Mitochondrial metabolism and ROS generation are essential for Kras-mediated tumorigenicity

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Otto Warburg’s theory on the origins of cancer postulates that tumor cells have defects in mitochondrial oxidative phosphorylation and therefore rely on high levels of aerobic glycolysis as the major source of ATP to fuel cellular proliferation (the Warburg effect). This is in contrast to normal cells, which primarily utilize oxidative phosphorylation for growth and survival. Here we report that the major function of glucose metabolism for Kras-induced anchorage-independent growth, a hallmark of transformed cells, is to support the pentose phosphate pathway. The major function of glycolytic ATP is to support growth under hypoxic conditions. Glutamine conversion into the tricarboxylic acid cycle intermediate alpha-ketoglutarate through glutaminase and alanine aminotransferase is essential for Kras-induced anchorage-independent growth. Mitochondrial metabolism allows for the generation of reactive oxygen species (ROS) which are required for Kras-induced anchorage-independent growth through regulation of the ERK MAPK signaling pathway. We show that the major source of ROS generation required for anchorage-independent growth is the Qo site of mitochondrial complex III. Furthermore, disruption of mitochondrial function by loss of the mitochondrial transcription factor A (TFAM) gene reduced tumorigenesis in an oncogenic Kras-driven mouse model of lung cancer. These results demonstrate that mitochondrial metabolism and mitochondrial ROS generation are essential for Kras-induced cell proliferation and tumorigenesis.

I

In the 1920s, Otto Warburg observed that tumor slices have elevated levels of glucose consumption and lactate production in the presence of ample oxygen (termed the Warburg effect) (1). He later postulated that cancer originates from irreversible injury to respiration followed by an increase in glycolysis to replace ATP loss due to defective oxidative phosphorylation (2). According to Warburg, this metabolic shift from oxidative phosphorylation to glycolysis converts differentiated cells into undifferentiated cells that proliferate as cancer cells. Although the observation that tumor cells exhibit high levels of aerobic glycolysis has been corroborated, the role of mitochondria in tumor cells has been contentious (3). While multiple investigators have demonstrated that mitochondria are indeed functional in most tumor cells, some argue that decreases in mitochondrial metabolism and respiratory rate are essential for tumor cell proliferation (4). However, the only tumor cells shown to exhibit mitochondrial dysfunction are those that have mutations in the tricarboxylic acid (TCA) cycle enzymes succinate dehydrogenase (SDH) or fumarate hydratase (FH) (5). Furthermore, oncogenic activation increases mitochondrial metabolism (6), correlating with metastatic potential (7).

An attractive model of tumor cell metabolism is that oncogene-induced transformation depends on elevated glycolysis for ATP synthesis and the diversion of glycolytic intermediates to the pentose phosphate pathway (8). Those intermediates are used to generate NADPH and ribose-5-phosphate for reductive biosynthesis reactions and nucleotide biosynthesis, respectively. Glycolysis generates ATP with lower efficiency but at a faster rate than oxidative phosphorylation (9). The increased production of ATP by glycolysis is postulated to provide metabolic advantage to proliferating cells, while the role of mitochondria has been restricted to providing TCA cycle intermediates as substrates for de novo synthesis of lipids and nonessential amino acids (10). Since Warburg’s initial observation, much progress has been made in elucidating the metabolic reprogramming of tumor cells, however, the role of glucose metabolism and mitochondrial metabolism in regulating oncogene-induced tumorigenicity are not fully understood. In the present study, we utilized a combination of biochemical, pharmacological, and genetic techniques to dissect the role of glucose and mitochondrial metabolism in Kras-mediated tumorigenicity.

