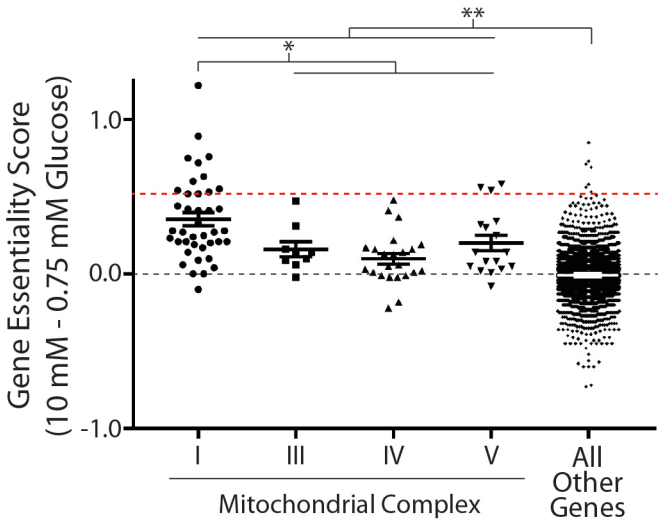

Figure S1: Screening Data by Mitochondrial Complex, Related to Figure 1A. Differential essentiality scores in 0.75 mM glucose, compared to 10 mM glucose, as reported in the Supplementary Table, organized by mitochondrial complex. Black dotted line indicates median gene score. Red dotted line indicates the median Log_2 fold change cutoff of 0.5 used in Figure 1B. Population mean and s.e.m. indicated for each group. * $p < 0.05$ for Complex I versus Complex III, IV, or V; ** $p < 0.002$ for comparisons between Complex I, III, IV, or V versus all other genes. Significance values calculated by heteroscedastic two-sided student's t test.

