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Supplemental information

Malic enzyme 2 connects the Krebs cycle intermediate fumarate to mitochondrial biogenesis

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Figure S1. Fumarate upregulates mitochondrial biomass

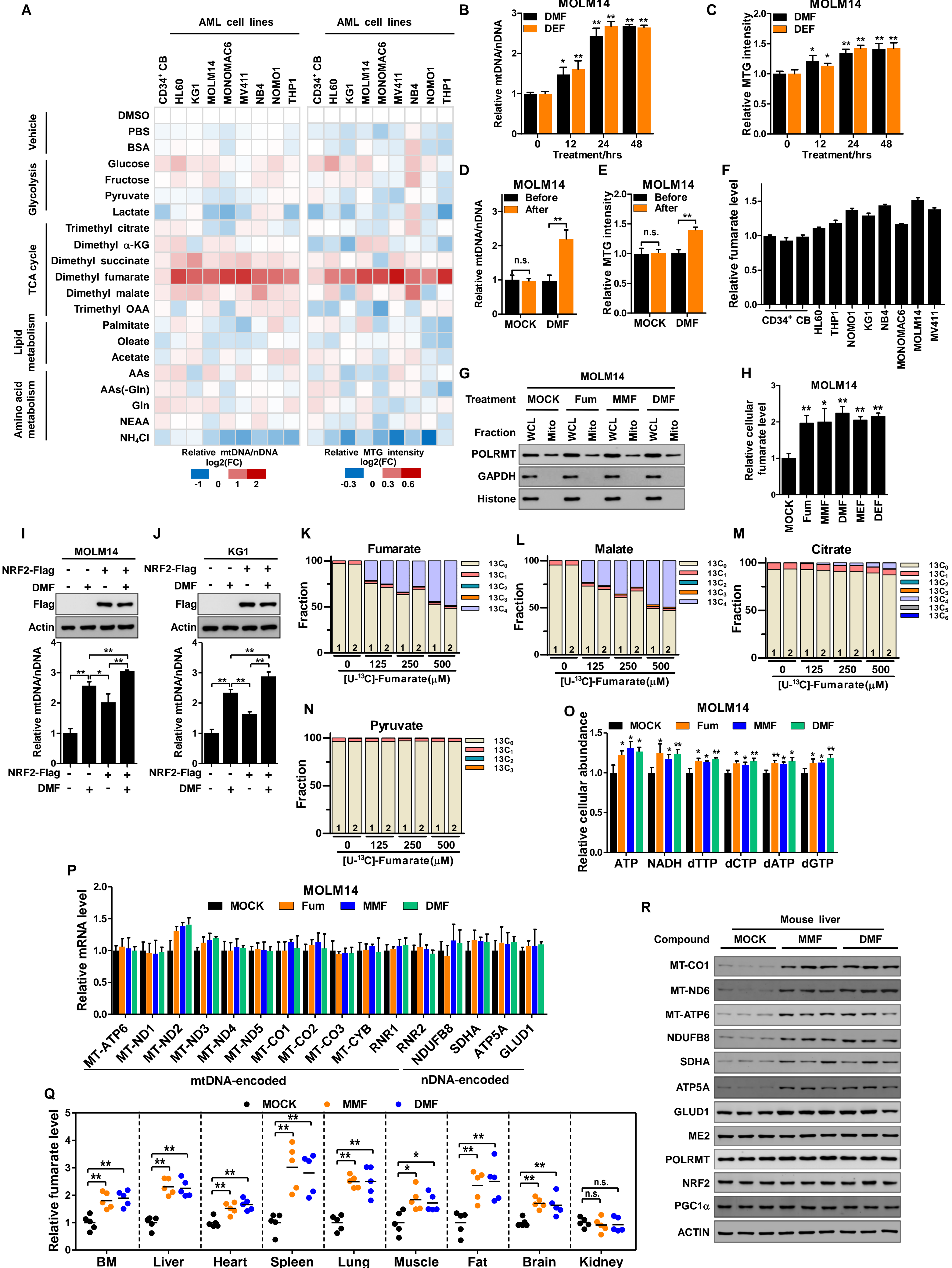


Figure S2. Fumarate relies on ME2 to increase mitochondrial mass