Results

The Pentose Phosphate Pathway, Not Glycolysis, Is Essential for Kras-Induced Growth Under Aerobic Conditions. To test whether high levels of aerobic glycolysis are required for Kras-induced cell proliferation, we assessed the ability of KrasG13D driven HCT116 colon cancer cells to grow in soft agar in media containing galactose or glucose. Galactose enters glycolysis through the Leloir pathway, which occurs at a significantly lower rate than glucose entry into glycolysis (11). Cells grown in media containing glucose or galactose exhibited growth in soft agar (Fig. L4). HCT116 cells grown in galactose displayed an increased ratio of oxygen consumption rate (OCR) to extracellular acidification rate (ECAR) (Fig. 1B). ECAR is an indirect measurement of lactate production. Similar results were observed with murine embryonic fibroblasts (MEFs) immortalized with a dominant negative p53 and transformed ectopically by either myristoylated AKT, HrasV12, KrasV12, or MCF-7 breast cancer cells (activated PI3K mutation) as well as MEFs isolated from mice containing one wild-type Kras allele and the other allele containing a Lox-STOP-Lox KrasG12 D (termed LSL-KrasG12D 3T3MEFs) (Fig. S1).

Galactose mainly supports cell proliferation by entering into the pentose phosphate pathway and not glycolysis (11). To further dissect the role of glycolysis and the pentose phosphate pathway for growth, we reduced glucose 6-phosphate isomerase (GPI) protein levels by shRNA. Diminishing GPI allows the pentose phosphate pathway to proceed normally while compro...
mising glycolysis. HCT116 cells infected with two lentiviral GPI shRNA died in the presence of the mitochondrial inhibitor rotenone (Fig. S2A) and displayed an increased OCR to ECAR ratio, indicating a dependency on mitochondrial metabolism for survival (Fig. 1C). The total number of soft agar colonies did not differ between HCT116 cells expressing GPI shRNA or control shRNA under normoxic conditions; however, there was a noticeable difference in colony number under hypoxic conditions (Fig. 1D). A closer examination of the colony size revealed that reducing GPI protein levels inhibited the ability of cells to grow in colonies to a size greater than 150 microns, the threshold at which cells encounter hypoxia (Fig. S2B). These results indicate that the major role of glucose metabolism for growth is to fuel the pentose phosphate pathway while the generation of glycolytic ATP is only necessary for growth under hypoxic conditions.

Glutamine Catabolism by the TCA Cycle Is Required for Oncogenic Kras-Induced Growth. Previous studies suggest that Hela cells and Myc-overexpressing tumor cells require glutamine metabolism for cell growth and proliferation (12–15). Glutamine can be converted to glutamate by glutaminase. Subsequently, glutamate can enter the TCA cycle through its conversion into alpha-ketoglutarate by either aminotransferases or glutamate dehydrogenase. To determine whether glutamine entry into the TCA cycle is a general requirement for the growth of tumor cells, we utilized aminooxycetic acid (AOA), a potent inhibitor of aminotransferase activity. Soft agar colony growth of HCT116 human colon cancer cells was inhibited in the presence of AOA, which was rescued with the addition of the cell permeable dimethyl a-ketoglutarate (DMK) (Fig. 2A). Similar results were observed in LSL-KrasG12D 3T3 MEFs and human MCF-7 breast cancer cells (Fig. S3 A and B). Glutamine addition to media stimulated oxygen consumption, which was inhibited by AOA and rescued by DMK (Fig. 2B). DMK did not further increase growth in the presence of glutamine indicating that DMK-induced colony growth was a specific rescue of AOA treatment (Fig. S3C). Glutamate conversion into alpha-ketoglutarate requires either pyruvate or oxaloacetate through the alanine or aspartate aminotransferases, respectively. We reasoned that pyruvate would be more abundant than oxaloacetate for carrying out the transamination reaction; thus, we tested whether diminishing mitochondrial alanine aminotransferase (ALT2) would attenuate anchorage-independent growth. Indeed, HCT116 cells expressing ALT2 shRNA had diminished colony growth compared to the control shRNA cells, an effect rescued by DMK (Fig. 2C). Interestingly, knockdown of glutaminase (GLS1) also decreased colony growth but was not effectively rescued by DMK (Fig. 2D). Glutamate, the product of GLS, is also required for generation of glutathione and NADPH to provide redox balance (15). These data suggest that the ability of glutamine to fuel the TCA cycle through ALT2 and GLS1 is critical for anchorage-independent growth.