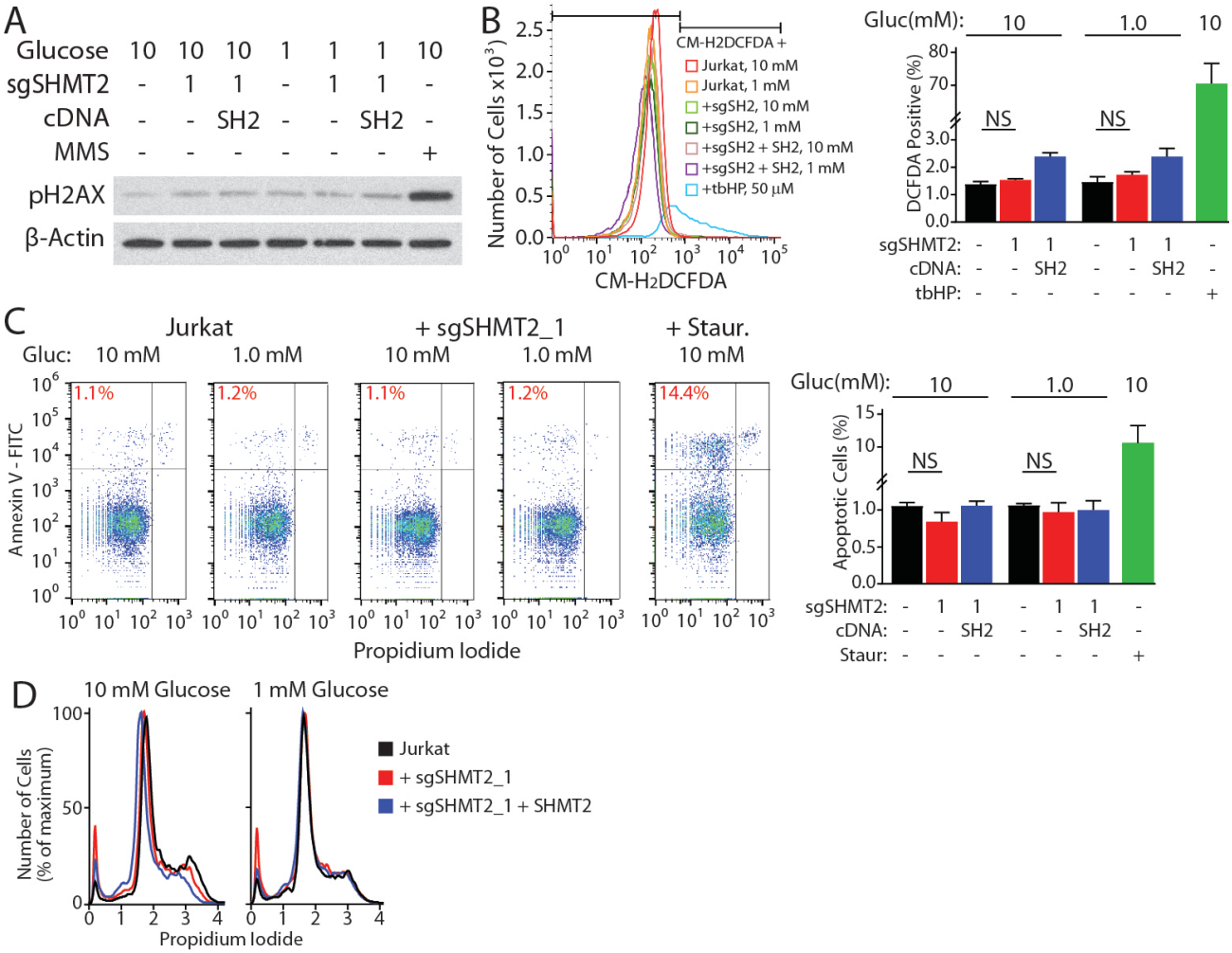

Figure S2: Characterization of SHMT2 knockout cells, Related to Figure 2. A, Immunoblot from cell lysates for the DNA damage marker phospho histone H2AX S139 (pH2AX) or β-actin. Jurkat cells or SHMT2-null clones with or without expression of an SHMT2 cDNA were grown at the indicated glucose concentrations. Methyl methanesulfonate (MMS, 150 µM) used as a positive control. B, (Left) Fluorescence intensity of the cell lines used in *A* culturedat the indicated glucose concentration upon incubation with the ROS reactive dye CM-H_2DCFDA, as measured by flow cytometry. The histogram is representative of three independent experiments. The bracketed region denotes the threshold for identifying cells as CM-H_2DCFDA positive. (Right) Average proportion of cells identified as CM-H_2DCFDA positive for the indicated cell lines and conditions. tert-butyl hydroperoxide (tbHP, 50 µM) used as a positive control. C, (Left) Fluorescence intensity of the cell lines used in *A* cultured at the indicated glucose concentrations upon staining with propidium iodide and FITC-conjugated annexin V, as measured by flow cytometry. The dot plot is representative of three independent experiments. The upper left region denotes cells undergoing apoptosis, and the proportion of cells in this region is indicated by red text. (Right) Average proportion of apoptotic cells identified for the indicated cell lines and conditions. Staurosporine (Staur, 1.5 µM) used as a positive control. D, Fluorescence intensity of the cell lines used in *A* cultured at the indicated glucose concentrations upon permeabilization and staining with propidium iodide, as measured by flow cytometry. Flow cytometry plots are representative of three replicate experiments. NS, not significant. Measurements are mean +/- s.e.m. Significance values calculated by heteroscedastic two-sided student's t test.