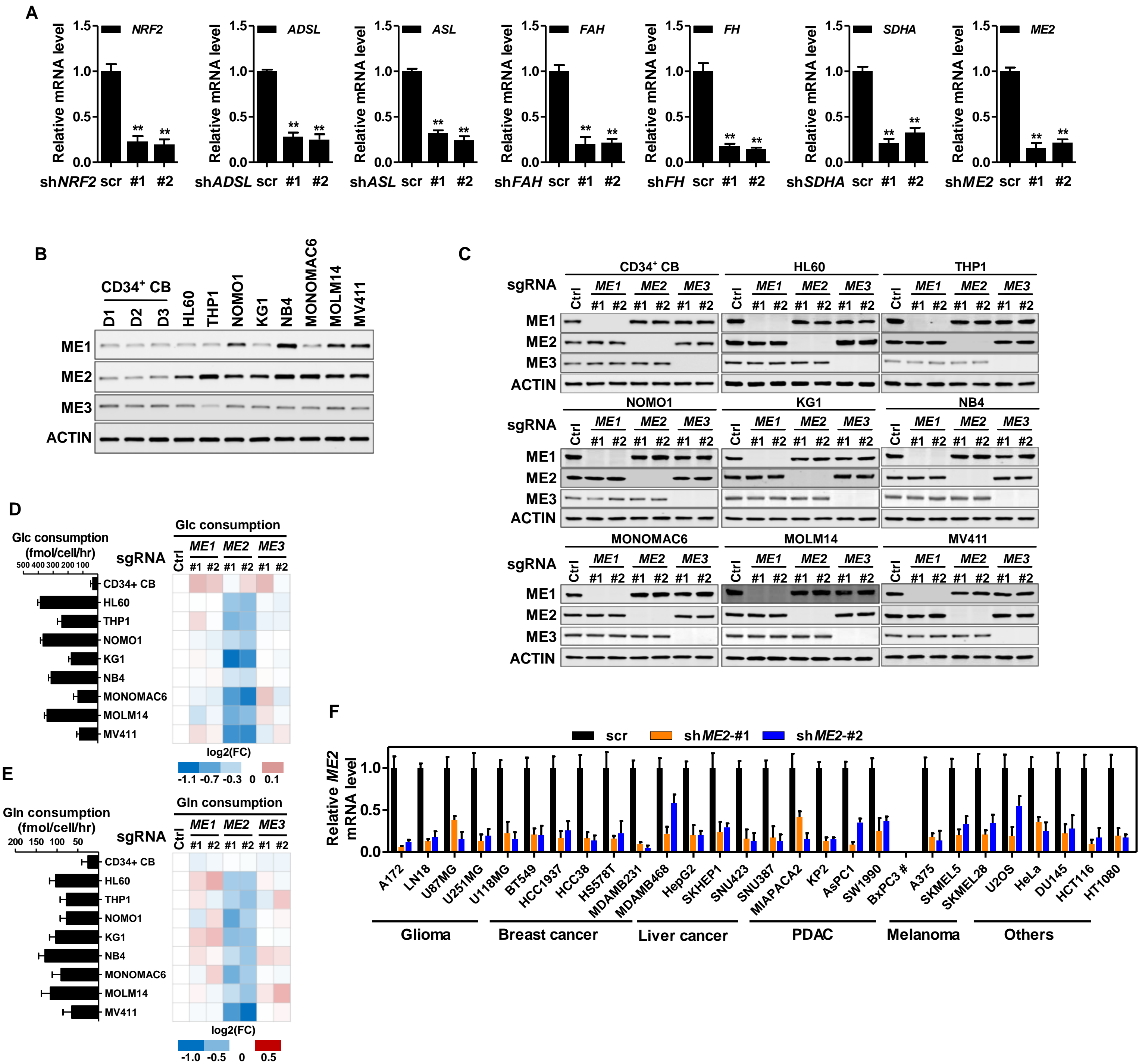


Figure S3. ME2 responds to fumarate by increasing DUT activity and mtDNA

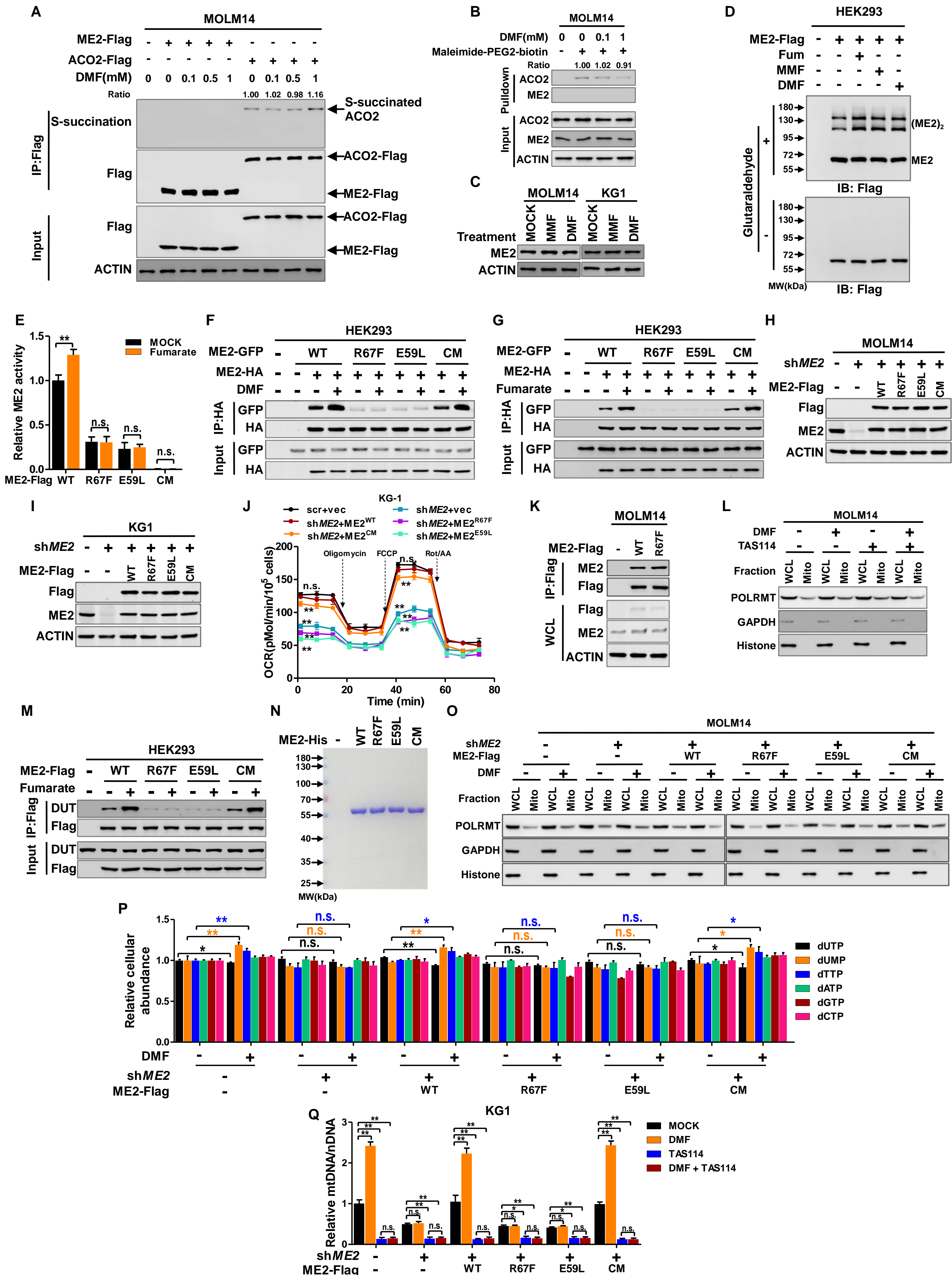


Figure S4. ME2 responds to fumarate by modulating mitoribosome assembly