Mitochondria Derived Reactive Oxygen Species (ROS) Are Required for Cell Proliferation. A consequence of mitochondrial metabolism is the generation of ROS by the electron transport chain. Previous reports indicate that cancer cells exhibit more oxidative stress than normal cells (16). Indeed, we observed an oncogene-induced increase in mitochondrial ROS as assessed by mitochondria-targeted redox sensitive GFP (mito-roGFP, Fig. 3A). This roGFP protein contains two surface-exposed cysteines that form a disulfide bond in the presence of an oxidant resulting in an increase in the excitation at 400 nm at the expense of the peak near 490 nm. These results were corroborated using Amplex Red, which detects intracellular H2O2 levels (Fig. 3B). We hypothesized that mitochondrial ROS is required to maintain oncogene-induced anchorage-independent growth. To test this, colony growth was evaluated in the presence of the mitochondria-targeted nitroxides,
Mitochondrial ROS Regulate Cellular Proliferation Through ERK1/2.

To determine whether mitochondrial ROS regulate cell proliferation, we assessed the ability of KrasV12-p53DN, HrasV12-p53DN, myr-Akt-p53DN MEFs, LSL-KrasG12D 3T3 MEFs, and HCT116 cells to proliferate following treatment with mitochondrion-targeted antioxidants. All cell lines showed a reduction in the rate of proliferation at 48-hours post-treatment suggesting that ablation of mitochondrial ROS by the mitochondrion-targeted antioxidants induced cell proliferation arrest (Fig. 4A and Fig. S5). The extracellular signal-regulated kinase (ERK1/2) MAP kinase pathway is an important regulator of proliferation. The ERK MAPKs are regulated by the upstream MEK kinase (MAPK kinase) and at low levels induce cellular proliferation but at higher levels cause growth arrest (18). The mito-nitroxides, but not untargeted nitroxides, induced an increase in phosphorylated ERK 1/2 in tumor cells (Fig. 4B and Fig. S6 A and C). The MEK inhibitor U0126 decreased the phosphorylation of ERK1/2 to levels similar to cells incubated in the absence of mito-nitroxides (Fig. 4C and Fig. S6 B and D). Additionally, cells treated with the MEK inhibitor U0126 rescued soft agar colony formation in the presence of mito-nitroxides (Fig. 4D and Fig. S7). These data suggest that oncogene-induced mitochondrial ROS serve as signaling molecules to dampen the ERK1/2 MAPK pathway to levels that are compatible with cellular proliferation and subsequent anchorage-independent growth.

Mitochondrial Complex III Regulates Anchorage Independent Growth Individually from Oxidative Phosphorylation. To genetically address the requirement of mitochondrial ROS for anchorage-independent growth, we utilized 143B osteosarcoma cells (oncogenic Kras mutation) that are depleted of mitochondrial DNA (ρ0 cells). These ρ0 cells were then reconstituted with either MCP and MCTPO, which act as superoxide dismutase mimetics and superoxide scavengers. These nitroxides are targeted to mitochondria by covalently coupling the nitroxide moiety to a triphenylphosphonium cation (TPP) (17). Cells treated with mito-nitroxides (MCP and MCTPO) demonstrated reduced colony growth as compared to control nitroxides (CTPO and CP), which were not targeted to the mitochondria, suggesting that mitochondria-derived ROS are critical for anchorage-independent growth (Fig. 3 C and D and Fig. S4 A–C). The antioxidants did not cause cell death (Fig. S4D).