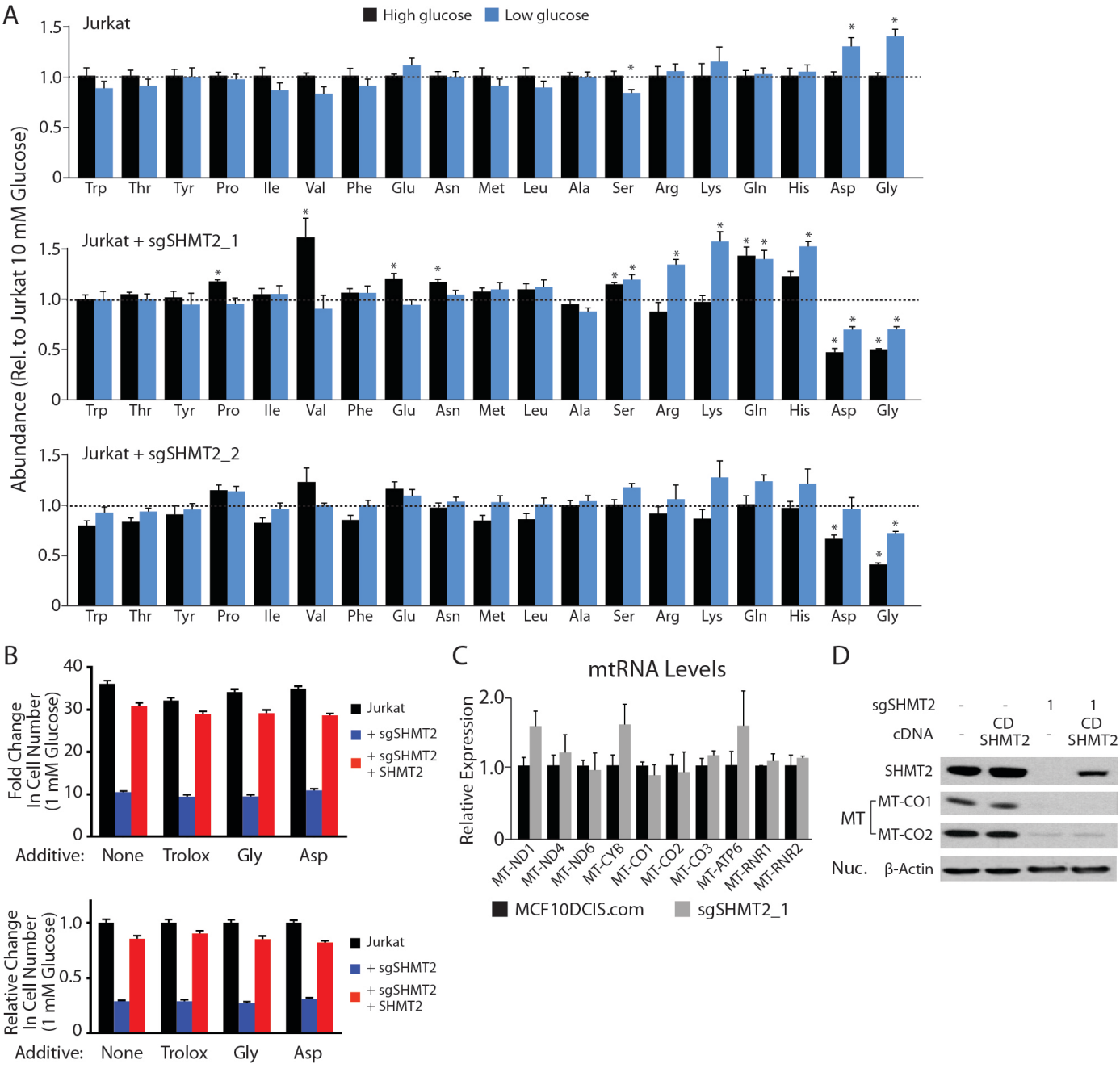

Figure S3: Further characterization of SHMT2 knockout cells, Related to Figure 2. A, Intracellular levels of the indicated amino acids in the indicated cell lines cultured in media containing 10 mM (high glucose) or 1.5 mM glucose (low glucose), as measured by LC/MS. * $P < 0.01$ compared to Jurkat cells cultured in 10 mM glucose. Measurements are mean +/- s.e.m. n=4. Significance values calculated by heteroscedastic two-sided student's t test. B, Above, proliferation of Jurkat cells or clones expressing an sgRNA targeting SHMT2 (shSHMT2) with or without expression of an SHMT2 cDNA. Below, data from above, normalized to Jurkat cells for each condition. Cells were grown for 5 days in media initially 1 mM glucose and with Trolox (100 μM), Glycine (500 μM), or Aspartate (10 mM) added to the media, as indicated. Measurements are mean +/- s.e.m. n=3. C, Relative mitochondrial expression in MCF10DCIS.com cells or a clone expressing sgSHMT2_1 as assessed by qPCR based measurement of the indicated mitochondrial genes. Data are normalized to MCF10DCIS.com and represent the average of three independent experiments. Measurements are mean +/- s.e.m. D, Immunoblots from cell lysates of Jurkat cells or clones expressing small guide RNAs targeting SHMT2 (sgSHMT2), or with introduction of a cDNA encoding a catalytically inactive mutant (K280A) of SHMT2 (CD SHMT2). MT-CO1 and MT-CO2 are encoded by the mitochondrial genome and translated in the mitochondria (MT) whereas β-actin is encoded by the nuclear genome and translated in the cytoplasm (Nuc.).

