQ YLIHCQWCEERIFQKDGNMVEENPYRQLPCNCHGSMPGKTAIELGPLWSSSLFNTGFLKRMLFESLHHG LDDIQTLIKTLIFESECTPQSQFSIHASSNVNKQEENGVFIKTTDDTTTDNYIAQGKRKSNEMITNLGK KQKTDVSTHPPFYNINHRHSIGMNMPKLKKFLCYLSQAGFRVSRTHFDPMGVRTDAPLMQFKSILLK YSTPTYTGGQSESHVQSASEDTVTERVEMS

VNDKAEASGCRRW

Targeting peptide

| Predicted | bipartite | NLS | Score | |
|------------------------|-----------|-----|-------|--|
| IAQGKRKSNEMITNLGKKQKTD | | | | |

B TRMT1

MPP Max charged amphiphilicity

MQGSSLWLSLTFRSARVLSRARFFEWQSPGLPNTAAMENGTGPYGEERPREVQETTVTEGAAKIAFPSANEV

 β σ φ β φ φ
 ΦχβΦφ

 Reduced letters composing statistically
 TOM20 recognition motif

 significant 6mer in presequence
 Φ (hydrophobic), β (basic), σ (polar)

C TRMT1

Mature protein

MQGSSLWLSLTFRSARVLSRARFFEWQSPGLPNTAAMENGTGPYGEERPREVQETVTEGAAKIAFPSANEV



Supplemental Figure 2. PHA assay on mitochondrial tRNAs. An increase in signal for the D-AC PHA probe was detected for mitochondrial tRNA-IIe-GAU isolated from TRMT1-KO cells. No change in PHA signal was detected for any of the other mitochondrial tRNAs containing G at position 26.



Supplemental Figure 3. *De novo* protein synthesis assays via puromycin labeling. (A) Nascent polypeptide chains labeled with puromycin from control-WT or TRMT1-KO cell lines were fractionated and detected by immunoblotting. Total protein serves as loading control. (B) Quantification of relative *de novo* protein synthesis as measured by the accumulation of puromycin-labeled polypeptides. The mean and standard deviation represents the puromycin signal of each cell line relative to control-WT after normalization to total protein from three independent labeling experiments. (C) Cells were incubated with the indicated concentration of puromycin in the absence or presence of cycloheximide (50 μ g/mL) to block protein synthesis followed by harvesting and immunoblotting. (*) denotes a loading control protein that was probed simultaneously with the puromycin-labeled polypeptides.



Supplemental Figure 4. TRMT1-KO cell lines exhibit defects in ROS homeostasis without major changes in mitochondrial membrane potential or mitochondrial ROS. (A) Flow cytometry scatter plots measuring mitochondrial potential using TMRE. The percentage of depolarized/dead, dead, depolarized/live and polarized/live cells (green quadrant) are denoted. As a control, cells were treated with FCCP to dissipate the mitochondrial membrane potential assays. Plotted are the live cells with polarized mitochondria (green quadrant of scatter plot). (C) Flow cytometry histogram plots of the indicated cell lines stained with the mitochondrial ROS detection stain, MitoSox Red with relative quantification shown in (D). (E) Flow cytometry histogram plots of the indicated cell lines stained with the standard deviation of three independent experiments. (*) p < 0.05; (**) p < 0.01.



Supplemental Figure 5. Analysis of TRMT1 function in oxidative stress survival. (A, B) Flow cytometry scatter plots of propidium iodide-positive (dead cells) versus nucleated (total) cells after mock treatment (A) or exposure to *t*-bu-OOH (B). (C) The indicated cell lines were transfected with either empty vector or TRMT1 expression constructs followed by splitting into 6-well plates at 24-hours post-transfection. At 48-hours post-transfection, cells from one well of each transfection was harvested for extract preparation and immunoblot analysis while other wells were treated with *t*-buOOH (see Fig. 6). Error bars for (A) represent the standard deviation of three independent experiments. (**) p < 0.01; (***) p < 0.001; (***) p < 0.001.