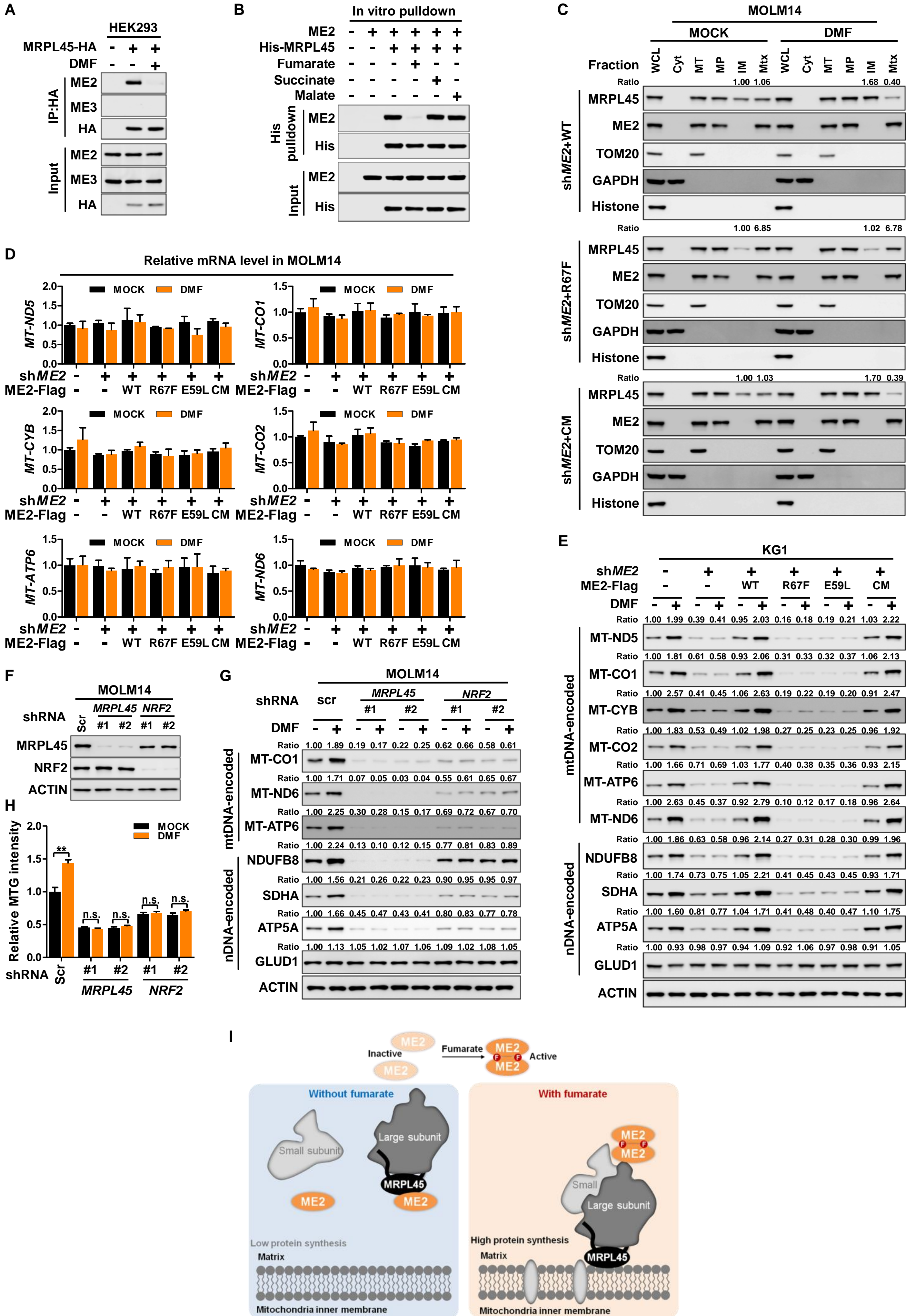


Figure S5. PRMT1 methylates ME2 inhibiting fumarate sensing

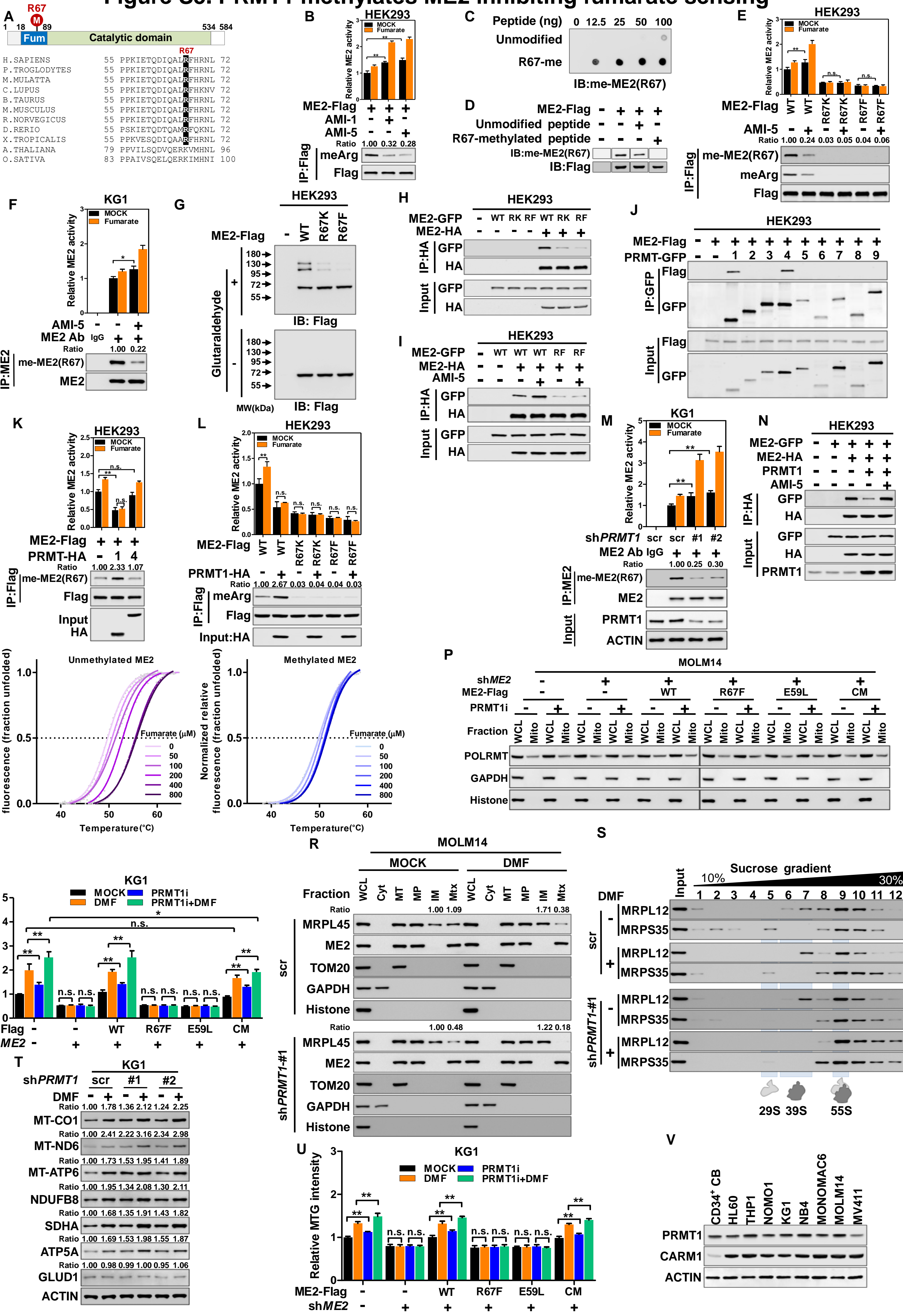
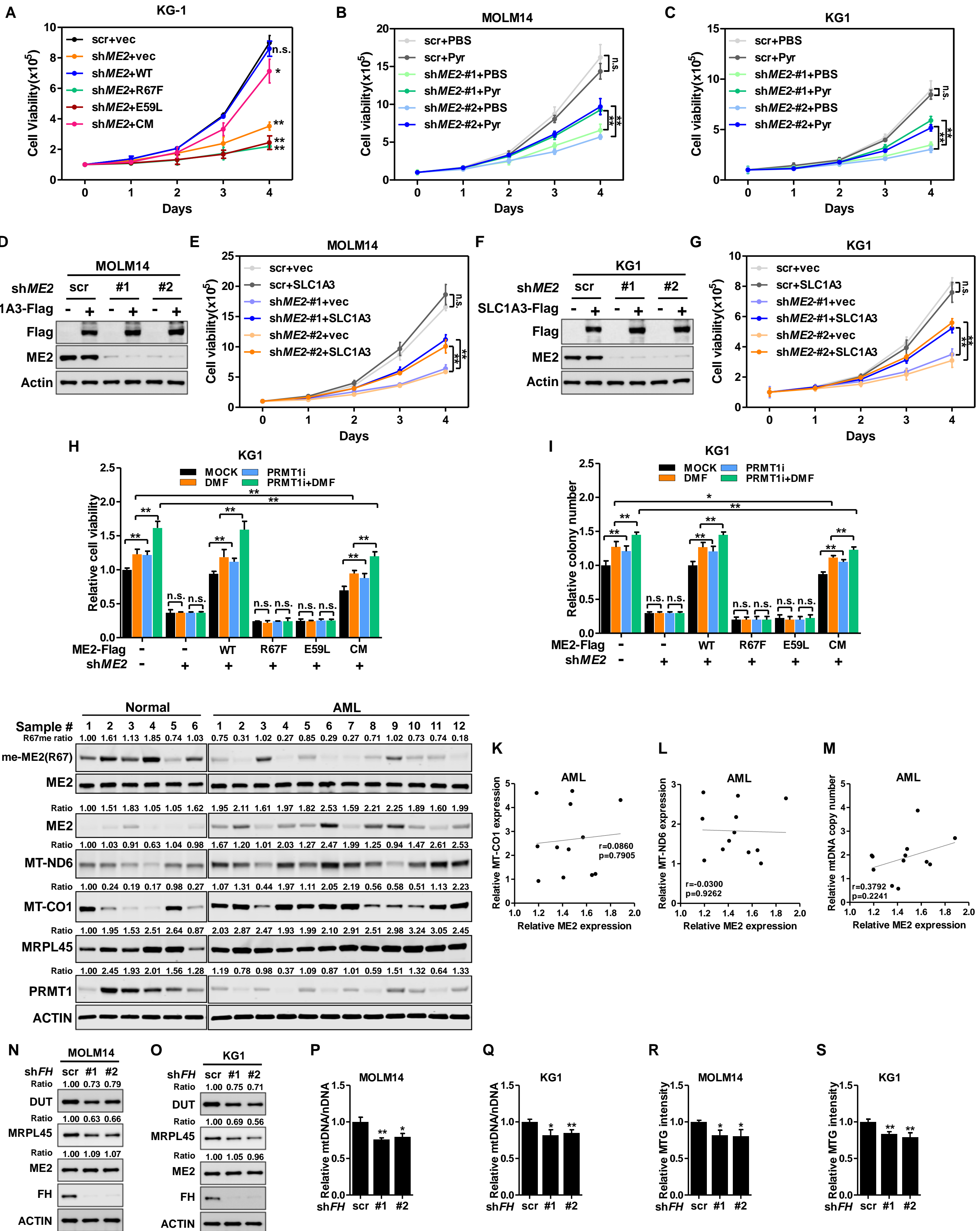


