Supporting Information

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SI Methods

Cell Culture. MEFs were cultured in high glucose DMEM supplemented with 5% Penicillin/Streptamycin, 1 mM Pyruvate, and 10% FBS and HEPES Buffer. Cells where immortalized with a dominant negative p53 mutant plasmid(Addgene; plasmid 9058) and transformed with either a construct containing a mutant KrasV12 insert (Addgene; plasmid 12544), a myristolated Akt insert (Addgene; plasmid 15294) or a mutant HrasV12 insert (Addgene; plasmid 1768). LSL-Kras G12D 3T3-MEFs were isolated from LSL-KrasG12D floxed mice (1). Subsequently, these MEFs were immortalized by 3T3 protocol and infected with a lentivirus containing Cre-recombinase linked to GFP (kind gift of Dr. Thomas Südhof, Stanford University, Palo Alto, CA) (2). Human cancer cells lines HCT116 and MCF-7 were all grown in DMEM. Wild-type 143B cells were cultured in DMEM, whereas the ρ° cells were cultured in DMEM supplemented with 100 μg/ml uridine and 1 mM pyruvate. The ρ° cells were generated as previously described (3). The wild-type and ΔCyt *b* 143B cybrid cells (provided by I.F.M. de Coo, University Medical Center, Rotterdam, Netherlands) were previously described and were cultured in DMEM supplemented with 100 μg/ml uridine and 1 mM pyruvate (4). For our metabolic studies, DMEM lacking glutamine, glucose, and sodium pyruvate was purchased from Invitrogen. Cells were cultured at 37 °C in 5% CO_2 humidified incubators. Dimethyl α-ketoglutarate, aminooxyacetic acid, and were all purchased from Sigma-Aldrich.

Anchorage-Independent Soft Agar Assay. Cells were plated in defined medium supplemented with 10% fetal bovine serum at a density of 500 cells/well in 96-well tissue culture dishes. A two-layer agar system was used (Millipore), in which the final concentration of the bottom layer agarose was 0.8% and 0.4% for the top layer that contained the cells. At 14 days cells were stained and imaged with an inverted microscope at 8× magnification (Leica; MZFLIII). The number of colonies over 100 μm were counted using Colony Counter 1.0 software (Microtec Nition). The colony sizes were typically between 100-200 μm in diameter.

Proliferation Assay. Cells were plated at a density of 5×10^4 cells/well in 6-well tissue culture dishes. After 24, 48, or 72 hours, cells were trypsinized and resuspended in medium. Cells were counted using a ViCell Counter (Becton Dickinson).

Cell Death Assay. Cells were plated at a density of 2×10^4 cells/well in 6 well tissue culture dishes. Cells were treated with antioxidants for 48 hours and then stained with 100 ng/mL Propidium Iodide. Cells were analyzed using a fluorescence-activated cell sorter (FACS) flow cytometer (Becton Dickinson), and the data were analyzed with CellQuest software.

Immunoblotting. Cells were scraped and lysed using 1x cell lysis buffer (Cell Signaling) supplemented with 1 mM phenylmethylsulfonyl fluoride, and the Bio-Rad protein assay was used to measure the protein concentration. Whole-cell lysates (25 μg) were resolved on a 10% sodium dodecyl sulfate-polyacrylamide gel (Bio-Rad) and transferred to a Hybond-ECL nitrocellulose membrane (Amersham). Membranes were blocked in 5% milk in Tris-buffered saline-Tween 20 buffer. Primary antibodies used were phosphorylated Erk1/2 antibody (Cell Signaling) at 1:1,000 and total Erk1/2 antibody (Cell Signaling) at 1:1,000. Secondary antibodies used were horseradish peroxidase-linked anti-rabbit IgG (Cell Signaling) 1:1,000. SuperSignal chemiluminescent substrate (Pierce) was used to develop the blot. For in vivo studies, lungs were isolated from mice and homogenized and sonicated using a mild RIPA Buffer containing a protease inhibitor cocktail (Roche). Lung lysate (50 μg) were resolved per the procedure above. Primary antibodies used were Tfam antibody (Gift from Dr. Gerry Shadel, Yale University, New Haven, CT) at 1:2,000, Cox I antibody (Molecular Probes) at 1:1,000, and α-Tubulin antibody (Sigma) at 1:2,000.