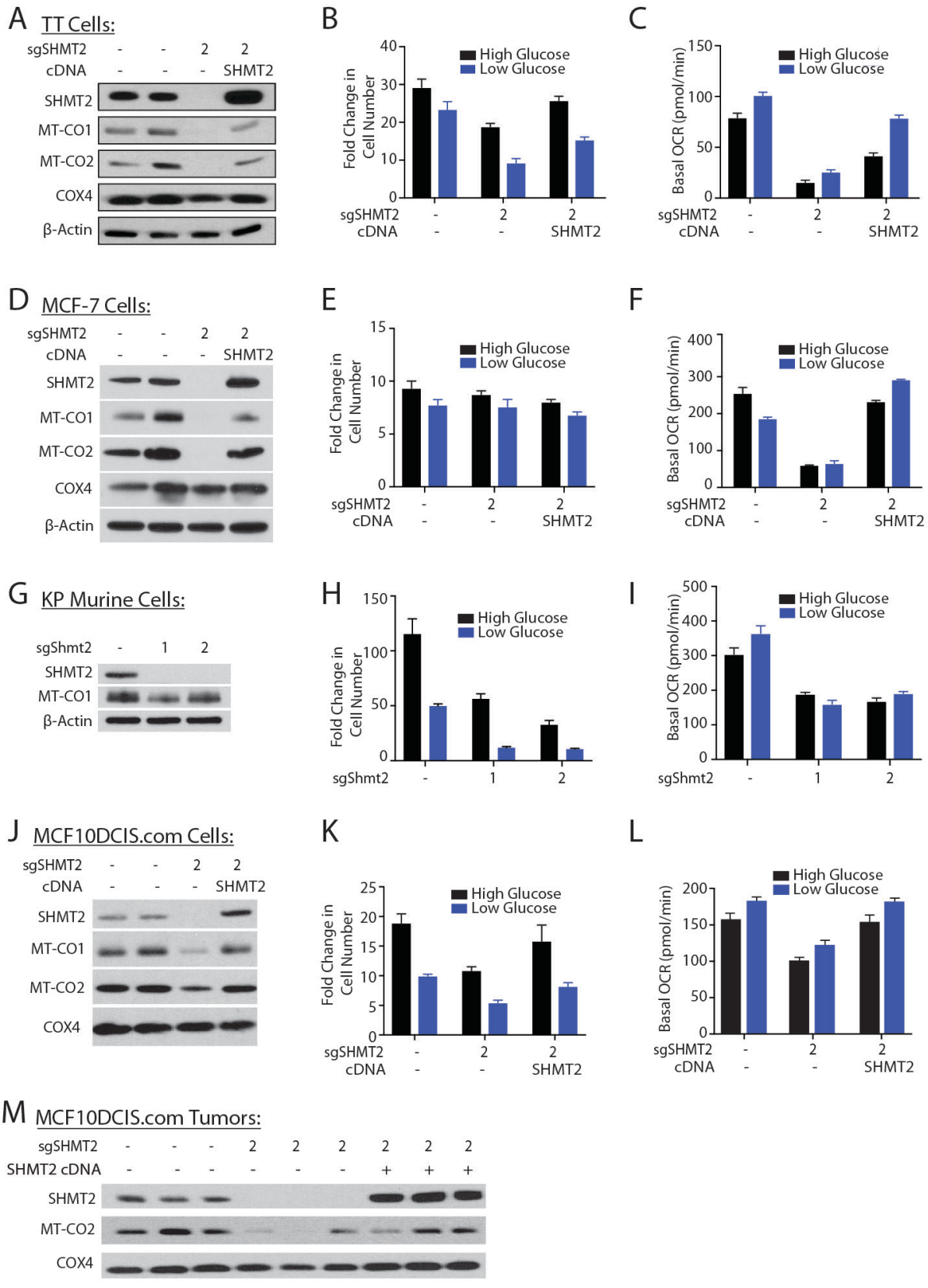

Figure S4: Deletion of SHMT2 limits mitochondrial function across cell types, Related to Figure 2. A, Immunoblot from cell lysates of TT esophageal cells for proteins encoded by the indicated genes. TT cell clones express sgSHMT2 or re-express SHMT2 via transduction with an SHMT2 cDNA. MT-CO1 and MT-CO2 are encoded by the mitochondrial genome and translated in the mitochondria whereas COX4 is encoded by the nuclear