Figure S6. ME2-mediated fumarate sensing supports leukemia growth



Supplementary Figure Legends

Figure S1. Fumarate upregulates mitochondrial biomass

(A) Human cord blood $CD34^+$ cells and eight different AML cell lines were treated with sugars, lipids, amino acids, or metabolic intermediates from glycolysis, Krebs cycle, and lipid metabolism for 48 hours. Total DNA was extracted. Mitochondrial DNA (mtDNA) copy number was determined by quantitative PCR (qPCR) and normalized to nuclear DNA (left). Cells were stained with mitotracker green (MTG), the fluorescent intensity of MTG was normalized to cell number (right). All data were normalized to DMSO-treated group. The fold change (FC) was presented on a log2 scale.

(B-C) MOLM14 cells were treated with 100 μM DMF or DEF at increasing durations up to 48 hours. mtDNA copy number was determined by qPCR and normalized to nuclear DNA **(B)**. Cells were stained with mitotracker green (MTG), the fluorescent intensity of MTG was normalized to cell number **(C)**.

(D-E) MOLM14 cells were treated with DMSO (MOCK) or 100 μM DMF for 24 hours. Cells were collected before and after the treatment. mtDNA copy number was determined by qPCR and normalized to nuclear DNA **(D)**. Cells were stained with mitotracker green (MTG), the fluorescent intensity of MTG was normalized to cell number **(E)**.

(F) The intracellular fumarate levels in $CD34^+$ cord blood cells and eight different AML cell lines were determined.

(G-H) MOLM14 cells were treated with DMSO (MOCK), 1 mM fumarate (Fum), or 100 μM fumarate esters (MMF and DMF) for 24 hours. Mitochondria were isolated after

treatment, POLRMT, GAPDH, and histone H3 (Histone) were included as markers for mitochondria, cytoplasm, and nucleus, respectively **(G)**. Intracellular fumarate level was determined and normalized to cell number **(H)**.

