Supplemental Experimental Procedures

Proliferation Rates

Adherent cell lines growing in log phase were trypsinized, counted, and plated onto 6 well dishes (Corning) in 2 mL complete DMEM and incubated overnight. Initial seeding density was 20,000 cells per well for 143B, A172, H1299, HeLa, 143B WT Cybrid, and MEFs and 30,000 cells for 143B CytB and U87 cells. The following day, one plate of cells was counted to determine starting cell number at the time of treatment. Cells were washed 2 times with 2 mL phosphate buffered saline (PBS) and 4 mL media premixed with the indicated metabolites or compounds was added. FL5.12 cells, a suspension cell line, were washed 2 times in PBS and added directly to IL-3-containing media with the indicated metabolites or compounds. Proliferation rates were measured in DMEM without pyruvate containing 10% dialyzed fetal bovine serum, penicillin-streptomycin, and 0.1 mg/mL uridine, unless otherwise noted. For all conditions the seeding densities used allowed exponential proliferation for 4 days and final cell counts were measured 4 days after treatment. Cells were counted using Cellometer Auto T4 Plus Cell Counter (Nexcelom Bioscience). Proliferation rate was determined using the following formula:

$$\text{Proliferation Rate (Doublings per day)} = \log_2 (\text{Final cell count (day 5)}/\text{Initial cell count (day 1)})/4 \text{ (days)}$$

Concentrations of all metabolites and compounds added to culture media for each cell line are included as a table in supplemental methods (Table S1).

Mitochondrial Oxygen Consumption

Oxygen consumption rates were determined using a Seahorse Bioscience Extracellular Flux Analyzer (XF24). Cells were plated in Seahorse Bioscience 24 well plates at 60,000 cells per well in 100 µl complete DMEM, allowed to attach for 1 hour, and 500 µl complete DMEM was added before overnight incubation.

The following day cells were washed 2 times in assay media: DMEM without phenol red or pyruvate containing 0.5% dialyzed FBS and 0.1 mg/mL uridine at pH 7.4 and incubated in 500 µL of the same media. Oxygen consumption measurements were compared between basal measurements and following injection of the compound (AKB, rotenone, antimycin), or between wells cultured overnight in their treatment conditions (aspartate, oligomycin, FCCP). These measurements were then subtracted from oxygen consumption measurements following addition of high dose rotenone and antimycin treatment (2 µM each) to determine the mitochondria specific oxygen consumption rate. Following measurements, cell number was determined, averaged per condition, and the mitochondrial oxygen consumption rates were normalized to 100,000 cells.

Lactate Dehydrogenase Assay

Immediately prior to the start of the assay 100 μl of a reaction buffer containing 50 mM HEPES-KOH pH 7.5, 20 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 180 μM NADH, 1 mM alpha-ketobutyrate (when used) was combined with LDH (Sigma) (when added) in 96-well plates. Lactate dehydrogenase activity was assayed by monitoring disappearance of NADH absorbance at 340 nm over time.

Viability

Cell viability was determined by propidium iodide (PI) exclusion by standard protocols. 143B WT cybrid and HeLa cells were seeded overnight at 50,000 cells per well on 6 well plates. The following day, cells were washed 2 times with PBS and complete DMEM or DMEM without glucose, glutamine, or pyruvate was added with the indicated supplements. After 48 hours (HeLa) or 72 hours (143B WT Cybrid) both attached and suspension cells were collected and resuspended in 1 μg/ml PI. PI incorporation was measured by flow cytometry (BD FACS Canto II) and quantified (FACS Diva Software).

