The Role of Respiration in Supporting Cell Proliferation

by

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B.S. Biological Science and Chemistry
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Abstract

Compared to non-proliferating cells, proliferating cells such as cancer cells have additional metabolic requirements for generating biomass. However, despite these additional requirements the components of the mammalian metabolic network in both proliferating and non-proliferating cells are largely the same. Thus, in order to balance the competing anabolic and catabolic needs of a proliferating cell, the same metabolic networks components must take on distinct roles. Understanding how the various network components support proliferation may lead to improvements in cancer therapy. It has long been known that mitochondrial respiration is essential for proliferation. However, the precise metabolic role that is filled by respiration is not well defined. This thesis focuses on understanding the role of respiration in supporting mammalian proliferation. In non-proliferating cells respiration is considered to be primarily an ATP-producing catabolic process. We find that in proliferating cells, respiration serves a crucial anabolic role by providing access to an electron acceptor in the form of molecular oxygen. Electron acceptor availability is required for maintaining NAD+/NADH homeostasis and supporting aspartate synthesis. In conditions where alternative electron acceptors are provided such that cells can maintain NAD+/NADH homeostasis through alternative pathways, or when exogenous aspartate is provided, respiration is dispensable for proliferation. These findings highlight that metabolic dependencies can be modified by environmental conditions. Consistent with this, we find that altering NAD+/NADH homeostasis through alternative pathways or providing exogenous aspartate can modulate cellular sensitivity to respiration inhibitors such as metformin. Collectively, these studies contribute to an understanding of how metabolism supports biomass generation for proliferation and offers insight to how metabolism could be targeted for cancer therapy.

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Chapter 1: Identifying and targeting metabolic requirements of proliferation

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Introduction

In 1947, Sidney Farber, one of the fathers of modern chemotherapy, discovered that aminopterin could cause disease remission in children with acute lymphoblastic leukemia (Farber and Diamond, 1948). Aminopterin was the precursor of the currently used drugs methotrexate and pemetrexed, all of which are folate analogues that inhibit one-carbon transfer reactions required for de novo nucleotides synthesis. The early clinical success of antifolate drugs led to the development of an entire class of drugs termed 'antimetabolites.' These compounds are small molecules that resemble nucleotide metabolites and inhibit the activity of enzymes involved in nucleotide base synthesis. Notable examples include the purine analogues 6-mercaptopurine and 6-thioguanine, which inhibit PRPP amidotransferase, the first enzyme in de novo purine biosynthesis, and have been successful in treating many cancers including childhood leukemia (Elion, 1989). The antipyrimidine 5-fluoruracil (5-FU) is a synthetic analogue of uridine and inhibits thymidylate synthase activity, limiting the availability of thymidine nucleotides. 5-FU and the related 5-FU prodrug capecitabine are among of the most widely used chemotherapies today and remain standard-of-care therapies for gastrointestinal cancers (Heidelberger et al., 1957; Wagner et al., 2006). Finally, the anti-metabolite nucleoside analogues, including gemcitabine and cytarabine, are incorporated into DNA, inhibit DNA polymerases and are also commonly used as first line agents for treating select cancers.

The clinical success of antimetabolites for treating cancer is a result of the increased metabolic demand of neoplastic cells for nucleotide biosynthesis and DNA replication. However, nucleotide metabolism is only one of many metabolic dependencies altered to

support cancer proliferation. Indeed, more than thirty years preceding Farber's work on antifolates, Otto Warburg discovered that cancer cells consume tremendous amounts of glucose and metabolize the majority of the glucose into lactate, even in the presence of oxygen. This phenomenon is now referred to as aerobic glycolysis or the Warburg effect and is one of the most widely observed examples of the metabolic differences between cancer cells and normal non-proliferating tissue.

Proliferating cells have different metabolic requirements than non-proliferating cells. While non-proliferating cells have primarily catabolic demands to maintain cellular homeostasis, proliferating cells must balance the divergent catabolic and anabolic demands of sustaining cellular homeostasis while duplicating cell mass. To satisfy the metabolic requirements of macromolecule synthesis, the metabolism of cancer cells must be rewired from what is observed in the normal tissue from which cancer arises.

From a therapeutic perspective, the aberrant metabolism of proliferating cancer cells presents numerous potential opportunities, and there has been a growing interest in studying how best to target the metabolism of cancer (Galluzzi et al., 2013; Martinez-Outschoorn et al., 2017; Vander Heiden, 2011). Given that the Warburg effect represents one of the most striking metabolic difference of cancer relative to many normal tissues, substantial work has been dedicated to develop ways to target increased glycolysis, including efforts to inhibit lactate production and excretion. One example of a compound that can block glucose metabolism is 2-deoxyglucose. 2-deoxyglucose is phosphorylated by hexokinase, and because downstream glucose metabolism enzymes cannot metabolize the resulting product 2-deoxyglucose-6-phosphate, it accumulates in the cell and competitively inhibits hexokinase to slow glucose uptake. Numerous

preclinical studies have demonstrated the anti-proliferative effects of 2-deoxyglucose (Zhang et al., 2014). In fact, early clinical use of 2-deoxyglucose yielded responses in some patients, although the use this drug was limited by significant hypoglycemia symptoms (Landau et al., 1958). Use of 2-deoxyglucose at more tolerable doses was revisited in several more recent Phase I/II studies, however at lower doses, patients continued to have progressive disease. Unfortunately, the relative lack of success of 2-deoxyglucose has been echoed by most other attempts at directly targeting glucose to lactate conversion. Efforts to target glucose uptake or lactate production have found various levels of success in preclinical studies, but to-date, clinical success has been far more limited (Vander Heiden and DeBerardinis, 2017).

One area where high glucose uptake by cancer has been exploited for patient care is the use of fluro-deoxyglucose positron-emission-tomography (FDG-PET) imaging to stage cancers and assess response to therapy. Notably, many non-cancerous tissues including the brain are also FDG-PET avid, illustrating that high glucose uptake is not a unique feature of cancer tissue and offering a potential explanation for the relative lack of success of targeting glucose metabolism for cancer treatment. Doses of 2-deoxyglucose that are needed to limit cancer growth are not tolerated due to disruption of glucose metabolism in normal tissues dependent on high rates of glucose uptake to carry out their normal function. Indeed, dose limiting toxicities of 2-deoxyglucose treatment include hypoglycemia and cardiac conduction anomalies.

Unlike glycolysis, other metabolic pathways offer potentially more specific targets for proliferating cells. However, even specifically targeting only proliferative metabolism may not offer an adequate therapeutic window, since many nonmalignant cells, including

those in bone marrow, intestinal crypts, and hair follicles, are rapidly proliferating. Furthermore, the normal physiological proliferation rate of myeloid and gut cells in most cases is far more rapid than the proliferation rate of cancer cells (Vander Heiden and DeBerardinis, 2017). Prominent side effects of anti-metabolite chemotherapy are thought to be due to the destruction of normal proliferating cells, and myeloid suppression or GI toxicity are often the dose limiting toxicity for these drugs. In spite of this, antimetabolites are standard in many modern chemotherapy regimens that increase patient survival and, in some cases, help cure disease.

Altered response pathways to DNA damage is one explanation for why a therapeutic window exists for these antimetabolite drugs, despite the fact that cancer cells proliferate more slowly than cells in certain normal tissues. However, these drugs are only effective against a subset of cancer types. Many of the resistant cancers have the same mutational spectrum as sensitive cancers, and defining genetic predictors of chemotherapy response for most malignancies has been elusive. Thus, the fact that chemotherapies are clinically effective strongly suggests that there exists a metabolic therapeutic window beyond proliferation rate and response to DNA damage. While the precise mechanisms underlying the differential efficacies of existing antimetabolite therapies are unknown, it is clear that a better understanding of these and other metabolic therapeutic windows may lead to the development of more effective and selective cancer treatments. Here we discuss recent advances in cancer metabolism that have identified metabolic targets, which might be exploited for improved cancer therapy with enhanced therapeutic window.

Altered Metabolic Enzyme Expression

The expression of metabolic genes is frequently altered in cancer. Some changes in metabolic enzyme expression are the result of gene amplification or deletion, while others are downstream of growth signaling pathways or the consequence of epigenetic changes. The activity of some metabolic enzymes can also be affected by mutations that impair or alter metabolic enzyme function. Regardless of the underlying mechanism, alterations in metabolic enzyme activity present potential vulnerabilities that can be targeted for cancer therapy.

Oncogenic mutations in isocitrate dehydrogenase

Recurrent somatic point mutations in the genes coding for isocitrate dehydrogenase 1 and 2 (IDH1, IDH2) are found in a wide variety of cancers, including glioblastoma multiforme (GBM) (Yan et al., 2009) and acute myeloid leukemia (AML; Dang et al., 2016; Mardis et al., 2009). Cancers expressing mutant IDH represent a unique case in which a metabolic enzyme can act as an oncogene and contribute to tumor development. Wild-type IDH1 and IDH2 catalyze the reversible oxidative decarboxylation of isocitrate to alpha-ketoglutarate (αKG) and CO_2. However, cancer associated mutations in IDH1 and IDH2 confer a neomorphic catalytic activity to the enzyme that favors reduction of αKG to (D)-2-hydroxyglutarate (D-2HG) (Dang et al., 2009; Ward et al., 2010). Though D-2HG is found at low levels in normal cells, D-2HG can accumulate to millimolar levels in cancer cells that express mutant IDH. At these high concentrations, D-2HG can inhibit αKG-dependent dioxygenases, including enzymes involved in histone and DNA demethylation (Chowdhury et al., 2011; Koivunen et al., 2012; Xu et al., 2011).