shRNA and Generation of Stable Cell Lines. The pLKO.1 vector was used to express shRNA targeting GPI, ALT2, and GLS1. Constructs were ordered from Sigma and for GPI, the following two validated hairpin sequences were used, 5' CCGGGCGGATGTTCAATGGTGAGAACTCGAGTTCTCACCATTGAACATCCGCTTTTTG 3' and 5' CCGGCGTCTGGTATGTCTCCAACATCTCGAGATGTTGGAGACATACCAGACGTTTTTG 3'. For ALT2 we screened five hairpin sequences and determined that the following two hairpin sequences had the most efficient knockdown, 5' CCGGGACGCCATCCAGGTGAATTACCTCGAGGTAATTCACCTGGATGGCGTCTTTTTG 3' and 5' CCGGCCATCAAATGGCTCCAGACATCTCGAGATGTCTGGAGCCATTTGATGGTTTTTG 3'. For GLS1 we screened five hairpin sequences and determined that the following two hairpin sequences had the most efficient knockdown, 5' CCGGGCCCTGAAGCAGTTCGAAATACTCGAGTATTTCGAACTGCTTCAGGGCTTTTTG 3' and 5' CCGGGCCCTGAAGCAGTTCGAAATACTCGAGTATTTCGAACTGCTTCAGGGCTTTTTG 3'. The nonsilencing (control) shRNA was ordered from Addgene (plasmid 1864), 5' CCTAAGGTTAAGTCGCCCTCGCTCTAGCGAGGGCGACTTAACCTTAGG 3'.

The pSiren retroviral vector (Clontech) was used to express short hairpin RNA (shRNA) sequences for the Rieske Fe-S (5'-AAGGTGCCTGACTTCTCTGAA -3') (5). A control shRNA vector from Clontech was also utilized. Additionally, we screened two hairpin sequences in the pGIPZ backbone vector and determined that the following hairpin sequence had the most efficient Rieske Fe-S knockdown 5' CTGATGTATTTGCAAACATTAA 3'.

The pLKO.1 vector targeting Rieske Fe-S sequence was CCGGCCTATTTGGTAACTGGAGTAACTCGAGTTACTCCAGTTACCAAATAGGTTTTTG. Stable cell lines were generated by lentiviral infection using the 293FT packaging cell line and puromycin selection. At 48 hours post transfection, medium containing virus was supplemented with 8μg/ml polybrene (Sigma) for cell line infection and applied to cells.

Measurements of OCR and ECAR. A Seahorse Bioscience instrument (model XF24) was used to measure the rate of change of dissolved O_2 and pH in media immediately surrounding HCT116 cells cultured in custom 24-well plates; 20,000 cells were seeded in the custom 24-well plate in complete media for 16 hours prior to beginning the XF24 assay. For the XF24 assay, cells were equilibrated with DMEM lacking bicarbonate at 37 °C for 1 hour in an incubator lacking CO_2. Measurements of O_2 concentration and pH were made over 2 min. Measurements are reported in pmol/min for oxygen consumption and mpH/min for extracellular acidification rate.

ROS Measurement. To measure ROS levels in cells, we used the roGFP previously described (6). Cells were infected with 100 PFU of adenovirus encoding roGFP targeted to the mitochondria. Cells were harvested for analysis and analyzed using a CyanADP

flow cytometry analyzer (Dako) 48 hours after being placed under test conditions. As internal controls, samples were fully reduced with 10 mM dithiothreitol (DTT) and fully oxidized with 1 mM H_2O_2. The mean fluorescent channel for the ratio of violet excitable to blue excitable was determined with Summit software, version 4.2 (Dako). Percentage oxidized probe was determined with the equation $(R - R_{DTT})/(R - R_{H_2O_2})$, where R is sample without DTT or H_2O_2 added, R_{DTT} is the fully reduced sample, and $R_{H_2O_2}$ is the fully oxidized sample. Relative intracellular H_2O_2 was measured using Amplex Red. Briefly, cells were lysed in Amplex Red solution (100 μM) supplemented with HRP (2 units/ml) and 200 mUnits/ml of superoxide dismutase (SOD, OXIS International) and incubated in the dark for 30 min. Fluorescence was measured in the Spectra Max Gemini plate reader with excitation of 540 nm and emission of 590 nm.

Mouse Colony Maintenance and Adenovirus Cre Administration. The protocol for the use of mice was approved by the Animal Care and Use Committee at Northwestern University. Six- to eight-week-old, male, *LSL-Kras G12D* mice (weighing 20–25 g) are on a mixed B6/129 background. These mice were obtained from National Cancer Institute Repository. *Tfam* floxed mice are on a C57BL/6 background were generated by Ozgene as previously described (7). *LSL-KrasG12D* were generated by Jackson et al. (1). We utilized littermate cohorts of *Tfam* floxed mice crossed to *LSL-Kras G12D* mice. Adenoviral virus containing Cre-recombinase or the null adenoviral virus was instilled into the lungs of mice as described previously (8). Briefly, the mice were anesthetized with sodium pentobarbital and intubated with a 20-gauge angiocath. A Hamilton syringe was used to instill 1×10^9 pfu of virus in 50% bovine surfactant (Infrasurf; Forest Pharmaceuticals) and balanced Tris-EDTA buffer through the angiocath. The virus was administered in two equal aliquots, 3–5 min apart, after which the animals were extubated and allowed to recover from anesthesia with the administration of supplemental oxygen as required to treat hypoventilation.