Mitochondrial ROS are required for oncogene-induced anchorage-independent growth. (A) Levels of oxidized mito-roGFP in p53DN, Myr-Akt-p53DN, HrasV12-p53DN, and KrasV12-p53DN cell lines. Mean ± SE (n = 4). *P < 0.05; Statistical comparisons were made between p53DN cells and Myr-Akt, HrasV12, or KrasV12 cells. (B) Intracellular H2O2 levels assessed by Amplex Red in cell lysates of p53DN, HrasV12-p53DN cells, LSL-KrasG12D 3T3 + Cre. Mean ± SE (n = 4). Data represented is KrasV12-p53DN cells/immortalized p53DN and LSLKrasG12D3T3 + Cre/immortalized LSLKrasG12D. Analysis of soft agar colonies of (C) LSL-Kras G12D, and (D) HCT116 cell lines treated with mitochondrial targeted antioxidants 1 μM MCTPO, 1 μM MCP, or the control compounds 1 μM CTPO, 1 μM CP, and 1 μM TPP. Mean ± SE (n = 9). **P < 0.01; Statistical comparisons are between MCTPO or MCP and TPP.

Fig. 3. Mitochondrial ROS are required for oncogene-induced anchorage-independent growth. (A) Levels of oxidized mito-roGFP in p53DN, Myr-Akt-p53DN, HrasV12-p53DN, and KrasV12-p53DN cells. Mean ± SE (n = 4). *P < 0.05; Statistical comparisons were made between p53DN cells and Myr-Akt, HrasV12, or KrasV12 cells. (B) Intracellular H2O2 levels assessed by Amplex Red in cell lysates of p53DN, HrasV12-p53DN cells, LSL-KrasG12D 3T3 + Cre. Mean ± SE (n = 4). Data represented is KrasV12-p53DN cells/immortalized p53DN and LSLKrasG12D3T3 + Cre/immortalized LSLKrasG12D. Analysis of soft agar colonies of (C) LSL-Kras G12D, and (D) HCT116 cell lines treated with mitochondrial targeted antioxidants 1 μM MCTPO, 1 μM MCP, or the control compounds 1 μM CTPO, 1 μM CP, and 1 μM TPP. Mean ± SE (n = 9). **P < 0.01; Statistical comparisons are between MCTPO or MCP and TPP.

Fig. 4. Mitochondrial ROS regulate anchorage-independent growth through the MAPK/ERK1/2 pathway. (A) Effects of mitochondrial targeted nitroxides and control compounds on LSL-KrasG12D 3T3 MEFs + Cre cellular proliferation at 24, 48, or 72 hours after treatment. *P < 0.05; **P < 0.01. Statistical comparisons are between MCTPO or MCP and TPP. (B) Western blot analysis of phosphorylated ERK1/2 and Total ERK in LSL-KrasG12D 3T3 MEFs cell lysates serum starved for 18 hours (0 time point) or after 15 min serum stimulation post-48-hours treatment with 1 μM MCTPO, 1 μM CTPO, 1 μM MCP, 1 μM CP, and 1 μM TPP. (C) Western blot analysis of phosphorylated ERK1/2 and Total ERK 48 hours after treatment with no drug or 1 μM MCP in the presence of either 0 nM, 100 nM, or 500 nM U0126. (D) Analysis of soft agar colonies of LSL-KrasG12D 3T3 MEFs treated with either 0 or 1 μM MCP in the presence of 0 nM, 100 nM, or 500 nM U0126. Mean ± SE (n = 9). **P < 0.01. Statistical comparison was made between cells treated with Mito CP and cells not treated with Mito CP.
wild-type mitochondria or mitochondria containing a 4-bp mutation in the mitochondrial-encoded cytochrome b gene (wt or Δcytochrome b 143B cybrids). Both ρ₀ cells and Δcytochrome b 143B cybrids are deficient in oxidative phosphorylation and did not survive in glucose-free media enriched with galactose (Fig. S8A). However, ρ₀ 143B cells cannot generate mitochondrial ROS, while the Δcytochrome b cybrids can generate superoxide at the Qₒ site of complex III (19). As predicted, the ρ₀ 143B cells had undetectable levels of mitochondrial ROS compared to Δcytochrome b cybrids as assessed by oxidation of the mito-roGFP probe (Fig. 5A). The ρ₀ 143B cells exhibited minimal growth in soft agar as compared to the Δcytochrome b 143B cybrids, highlighting the importance of mitochondrial ROS in tumor cell proliferation (Fig. 5B). The Δcytochrome b 143B cybrids had less growth in soft agar than the wild-type 143B cybrids, suggesting that a defect in oxidative phosphorylation also diminishes anchorage-independent growth. Wild-type cybrids and Δcytochrome b cybrids were both dependent on glutamine entry into the TCA cycle as AOA abolished soft agar colony formation (Fig. 5C). These results indicate that the Δcytochrome b cybrids still require glutamine catabolism by the TCA cycle even though they cannot generate ATP by oxidative phosphorylation.