Purine Nucleotide Metabolite Extraction and LCMS Analysis

143B CytB cells were seeded at 400,000 cells/well in 6 well dishes overnight. The following day, cells were washed 2 times in PBS and media was changed to proliferation assay media with or without the indicated treatments. After 15 hours, polar metabolites were extracted from cells using 250 μl of ice cold 80% methanol. After scraping the cells, 250 μl of chloroform was added before vortexing for 10 min at 4 °C and centrifugation for 10 min at 4 °C at 16,000g. 40 μl of the top, water-methanol layer was transferred into a LCMS tube prior to sample analysis. A Dionex UltiMate 3000 ultra-high performance liquid chromatography system connected to a Q Exactive benchtop Orbitrap mass spectrometer, equipped with an Ion Max source and a HESI II probe (Thermo Fisher Scientific) was used to quantify metabolites. Samples were separated by chromatography by injecting 10 μl of sample on a SeQuant ZIC-pHILIC Polymeric column (2.1 × 150 mm 5 µM, EMD Millipore). Flow rate was set to 150 μl/min, temperatures were set to 25 °C for column compartment and 4 °C for autosampler sample tray. Mobile Phase A consisted of 20 mM ammonium carbonate, 0.1% ammonium hydroxide. Mobile Phase B was 100% acetonitrile. The mobile phase gradient (%B) was set in the following protocol: 0-20 min.: linear gradient from 80% to 20% B; 20-20.5 min.: linear gradient from 20% to 80% B; 20.5-28 min.: hold at 80% B. Mobile phase was introduced into the ionization source set to the following parameters: sheath gas = 40, auxiliary gas = 15, sweep gas = 1, spray voltage = –3.1kV, capillary temperature = 275 °C, S-lens RF level = 40, probe temperature = 350 °C. Metabolites were monitored using full scan in negative mode in the range of 285-700 m/z, with the resolution set at 140,000, the AGC target at 1,000,000, and the maximum injection time at 250 msec. Relative quantitation of metabolites was performed with XCalibur QuanBrowser 2.2 (Thermo Fisher Scientific) using a 5 ppm mass tolerance and referencing an in-house retention time library of chemical standards.

Measurement of NAD+/NADH

NAD+/NADH measurements were done using a modified version of manufacturer instructions supplied with the NAD/NADH Glo Assay (Promega). Cells were plated as done for proliferation assays and treated as indicated prior to preparation of cell extracts 6 hours after treatment. For extraction, cells were washed 3 times in ice cold PBS, extracted in 100 µL ice cold lysis buffer (1% Dodecyltrimethylammonium bromide (DTAB) in 0.2 N NaOH diluted 1:1 with PBS), and immediately frozen at -80°C. To measure NADH, 20 µL of sample was moved to PCR tubes and incubated at 75°C for 30 min where basic conditions selectively degrade NAD+. To measure NAD+, 20 µL of the samples was moved to PCR tubes containing 20 µL lysis buffer and 20 µL 0.4 N HCl and incubated at 60°C for 15 min, where acidic conditions selectively degrade NADH. Following incubations, samples were allowed to equilibrate to room temperature and then quenched by neutralizing with 20 µL 0.25 M Tris in 0.2 N HCl (NADH) or 20 µL 0.5 M Tris base (NAD+). Manufacturer instructions were followed thereafter to measure NAD+/NADH.

Statistical Analysis

Data are presented as the mean ± standard error of the mean (SEM). Sample size (n) indicates experimental replicates from a single representative experiment, the results of all experiments were validated by independent repetitions. Statistical significance was determined using a two-tailed Welch's t test where significance is $p \leq 0.05$.

Table S1. Concentrations of Media Supplements and Inhibitors Used, Related to Experimental Procedures.

Unless otherwise noted, the concentrations of all compounds were as follows:

Cell Line	AKB (mM)	Asp (mM)	Rot (nM)	Ant (nM)	Oligo (nM)	Phen (μM)	Myxo (μM)	N_3 (μM)	FCCP (nM)
143B	1	20	30	100	5	20	5	600	250
A172	1	10	75	2000	1				
H1299	1	10	50	5000	1				
HeLa	1	20	30	800	1				
MEF	1	20	200	10000	3				
FL5	1	20	30	500	0.5				
U87	1	20	40	250	5				
143B CytB	1	20							
143B WT cybrid	1	20							

Abbreviations: AKB, alpha-ketobutryate; Asp, aspartate; Rot, rotenone; Ant, Antimycin; Oligo, Oligomycin; Phen, phenformin; Myxo, Myxothiazol; N_3, Sodium Azide; FCCP, Carbonyl Cyanide-4-(Trifluoromethoxy) Phenylhydrazone.