Lung Tumor Load and Lesion Numbers. Twelve weeks post-infection, mouse lungs were inflated with 4% paraformaldehyde (15 cm H_2O) then fixed (24 hours) and paraffin embedded. Coronal lung sections from the midlung field were stained using hematoxylin and eosin. Tumor load was quantified using Olympus DP2-BSW software. One section per animal was systematically analyzed by an operator blinded to genotype. Regions positive for tumor and the total area of the lung were identified at 400× and 40×, respectively, according to the Olympus DP-2-BSW software. Tumor load was defined as the area of the regions identified as being tumor positive divided by the total area. Lesion number was defined as the number of positive regions divided by the total area of the lung.

Ki67 Proliferation Index in Lungs. Lungs were sectioned as described in the previous section and stained for hematoxylin and Ki67 (Dako; Clone Tec3). Lung sections were analyzed at 20× magnification using a Zeiss Axio Microscope. All 20× images from each lung where arranged using TissueFAXs (TissueGnostics) software to obtain a high resolution image of each lung. The percentage of Ki67 positive cells in each lung was quantified using HistoQuest (TissueGnostics) software. The number of Ki67 positive cells in each lung section was divided by the total number of cells in each lung section (hematoxylin positive counterstain).

Statistical Analysis. Data are presented as means ± SEM. One-way analysis of variance was performed in Origin 7 to determine the presence of significant differences in the data. When analysis of variance indicated that a significant difference was present, two-sample Student's t tests were performed to compare experimental data with appropriate controls (as indicated in each figure legend). Statistical significance was determined at a value of $P < 0.05$ or $P < 0.01$.

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Fig. S1. Galactose can support oncogene-induced anchorage-independent growth. Comparison of anchorage-independent cell growth in soft agar of (*A*) Akt-myr cells, HrasV12 cells, and KrasV12 cells; (*B*) LSL-Kras G12D 3T3 MEFs± Cre and (*C*) MCF-7 cells when treated with either 20 mM glucose or 20 mM galactose and ± 4 mM glutamine or ± 1 mM sodium pyruvate. Bars represent the mean ± SE (n = 9).

Fig. S2. (*A*) HCT116 infected with lentivirus encoding control shRNA or glucose phosphate isomerase (GPI) shRNA were incubated with 2 μM rotenone for 24 hours and cell death was assessed by propidium iodide (PI) staining. (*B*) Diminishing GPI levels decreases anchorage-independent growth under hypoxia. Growth in soft agar of HCT116 cells under normoxia (21% O2) or hypoxia (3% O2).

A

Human MCF-7 breast cancer cells

# of Colonies

2mM Aminooxyacetic Acid: - + + - + +

7mM dimethyl α-ketoglutrate: - - + - - +

20mM Galactose 4mM Glutamine | 20mM Glucose 4mM Glutamine

B

LSL-KrasG12D 3T3 MEFs +Cre

# of Colonies

Aminooxyacetic Acid: - + + - + +

Dimethyl α-ketoglutrate: - - + - - +

20mM Galactose 4mM Glutamine | 20mM Glucose 4mM Glutamine

C

4mM Glutamine

7mM DMK

4mM Glutamine + 7mM DMK

# of Colonies

0mM Pyruvate | 1mM Pyruvate

Fig. S3. Glutamine catabolism by the TCA cycle is essential for anchorage-independent growth of human MCF-7 breast cancer cells. Comparison of anchorage-independent cell growth in soft agar of (*A*) MCF-7 cells and (*B*) LSL-Kras G12D 3T3 MEFs + Cre when treated with either 20 mM glucose or 20 mM galactose with 4 mM glutamine and ± 2 mM aminooxyacetic acid and 7 mM dimethyl α-ketoglutarate. (*C*) Anchorage-independent cell growth in soft agar of HCT116 cells incubated with 4 mM glutamine ± 7 mM dimethyl α-ketoglutarate (DMK) or DMK without glucose.

Fig. S4. Mitochondria targeted nitroxides MCTPO and MCP inhibit colony growth in soft agar without inducing cell death. Effects of treatment with either no drug (Untreated), 1μM MCTPO, 1μM MCP, or the control compounds, 1μM CTPO, 1μM CP, and 1μM TPP on anchorage-independent cell growth in soft agar in (*A*) Akt-myr cells, (*B*) HrasV12 cells, and (*C*) KrasV12 cells. Bars represent the mean ± SE ($n = 9$). $^{*}P < 0.05$; $^{**}P < 0.01$. (*D*) Effects of (Untreated), 1 μM MCTPO, 1 μM MCP, or the control compounds, 1 μM CTPO, 1 μM CP, and 1 μM TPP on percentage of propidium iodide positive cells in Akt-myr, HrasV12, KrasV12, and LSL-Kras G12D 3T3 MEFs at t = 48 hours.

