Supplemental Figures

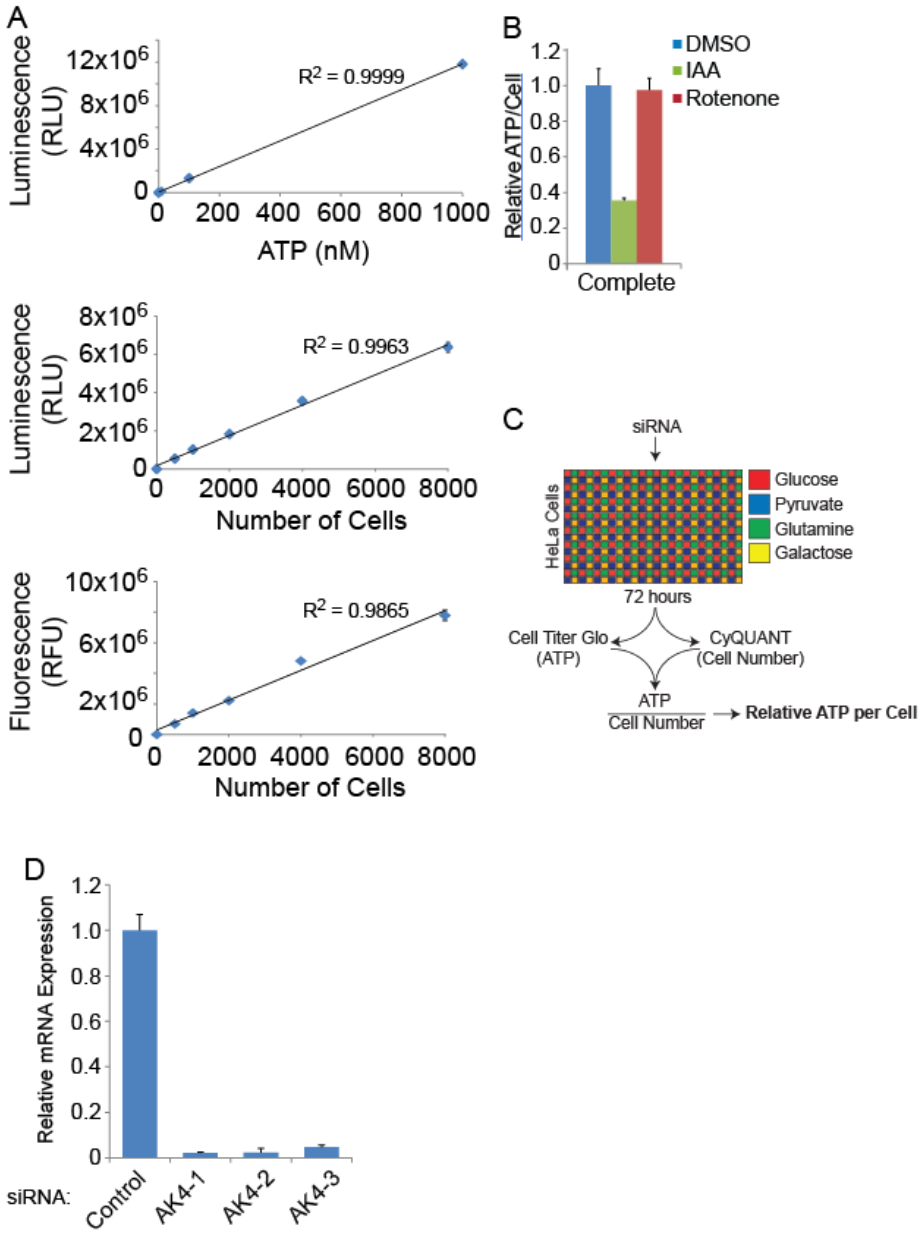

Figure S1, related to Figure 1: ATP/cell measurements, glycolytic and OXPHOS proliferation rates, and knockdown efficiency.

A. Cell Titer-Glo measurements were obtained with increasing concentrations (0 nM, 10 nM, 100 nM, 1000 nM) of ATP (top). Cell Titer-Glo measurements were obtained with increasing

numbers of HeLa cells (middle). CyQUANT measurements were obtained with increasing numbers of cells (bottom, same population of cells as Cell Titer-Glo measurements above). B. Relative ATP/cell measurements were obtained from HeLa cells maintained in the indicated medias after a four hour treatment with DMSO, iodoacetic acid (IAA, 10 µM), or rotenone (500 nM). (n >3 for each condition). Data are represented as means ± s.d. C. Schematic representing RNAi screen. D. Knockdown analysis of pooled siRNAs (two siRNA duplexes). Total RNA was isolated from HeLa cells and three separate primer sets were designed to specifically amplify the indicated gene.