Thus, D-2HG accumulation in cancer cells expressing mutant IDH results in hypermethylation of histones and CpG islands in DNA (Figueroa et al., 2010; Lu et al., 2012; Turcan et al., 2012). These epigenetic changes caused by D-2HG accumulation contribute to cancer phenotypes (Losman et al., 2013; Rohle et al., 2013; Saha et al., 2014; Wang et al., 2013), and have been proposed to promote oncogenesis by preventing normal cellular differentiation (Losman and Kaelin, 2013).

Pharmacological agents that inhibit mutant IDH1 and IDH2 enzyme activity are being developed. One of the first compounds reported is AGI-5198, which targets mutant IDH1. AGI-5198 reduces intratumoral D-2HG levels, induces expression of genes involved in glial cell differentiation and suppresses growth of IDH1-mutant human glioma cells in a xenograft model (Rohle et al., 2013). A specific inhibitor of mutant IDH2, AG-221, confers significant survival benefit in a mouse model of IDH2-mutant AML (Yen et al., 2017) and in 2014 became the first compound targeting mutant IDH to enter Phase I clinical trials. Early results in patients with IDH2-mutant AML have suggested AG-221 can provide clinical benefit (DiNardo et al., 2015), and this drug is also being evaluated in solid tumors. The mutant IDH1 inhibitors AG-120 and IDH305, as well as the pan-mutant IDH inhibitor AG-881 are also currently in Phase I/Phase II clinical trials to treat both hematologic malignancies and solid tumors and will further inform whether targeting mutant IDH is a successful strategy for controlling disease where mutations in IDH are prevalent.

Despite the preliminary success in some preclinical models and in patients with AML, IDH inhibitors may not be effective as single agents in all IDH-mutant cancers. For example, these drugs are unable to reverse epigenetic changes or inhibit tumor

proliferation in many models of IDH mutant glioma, despite a robust ability to lower 2-HG in cells and tumors (Tateishi et al., 2015; Turcan et al., 2013)). IDH1 mutations are early events in the development of glial cancer Watanabe 19246647, and accumulation of additional oncogenic mutations in glioblastoma may cause these tumors to be less dependent on mutant IDH1(Johnson et al., 2014; Wakimoto et al., 2014)). This has raised the possibility that in some cancers IDH mutations are more important for tumor initiation or early stage disease and not constitutively required for proliferation. Nevertheless, the presence of an IDH mutation and high levels of 2-HG might still drive dependencies on some pathways and introduce therapeutic vulnerability. For example, harboring an IDH mutation may render cells more sensitive to hypomethylation agents (Turcan et al., 2013), electron transport chain inhibitors (Grassian et al., 2014), depletion of the coenzyme NAD+ (Tateishi et al., 2015), and chemoradiotherapy (Cairncross et al., 2014). Other evidence suggests that targeting IDH mutational status may confer resistance to conventional therapy. For example, it has been shown that inhibition of mutant IDH1 might antagonize the effects of radiation therapy in glioma (Molenaar et al., 2015). Thus, testing whether combination therapies are synergistic or antagonistic with inhibition of mutant enzyme function will be important. Additionally, further efforts to identify synthetic lethal targets with IDH mutations are ongoing and can serve as an alternative therapy to direct targeting of mutant IDH.

Glutamine metabolism is upregulated in some cancers

Glutamine is a non-essential amino acid, and yet cancer cells proliferating *in vitro* consume glutamine far in excess of any other amino acid and are often dependent on

extracellular glutamine for survival (Jain et al., 2012). Glutamine is an important nitrogen donor for amino acids and nucleotides (Hosios et al., 2016), but glutamine uptake can exceed the nitrogen requirement of some cancer cells (DeBerardinis et al., 2007). Glutamine carbon has been found to contribute to aspartate, glutamate and TCA cycle metabolites via a process termed glutaminolysis (Altman et al., 2016). High rates of glutaminolysis have been proposed to support rapid proliferation by supplying precursors to low-flux biosynthetic pathways (Newsholme et al., 1985). Providing cells with αKG, oxaloacetate, or pyruvate is sufficient to rescue cancer cell proliferation and/or in conditions of glutamine starvation, confirming that glutamine supports proliferation by replenishing depleted TCA cycle intermediates (Altman et al., 2016; Yuneva et al., 2007).

Glutamine metabolism is upregulated downstream of various oncogenic signaling pathways (Altman et al., 2016). Among them, the MYC oncogene signaling pathway has one of the most-well characterized associations with increased glutamine metabolism. In certain contexts, MYC-transformed cancers become glutamine dependent and undergo apoptosis in the absence of glutamine (Yuneva et al., 2007). MYC has been found to increase mRNA and protein levels of glutamine transporters as well as expression of the enzyme glutaminase, which catalyzes the cleavage of glutamine to form glutamate, the first step in glutaminolysis (Gao et al., 2009; Wise et al., 2008). Glutamate is further converted to αKG, an anaplerotic TCA cycle intermediate (Moreadith and Lehninger, 1984). Importantly, inhibiting glutamine entry into the TCA cycle and was found to blunt tumor progression in a MYC-driven cancer model of hepatocellular carcinoma (Le et al., 2012; Gross et al., 2014; Xiang et al., 2015).

The dependence of cancer cells on glutamine has made glutaminolysis an attractive cancer therapy target (Altman et al., 2016; Vander Heiden, 2011). Clinical trials using glutamine analogues to treat cancers were initiated decades ago, but those trials were abandoned due to lack of efficacy and/or severe patient toxicity (Livingston et al., 1970). The absence of a therapeutic window for these studies can likely be attributed to fact that the drugs used were relatively non-specific and there is a panoply of glutamine utilizing enzymes that were likely affected. Current attempts to target glutaminolysis clinically have largely focused on inhibiting glutaminase. Mammals have two glutaminase genes, GLS and GLS2 and targeting the enzymes encoded by these genes with chemical inhibitors has been found to decrease cancer cell proliferation in both in vitro and in vivo models (Gross et al., 2014; Jacque et al., 2015; Xiang et al., 2015). One highly potent glutaminase inhibitor, CB-839, is currently in being evaluated in cancer trials in patients, although the exact disease context where glutaminase inhibition will be most effective remains an area of active investigation. There is also data that GLS2 activity can be tumor suppressive (Hu et al., 2010), underscoring the importance of defining the right patient population that is likely to benefit from glutaminase inhibition.

Increased serine synthesis is a feature of many cancers

Increased activity of de novo serine synthesis enzymes in cancer has been known for more than 30 years (Snell 1984, Snell 1988). More recently it was discovered that in some cancers increased expression of the serine synthesis enzyme phosphoglycerate dehydrogenase (PHGDH) is the result of copy number gain of a genetic region on

chromosome 1p (Beroukhim et al., 2010; Locasale et al., 2011; Possemato et al., 2011). Additionally, PHGDH can be overexpressed in certain tumors as a consequence of aberrant NRF2 or ATF4 signaling (DeNicola et al., 2015).

The PHGDH gene encodes the enzyme that catalyzes conversion of the glycolytic intermediate 3-phosphoglycerate into 3-phosphohydroxypyruvate, and 3-phosphohydroxypyruvate is converted to serine via two subsequent reactions. Increased *PHGDH* gene expression leads to increased production of serine from glucose and is associated with specific subsets of breast cancer and melanoma (Locasale et al., 2011; Possemato et al., 2011). While serine is present in plasma and can be taken up by cells via amino acid transporters, expression of PHGDH and increased serine biosynthesis have been shown to be important for supporting cancer cell proliferation and survival in both in vitro and in vivo settings (Locasale et al., 2011; Possemato et al., 2011).

Why some cancers are dependent on increased serine synthesis is unknown, but increased flux through this pathway may serve to maintain adequate intracellular serine levels. Serine is an amino acid, and thus is required for protein synthesis, but serine can also support many other important critical metabolic processes including synthesis of glycine, glutathione, and phospholipids. With respect to proliferating cells such as cancer, serine is the primary carbon donor to the tetrahydrofolate (THF) cycle, which is required for both purine and pyrimidine nucleotide biosynthesis. Serine can also contribute to NADPH production via the folate cycle, which serves to maintain redox homeostasis and support reductive anabolic reactions (Fan et al., 2014; Lewis et al., 2014; Ye et al., 2014). Additionally, emerging work suggests that serine synthesis may

be particularly important for maintenance of redox homeostasis during metastasis (Piskounova et al., 2015).

Given the requirements of cancer cells for high levels of intracellular serine, there have been efforts to target de novo serine synthesis for cancer therapy. Functional PHGDH loss is selectively toxic to tumor cells with PHGDH amplification or high serine biosynthetic flux (Possemato et al., 2011; Locasale et al., 2011; Mattaini et al., 2015), and small-molecule inhibitors targeting PHGHDH have been shown to inhibit serine synthesis and tumor proliferation in xenograft cancer models (Pacold et al., 2016). However, de novo serine synthesis also has an important physiological role in the central nervous system (Hirabayashi and Furuya, 2008). Thus, compounds that with decreased distribution in the brain may have a better therapeutic index.