(I-J) Flag-tagged NRF2 was stably expressed in MOLM14 **(I)** and KG1 **(J)** cells. Cells were treated with DMSO or 100 μM DMF for 24 hours. mtDNA copy number was determined by qPCR and normalized to nuclear DNA.

(K-N) MOLM14 cells were incubated with increasing concentrations of [U-^{13}C]-fumarate for 24 hours. Cellular metabolites were extracted, the isotope distribution in fumarate **(K)**, malate **(L)**, citrate **(M)**, and pyruvate **(N)** was quantified by liquid chromatography with tandem mass spectrometry (LC-MS/MS). Shown are two independent experiments (1 and 2).

(O-P) MOLM14 cells were treated with DMSO (MOCK), 1 mM fumarate (Fum), or 100 μM fumarate esters (MMF and DMF) for 24 hours. Cellular metabolites were extracted, the abundance of ATP, NADH, and dNTPs were quantified **(O)**. The mRNA expression of mtDNA and nDNA-encoded mitochondrial genes was quantified by qPCR and normalized to actin **(P)**.

(Q-R) Mice were injected intraperitoneally with DMSO (MOCK), MMF, or DMF for seven days. Tissues were homogenized and metabolites were extracted, the abundance of fumarate was determined and normalized to total protein (n=5) **(Q)**. The expression of mitochondrial proteins in liver tissues from three independent mice was determined by western blotting **(R)**.

All data are shown as mean ± SEM from three independent experiments. *$p < 0.05$,

**p < 0.01, n.s. indicates not significant.

Related to Figure 1

Figure S2. Fumarate relies on ME2 to increase mitochondrial mass

(A) The knockdown efficiencies of shRNAs targeting *NRF2*, *ADSL*, *ASL*, *FAH*, *FH*, *SDHA*, and *ME2* in MOLM14 cells were determined by qPCR.

(B) Whole cell lysate of eight different AML cell lines and human $CD34^+$ cord blood (CB) cells from three different donors (D1-D3) was subjected to western blotting. Protein expression of ME1, ME2, and ME3 was determined, β-actin was included as the loading control.

(C-E) Control sgRNA or two different sgRNAs targeting each malic enzyme was stably expressed in human $CD34^+$ cord blood cells or eight different AML cell lines. The expression of ME1, ME2, and ME3 was determined by western blotting **(C)**. The consumption rate of glucose **(D)** and glutamine **(E)** was determined. All data were normalized to the control group and presented on a log2 scale.

(F) A panel of solid tumor cell lines from glioma, breast cancer, liver cancer, pancreatic ductal adenocarcinoma (PDAC), and melanoma was transduced with scrambled control or two different shRNAs targeting *ME2*. The mRNA expression of *ME2* was determined by qPCR. # denotes that BxPC3 is a *ME2*-null cell line.

All data are shown as mean ± SEM from three or four independent experiments. **p < 0.01.

Related to Figure 2

Figure S3. ME2 responds to fumarate by increasing DUT activity and mtDNA

(A) Flag-tagged ME2 and ACO2 were transduced into MOLM14 cells. Cells were treated with increasing doses of DMF as indicated. ME2 and ACO2 were immunopurified with Flag beads. The succination of proteins were detected by western blotting.

(B) MOLM14 cells were treated with increasing doses of DMF as indicated for 24 hours. Cells were lysed and incubated with maleimide-PEG2-biotin to capture free thiols in cellular protein. Labeled protein was further pulled down with streptavidin agarose beads and subjected to western blotting.

(C) MOLM14 and KG1 cells were treated with MMF and DMF for 24 hours, the protein level of ME2 was assayed by western blotting, β-actin was included as the loading control.

(D) ME2-Flag was expressed in HEK293 cells, which were treated with fumarate and its esters for 24 hours. Whole cell lysate was crosslinked with glutaraldehyde and subjected to western blotting.

(E) Wildtype ME2-Flag and its mutants were immunopurified from HEK293 cells. The catalytic activity of ME2 was assayed with or without fumarate and normalized to ME2 protein.

(F-G) HA-tagged ME2 was co-expressed with GFP-tagged wildtype ME2 and its mutants (R67F, E59L, and CM). Cells were treated with DMF **(F)** or fumarate **(G)** for 24 hours. HA-tagged ME2 was immunoprecipitated using an HA antibody. The interaction between differently tagged ME2 was determined by western blotting.

(H-J) MOLM14 and KG1 cells were transduced with shRNA targeting *ME2* and

re-expressed with wildtype ME2 or its mutants. The knockdown and re-expression efficiency of ME2 was determined by western blotting **(H, I)**. The oxygen consumption rate of KG1 cells was determined by Seahorse flux analyzer **(J)**.

(K) Wildtype ME2-Flag and its R67F mutant were stably expressed in MOLM14 cells. ME2 protein was immunoprecipitated by Flag beads and subjected to mass spectrometry analysis to identify ME2 interactors.

(L) MOLM14 cells were treated with DMF and TAS114 as indicated for 24 hours. Mitochondria were isolated after treatment. POLRMT, GAPDH, and histone H3 (Histone) were included as markers for mitochondria, cytoplasm, and nucleus, respectively.