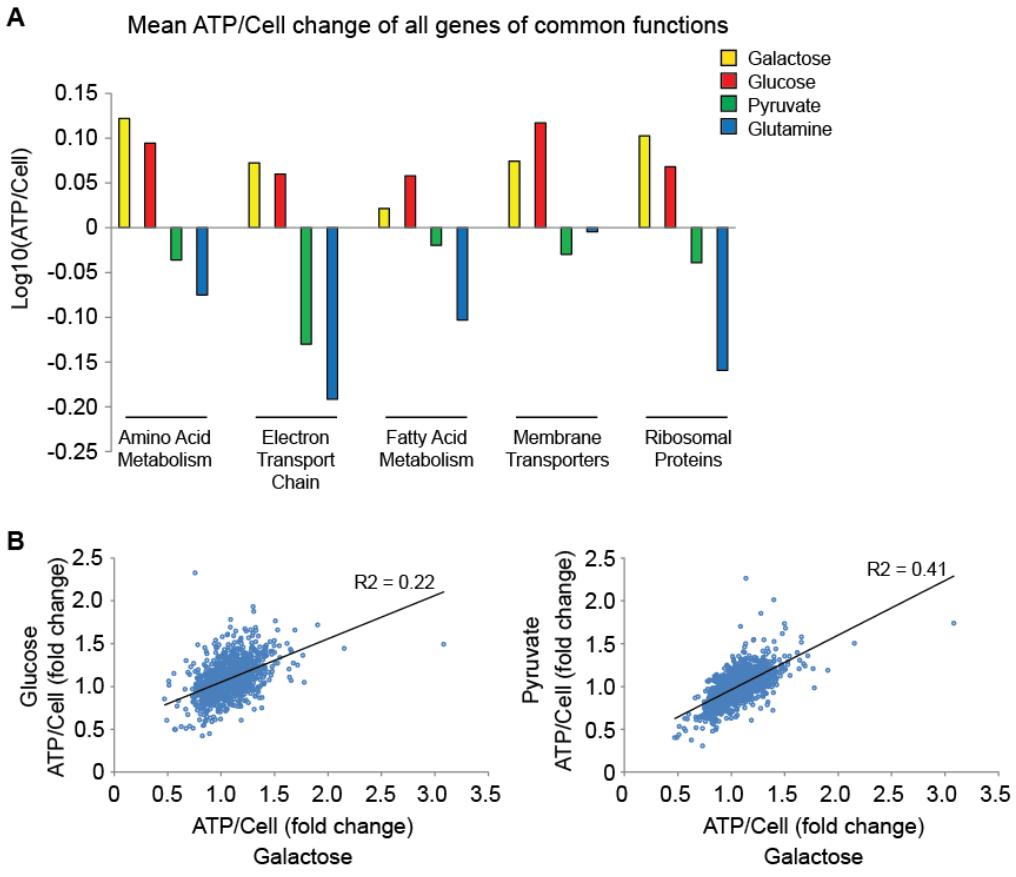

Figure S2, related to Figure 2: Comparison of mitochondrial functions and genes from RNAi screen.

A. Average ATP/Cell changes for all genes represented in each Cytoscape function. B. Gene-by-gene comparisons of screen data for galactose vs. glucose and galactose vs. pyruvate conditions.