While some tumors exhibit high rates of serine synthesis, others are dependent on uptake of environmental serine (Jain et al., 2012; Maddocks et al., 2013), and limiting plasma serine availability can be beneficial for patients with these cancers. Removing serine from culture media limits incorporation of one-carbon units into nucleotides and impairs proliferation (Labuschagne et al., 2014). Additionally, serine deprivation by dietary restriction is sufficient to slow growth of both xenograft (Maddocks et al., 2013; Gravel et al., 2014), and autochthonous models of cancer, although efficacy of serine deprivation appears to be influence by both the oncogenic driver mutation and tissue context. Combining serine restriction with other drugs might also potentiate antitumor responses (Gravel et al., 2014; Maddocks et al., 2013). Further understanding the roles of de novo serine synthesis and serine uptake in different tumor contexts can yield important insights into how to target serine metabolism for the benefit of cancer patients.

FH and SDH loss in heritable cancer predispositions syndromes

In addition to genetic events that increase metabolic enzyme expression, some cancers select for deletion of metabolic enzymes that promote tumor growth. Familial cancer syndromes are caused by deletion of fumarate hydratase (FH) or succinate dehydrogenase (SDH), suggesting that these TCA enzymes can behave as classical tumor suppressors. Affected families inherit one defective copy of either FH or SDH, and develop an aggressive form of cancer upon loss of heterozygosity (Baysal et al., 2000; Tomlinson et al., 2002). Loss of FH activity and SDH activity results in disruption of the TCA cycle and accumulation of very high concentrations of fumarate or succinate, respectively. Like D-2HG, fumarate and succinate accumulation can inhibit select αKG-dependent dioxygenases resulting in hypermethylation of DNA and histones in tumors with loss of FH or SDH (Hoekstra et al., 2015; Letouze et al., 2013; Xiao et al., 2012). Additionally, succinate and fumarate inhibit the prolyl hydroxylase domain-containing (PHD) enzymes that regulate stability of hypoxia-inducible factors (HIFs), such that the hypoxic gene expression program is active in FH and SDH deficient cancers even under normoxic conditions (Hewitson et al., 2007; Pollard et al., 2005; Selak et al., 2005). Both the epigenetic changes and HIF activation can contribute to tumor initiation and progression in these cancers.

Loss of FH and SDH results in changes in cell metabolism that introduces vulnerabilities that may be amenable to therapeutic targeting. *In silico* modeling of metabolic networks suggests that FH-null cells can upregulate heme metabolism to enable their survival (Frezza et al., 2011). The heme biosynthesis pathway uses succinyl-CoA to generate

heme, which can be degraded to bilirubin and excreted from cells, allowing cells to dispose of excess TCA cycle carbon. Thus, FH-null cells may upregulate heme metabolism as a mechanism to generate NADH and deplete TCA intermediates that accumulate upon FH loss. Consequently, FH deletion renders mouse and human cells more sensitive to genetic and pharmacological inhibition of heme oxygenase 1 (Hmox1), an enzyme involved in heme degradation.

Another potentially targetable metabolic liability of renal cell cancers (RCC) that have lost FH expression is a dependence on exogenous arginine. The high levels of fumarate resulting from FH deficiency drives arginosuccinate lyase (ASL) and arginosuccinate synthase (ASS1) in a direction that reverses the arginine synthesis pathways and consumes arginine. Depletion of intracellular arginine causes these cells to become arginine auxotrophs (Adam et al., 2013; Zheng et al., 2013), and therapies that deplete exogenous arginine may be effective against malignancies where FH is lost (Phillips et al., 2013).

Loss of SDH also results in TCA cycle dysfunction, leading to the accumulation of succinate and depletion of aspartate, fumarate, citrate, and malate. In this context, aspartate cannot be synthesized from glutamine or other sources of aKG, and aspartate production is dependent on pyruvate carboxylase (PC) activity. PC catalyzes carboxylation of pyruvate to oxaloacetate, which can then undergo transamination to form aspartate. To cope with TCA cycle truncation and synthesize aspartate, SDH defective cells upregulate PC protein expression in culture and in tumors to synthesize aspartate from glucose (Cardaci et al., 2015; Lussey-Lepoutre et al., 2015). Ablation of

PC impairs the ability of SDH-deficient cells to proliferate and to form tumors, and thus targeting PC might be a liability that can be exploited to treat these types of cancers.

Arginosuccinate synthase 1 expression changes

Somatic loss of metabolic enzyme expression may be selected for in other tumor types. For example, some melanoma, lymphoma, glioma, and prostate cancers reduce or lose the expression of the urea cycle enzyme arginosuccinate synthase 1 (ASS1) (Delage et al., 2010). As noted above, ASS1 is involved in arginine synthesis, catalyzing the conversion of citrulline and aspartate to arginine and urea. One advantage to genetic or epigenetic silencing of ASS1 expression in tumor cells is conservation of aspartate (Rabinovich et al., 2015) which can be a critical output of the TCA cycle to support *de novo* nucleotide synthesis and cell proliferation (Sullivan et al., 2015).

A potential liability of ASS1 deficient cancers is that, like FH null cancers, they are unable to synthesize arginine de novo. As functional arginine auxotrophs, these cells are reliant on exogenous arginine for proliferation and survival and may be sensitive to therapies that lower arginine availability. Arginine deiminase (ADI) is a microbial enzyme that catabolizes arginine and can be used deplete extracellular arginine levels. Recombinant pegylated arginine deiminiase (ADI-PEG20) has been tested in phase I/II clinical trials to treat melanoma and hepatocellular carcinoma with some therapeutic benefit (Ascierto et al., 2005; Izzo et al., 2004; Szlosarek et al., 2013; Ott et al., 2013; Yang et al., 2010). However, the use of arginine-catabolizing enzymes may not be an effective therapeutic strategy against all ASS1-deficient cancer, as some ADI-treated

tumors have been found to re-express ASS1 (Feun et al., 2012; Long et al., 2013; Shen et al., 2003). Whether some tumors are more dependent on loss of ASS1 expression to proliferate is an area of active investigation as these may be more responsive to arginine depleting drugs. The combination of arginine depletion with other therapies might also limit resistance, and the identification of synthetic lethality targets with ASS1-loss is another approach being evaluated to increase the clinical efficacy of therapies that lower circulating levels of arginine (Bean et al., 2016; Locke et al., 2016; Kremer et al., 2017).

Metabolic collateral lethality

Loss of metabolic enzyme expression can also occur as a passenger event. For example, genomic deletions leading to loss of tumor suppressor genes can also lead to loss of adjacent non-essential metabolic genes. Because cells often exhibit redundancy in essential pathways, this phenomenon can limit redundancy in cancer cells and present a therapeutic opportunity. This loss of redundancy leading to a vulnerability has been termed collateral lethality (Muller et al., 2015). Recently, it has been determined that pancreatic ductal adenocarcinoma (PDAC) cells exhibiting homozygous deletion of the tumor suppressor SMAD4 often lose malic enzyme 2 (ME2) expression due to the chromosomal proximity of the two genes. Targeting the malic enzyme 3 (ME3) isoform was found to impair tumor proliferation of PDAC xenografts that lacked ME2 expression, but had no effect on tumors with intact ME2 (Dey et al., 2017) . Similarly, the gene encoding enolase 1 (ENO1) is on the tumor-suppressor locus 1p36, and is homozygously deleted in 1-5% of glioblastoma cancers (GBM). Knockdown of enolase 2 (ENO2) in ENO1-null GBM cells resulted in significant inhibition of proliferation and intracranial tumorigenesis, whereas ENO1 expressing cancer cells were insensitive to

ENO2 ablation. Furthermore, ENO1 loss results in extreme sensitivity to the pan-enolase inhibitor phonoactohydroxamate (PHAH) (Muller et al., 2012). In both examples, decreased metabolic enzyme redundancy rendered the cells dependent on a specific isoform of an enzyme that could be selectively targeted. It also decreases the basal enzyme activity to catalyze a specific step in a metabolic pathway, thereby lowering the threshold for toxicity to targeting that enzyme.

Passenger deletion of metabolic genes can introduce vulnerabilities involving other pathways as well. Deletion of the tumor suppressor CDKN2A results in concomitant deletion of the methionine salvage pathway enzyme methylthioadenosine phosphorylase (MTAP) in many cancers, including 53% of glioblastomas and 26% of pancreatic cancers (Mavrakis et al., 2016). MTAP cleaves methylthioadenosine (MTA), a product of polyamine biosynthesis, into 5-methylthioribose-1-phosphate and adenine, which are further metabolized to methionine and adenosine monophosphate (AMP), respectively. MTAP deficient cells are more reliant on *de novo* purine biosynthesis to generate AMP, since they are unable to cleave MTA to salvage adenine, and MTAP loss makes cells more susceptible to inhibitors of purine biosynthesis as well as to methionine depletion (Hori et al., 1996). Co-administration of MTA with toxic adenosine analogues has been shown to be selectively lethal to MTAP deficient cancer cells, since MTAP expressing normal tissues are able to convert MTA to adenine to competitively inhibit the effects of toxic adenine analogues (Lubin and Lubin, 2009). L-alanosine inhibits conversion of inosine monophosphate (IMP) to AMP and has shown selective toxicity towards MTAP-null cancer cells (Batova et al., 1999; Efferth et al., 2002; Harasawa et al., 2002), but was found in a phase II trial to be clinically ineffective in patients with advanced MTAP-deficient tumors (Kindler et al., 2009). Pharmacokinetic analyses confirming successful

purine biosynthesis inhibition by doses of L-analosine used in the study were not reported, so further exploration of why this approach failed my yield insight into how to exploit MTAP deficiency to treat cancer.

