Cell

Supplemental Information

An Essential Role of the Mitochondrial Electron Transport Chain in Cell Proliferation Is to Enable Aspartate Synthesis

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Supplemental Experimental Procedures

Cell lines, constructs and antibodies

Materials were obtained from the following sources: antibodies to GOT1 from Novus (NBP1-54778), to MDH1 from Proteintech (15904-1-AP), to PC from Novus (NBP1-49536), to Raptor, mTOR, and pan-Akt from Cell Signaling Technologies; HRP-conjugated anti-rabbit antibody from Santa Cruz; Cell-Tak from BD Biosciences; sodium pyruvate, aspartic acid, polybrene, puromycin from Sigma; and blasticidin from Invivogen.

The Jurkat and Raji cell lines were purchased from ATCC and KMS-26 cells from the JCRB Cell Bank. 143B ρ^0, wild type, and CYTB cybrids were kindly provided by Navdeep Chandel (Northwestern University). MERRF cybrids were kindly provided by Giovanni Manfredi (Kwong et al., 2007; Wallace et al., 1988). All cell lines were grown in RPMI base medium containing 10% heat inactivated fetal bovine serum, 1 mM glutamine, penicillin, and streptomycin, unless otherwise indicated. For tracing experiments, RPMI without glucose and glutamine (US Biologicals-R9011), dialyzed fetal bovine serum (Sigma) and [U-^{13}C]-L-glutamine (CIL, CLM-1822-H-PK) were used. For cybrid and 143B ρ^0 proliferation experiments, RPMI without amino acids (US Biologicals-R8999) was used. Mouse embryonic fibroblasts were cultured in DMEM with 10% heat inactivated fetal bovine serum. Individual amino acids were reconstituted to RPMI amino acid concentrations except for aspartate and asparagine for the experiment in Figure 7D.

The lentiviral sgGOT1, sgMDH1 and sgPC vectors were generated via ligation of hybridized oligos (below) into lentiCRISPR-v1 vector linearized with BsmBI using Gibson assembly (NEB).

sgGOT1_10F, caccgGATAGGCTGAGTCAAAGAAG
sgGOT1_10R, AAACCTTCTTTGACTCAGCCTATCC
sgMDH1_1F, caccgGACATCTGGATACTGAGTCG
sgMDH1_1R, aaacCGACTCAGTATCCAGATGTCc
sgPC_1R, caccgCAGGCCCGGAACACACGGA
sgPC_1R, aaacTCCGTGTGTTCCGGGCCTGc

The retroviral *GOT1 and MDH1* vectors were generated by cloning sgGOT1_10 and sgMDH1_1 resistant GOT1 and MDH1 cDNAs synthesized by IDT (Geneblock) into the pMXS-ires-blast vector via Gibson Assembly. The retroviral *SLC1A3* vector was generated by cloning an SLC1A3 PCR fragment into the pMXS-ires-blast vector by Gibson Assembly. Primers for SLC1A3 PCR are below.

SLC1A3F,GCCGGATCTAGCTAGTTAATTAAGccaccATGACTAAAAGCAATGGAGA AGAGCCC;
SLC1A3R, GGGCGGAATTTACGTAGCCTACATCTTGGTTTCACTGTCGATGGG

CRISPR screens

The metabolism-focused sgRNA library was designed as previously described . Oligonucleotides for sgRNAs were synthesized by CustomArray Inc. and amplified by PCR (Wang et al., 2014). Amplicons were inserted into lentiCRISPR-v1, linearized by BsmBI digestion, by Gibson Assembly (NEB). Gibson Assembly products were then transformed into E. coli 10G SUPREME electrocompetent cells (Lucigen). This plasmid pool was used to generate lentivirus-containing supernatants. The titer of lentiviral supernatants was determined by infecting targets

cells at several amounts of virus in the presence of polybrene (4 ug/ml), counting the number of drug resistant infected cells after 3 days of selection. 40 million target cells were infected at an MOI of ~0.5 and selected with puromycin (4 ug/ml) 72 hours after infection. An initial pool of 40 million cells was harvested for genomic DNA extraction. The remaining cells were cultured for 14 doublings (with/without phenformin), after which cells were harvested for genomic DNA extraction. sgRNA inserts were PCR amplified, purified and sequenced on a HiSeq 2500 (Illumina) (primer sequences provided below). Sequencing reads were mapped and the abundance of each sgRNA was tallied. Gene score is defined as the median log2 fold change in the abundance between the initial and final population of all sgRNAs targeting that gene. The differential gene score is the difference between the untreated and phenformin-treated gene scores.