genome and translated in the cytoplasm. B, Proliferation of TT cells from *A*. Cells were grown for 5 days in media initially containing 10 mM (high glucose) or 1.5 mM glucose (low glucose). C, Basal oxygen consumption rate (OCR) of the cell lines from *A*, cultured in 10 mM (high glucose) or 1.5 mM glucose (low glucose). D, Immunoblot from cell lysates of MCF-7 breast cells for proteins encoded by the indicated genes. MCF-7 cell clones express sgSHMT2 or a re-express SHMT2 via transduction with an SHMT2 cDNA. E, Proliferation of cells from *D*. Cells were grown for 5 days in media initially containing 10 mM (high glucose) or 1.5 mM glucose (low glucose). F, Basal oxygen consumption rate (OCR) of the cell lines from *D*, cultured in 10 mM (high glucose) or 1.5 mM glucose (low glucose). G, Immunoblot from cell lysates of KP murine lung adenocarcinoma cells for proteins encoded by the indicated genes. KP cell clones express sgShmt2_1 or sgShmt2_2. H, Proliferation of cells from *G*. Cells were grown for 5 days in media initially containing 10 mM (high glucose) or 1.5 mM glucose (low glucose). I, Basal oxygen consumption rate (OCR) of the cell lines from *G*, cultured in 10 mM (high glucose) or 1.5 mM glucose (low glucose). J, Immunoblot from cell lysates of MCF10DCIS.com breast cells for proteins encoded by the indicated genes. MCF10DCIS.com cell clones express sgSHMT2 or a re-express SHMT2 via transduction with an SHMT2 cDNA. K, Proliferation of cells from *J*. Cells were grown for 5 days in media initially containing 10 mM (high glucose) or 1.5 mM glucose (low glucose). K, Basal oxygen consumption rate (OCR) of the cell lines from *J*, cultured in 10 mM (high glucose) or 1.5 mM glucose (low glucose). M, Immunoblot from lysates of MCF10DCIS.com xenograft tumors derived from the cell lines described in *J* for proteins encoded by the indicated genes. Measurements are mean +/- s.e.m. n=3.

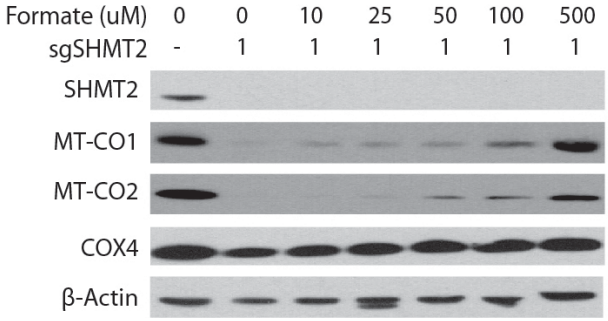

Figure S5: Formate restores mitochondrially translated proteins to MCF-7 cells, Related to Figure 4E. Immunoblot from cell lysates of MCF-7 cell lines for proteins encoded by the indicated genes, upon addition of the indicated concentrations of sodium formate for 3 days.