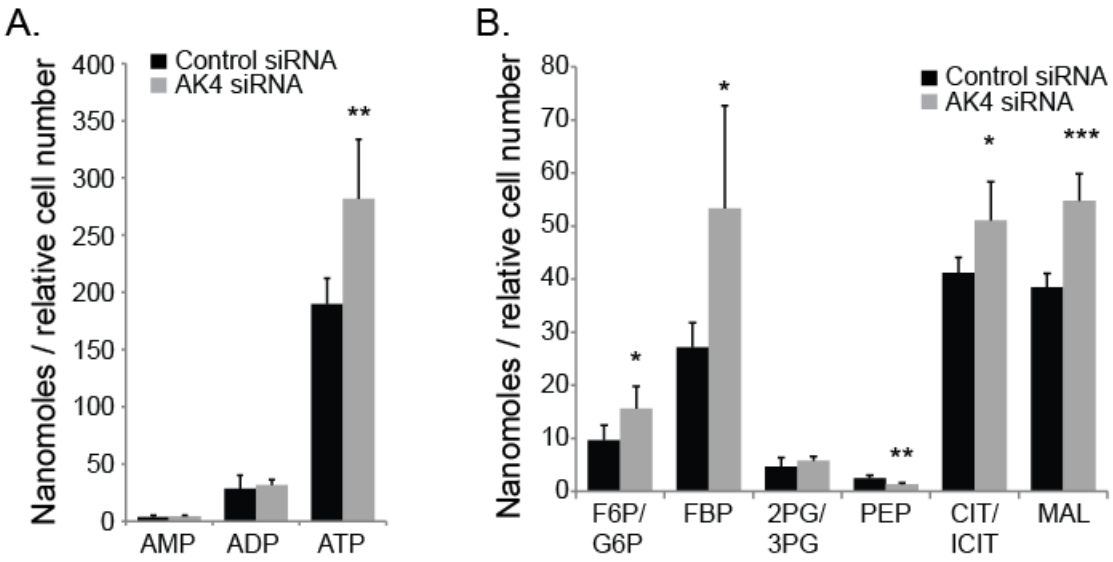

Figure S3, related to Figure 6: Adenosine nucleotide pool and central carbon metabolite analysis of AK4-silenced cells.

LC-MS analysis of adenosine nucleotide pools (A) and central carbon metabolites (B) from cells transfected with control or AK4 siRNAs.

Supplemental Tables

Table S1, related to Figure 1.

RNAi screen siRNA sequences and gene annotation.

Table S2, related to Figure 1.

RNAi screen ATP/cell measurements.

Table S3, related to Figure 1, Table 1.

RNAi screen ATP/cell measurements for genes altering ATP/cell by ≥25%.

Table S4, related to Figure 4.

Phosphorylation sites on electron transport chain machinery.

Supplemental Experimental Procedures

Cell and reagents

HeLa, U20S, and U251 cells were maintained in media formulated from DMEM lacking glucose, glutamine, and pyruvate (Sigma-Aldrich, St. Louis, MO), and supplemented with 10 mM glucose, glutamine, pyruvate, or galactose as indicated. All media was supplemented with 10% fetal bovine serum (FBS). Cells were grown in normal tissue culture-treated plates under standard growth conditions. All small interfering RNAs (siRNAs) were from Qiagen and were transfected into cells with oligofectamine (Invitrogen). siRNA sequences are provided in Supplementary Table S1. Knockdown levels were determined using quantitative reverse transcription-PCR (qRT-PCR) as previously described (MacKeigan et al, 2005).

ATP/cell measurements

With the exception of the siRNA screen (see below), all ATP/cell assays were performed in 96-well black-walled, clear bottom plates. Growth media was removed following treatments, and 100 µl of a room-temperature solution of Cell Titer-Glo, Opti-MEM, and CyQUANT was immediately added to each well. Luminescence and fluorescence readings were consecutively taken after 10 minute room-temperature incubation. Student's t-test was used to determine significance of gene knockdown values compared to non-targeting siRNA values.

RNAi Screen

siRNA transfection and data collection. HeLa cells were plated at a density of 500 cells per well in black-walled, clear bottom 384-well plates in DMEM containing 25 mM glucose, 1 mM pyruvate, and 4 mM glutamine, and 10% FBS. 8 hours after cells were plated, the media was switched to DMEM containing 10% FBS and either 10 mM glucose, 10 mM pyruvate, 10 mM glutamine, or 10 mM galactose. 16 hours after the media change, cells were transfected in each nutrient condition in duplicate with a pool of 2 siRNAs per gene at 25 nM per siRNA and 2 µl/ml HiPerfect. 24 and 48 hours after siRNA transfection, fresh media was added to the cells. 72 hours after siRNA transfection, growth media was removed and 30 µl of a room-temperature solution of Cell Titer-Glo, Opti-MEM, and CyQUANT was immediately added to each well. Luminescence and fluorescence readings were consecutively taken after a 10 minute room-temperature incubation. *Data analysis.* 8 wells of each plate were transfected with cell death control siRNAs (AllStars Hs Cell Death siRNA) for transfection quality control. 8 additional wells were transfected with non-targeting siRNAs (AllStars Negative Control siRNA) for plate normalization. Pooled siRNA for each gene were transfected in each nutrient condition in duplicate, with duplicate transfections occurring in separate plates. Luminescence and fluorescence values for each well were normalized to the average value of non-targeting siRNAs for each respective nutrient condition in each plate. Only siRNAs with s.d. <2 between replicates were included in further analyses.

Electron transport chain inhibitor treatment

Cells were cultured in glucose or pyruvate media for 72 hours prior to treatment. Cells were exposed to the indicated doses of electron transport chain inhibitors or 0.1% DMSO for 4 hours. The following ranges of doses were used (5 treatments for each inhibitor): 10nM – 125nM Rotenone; 10nM – 125nM Antimycin; 625 µM – 20 mM Azide; 62.5 nM – 2.0 µM Oligomycin.