The Δcytochrome b 143B cybrids have a disrupted Complex III, however there are residual levels of Rieske iron sulfur protein (RISP), a component required for ROS generation at the Qₒ site of complex III (20). In the absence of RISP, ROS are not generated at the Qₒ site of complex III (19). The wild-type 143B cybrids and Δcytochrome b 143B cybrids were stably infected with shRNA against the RISP or a negative control shRNA (Fig. 5D). In the absence of RISP, anchorage-independent growth was ablated in both wild-type 143B cybrids and Δcytochrome b 143B cybrids, indicating that the ROS generated from the Qₒ site of complex III is required for anchorage-independent growth (Fig. 5E). Similar results were obtained with a second shRNA against RISP (Fig. S8B).

Mitochondrial Metabolism Is Required for in Vivo Tumorigenesis. The inability of ρ₀ cells to grow in an anchorage-independent manner suggests that mitochondrial function is crucial for tumor cell proliferation. To address whether loss of mitochondrial function in vivo impacts tumorigenesis, we crossed LSL-Kras G12D mice with mice harboring floxed alleles of mitochondrial transcription factor A (Tfam). Tfam is required for mitochondrial DNA replication and transcription and previous studies demonstrated that Tfam-null tissues are deficient in electron transport and oxidative phosphorylation (21). After crossing these animals, we verified that Cre recombinase reduced Tfam protein levels and the mitochondrial DNA encoded protein, cytochrome c oxidase subunit I (COXI) (Fig. 6A). The recombination frequency of LSL-KrasG12D allele was not influenced by the presence of Tfam floxed alleles (Fig. S9). Intratracheal adenoviral Cre-treated LSL-Kras G12D⁺β mice formed fewer lesions per unit area, had smaller tumors, and displayed fewer Ki67 positive cells compared to adenoviral Cre-treated LSL-Kras G12D⁺β mice (Fig. 6B–E).
This indicates that a functional mitochondrial electron transport chain is required for tumorigenesis.

Discussion

Recent studies demonstrate that a gain of function in oncopgenes, loss or mutation of tumor suppressors, and the activation of phosphoinositide 3-kinase (PI3K) are major regulators of the high levels of aerobic glycolysis observed in tumor cells (22). Our present studies suggest that this high glycolytic flux is required to provide continuous glycolytic intermediates for the pentose phosphate pathway in order to generate nucleotides and phospholipids for rapidly proliferating tumor cells. Our premise that enhanced aerobic glycolysis is more important for anabolic processes such as nucleotide and phospholipid synthesis, as opposed to the generation of ATP, is supported by the observation that diminishing GPI protein levels did not prevent colony survival of these tumor cells under hypoxia. These results are consistent with previous studies that showed the inhibition of the glycolytic enzyme lactate dehydrogenase A (LDH-A) prevents anchorage-independent growth of Myc tumor cells and xenograft tumor formation of mouse mammary tumor cells by preventing the survival of these tumor cells under hypoxia (24, 25).