Loss of MTAP expression can result in another vulnerability for cancer cells because the MTA that accumulates following MTAP loss can act as a potent and selective inhibitor of the enzyme arginine methyltransferase 5 (PRMT5). MTAP loss results in reduced PRMT5 activity and makes MTAP-null cancer cell more sensitive to PRMT5 depletion than isogenic counterparts that express MTAP (Kryukov et al., 2016; Marjon et al., 2016; Mavrakis et al., 2016). Additionally, ablation of methionine adenosyltransferase II alpha (MAT2A), the enzyme that produces the canonical and high-affinity PRMT5 substrate, S-adenosylmethionine (SAM), also reduces PRMT5-dependent methylation and proliferation in MTAP-deleted cancer cells (Marjon et al., 2016). However, PRMT5 inhibitors that are in current clinical trials are not effective in MTAP null cancers, in part because the high levels of MTA in these cells compete for binding of the inhibitors to the enzyme. Future studies will better define whether reducing PRMT5 activity using drugs that act via a different mechanism could be used as effective treatments in tumors where MTAP is co-deleted with CDKN2A.

Emerging Metabolic Targets

Numerous metabolic differences between cancer cells and normal cells have been described, and many present opportunities for therapeutic intervention. Some recent examples to illustrate how metabolism might be exploited for therapy are highlighted, but many other targets have been proposed and are discussed elsewhere (Galluzzi et al., 2013; Martinez-Outschoorn et al., 2017).

Targeting de novo lipid synthesis

Several lines of evidence suggest that targeting de novo fatty acid synthesis might be effective in some cancers. Fatty acids are a key component of cell membranes and can also act as energy storage or signaling molecules. Medes et al. first discovered in the 1950s that tumors are able to synthesize lipids, and a subsequent study determined that the large majority of lipids in tumors cells are synthesized de novo, rather than generated by other lipogenic tissues and transported to the cancer (Medes et al., 1953; Ookhtens et al., 1984). Since then, numerous studies have identified de novo fatty acid biosynthesis as a key metabolic requirement for some cancers, and it has been dubbed by some as a distinct metabolic "hallmark of the transformed phenotype." Under normal physiological conditions, adult tissues other than the liver, adipose tissue, and lactating breast generally do not de novo synthesize fatty acids. Thus, inhibition of de novo fatty acid synthesis might have relatively minimal effects on normal tissues, since most tissues do not rely on this pathway.

Fatty acid synthesis is a multi-step process that primarily occurs in the cytosolic compartment of the cell. First, acetyl-CoA groups are converted to malonyl-CoA via the enzyme acetyl-CoA carboxylase (ACC). Next, the multidomain enzyme fatty acid

synthase (FASN) generates a fatty acid chain by catalyzing the serial condensation of malonyl-CoA followed by desaturation of the double bonds to produce fatty acids, primarily palmitate. Of note, while synthesis of the fatty acid chain occurs in the cytosol, production of cytosolic acetyl-CoA from glucose involves first producing citrate in the mitochondria, which must be exported out of the mitochondria and cleaved by cytosolic ATP-citrate lyase (ACLY) to regenerate acetyl-CoA and oxaloacetate. Recent work suggests that under certain contexts some cancers may also be able to generate acetyl-CoA from free cytosolic acetate (Kamphorst et al., 2014; Mashimo et al., 2014; Gao et al., 2016), however, acetate is not always abundant in blood and in some cells acetate is not the predominate source of acetyl-CoA used for bulk fatty acid synthesis (Hosios and Vander Heiden, 2014). Nevertheless, some cancers are dependent on expression of ACSS2, the enzyme that allows cells to scavenge acetyl-coA from acetate, and this represents a potential therapeutic target for some cancers.

Numerous strategies for targeting fatty acid synthesis have been attempted. The primary approaches have focused on limiting the availability of cytosolic acetyl-CoA via inhibition of ACLY as well as direct targeting of the enzymes ACC and FASN that are used in fatty acid synthesis. Targeting ACLY genetically or chemically prevents xenograft tumor formation and proliferation (Bauer et al., 2005), (Hatzivassiliou et al., 2005). Genetic knockdown of ACC induces apoptosis in cancer cell lines (Brusselmans et al., 2005; Chajes et al., 2006), and a recently identified allosteric inhibitor of ACC, ND-646, has shown antitumor efficacy in autochthonous lung tumor mouse models (Svensson et al., 2016). The compound TVB-2640 is the first compound targeting FASN to enter clinical trials, and when combined with paclitaxel, treatment resulted in some cases of partial responses or prolonged stable disease.

Differential requirements for NAD+/NADH homeostasis

Warburg's observation that cancer cells have dramatically increased glucose consumption and lactate production, even in the presence of oxygen, led him to initially hypothesize that cancer cells have diminished mitochondrial function (Warburg et al., 1956). However, subsequent work found that despite engaging in aerobic glycolysis, cancer cells consume oxygen at levels comparable to normal tissue (Weinhouse et al., 1956; Zu et al., 2004). Moreover, exposing cancer cells to respiration inhibitors blocks proliferation, suggesting that most cancer cells require respiration in order to proliferate (Harris et al., 1980; Howell and Sager, 1979; Kroll et al., 1983; Loffer et al., 1982). Respiration is also needed to form tumors, as tumor cells with genetic impairment of oxidative phosphorylation due to depletion of mitochondrial DNA exhibit increased tumor latency upon subcutaneous transplantation. In fact, cells derived from these tumors acquire host mtDNA to regain the ability to do respiration, providing compelling evidence that respiration is required and strongly selected for tumorigenesis (Tan et al., 2015a).

Despite the ability of respiration inhibitors to block proliferation, on the surface respiration would not appear to be a promising therapeutic target. Because most cells in the body generate ATP via respiration targeting respiration might be expected to be highly toxic with a limited therapeutic window. However, metformin, one of the commonly prescribed drugs in the clinic for treating type II diabetes is very safe and acts as a mitochondrial complex I inhibitor blocking respiration (Bridges et al., 2014; El-Mir et al., 2014; Owen et al., 2000; Wheaton et al., 2014). Furthermore, retrospective clinical studies have revealed that metformin use is associated with improved cancer outcomes, with

reductions in cancer incidence and decreased cancer mortality (Evans et al., 2005; Gandini et al., 2014; Lee et al., 2012). Additionally, metformin can cooperate with neo-adjuvant chemotherapy to increase pathological complete responses in patients undergoing surgery for breast cancer (Jiralerspong et al., 2009). The anti-tumorigenic properties of metformin and other biguanides have since been modeled in various mouse models of cancer (Buzzai et al., 2007; Huang et al., 2008; Shackelford et al., 2013; Wheaton et al., 2014), although the precise mechanism of metformin remains controversial (Luengo et al., 2014), recent work has further shown that the anti-tumorigenic effect of metformin can at least be partially accounted for by its action at complex I inhibiting (Wheaton et al., 2014; Gui et al., 2016). Consistent with complex I being a viable anti-tumor target, other complex I inhibitors have shown efficacy as anti-tumor agents (Schockel et al., 2015), and may show selective toxicity against oncogene-ablation resistant cells (Viale et al., 2014) and cancer stem cells (Sancho et al., 2015). Due to the promising early clinical results from metformin, developing other complex I inhibitors is currently an area of active research (Bastian et al., 2017). Other compounds targeting respiration or mitochondrial metabolism, including the lipoic acid derivative CPI-613, are also being assessed in clinical trials (Lycan et al., 2016; Pardee et al., 2014).

Recent work studying the role of respiration in supporting proliferation has shed some light as to why there may exist a potential therapeutic window in treating tumors with respiration inhibitors. Mitochondria are typically viewed as the powerhouse of the cell, and respiration is typically considered primarily an ATP-producing catabolic process. However, in proliferating cells the ATP-producing function of respiration appears dispensable for supporting proliferation (Sullivan et al., 2015; Birsoy et al., 2015; Titov et al., 2016). Instead, respiration serves an alternative anabolic role for proliferating cells by

providing access to electron acceptors in order to regenerate the oxidized form of nicotinamide adenine dinucleotide (NAD+), from the reduced form (NADH) (Sullivan et al., 2015; Birsoy et al., 2015; Titov et al., 2016). The specific of this topic investigated in depth in chapter 2 of this thesis. Maintenance of intracellular NAD+ is required for many cellular processes, including protein deacetylation, ADP ribosylation and calcium signalling (Chiarugi et al., 2012). Additionally, NAD+ serves as a critical redox cofactor required to generate oxidized molecules, such as nucleotides, for biomass accumulation. Proliferating cells often require a high NAD+/NADH ratio in order to support anabolic reactions, and in some contexts the NAD+/NADH ratio directly correlates with proliferation rate (Gui et al., 2016). Importantly, for catabolic ATP production the NAD+/NADH ratio inversely correlates with energy charge, meaning low NAD+/NADH ratio favors ATP production. Thus, one possible explanation for the potential therapeutic window of respiration inhibitors in targeting cancer is that the NAD+/NADH ratio regime required for proliferation is distinct from that which is required to maintain ATP production.