Proliferation Index

Real-time cell proliferation was determined using the xCelligence system, which utilizes an electric current to determine cellular attachment to an electrode-containing plate. HeLa and HK2 cells were plated in glucose media in a 96-well electrode-containing plate, allowed to enter exponential growth, then transfected with the indicated siRNAs. The electrical impedance, caused by cell adhesion to the plate, is directly proportonial to cell number and is what is numerically reported. Data from the xCelligence was collected using software provided with the system, and values were normalized at the point of transfection for all cell lines.

Cell number assay

All cell number assays were CyQUANT measurements, and were performed in 96-well black-walled, clear bottom plates. Growth media was removed following indicated treatments and cells were processed according to manufacturer's protocol. Student's t-test was used to determine significance of specified gene knockdown values from non-targeting siRNA values.

[3-13C] Lactate measurement

Lactate production was measured with gas chromatography-mass spectrometry. Cells were cultured for 6 hr in glucose free medium containing dialyzed FBS supplemented with 10 mM D-[1,6-^{13}C]-glucose (Cambridge Isotope Labs), to allow the glucose-derived lactate pool to become enriched with 13C on C-3. Lactate was analyzed in 50ul of media. After a phenol-chloroform extraction of the media to remove proteins and lipids, a uniformly labeled ^{13}C lactate standard was added to the aqueous phase (internal standard). The sample was then evaporated to completion and derivatized by adding 100 microliters of a trimethylsilyl donor (Tri-Sil, Thermo scientific) for 1 hour at 42C. After being derivatized, 5 microliters of the sample was injected onto an Agilent 6890 GC networked to an Agilent 5975 Mass Selective Detector. The

abundance of [3-13C]lacate was calculated by comparing the abundance of the internal standard to the size of the total lactate pool and the fractional enrichment on C-3.

Repository of Molecular Brain Neoplasia Data (REMBRANDT) analysis

Data were downloaded from http://caintegrator-info.nci.nih.gov/rembrandt on February 25, 2014. Kaplan-Meier survival plots for gene expression data were derived from genes up-regulated or down-regulated ≥ 2 folds using the median value of all reporters. Intermediate indicates neither up-regulated nor down-regulated. Kaplan-Meier survival plots for gene copy number data were derived from genes with ≥ 2.2 copies (amplified) or ≤ 1.8 copies (deleted). Log-rank *p*-value indicates the significance of the difference in survival between any two groups of samples segregated based on gene expression or copy number of the gene of interest. The log rank *p*-value is calculated using Mantel-Haenszel procedure.

Oxygen consumption and extracellular acidification rates (OCR and ECAR)

HeLa cells transfected with 25nM of control or PPTC7 siRNAs were plated at a density of 40,000 cells per well in a Seahorse 96-well assay plate in media containing 10mM glucose, 1mM pyruvate, 1 mM glutamine. Five simultaneous OCR and ECAR measurements were taken over a 30 minute period.

Antibodies and Immunoblotting

Phospho-specific antibodies were obtained from Cell Signaling Technologies (Danvers, MA) and β-tubulin antibody was from Sigma-Aldrich (St. Louis, MO). Cells grown in the indicated culture conditions were washed with cold PBS and harvested on ice in cold pH 7.5 lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 50 mM sodium fluoride, 1 mM Na_3VO_4, 1% Triton-X100, 1 mM DTT)

supplemented with protease inhibitor cocktail (Sigma-Aldrich). Soluble protein from lysates was quantified by Bradford assay (Bio-Rad, Hercules, CA). After normalization of concentration, samples were diluted with Laemmli buffer and denatured by boiling. Samples were then separated on Tris-glycine polyacrylamide gels and transferred overnight to nitrocellulose membranes in a wet transfer apparatus (Hoefer, Holliston, MA). Membranes were blocked in 3% non-fat dry milk in Tris-buffered saline/0.1% Tween (TBS-T) and probed with primary antibodies overnight at 4°C. After washing in TBS-T buffer and incubation with a horseradish peroxidase-coupled secondary antibody, membranes were incubated in enhanced chemiluminescent reagent, exposed to film and developed for signal using an *X-omat* processing machine (Kodak, Rochester, NY).

Metabolomics

Sample preparation: 1.5ml of extraction solvent (mixture of methanol, chloroform and water) with internal standards is added to cell culture plates and cells are scraped off from plate and transferred to eppendorf tubes. Sample tubes were vertex briefly, sitting on ice for 5min, then centrifuge at 15000 xg for 5min. Supernatant containing metabolites are transferred to autosampler vials for LC-MS analysis. A series of calibration standards were prepared along with samples to quantify metabolites.

LC-MS analysis: Agilent 1200 chromatographer, Luna NH2 HILIC (hydrophilic interaction chromatography) column was used for chromatographic separation. Mass spectrometer: Agilent 6520 series time-of-flight mass spectrometer.

Data were processed by MassHunter workstation software, version B.06.