| TRMT1 cloning | Sequence (5'-3') |
|-----------------------|---|
| TRMT1 HindIII F | GAACT AAGCTT ATGCAAGGATCGTCTCTGTGGCTAA |
| TRMT1 NotI R | CATGA GCGGCCGC TCAGTCTATGCCTGGCCCAGC |
| TRMT1 KpnI F | GAACT GGTACC ATGCAAGGATCGTCTCTGTGGCTAA |
| TRMT1 NotI R nostop | CATGA GCGGCCGC GTCTATGCCTGGCCCAGCGG |
| TRMT1 AMTS HindIII F | GAACT AAGCTT ATGGAGAACGGCACCGGGCC |
| TRMT1 AMTS KpnI F | GAACTGGTACCATGGAGAACGGCACCGGGCC |
| silent gs1 frag1 fwd | ccaagctggctagcgtttaaacttaagcttATGCAAGGATCGTCTCTG |
| silent gs1 frag1 rev | aattettggaetggattgTAAAAGACCTCGTTGGCAC |
| silent gs1 frag2 fwd | acgaggtcttttacaatccagtccaagAATTCAATCGGGACCTGACATG |
| silent gs1 frag2 rev | ttaaacgggccctctagactcgagcggccgcTCAGTCTATGCCTGGCCC |
| C348R frag1 fwd | ccaagctggctagcgtttaaacttaagcttATGCAAGGATCGTCTCTGTG |
| C348R frag1 rev | ccgcagcccacacgCTGGAACACCAGCGCCTG |
| C348R frag2 fwd | gctggtgttccagcGTGTGGGCTGCGGGGCCT |
| C348R frag2 rev | ttaaacgggccctctagactcgagcggccgcTCAGTCTATGCCTGGCCCAGCG |
| TRTM1 mut F1(Q219) | gccagcgggagcccaagcttATGCAAGGATCGTCTCTG |
| TRTM1 mut R1(Q219) | cagatcgaTGGTACATCAGCATCCGG |
| TRTM1 mut F2(Q219) | gatgtaccaTCGATCTGGACCCCTATG |
| TRTM1 mut R2(Q219) | cctctagactcgagcggccgcTCAGTCTATGCCTGGCCC |
| Y445L fs frag1 fwd | caccctcagggccagcgggagcccaagcttATGCAAGGATCGTCTCTGTGGC |
| Y445L fs frag1 rev | tccagggtgtagtAGAGGCACGTCCGGGAGC |
| Y445L fs frag2 fwd | cggacgtgcctctACTACACCCTGGACCAGC |
| Y445L fs frag2 rev | ttaaacgggccctctagactcgagcggccgcTCAGTCTATGCCTGGCCC |
| CRISPR mutagenesis | |
| TRMT1 gs F1 | CACCGGGTCTTTTATAACCCGGTGC |
| TRMT1 gs R1 | AAACGCACCGGGTTATAAAAGACCC |
| TRMT1 gs F3 | CACCGCGTGGACGTTCTTCTCCGTA |
| TRMT1 gs R3 | AAACTACGGAGAAGAACGTCCACGC |
| TRMT1-gPCR-HIII-F2 | GAACT AAGCTT agtcatccccaaaacgaggg |
| TRMT1-gPCR-BHI-R6 | CGC GGATCC cccaggcagggagataaactt |
| T-loop (nuclear encod | led tRNAs) |
| Ala-AGC-G57 | TGGAGAATGYGGGCGTCGATCCC |
| Arg-ACG-G59 | GAGCCAGCCAGGAGTCGAACCT |
| Asn-GTT-G56 | CGCTaACCGATTGCGCCACAGAGAC |
| Gly-CCC | TGATACCACTACACCAGCGGCGC |
| Leu-CAA | TGTCAGAAGTGGGATTCGAACCCACGC |
| Met-CAT | AACTCACGACCTTCAGATTATG |
| Phe-GAA | GGGATCGAACCAGGGACCTTTAGATC |
| Ser-AGA | GCGCGGGGAGACCCCAATGGATT |
| Thr-CGT | AGGCACGGACGGGGTTCGAACC |
| Trp-CCA | CCCCGACGTGATTTGAACACGCAa |
| Val-CAC | GGaCCTTTCGCGTGTGAGGCGA |
| Ile-TAT 5'-exon | TAT AAG TAC CGC GCG CTA AC |
| D-AC probes (nuclear | encoded tRNAs) |
| Ala-AGC-G57 | TGCTAAGCACGCGCTCTACCACT |
| Arg-ACG-G59 | TCCGTAGTCAGACGCGTTaTCCAT |
| Asn-GTT-G56 | ACAGCCGAACGCGCTaACC |
| Gly-CCC | AATGGGAATCTTGCATGATACCACT |
| Leu-CAA | CTTGAGTCTGGCGCCTTAGAC |
| Met-CAT | GAGACTGACGCGCTGCCTACT |
| Phe-GAA | TCTTCAGTCTAACGCTCTCCCAAC |
| Ser-AGA | ATTTCTAGTCCATCGCCTTaACCAC |

 Table S1. Oligonucleotides used in this study.