Targeting NAD+ synthesis could be another mechanism to limit NAD+ pools and target proliferative metabolism. NAD+ is produced via multiple synthesis and salvage pathways and can utilize nicotinamide, nicotinic acid, or tryptophan as a nutrient precursor. A major source of NAD+ in proliferating cells is salvage from nicotinamide, and the rate-limiting enzyme in this pathway is NAMPT. NAMPT has been found to be highly expressed in tumors arising in diverse tissues, and small-molecules targeting NAMPT have been effective anti-tumor agents in vitro and in xenograft models of cancer (Nahimana et al., 2009; Tan et al., 2015b; Watson et al., 2009). In clinical trials, use of these inhibitors as single agents has not resulted tumor remission (von Heideman et al., 2010), but

additional studies may uncover how best to safely use these drugs and help patients. For example, NAMPT inhibitors may be more effective in contexts of MYCN-amplified glioblastoma (Tateishi et al., 2016) or when used in combination with other drugs known to deplete NAD+ pools (Bajrami et al., 2012; Chan et al., 2014).

Increased requirement for detoxification of reactive metabolites

Beyond their well-characterized canonical activity, some metabolic enzymes can generate so-called by-products or error products of metabolism. These metabolites can be formed enzymatically or by non-enzymatic chemical reactions due to the intrinsic reactivity of certain metabolic intermediates. Some of these by-products, including reactive oxygen species and methylglyoxal, are themselves chemically unstable and, thus, have been described as "reactive metabolites". Reactive metabolites can covalently modify both amino acids and nucleic acids. At high levels, these reactive metabolites become toxic and can eventually kill cells due to the resulting accumulated damage to the proteome and genome. In order to prevent the accumulation of excess reactive metabolites, cells rely on several pathways to prevent damage.

Glutathione, a cysteine containing tripeptide, plays a crucial role in many of these detoxification pathways. Glutathione is one of the most abundant metabolites in the cell and is able to buffer the level of reactive metabolites by serving as an abundant substrate for electrophilic attack with its nucleophilic sulfhydryl group. These reactions typically result in the formation of a glutathione dimer or a glutathione adduct. These

initial products generally then serve as substrates for various detoxification enzymes, which cleave the adducts to yield less reactive byproducts and regenerate glutathione. For example, methylglyoxal can be generated from intermediates in glucose or glycine metabolism, and accumulation of methylglyoxal can be a liability for glioma progression (Kim et al., 2015). In the methylglyoxal detoxification pathway, methylglyoxal spontaneously reacts with glutathione to form glutathione-methylglyoxal hemiacetal. Subsequently, glyoxylase 1 (GLO1) isomerizes the initial glutathione adduct to form S-D-lactoyl-glutathione, which is then cleaved by glyoxylase 2 (GLO2), to produce D-lactate and regenerate glutathione (Sousa Silva et al., 2013). Glo1 has been described as a metabolic oncogene (Hosoda et al., 2015), and chemical inhibition of GLO1 has been shown to inhibit cancer cell proliferation in both in vitro and in vivo models (Sakamoto et al., 2001).

Cancer cells generally have increased levels of reactive metabolites (Sullivan et al., 2016; DeBerardinis et al., 2016). While the increased levels of reactive metabolites have been described to support oncogenesis in certain contexts, it also leads to a greater dependence on reactive metabolite detoxification pathways to prevent accumulation of excess cellular damage. Disruption of these detoxification pathways is predicted to have a more deleterious effect on cancer cells than normal cells, and thus serves as a potential targetable metabolic liability.

Detoxification pathways can be disrupted by selective inhibition of enzymes that are involved in the detoxification of specific reactive metabolites. An alternative strategy for disrupting detoxification pathways is to target the synthesis or utilization of glutathione itself. Several small-molecule screens for drugs that selectively kill transformed cells

have identified compounds, which decrease glutathione levels. Additionally, most cancer cells are auxotrophic for cysteine, the key reactive residue of glutathione. Thus, lowering whole body cysteine levels with bacterial cysteineases is another potential approach to inhibiting glutathione synthesis that is currently in clinical trial.

Lineage and Environment Specific Vulnerabilities:

Analysis of human metabolic gene expression across different cancer types found that metabolic enzymes are heterogeneous across tumors, and there is no universal metabolic transformation common to all cancers. In fact, tumors retain many features that correspond to their parental normal tissue (Gaude and Frezza, 2016; Hu et al., 2013). It is not known whether this effect is a result of the rigidity of lineage specific metabolic expression programs or the similar local microenvironment experienced by the tumor and normal tissue. In any event, these analyses suggest that metabolic vulnerabilities may not be universal across all cancers. Importantly, this highlights the possibility that some metabolic dependencies of cancer may be modulated by the environment or defined by tumor lineage.

Differential utilization of amino acids

Acquisition of amino acids is an important biosynthetic requirement for cancer cells to support their rapid proliferation (Hosios et al., 2016). How cells acquire amino acids can vary according to cell type and environment (Mayers et al., 2016), and therefore targeting amino acid metabolism could potentially offer a large therapeutic window for

cancer treatment. Recently, it has been determined that mouse lung and pancreatic tumors exhibit differences in amino acid metabolism, even when harboring the same genetic lesion (Mayers et al., 2016). Lung tumors support their nitrogen requirement by catabolizing free branch chain amino acids (BCAA), whereas pancreatic tumors expressing the same driver mutation do not. Interestingly, targeting BCAA transaminases inhibits xenograft tumor formation of cell lines derived from lung tumors but not of those derived from pancreatic tumors, suggesting that the dependence branch chain amino acid catabolism is determined by cell lineage rather than tumor site (Mayers et al., 2016). These observations suggest that tissue of origin can have important implications for metabolic requirements and vulnerabilities.

Asparagine auxotrophy in acute lymphoblastic leukemia

Though non-essential amino acids are often synthesized in excess of biosynthetic demand, in some contexts depletion of these nutrients results in impaired proliferation. Acute lymphoblastic leukemia (ALL) and related lymphomas are auxotrophic for asparagine. Bacterial L-asparaginase deamidates asparagine to aspartic acid, thereby limiting the availability of asparagine for cancer cells. L-asparaginase and has been found to be highly effective in the treatment of ALL and is now a standard part of the chemotherapy regimen for this disease.

Though the mechanism of asparagine auxotrophy in ALL cells was assumed to be genetically defined, studies have shown that clinical response to L-asparaginase treatment is independent of expression levels of asparagine synthase (ASNS), the enzyme that converts glutamine to asparagine (Stams et al., 2003; Appel et al., 2006). In

fact, genome-wide analysis has shown that there is no consistent expression pattern that dictates sensitivity to L-asparaginase (Fine et al., 2005). The absence of a genetic driver for asparagine auxotrophy, combined with the fact that L-asparaginase has had little clinical utility outside of the context of ALL, suggests that this metabolic vulnerability may be lineage dependent.

Inducing differentiation in myeloid cells

A common feature of acute myeloid leukemia (AML) is that leukemic myeloblasts arrest at an immature stage of differentiation. This differentiation blockade is a hallmark of AML even though there are multiple driver mutations for this disease. Inhibitors of mutant IDH1 or IDH2 have been shown to induce differentiation in AML cells harboring mutant IDH (Okoye-Okafor et al., 2015). The transcription factor HoxA9 is overexpressed in 70% of AML cases, and overexpression of this transcription factor alone is sufficient to immortalize myeloid cells in derived from murine bone marrow (Ayton and Cleary, 2003) (Golub et al., 1999; Kroon et al., 1998). Recently, a phenotypic screen performed myeloid cells with HoxA9-enforced differentiation arrest found that compounds targeting dihydroorotate dehydrogenase (DHODH), an enzyme in the pyrimidine biosynthesis pathway, were able to induce differentiation (Sykes et al., 2016). Inhibition of DHODH renders cells unable to synthesize uridine *de novo*, and results in depletion of uridine and downstream metabolites. Providing immortalized bone marrow cells with extracellular uridine permits uridine salvage and is sufficient to maintain myeloid arrest, even in the presence of compounds targeting DHODH. Treatment with brequinar sodium (BRQ), an inhibitor of DHODH that is approved to treat autoimmune disease, is able to overcome myeloid differentiation blockade, and reduces leukemic cell burden in patient-

derived xenografts and syngeneic mouse models of diverse genetic subtypes. BRQ has previously been evaluated to treat advanced solid tumors but was not shown to be effective in clinical trials (Arteaga et al., 1989; Noe et al., 1990; Peters et al., 1990)). However, the effect of BRQ on patients with hematologic malignancies has not yet been evaluated. Future trials will determine whether targeting DHODH could exploit a cell lineage-dependent liability in myeloid-derived cancers.

Non-tumor cells can influence tumor cell metabolism

Metabolic symbiosis likely exists between tumor cells and the surrounding stroma, and this interaction could also contribute to tumor growth and proliferation in a way that might be exploited for therapy. Typically, lactate is excreted at high rates by tumor cells, but lactate has also been described as an oxidative substrate for some cancers. It has been reported that lactate produced by cancer-associated fibroblasts (CAFs) can enter the TCA cycle of cancer cells(Bonuccelli et al., 2014), and lactate produced in hypoxic regions of a tumor can fuel respiration in well-oxygenated tumor cells (Sonveaux et al., 2008; Kennedy et al., 2013). Intraoperative isotopically-labeled glucose infusions performed on non-small cell lung cancer (NSCLC) patients revealed higher labeled carbon in TCA intermediates than glycolytic intermediates in tumors, but not in normal lung, leading the authors to conclude that lactate could serve as an anaplerotic substrate in this context as well (Hensley et al., 2016). Lactate utilization by tumors is dependent on monocarboxylate transporters (MCTs), which are highly expressed in a wide variety of tumors (Pinheiro et al., 2012), and compounds targeting MCT are currently being evaluated for antitumor efficacy (Marchiq and Pouyssegur, 2016).