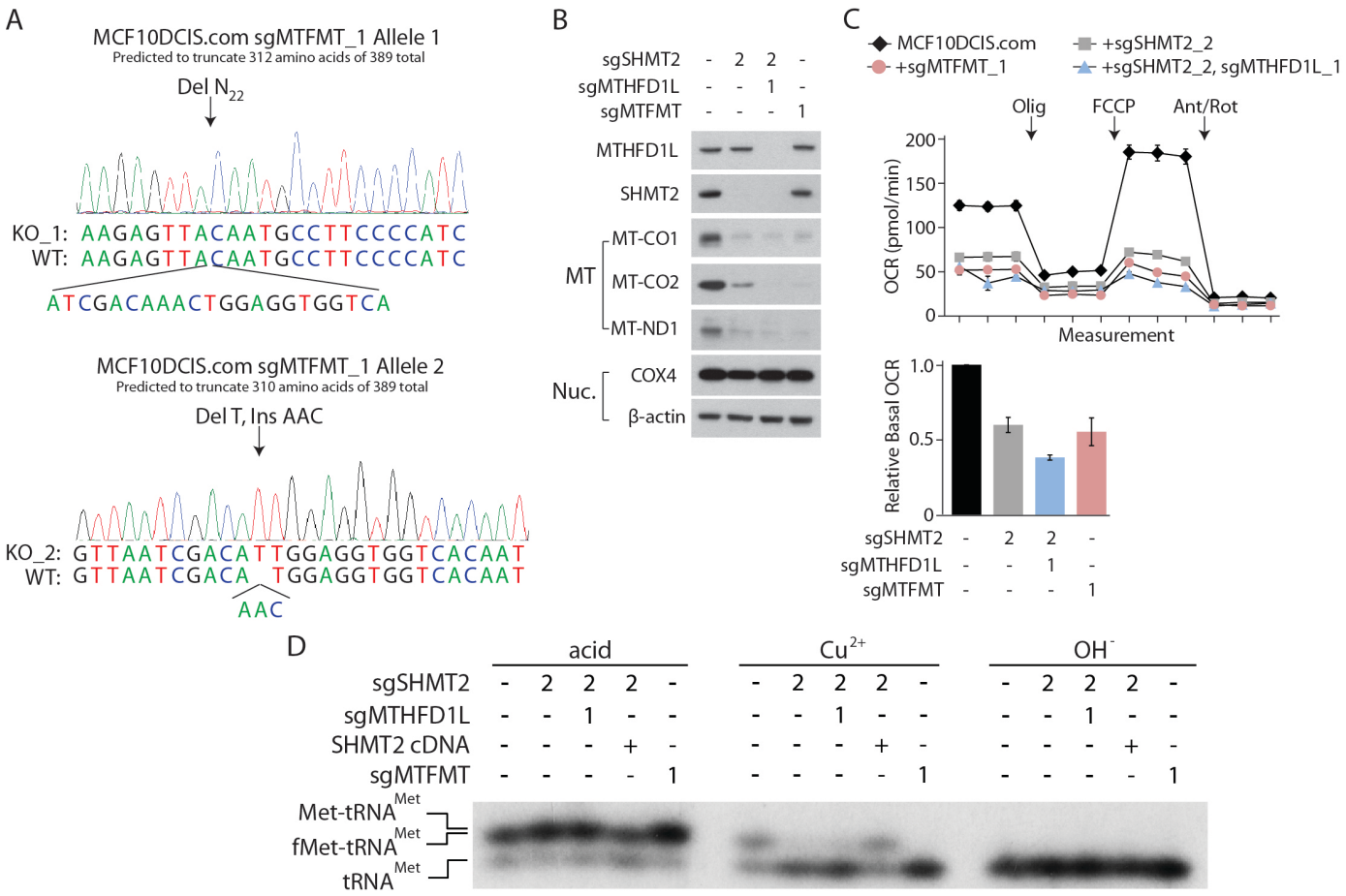

Figure S6: Deletion of MTFMT phenocopies deletion of SHMT2, Related to Figure 5. A, Sanger sequencing traces of a single cell clone of MCF10DCIS.com cells transduced with sgMTFMT_1. Traces represent the two alleles identified in this clone, isolated by PCR-based cloning and sequencing of the gDNA region targeted by the sgRNA. A is green, G is black, C is blue, T is red. The sequence observed (KO_1 or KO_2) and the corresponding wild-type sequence are shown below each trace. The inferred nucleotide change is indicated above the trace. In each case, a frameshift mutation near amino acid 77-79 of MTFMT results in an early stop codon being introduced (not shown). B, Immunoblots for the indicated proteins in MCF10DCIS.com single cell clones expressing sgMTFMT_1, sgSHMT2_1, or a combination of sgSHMT2_1 and sgMTHFD1L_1. MT, mitochondrially encoded proteins. Nuc, nuclear encoded loading controls. An MTFMT antibody was not available to confirm loss of full-length protein by immunoblot, however the Sanger sequencing traces from *A* demonstrate the introduction of two frameshift mutations and the lack of a wild-type allele. C, Above, representative oxygen consumption rate (OCR) measurements of cell lines from *B*, 10 mM glucose. The complex V inhibitor oligomycin (Olig), the uncoupler carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), and the complex I and III inhibitors Antimycin (Ant) and Rotenone (Rot) were added sequentially to a final concentration of 1 μM at the indicated time points. Measurements are mean +/- s.e.m, for four biological replicates. Below, basal OCR from the lines shown above, relative to the parent MCF10DCIS.com cell lines. Measurements are mean +/- s.e.m, for three biological replicates. D, Detection of mitochondrial $tRNA^{Met}$ species by northern blot using a probe specific to the mitochondrial $tRNA^{Met}$ and RNA isolated from MCF10DCIS.com cell clones from *B*. Met and fMet modification of the tRNA alters its mobility as indicated on the left. RNA was isolated under acidic conditions to preserve fMet or Met charged species (left) or treated to selectively hydrolyze Met (Cu^{2+}, middle) or non-selectively hydrolyze both fMet and Met (OH^-, right).