Fig. S5. Mitochondrial targeted antioxidants prevent cellular proliferation. Effects of the mitochondrial targeted antioxidants on cellular proliferation at 24, 48, or 72 hours after treatment in (*A*) Myr-Akt cells, (*B*) HrasV12 cells, (*C*) KrasV12, and (*D*) HCT116 cells. Bars represent the mean ± SE (n = 3). *$P < 0.05$; **$P < 0.01$. Statistical comparisons were made between cells treated with the mito-nitroxides (MCTPO and MCP) and the control compound (TPP).

Fig. S6. (*A* and *C*) Mitochondrial targeted nitroxides activate phosphorylated ERK1/2. Western blot analysis of phosphorylated ERK1/2 and Total ERK in HrasV12 and KrasV12 cell lysates serum starved for 18 hours (0 time point) or after 15 min serum stimulation post-48-hours treatment with either 1μM MCTPO, 1μM CTPO, 1μM MCP, 1μM CP, or 1μM TPP. (*B* and *D*) ERK1/2 phosphorylation induced by mitochondrial targeted nitroxides is reduced with the MEK inhibitor UO126. Western blot analysis of phosphorylated ERK 1/2 and Total ERK in HrasV12 and KrasV12 cell lysates serum starved for 18 hours (0 time point) or after 15 min serum stimulation post-48-hours treatment with 0 or 1 μM MCP along with either 0 nM, 100 nM, or 500 nM U0126.

Fig. S7. Treatment with the MEK inhibitor U0216 rescues mitochondrial targeted nitroxide (MCP)-mediated inhibition of soft agar colony formation. Analysis of anchorage-independent cell growth in soft agar in HrasV12 cells treated with either no drug or 1 μM MCP in addition to either 0 nM, 100 nM, or 500 nM U0216. Statistical comparison was made between cells treated with Mito CP and cells not treated with Mito CP.

Fig. S8. (*A*) Cells with electron transport deficiencies die in 20 mM galactose. Cell death was measured by propidium iodide staining (PI) in 143B, ρ°143B, wild-type 143B cybrids, and Δcytochrome *b* 143B cybrids cultured in complete media containing either 20 mM glucose (black bars) or 20 mM galactose (white bars). Bars represent the mean ± SE ($n = 4$). $^{**}P < 0.01$. Statistical comparisons were made between ρ°143B or cytochrome *b* 143B cybrids incubated in glucose media and galactose media. (*B*) Soft agar colony growth of WT143B and Δcytochrome *b* 143B cybrids infected with pLKO.1 control and RISP shRNA. Bars represent the mean ± SE ($n = 9$). $^{**}P < 0.01$.

A

Adenovirus Null-Rosa26R Adenovirus Cre-Rosa26R

B. PCR of wild-type Kras and LSL-Kras recombined locus. DNA was isolated from tumor sections.

Null Adenovirus

Cre Recombinase Adenovirus

$Kras^{fl/+}$ $Tfam^{fl/fl}$ $Kras^{fl/+}$

$Kras^{fl/+}$ $Tfam^{fl/fl}$ 1 2 3

$Kras^{fl/+}$ 1 2 3

800bp

700bp

600bp

650bp-Recombined LSL-Kras

622bp-Wildtype Kras

.40 .63 .76 .47 .51 .70 Ratio of 650bp/622bp

.60 ± .11 .56 ± .07

Fig. S9. (*A*) *Rosa 26R LacZ* reporter mice were administered Adenovirus null or Cre recombinase. After 30 days we examined β-galactosidase staining. (*B*) Recombination frequency of LSL-KrasG12D allele in $Kras^{fl/+}$ and $Kras^{fl/+}$ $Tfam^{fl/fl}$. DNA was isolated from 30 μm whole lung paraffin embedded sections using a QIAamp DNA FFPE Tissue Kit (Qiagen). Three different $Kras^{fl/+}$ and $Kras^{fl/+}$ $Tfam^{fl/fl}$ mice were utilized. PCR was performed using GoTaq (Promega) and primers that recognize the wild-type Kras allele and the recombined allele upon Cre recombinase treatment. These primers, GTCTTTCCCCAGCACAGTGC and CTCTTGCCTACGCCACCAGCTC detect a wild-type band at 622 bp and a recombined band at 650 bp (larger due to the presence of a *loxP* site indicative of recombination). Densitometry between recombined allele and wild-type allele was performed using the Image J software.