Alanine is rarely limiting for proliferation, as cancer cells typically excrete alanine, rather than consume it (DeBerardinis et al., 2007; Jain et al., 2012: Hosios et al., 2016). However, in some contexts alanine may not be synthesized in excess of the proliferation requirements of cells. Pancreatic ductal adenocarcinoma (PDAC) cells can consume alanine produced by pancreatic stellate cells *in vitro*, and the secreted alanine appears to help promote tumor cell proliferation and survival under nutrient-limited conditions (Sousa et al., 2016). An improved understanding of the interaction between PDAC tumors and stroma can help determine what the metabolic limitations of pancreatic tumor growth are and guide target selection to exploit this symbiotic relationship for therapy.

Metabolic dependencies can be modulated by environment

The ability of a given drug to inhibit its protein target is often used as a proxy for the efficacy and potency of the drug to treat disease. However, when targeting metabolism, it is important to recognize that metabolic dependencies of cells can be influenced by environment and differences in nutrient availability may contribute to sensitivity and resistance of cancer cells to some drugs. For example, while the glutaminase protein is expressed in most cancers, targeting glutaminase is not an effective cancer therapy in all contexts. Glutamine tracing studies have demonstrated that in some tumor tissues, glutamine contributes minimally to TCA cycle intermediates and those cancers can be resistant to glutaminolysis inhibition (Marin-Valencia et al., 2012). Importantly, cell lines derived from resistant lung tumors become sensitive to glutaminase inhibition in vitro, highlighting that environment must be a contributor to glutaminase sensitivity (Davidson et al., 2016). What factors determine the differential use of glutamine in different

environments remains an active question, but at least one factor that influences glutamine utilization appears to be environmental amino acid levels.

Another example where inhibition of the metabolic protein target fails to predict biologic efficacy is targeting redox homeostasis with the drug metformin. Recently, it has been shown that the environmental context can decouple the biological effect of decreased proliferation from inhibition of complex I (Gui et al., 2016). In fact, despite evidence arguing complex I is the relevant target of metformin to inhibit tumor growth (Wheaton et al., 2014), inhibition of complex I activity can be a poor predictor of the anti-proliferative effects of metformin (Gui et al., 2016). Furthermore, altering the culture media conditions is sufficient to change the effective anti-proliferative effect of metformin, again highlighting the importance of environment in modulating metabolic dependencies. A detailed investigation of how metformin sensitivity is modulated by the environment is described in chapter 3 of this thesis.

What Dictates Metabolic Dependencies:

One of the biggest hurdles for drug development is identifying the patients who are most likely to respond to a given therapy. With the recent success of some targeted cancer therapies, it has become conventional wisdom that sensitivity of cancer cells to drug is a genetically encoded intrinsic property. This is based on the fact that sensitivity to drugs targeting oncogenic signaling proteins is predicated on expression of the oncogene or the increased activity of the downstream signaling pathway. Thus, patient selection can

be based on the presence of mutations or evidence of activated signaling within the cancer cell.

While for many cases this target expression based strategy has been very powerful in identifying patients likely to respond, the same approach may not identify responders for all metabolic cancer therapies. Indeed, response to chemotherapies that target metabolism has not been well predicted by this approach. The protein targets of 5-FU and methotrexate are present in almost all cancers, but the efficacy of these drugs varies dramatically across malignancies. Many of the emerging metabolic targets discussed in this review are also expressed widely in cancer, but dependency on these metabolic pathways is not always universal. For example, while targeting ACC to limit fatty synthesis can suppress lung tumor progression in some models (Svensson et al., 2016), in other cancer models loss of ACC activity accelerates tumor growth. Even for cancers with mutant IDH where altered metabolic enzyme activity is a driver event, sensitivity to IDH inhibitors appears to differ between leukemia and solid tumor models.

There is growing evidence that metabolic dependencies in cancer are influenced by tissue environment and cancer lineage as well as genetic events. Cancer tissue-of-origin has served as a successful way to select patients for chemotherapies targeting metabolism for decades, and cancer cell dependency on some investigational targets such as DHODH, mitochondrial complex I, and glutaminase is also influenced by cancer cell lineage and environment. Unraveling the complexity for how lineage, environment and genetics interact will be a challenge, but offers a path to exploit metabolism in a way that can be transformative for patients. Many drugs targeting metabolism are among the most active drugs in the clinic for particular diseases, and many newer metabolic

therapies are limited more by toxicities than by their ability to kill cancer cells. A better understanding of the factors that constrain metabolism in specific cancer holds the key for defining the aspects of metabolism most limiting for tumor growth and finding a therapeutic window to exploit those vulnerabilities for better cancer treatment.

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Chapter 2: Supporting aspartate biosynthesis is an essential function of respiration in proliferating cells

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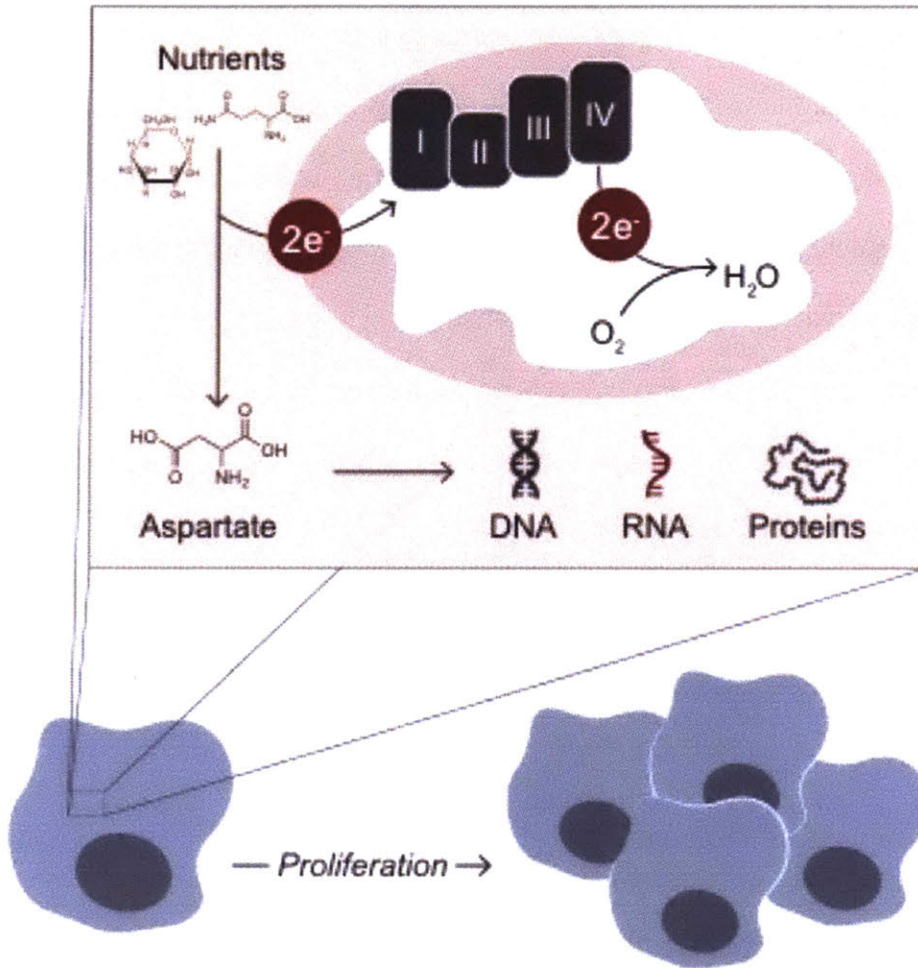
Summary

Mitochondrial respiration is important for cell proliferation, however the specific metabolic requirements provided by respiration to support proliferation have not been defined. Here we show that a major role of respiration in proliferating cells is to provide electron acceptors for *de novo* aspartate synthesis. This finding is consistent with the observation that cells lacking a functional respiratory chain are auxotrophic for pyruvate, which we confirm serves as an exogenous electron acceptor. Further, the requirement for pyruvate can be replaced with an alternative electron acceptor, alpha-ketobutyrate, which provides neither carbon nor ATP to cells. Alpha-ketobutyrate supplementation restores proliferation when respiration is inhibited, suggesting that an alternative electron acceptor can substitute for respiration to support proliferation. We find that electron acceptors are most limiting for producing aspartate, and supplying aspartate enables proliferation of respiration deficient cells in the absence of exogenous electron acceptors. Taken together, these data argue that the major function of respiration in proliferating cells is to support aspartate synthesis.

Graphical Summary



Introduction

In mammalian cells, mitochondrial respiration allows coupling of nutrient oxidation to ATP production. Respiration involves a series of oxidation-reduction reactions, where ultimately electrons from a reduced substrate are transferred to molecular oxygen as the final electron acceptor. This results in oxidation of consumed nutrients and reduction of molecular oxygen to water. The free energy released from this series of oxidation-reduction reactions is coupled to production of an electrochemical gradient that can be used to drive ATP synthesis, membrane transport, and thermogenesis (Harms and Seale, 2013; Mitchell, 1961; Schleyer et al., 1982).