Previous studies indicate that cells overexpressing Myc utilize glutamine metabolism for their proliferation (13, 14). Myc represses microRNA mir23a/b, which allows for the expression of glutaminase (15). Our current results indicate that glutamine addiction is likely a general phenomenon as Ras-, Myc-, and AKT-dependent tumor cells require glutamine-fueled mitochondrial metabolism. Glutamine catabolism by the TCA cycle is essential for tumor cell growth and proliferation in the absence or presence of glucose. Glutamine is converted to glutamate by glutaminase, which can be converted to alpha-ketoglutarate by alanine or aspartate aminotransferases as well as glutamate dehydrogenase. The inhibition of mitochondrial alanine aminotransferase by shRNA prevented anchorage-independent growth in oncogenic Kras-transformed cells, which was rescued by cell permeable alpha-ketoglutarate. An important consequence of glutamine catabolism by the TCA cycle is the production of reducing equivalents for the generation of ROS by complex I, II, and III of the electron transport chain. Our data indicate that oncogenic Kras induced an increase in mitochondrial ROS to control cellular proliferation. These results are consistent with previous studies suggesting that ROS regulate cell cycle progression (26). We
propose that oncogenes induce mitochondrial ROS to serve as signaling molecules to regulate cell proliferation.

The premise that ROS are required for tumor cell proliferation is further supported by our observations that cells deficient in mitochondrial DNA (p<sub>0</sub>) cells) do not generate ROS nor grow in an anchorage-independent manner. By contrast, cells with a deletion in the mitochondrial DNA encoded cytochrome b gene were able to generate ROS and grow in an anchorage-independent manner. Both p<sub>143B</sub> cells and cytochrome b null 143B cells are deficient in oxidative phosphorylation. Preventing the generation of ROS from complex III in wild-type or cytochrome b null cells by shRNA targeting of the complex III subunit RISP diminishes growth of cytochrome b null cells in soft agar. These results indicate that complex III-generated ROS are crucial to maintain the transformed cancer cell phenotype. The essential role of mitochondria in regulating tumorigenesis is further corroborated by our observation that loss of the TFAM protein, which is required to replicate and maintain mtDNA copy number, diminished tumorigenicity in an oncogenic Kras-driven mouse model of lung adenocarcinoma.

In summary, our data indicate that the majority of cancer cells have evolved to utilize glycolysis, pentose-phosphate pathway, and mitochondrial metabolism to provide the necessary resources for rapid cell proliferation. We suggest that the major role of aerobic glycolysis in cancer cells is likely to provide glycolytic intermediates to the pentose phosphate pathway for nucleotide and phospholipid synthesis, while glycolytic ATP generation is likely to be important for survival under hypoxic conditions.

The glutamine-fueled TCA cycle results in generation of ATP, ROS, NADPH, amino acids, nucleotides, and lipids. We propose that feeding substrates to the TCA cycle through amino acids, as well as other sources such as fatty acid oxidation, is critical for oncogene-induced tumorigenicity. Deciphering the pathways that fuel the TCA cycle differentially in cancer cells versus normal cells will enable design of targeted therapies.

**Methods**

Full methods are available as SI Methods.

Anchorage-independent growth was assessed by plating cells in a two-layer agar system (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. The protocol for the use of mice was approved by the Animal Care and Use Committee at Northwestern University. LSL-Kras G12D mice are on a mixed B6/129 background and were obtained from National Cancer Institute Repository (Bethesda, MD). Tfam floxed mice are on a C57BL/6 background and were generated by Oogene as previously described (21). We utilized litter-mate cohorts of Tfam floxed mice crossed to LSL-Kras G12D mice.