PCR primers for library amplification:
F-GGCTTTATATATCTTGTGGAAAGGACGAAACACCG
R-CTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC
Primer sequences for sgRNA quantification are:
F-AATGATACGGCGACCACCGAGATCTAGAATACTGCCATTTGTCTCAAG
R-CAAGCAGAAGACGGCATACGAGATCnnnnnnTTTCTTGGGTAGTTTGCAGTTTT
(nnnnn denotes the sample barcode).
Illumina sequencing primer
isCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTT
CTA GCTCTAAAAC.
Illumina indexing primer
isTTTCAAGTTACGGTAAGCATATGATAGTCCATTTTAAAACATAATTTTAAAACTG
CAA ACTACCCAAGAAA.

Seahorse measurements
Oxygen consumption of intact cells was measured using an XF24 Extracellular Flux Analyzer (Seahorse Bioscience). For Jurkat cells, seahorse plates were coated with Cell TAK (BD, 0.02 mg/ml in 0.1 μM $NaHO_3$) for 20 minutes to increase adherence of suspension cells. 200,000 cells were then attached to the plate by centrifugation at 2,200 rpm without brakes for 5 min. RPMI 8226 (US biological #9011) assay media was used as previously described (Birsoy et al., 2014). For basal oxygen consumption measurements the cell number was used to normalize. In order to increase phenformin entry, OCR measurements were taken 20 minutes after phenformin injection.

Gene set enrichment analysis
To study the association of gene sets with sensitivity to phenformin, we used the GSEA tool developed by the Broad Institute (Subramanian et al., 2005). The enrichment scores (ES) were computed for the ranked genes from the phenformin screen.

NAD^+ and NADH measurements:
The NAD+/NADH ratio was measured by modification of manufacturer instructions for NAD^+/NADH Glo Assay (Promega). Jurkat cells were incubated in RPMI under the conditions listed for 8 hours before cell extracts were taken. To extract

NAD^+/NADH, cells were centrifuged for 1 minute at 300 x g, and washed 3 times by quickly resuspending in 15 mL PBS and centrifuging for 1 minute at 300 x g. Pelleted samples were extracted in 100 µL ice cold lysis buffer (1% Dodecyltrimethylammonium bromide (DTAB) in 0.2 N NaOH diluted 1:1 with PBS) and frozen at -80°C. To measure NADH, 20 µL of extracts were heated to 75°C for 30 min in the basic lysis buffer to degrade NAD^+. To measure NAD^+, samples were diluted 1:1 with 0.4 N HCl and incubated at 60°C for 15 min where acidic conditions will degrade NADH. Following incubations, samples were quenched by adding 20 µL of 0.25 M Tris, 0.2 N HCl (NADH) or 20 µL of 0.5 M Tris base (NAD^+). Manufacturer instructions were then followed to measure NAD^+/NADH.

Immunoblotting

1.5 million Jurkat cells were rinsed twice in ice-cold PBS and harvested in a standard lysis buffer containing 50 mM Hepes, pH 7.4, 40 mM NaCl, 2 mM EDTA, 1.5 mM orthovanadate, 50 mM NaF, 10 mM pyrophosphate, 10 mM glycerophosphate, protease inhibitors (Roche) and 1% Triton-X-100. Proteins from total lysates were resolved by 12% SDS-PAGE, and analyzed by immunoblotting as described (Birsoy et al., 2014).

Mouse studies

All animal studies and procedures were approved by the MIT Institutional Animal Care and Use Committee. *TFAM*$^{loxP/loxP}$ mice were a generous gift from Dr. Navdeep Chandel and were generated as described previously (Larsson et al., 1998). *Ckmm-Cre/+* B6.FVB(129S4-Tg(Ckmm-cre)5Khn/J mice were obtained from The Jackson Laboratory and mated to *TFAM*$^{loxP/loxP}$ mice to generate *Ckmm-Cre/+, TFAM*$^{loxP/loxP}$ mice. All mice were maintained on a standard light-dark cycle with food and water *ad libitum*. Genotyping primers were designed to distinguish between the native wild type and loxP-containing alleles: mTFAMF1, CTGCCTTCCTCTAGCCCGGG; mTFAMR1, GTAACAGCAGACAACTTGTG; mTFAMR2, CTCTGAAGCACATGGTCAAT. When all three primers are included in the genotyping reaction, a wild type and loxP-containing allele produce a 404 and 437 bp band, respectively. Hearts were isolated from 14-16 week old animals and amino acid levels were determined from lysates as described above.

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