(M) ME2-Flag was expressed in HEK293 cells, which were treated with fumarate for 24 hours. The interaction between ME2-Flag and endogenous DUT was determined by co-immunoprecipitation and western blotting.

(N) His-tagged ME2 and its mutants were purified from *E.coli*, resolved on SDS-PAGE, and visualized by Coomassie Blue staining.

(O-P) *ME2*-knockdown and re-expression MOLM14 cells were treated with DMF for 24 hours. Mitochondria were isolated from MOLM14 cells after DMF treatment. Whole cell lysate and mitochondrial fraction were analyzed by western blotting to determine the isolation efficiency **(O)**. The cellular abundance of dUTP, dUMP and four dNTPs were quantified and normalized to cell number **(P)**.

(Q) *ME2*-knockdown and re-expression KG1 cells were treated with DMF and TAS114 for 24 hours. Total DNA was extracted; mtDNA abundance was determined

by qPCR.

All data are presented as mean ± SEM from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, n.s. indicates not significant.

Related to Figure 3

Figure S4. ME2 responds to fumarate by modulating mitoribosome assembly

(A) HA-tagged MRPL45 was expressed in HEK293 cells. Cells were treated with or without DMF for 24 hours. HA-tagged MRPL45 was immunopurified and subjected to western blotting to determine its interaction with mitochondrial malic enzymes (ME2 and ME3).

(B) Recombinant ME2 and His-tagged MRPL45 were incubated with 500 μM fumarate, succinate, or malate for 24 hours in vitro. MRPL45 was pulled down using nickel beads and subjected to western blotting.

(C) Wildtype ME2 and its mutants (R67F and CM) were re-expressed in *ME2*-knockdown MOLM14 cells. Cells were treated with or without DMF. Isolated mitochondria were fractionated and subjected to western blotting to determine MRPL45 localization. WCL, whole cell lysate; Cyt, cytoplasm; MT, mitochondria; MP, mitoplast; IM, inner membrane; Mtx, mitochondrial matrix.

(D) *ME2*-knockdown and re-expression MOLM14 cells were treated with DMF for 24 hours, the mRNA expression of multiple mtDNA-encoded genes were quantified by qPCR.

(E) *ME2*-knockdown and re-expression KG1 cells were treated with or without DMF for 24 hours. Whole cell lysate was subjected to western blotting to detect mtDNA and

nDNA-encoded proteins. Band intensity was quantified and normalized to β-actin (Ratio).

(F-H) shRNAs targeting *MRPL45* and *NRF2* were transduced into MOLM14 cells. The knockdown efficiency of *MRPL45* and *NRF2* were determined by western blotting **(F)**. Cells were treated with DMF for 24 hours. Protein expression of mtDNA and nDNA-encoded genes was detected by western blotting **(G)**. The MTG intensity of treated cells was quantified **(H)**.

(I) Schematic overview of fumarate-induced mitoribosome assembly. Left, ME2 monomer binds to MRPL45 and reduces its inner membrane attachment, leading to mitoribosome disassembly; Right, fumarate promotes the dimerization of ME2 and freeing MRPL45 to enhance mitoribosome activity.

All data are presented as mean ± SEM from three independent experiments. $^{**}p < 0.01$, n.s. indicates not significant.

Related to Figure 4

Figure S5. PRMT1 methylates ME2 inhibiting fumarate sensing

(A) R67 is an evolutionarily conserved residue. R67 resides in the fumarate-binding domain, but not the catalytic center (top). Amino acid sequences adjacent to R67 across different species were analyzed with multiple alignments (bottom).

(B) ME2-Flag was expressed in HEK293 cells. Cells were treated with AMI-1 or AMI-5 for 24 hours. ME2-Flag protein was immunoprecipitated and subjected to western blotting to detect arginine methylation. ME2 activity was assayed with or without fumarate.

(C) Nitrocellulose membrane was spotted with increasing amounts of monomethyl-R67 peptide (R67-me) or unmodified peptide as indicated. The membrane was blotted with site-specific antibody against R67 methylation [α-me-ME2 (R67)] to determine its specificity.

(D) Methylated R67 peptide, but not the unmodified peptide, blocks the recognition of immunopurified ME2 protein by α-me-ME2(R67) antibody.

(E) Flag-tagged ME2 and its mutants (R67K and R67F) were expressed in HEK293 cells. Cells were treated with or without AMI-5 for 24 hours. Immunopurified ME2 was subjected to western blotting and enzymatic activity assay.

(F) KG1 cells were treated with or without AMI-5 for 24 hours. R67 methylation of immunoprecipitated endogenous ME2 was determined by a site-specific methylation antibody [α-me-ME2(R67)]. ME2 activity was assayed in the presence or absence of fumarate.

(G) ME2-Flag and its mutants (R67K and R67F) were expressed in HEK293 cells. Cell lysate was cross-linked with glutaraldehyde and analyzed by western blotting.

(H) HA-tagged ME2 was co-expressed with GFP-tagged wildtype ME2 or its mutants (R67K and R67F). Interaction between differently tagged ME2 was determined by co-immunoprecipitation and western blotting.