Supporting bioenergetics is a critical function of respiration in mammalian cells. However, many proliferating cells display increased fermentation, which alone can be sufficient to supply ATP (Gottlieb and Tomlinson, 2005). In contrast to most normal tissues, cancer cells consume increased amounts of glucose and metabolize much of this glucose to lactate even in the presence of ample oxygen (Koppenol et al., 2011; Warburg et al., 1924). It was initially hypothesized that this phenotype, termed aerobic glycolysis or the Warburg effect, was the result of diminished mitochondrial function (Warburg, 1956). However, despite utilizing aerobic glycolysis, most cancer cells also consume oxygen (Weinhouse, 1956; Zu and Guppy, 2004). Notably, in cancer cell lines the primary substrate for oxidation is often not glucose but rather glutamine, one of the most heavily consumed nutrients by cells in culture (Fan et al., 2013; Kovacevic, 1971; Zielke et al., 1984). Hence, it appears that aerobic glycolysis does not replace

mitochondrial respiration, but rather, in rapidly proliferating cells these processes occur in parallel.

Most cells that engage in aerobic glycolysis are not only capable of respiration but also require respiration for proliferation. Exposure of cancer cells in culture to mitochondrial respiration inhibitors blocks proliferation (Harris, 1980; Howell and Sager, 1979; Kroll et al., 1983; Loffer and Schneider, 1982). *In vivo*, maintenance of mitochondrial DNA (mtDNA) is required for autochthonous tumor formation (Weinberg et al., 2010) and inhibition of respiration in cancer cells suppresses tumor growth in xenografts (Wheaton et al., 2014; Zhang et al., 2014). These findings argue that mitochondrial respiration is essential for rapid proliferation, but whether respiration is advantageous for proliferation beyond producing ATP is less clear.

Despite the importance of respiration in mammalian cell proliferation, under specific culture conditions proliferation is possible in the absence of mitochondrial respiration. Serial passage in low dose ethidium bromide produces cells devoid of mtDNA (ρ^0 cells) (King and Attardi, 1989, 1996). ρ^0 cells lack a functional mitochondrial electron transport chain (ETC), and these respiration-incompetent cells fail to proliferate unless supra-physiological levels of uridine and pyruvate are present in the culture media (King and Attardi, 1989). The uridine auxotrophy is explained by the fact that the *de novo* pyrimidine biosynthesis enzyme dihydroorotate dehydrogenase (DHODH) transfers electrons directly to the mitochondrial ETC to convert dihydroorotate to orotate. Therefore, loss of electron transport to O_2 prevents this reaction and exogenous uridine is needed to produce pyrimidines (Gregoire et al., 1984). The requirement for pyruvate,

however, was initially unexpected because cells deficient in mtDNA are highly glycolytic and capable of generating large amounts of pyruvate (King and Attardi, 1989).

The fact that supplementation of specific nutrients can substitute for respiration suggests respiration fulfills specific metabolic requirements for proliferating cells. While ATP synthesis via oxidative phosphorylation is often assumed to be the critical output of respiration, neither exogenous uridine nor pyruvate can be oxidized to supply ATP in the absence of respiration. However, other than dihyroorotate to orotate conversion, the metabolic function(s) that become limiting for proliferation in the absence of respiration are unknown.

Here we show that loss of mitochondrial respiration causes proliferating cells to become functionally limited for electron acceptors. This lack of electron acceptors impairs *de novo* aspartate synthesis and inhibits proliferation. Strikingly, this proliferation block can be overcome by supplementing cells with exogenous electron acceptors or by high levels of aspartate. This requirement for respiration to support aspartate synthesis is present in a diverse array of proliferating mammalian cells, including both transformed and non-transformed cells. Taken together our data argue that the most essential metabolic function for proliferation provided by mitochondrial respiration is to provide access to electron acceptors to support aspartate biosynthesis.

Results

Alpha-ketobutyrate can substitute for pyruvate to support proliferation in respiration-incompetent cells.

Cells lacking a functional mitochondrial ETC, including ρ^0 cells that lack mtDNA, require pyruvate for proliferation (King and Attardi, 1989). This suggests that pyruvate substitutes for an essential metabolic function of respiration. We reasoned that better understanding the role of pyruvate in these cells would allow us to gain insight into how respiration supports the metabolic needs of proliferating cells. To eliminate respiration-independent effects of mtDNA depletion, we used 143B ρ^0 cells that were repopulated with mtDNA harboring a frame shift deletion in cytochrome B (143B CytB), an essential component of complex III of the ETC (Rana et al., 2000). 143B CytB cells have otherwise wild-type mitochondria, but are respiration-incompetent due to lack of cytochrome B in complex III and, therefore, lack a functional ETC. As a control we utilized 143B ρ^0 cells which were repopulated with wild-type mtDNA (143B WT cybrid) and are respiration-competent with a functional ETC. 143B CytB cells are indeed auxotrophic for pyruvate as they failed to proliferate in the absence of pyruvate (Fig 1A). Conversely, 143B WT cybrid cells cultured with or without pyruvate divide at a similar rate (Fig S1A), confirming that pyruvate auxotrophy accompanies a loss of mitochondrial respiration.

Several hypotheses have been proposed to explain pyruvate auxotrophy (Howell and Sager, 1979; King and Attardi, 1996; Morais et al., 1994; van den Bogert et al., 1992). Pyruvate carbon has many metabolic fates including conversion to oxaloacetate via pyruvate carboxylase, malate via malic enzyme, and acetyl-CoA via the pyruvate dehydrogenase complex. Thus, one hypothesis is that pyruvate acts as a carbon

substrate for synthesis of biosynthetic intermediates that are normally dependent on respiration. An alternative hypothesis is that in the absence of a functional ETC, cells cannot adequately oxidize cellular NADH. Hence, pyruvate may be required to function as an exogenous electron acceptor to regenerate NAD+ via the lactate dehydrogenase (LDH) reaction (Fig 1B). Importantly, the continued production of pyruvate from glycolysis requires NAD+ and, therefore, the use of glucose-derived pyruvate as a biosynthetic intermediate requires a source of NAD+ regeneration to maintain redox balance.

In order to decouple the potential roles of pyruvate as a precursor for other metabolites and as an electron acceptor to regenerate NAD+, we sought alternative substrates that could support 143B CytB cell proliferation in the absence of pyruvate. One candidate we identified is the four-carbon metabolite alpha-ketobutyrate (AKB), which, similar to pyruvate, can act as a substrate for LDH or other intracellular dehydrogenases (Fig 1C). While not the preferred substrate for LDH, we confirmed that LDH can utilize AKB to regenerate NAD+ from NADH with reasonable kinetics (Fig S1B). We then tested whether AKB was sufficient to support proliferation of 143B CytB cells in the absence of pyruvate. Indeed, AKB restores proliferation of 143B CytB cells to levels similar to that observed when the cells are cultured in the presence of pyruvate (Fig 1D). Supplementation with AKB does not restore oxygen consumption (Fig S1C), and AKB addition does not alleviate uridine auxotrophy (Fig S1D). Like pyruvate, AKB also does not impact proliferation of 143B WT cybrids (Fig S1E), demonstrating that AKB acts in a manner similar to, and can substitute for, pyruvate to support proliferation of respiration deficient 143B CytB cells.

As electron acceptors, AKB and pyruvate are both expected to regenerate NAD+. To test whether AKB and pyruvate are sufficient to alter the NAD+/NADH ratio in cells, we measured cellular NAD+/NADH ratios in 143B CytB cells cultured in the absence or presence of either pyruvate or AKB. As expected, addition of either pyruvate or AKB is sufficient to increase the cellular NAD+/NADH ratio, consistent with these molecules serving as exogenous electron acceptors (Fig 1E). Given that AKB and pyruvate have different metabolic carbon-fates, we hypothesized that AKB is used predominantly as an electron acceptor and not as a carbon substrate in other metabolic pathways. If metabolized by LDH, or other dehydrogenases that use NADH to reduce the alpha-ketone to a hydroxyl and regenerate NAD+, the expected product is alpha-hydroxybutyrate (AHB) (Fig 1C). To test whether this is the fate of AKB in 143B CytB cells under conditions where this metabolite is required for proliferation, we quantitatively measured the consumption of AKB and excretion of AHB in the media of 143B CytB cells cultured in the absence of pyruvate. We found that the consumption of AKB matched the excretion of AHB (Fig 1F). Since AKB and AHB are both four-carbon metabolites that differ only by oxidation state, and levels of these metabolites in the culture media are negligible when AKB is not added, this result strongly suggests that AKB only acts as an electron acceptor and does not directly contribute carbon to metabolism. Since AKB is sufficient to replace pyruvate in supporting 143B CytB cell proliferation, the pyruvate auxotrophy of these respiration incompetent cells is best explained as a requirement for exogenous electron acceptors. Taken together, these data suggest that access to exogenous electron acceptors provided by respiration (O_2), pyruvate, or AKB are required to support proliferation.