| Thr-CGT | AGACCGACGCCTTACCACTT | | | |
|--|-------------------------------------|--|--|--|
| Trp-CCA | TCTGGAGTCAGACGCGCTACCG | | | |
| Val-CAC | TGTGAGGCGAACGTGATaACCACT | | | |
| IleTAT 3'exon | CGAACTCACAACCTCGGCAT | | | |
| T-loop probe (mitochondrial-encoded tRNAs) | | | | |
| Ala-TGC | ACTCTGCATCAACTGAACGCAAATCA | | | |
| Arg-TCG | TAAATATGATTATCATAATTTAATGAGTCGAAATC | | | |
| Asn-GTT | CCCTAATCAACTGGCTTCAATCTA | | | |
| Gln | GCCACCTATCACACCCCATCCTA | | | |
| Glu | TCGCACGGACTACAACCACGA | | | |
| Ile-GAT | GGGTTTAAGCTCCTATTATTTACTCTATCAAA | | | |
| Ser-TGA | CAAAAAGGAAGGAATCGAACCCC | | | |
| D-AC probe (mitochondrial encoded) | | | | |
| Ala-TGC | GAACGCAAATCAGCCACTTTAATTAA | | | |
| Arg-TCG | GAGTCGAAATCATTCGTTTGTTTAAA | | | |
| Asn-GTT | GTTAACAGCTAAGCACCCTAATCA | | | |
| Gln | ATCCAAAATTCTCCGTGCCACCTAT | | | |
| Glu | ATATGAAAAACCATCGTTGTATTTCAACT | | | |
| Ile-GAT | TCTATCAAAGTAACTCTTTTATCAGAC | | | |
| Ser-TGA | AAGCCAACCCCATGGCCTCC | | | |
| Primer extension | | | | |
| IleTAT 55-34 | AACTCACAaCCTCGGCATTATA | | | |
| AlaAGC 54-36 | TCCCGCTACCTCTCGCATG | | | |
| Met-CAU_55-34 | AACTCACGACCTTCAGATTATG | | | |
| Phe-GAA_63-37 | CGGGATCGAACCAGGGACCTTTAGATC | | | |
| Ile-GAU mito 54-30 | TTAAGCTCCTATTATTTACTCTATC | | | |
| Additional Northern | | | | |
| blot probes | | | | |
| tRNA-Glu-UUC | CCAGGAATCCTAACCGCTAGACCATRTGGGA | | | |
| U6 snRNA | CGTTCCAATTTTAGTATATGTGCTGCCGAAGCGA | | | |

| | | | Ret | Delta | | Collision | Cell | |
|-----------|----------|---------|-------|-------|-----------|-----------|------------|----------|
| Compoun | Precurso | Product | Time | Ret | Fragmento | Energy | Accelerato | |
| d Name | r lon | lon | (min) | Time | r Voltage | (eV) | r Voltage | Polarity |
| 15N dA | | | | | | | | |
| (internal | 0.5-7 | | 7.0 | | | 10 | | . |
| standard) | 257 | 141 | 7,3 | 2 | 380 | 10 | 2 | Positive |
| ac4C | 286 | 154 | 6,8 | 2 | 380 | 10 | 2 | Positive |
| Am | 282 | 136 | 8,8 | 2 | 380 | 10 | 2 | Positive |
| Cm | 258 | 112 | 4,4 | 2 | 380 | 10 | 2 | Positive |
| cm5U | 303 | 171 | 1,9 | 1 | 380 | 10 | 2 | Positive |
| cm5Um | 317 | 171 | 4,4 | 2 | 380 | 10 | 2 | Positive |
| D | 247 | 115 | 1,7 | 1 | 380 | 10 | 2 | Positive |
| Gm | 298 | 152 | 6,5 | 2 | 380 | 10 | 2 | Positive |
| I | 269 | 137 | 4,7 | 2 | 380 | 10 | 2 | Positive |
| i6A | 336 | 204 | 14,2 | 2 | 380 | 10 | 2 | Positive |
| m1A | 282 | 150 | 2,8 | 2 | 380 | 10 | 2 | Positive |
| m1G | 298 | 166 | 6,4 | 0,5 | 380 | 10 | 2 | Positive |
| m2,2G | 312 | 180 | 8,1 | 2 | 380 | 10 | 2 | Positive |
| m2G | 298 | 166 | 6,8 | 0,5 | 380 | 10 | 2 | Positive |
| m3C | 258 | 126 | 3,3 | 1 | 380 | 10 | 2 | Positive |
| m3U | 259 | 127 | 5,9 | 1 | 380 | 10 | 2 | Positive |
| m5C | 258 | 126 | 4 | 1 | 380 | 10 | 2 | Positive |
| m5s2U | 275 | 143 | 7,5 | 2 | 380 | 10 | 2 | Positive |
| m5U | 259 | 127 | 5,1 | 1 | 380 | 10 | 2 | Positive |
| m62A | 296 | 164 | 12,5 | 2 | 380 | 10 | 2 | Positive |
| m6t6A | 427 | 295 | 10 | 2 | 380 | 10 | 2 | Positive |
| m7G | 298 | 166 | 4 | 2 | 380 | 10 | 2 | Positive |
| mcm5U | 317 | 185 | 6,5 | 2 | 380 | 10 | 2 | Positive |
| mcm5Um | 331 | 153 | 8,9 | 2 | 380 | 10 | 2 | Positive |
| ms2i6A | 382 | 250 | 16 | 2 | 380 | 10 | 2 | Positive |
| Psi | 245 | 191 | 1,7 | 2 | 380 | 10 | 2 | Positive |
| Q | 410 | 295 | 5,7 | 2 | 380 | 10 | 2 | Positive |
| t6A | 413 | 281 | 8,4 | 2 | 380 | 10 | 2 | Positive |
| Um | 259 | 113 | 5,7 | 2 | 380 | 10 | 2 | Positive |

 Table S2. Mass spectrometer parameters of all analyzed nucleosides