(I) HA-tagged ME2 was co-expressed with GFP-tagged wildtype ME2 or R67F mutant. Cells were treated with AMI-5 for 24 hours. Interaction between differently tagged ME2 was determined by co-immunoprecipitation and western blotting.

(J) ME2-Flag was co-expressed with GFP-tagged PRMTs in HEK293 cells.

GFP-PRMT was immunoprecipitated using a GFP-specific antibody. The interaction between ME2 and PRMTs was determined by western blotting.

(K) ME2-Flag was co-expressed with HA-tagged PRMT1 or PRMT4. Immunopurified ME2-Flag was subjected to western blotting and enzymatic activity assay.

(L) Wildtype ME2-Flag and its mutants were co-expressed with HA-tagged PRMT1. ME2 protein was purified with Flag beads and subjected to western blotting.

(M) Scrambled control or two different shRNAs against *PRMT1* were stably expressed in KG1 cells. Immunopurified endogenous ME2 was subjected to western blotting and enzymatic activity assay

(N) PRMT1 was co-expressed with differently tagged ME2 in HEK293 cells. Cells were treated with or without AMI-5 as indicated. HA-ME2 was immunoprecipitated and subjected to western blotting.

(O) Unmethylated ME2 (left) and in vitro methylated recombinant ME2 (right) were subjected to protein thermal shift assay at the presence of increasing doses of fumarate as indicated.

(P) Wildtype ME2-Flag and its mutants were re-expressed in *ME2*-knockdown MOLM14 cells. Mitochondria were isolated after cells were treated with or without PRMT1-specific inhibitor. Whole cell lysate and mitochondrial fraction were analyzed by western blotting to determine the isolation efficiency.

(Q) *ME2*-knockdown and re-expression KG1 cells were treated with PRMT1-specific inhibitor (PRMT1i) and DMF as indicated for 24 hours. Total DNA was extracted and mtDNA copies were quantified by qPCR.

(R-S) Control and *PRMT1*-knockdown MOLM14 cells were treated with or without DMF. Isolated mitochondria were fractionated and subjected to western blotting to determine MRPL45 localization. WCL, whole cell lysate; Cyt, cytoplasm; MT, mitochondria; MP, mitoplast; IM, inner membrane; Mtx, mitochondrial matrix **(R)**. Mitochondrial lysate was loaded on a sucrose gradient to fractionate mitoribosome. MRPL12 and MRPS35 were included as markers for the large subunit and small subunit for mitoribosome, respectively **(S)**.

(T) Control and *PRMT1*-knockdown KG1 cells were treated with DMF for 24 hours. Whole cell lysate was subjected to western blotting to determine the expression of mtDNA and nDNA-encoded proteins.

(U) *ME2*-knockdown and re-expression KG1 cells were treated with PRMT1-specific inhibitor (PRMT1i) and DMF as indicated. MTG intensity was determined.

(V) Protein expression of PRMT1 and CARM1 in the whole cell lysate of eight different AML cell lines was determined by western blotting. Human $CD34^{+}$ cord blood cells were included as normal control.

All data are presented as mean ± SEM from three independent experiments. *p< 0.05, **p < 0.01, n.s. indicates not significant.

Related to Figure 5

Figure S6. ME2-mediated fumarate sensing supports leukemia growth

(A) Wildtype ME2 and its mutants were re-expressed in *ME2*-knockdown KG1 cells. Growth curves were determined by cell counting.

(B-C) PBS or pyruvate (2 mM) was added to the culture of scrambled control and

ME2-knockdown cells. Growth curves of stable MOLM14 **(B)** and KG1 **(C)** cells were determined by cell counting.

(D-G) Flag-tagged SLC1A3 was stably expressed in control or *ME2*-knockdown cells. The expression of SLC1A3-Flag in MOLM14 and KG1 cells was detected by western blotting **(D**, **F)**. Growth curves were determined by cell counting **(E**, **G)**.

(H-I) *ME2*-knockdown and re-expression KG1 cells were treated with PRMT1 inhibitor and DMF. Cell viability was determined by cell counting after four days of culture **(H)**. Colonies of KG1 cells were counted seven days after treatment **(I)**.

(J) The protein expression of ME2, MT-ND6, MT-CO1, MRPL45, and PRMT1 in normal and leukemic human bone marrow samples was determined by western blotting. The ratio indicates relative expression level after normalizing to β-actin. ME2 protein was immunoprecipitated and blotted with site-specific methylation antibody to determine R67 methylation level. The catalytic activity of immunopurified ME2 enzymes was assayed in the presence of fumarate.

(K-M) Pearson's correlation of ME2 protein expression with MT-CO1 protein **(K)**, MT-ND6 protein **(L)**, and mtDNA abundance **(M)** in AML samples was determined.

(N-S) shRNAs targeting *FH* were stably expressed in MOLM14 and KG1 cells, respectively. Protein expression of DUT, MRPL45, ME2, and FH was determined by western blotting **(N** and **O)**. Ratio indicates the quantification of corresponding protein after normalizing to actin. Relative mtDNA copy number **(P** and **Q)** and MTG intensity **(R** and **S)** of scrambled control and *FH*-knockdown cells were determined.

Related to Figure 6