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Supporting Information

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SI Methods

Cell Culture. MEFs were cultured in high glucose DMEM supplemented with 5% Penicillin/Streptomycin, 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 myristylated Akt insert (Addgene; plasmid 15294) or a mutant HrasV12 insert (Addgene; plasmid 1766). 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, supplemented with 5% Penicillin/Streptomycin, 1 mM Pyruvate, and pH in media immediately surrounding HCT116 cells was monitored. Cells were harvested for analysis and analyzed using a Coulter Counter (Becton Dickinson).

Anchorage-Independent Soft Agar Assay. Cells were plated in defined medium supplemented with 10% fetal bovine serum that contained 500 cells/well in 96-well tissue culture dishes. A twolayer 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; M2FLIII). 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 × 10^6 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 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 lysates (50 μg) were resolved per procedure above. Primary antibodies used were Tiam 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 for and for GPI, the following two validated hairpin sequences were used, 5′ CCGGCACGATGCCATCACTCTGGTATGTCTCCAA-3′ and 5′ CCGGGTCTGTGCTATGTTGGAGACATACCAGCTTTTTG 3′. For ALT2 we screened five hairpin sequences and determined that the following two hairpin sequences had the most efficient knockdown, 5′ CCGGGACGCCATCCAGGTTAACTCTCGCT-TAGTAATTCCTGATTGATGTTGGAGACATACCAGCTTTTTG 3′ and 5′ CGGGCATCAAAATGGCTCAGACATCTCGGAGATGTTGGAGACATACCAGCTTTTTG 3′. For GLS1 we screened five hairpin sequences and determined that the following two hairpin sequences had the most efficient knockdown, 5′ CGGGGCCCTGAAGCAGTGTCAACTCTGGGCTTTTTG 3′ and 5′ CCGGGCTCTGTGCTATGTTGGAGACATACCAGCTTTTTG 3′. The nonsilencing (control) shRNA was ordered from Addgene (plasmid 1864), 5′ CCTAAGCTTAAGGTTACCTGCTGAGCTTACCGGGCATTCATGTTTTTGG 3′. The pSiren retroviral vector (Clontech) was used to express short hairpin RNA (shRNA) sequences for the Rieske Fe-S (5′-AAGGTGCTACACTAGCATTGACGGCGCTTTTTG 3′) (5). A control shRNA sequence for Rieske Fe-S was obtained 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 knockdown, the following two hairpin sequences had the most efficient knockdown, 5′ CGGGGCCCTGAAGCAGTGTCAACTCTGGGCTTTTTG 3′ and 5′ CCGGGCTCTGTGCTATGTTGGAGACATACCAGCTTTTTG 3′. The pLKO.1 vector targeting Rieske Fe-S sequence was CCGGCCTTATTTGTAACACTGAGTAACTCGAGTAGTCTTAC-GAGTTCAAAATAGTGGTTTTG. 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 O2 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 CO2. Measurements of O2 concentration and pH were made over 2 min. Measurements are reported in pmol/min for oxygen consumption and pmH/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$_2$O$_2$. 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$_2$O$_2$ added, $R_{DTT}$ is the fully reduced sample, and $R_{H_2O_2}$ is the fully oxidized sample. Relative intracellular H$_2$O$_2$ 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 and 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 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 hypventilation.

**Lung Tumor Load and Lesion Numbers.** Twelve weeks post-infection, mouse lungs were inflated with 4% paraformaldehyde (15 cm H$_2$O) 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 Axiol Microscope. All 20× images from each lung were 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 $P < 0.01$.

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).
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), 1 μM MCTPO, 1 μM MCP, or the control compounds, 1 μM CTPO, 1 μM CP, and 1 μM 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 U0126. 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.
Fig. 59. (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<sup>fl/+</sup> and Kras<sup>fl/+Tfam<sup>fl/fl</sup></sup>. DNA was isolated from 30 μm whole lung paraffin embedded sections using a QIAamp DNA FFPE Tissue Kit (Qiagen). Three different Kras<sup>fl/+</sup> and Kras<sup>fl/+Tfam<sup>fl/fl</sup></sup> 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.