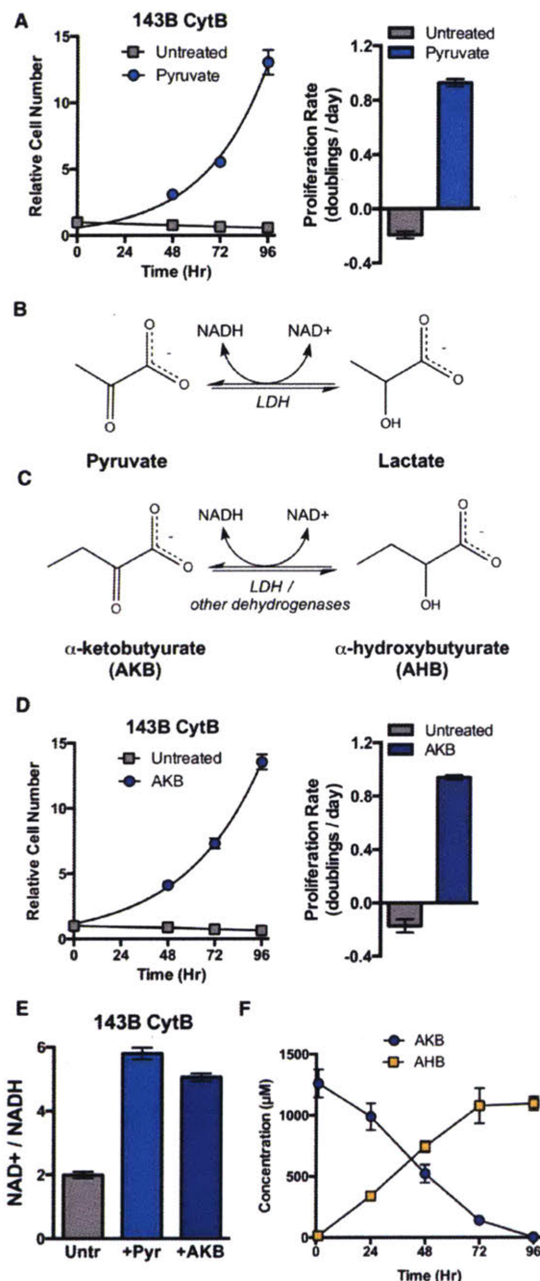


Figure 1. Cytochrome B Mutant 143B Cybrid Cells are Auxotrophic for Electron Acceptors that can Regenerate NAD+.

(A) Proliferation rate of 143B CytB cells was determined in the presence or absence of pyruvate. (left) Cell counts, normalized to cell number at t=0 when media conditions were applied, were assessed over time and used to calculate proliferation rate (right). (B) Pyruvate is a substrate of lactate dehydrogenase (LDH), accepting electrons from NADH to produce NAD+ and lactate. (C) Alpha-ketobutyrate (AKB) can also act as an electron acceptor from NADH and yield NAD+ and alpha-hydroxybutyrate (AHB). (D) Proliferation rate for 143B CytB cells in the presence or absence of AKB was determined as in (A). (E) Intracellular ratio of NAD+/NADH was determined in 143B CytB cells in untreated media or in the presence of pyruvate (Pyr) or AKB. (F) The concentration of AKB and AHB in the media of 143B CytB cells cultured in the presence of AKB was determined over time by GCMS analysis. Values in all figure panels denote mean ± standard error of the mean (SEM), n=3. See also Figure S1.

Inhibition of respiration causes auxotrophy for electron acceptors.

Generation of 143B CytB cells involves a selection process that might result in additional uncharacterized changes that alter the metabolic requirements provided by respiration. To test whether parental cells with a functional ETC have the same requirements for respiration, we treated wild-type 143B cells (143B) with an array of respiration inhibitors. Given the known DHODH requirement for respiration and the finding that AKB can substitute for pyruvate as an electron acceptor, this and all subsequent experiments were performed in the presence of uridine and in the absence of pyruvate. Inhibitors of mitochondrial complex I, complex III, complex IV, and ATP synthase were all sufficient to suppress proliferation and most resulted in decreased cell count over time (Fig 2A). Exposure to ETC inhibitors at these doses decreased oxygen consumption (Fig S2) and, thus, decreased access to electron acceptors. To test the hypothesis that respiration is necessary for proliferation in these cells because it provides access to electron acceptors, we tested if AKB supplementation could restore proliferation. In all cases, AKB supplementation rescued proliferation, with proliferation rates comparable to that observed in untreated cells (Fig 2A). To confirm that AKB also acts as an electron acceptor in this context, we measured the NAD+/NADH ratio in cells treated with ETC inhibitors in the absence or presence of AKB. ETC inhibitors all resulted in a decreased cellular NAD+/NADH ratio in the absence of AKB (Fig 2B). Treatment with AKB restored NAD+/NADH, while having no effect on O_2 consumption, confirming that AKB is sufficient to increase oxidized cofactor pools (Fig 2B, S2).

To determine whether a similar dependence on respiration exists in other proliferating cells, we treated a panel of genetically diverse cell lines including both transformed and

non-transformed cells with three representative respiration inhibitors: rotenone (complex I inhibitor), antimycin (complex III inhibitor), and oligomycin (ATP synthase inhibitor). In all cell lines tested, treatment with any of these inhibitors blocked proliferation and addition of AKB was sufficient to restore proliferation (Fig 2C). Taken together, these data suggest that the ability to transfer electrons to oxygen is a primary function of respiration to support proliferation.

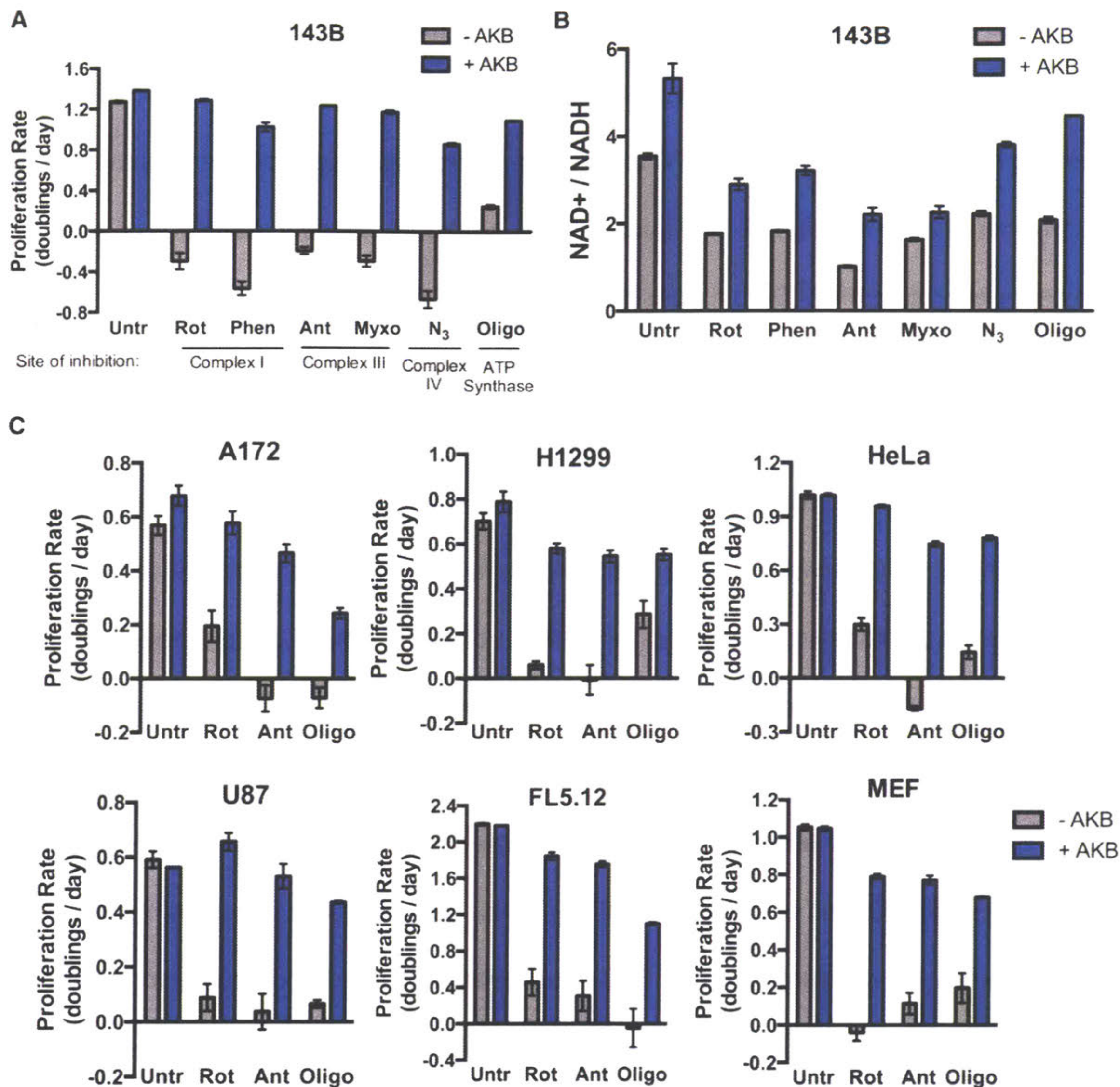


Figure 2. Proliferation of Respiration-Inhibited Cells is Restored by Exogenous Electron Acceptors.

(A) The proliferation rate of 143B cells was determined in media with or without AKB supplementation in the absence (Untr) or in the presence of the mitochondrial respiration inhibitors rotenone (Rot), phenformin (Phen), antimycin (Ant), myxothiazol (Myxo), azide (N_3), or oligomycin (Oligo). (B) The ratio of NAD+/NADH was determined in 143B cells with or without AKB supplementation in the absence or presence of respiration inhibitors as in A. (C) The proliferation rate of A172, H1299, HeLa, U87, FL5.12, and MEF cell lines was determined with or without AKB supplementation in the absence or presence of rotenone, antimycin, or oligomycin. Values in all figure panels denote mean ± standard error of the mean (SEM), n=3. See also Figure S2.

The use of oxygen as a terminal electron acceptor by mitochondrial respiration is well described. However, this role for oxygen is classically considered in the context of enabling NADH oxidation by the ETC to produce a membrane potential across the mitochondrial inner membrane and support ATP synthesis. Notably, reduction of AKB allows regeneration of NAD+ but does not support ATP production, arguing that mitochondrial ATP production is not required for proliferation. To further test this idea, we utilized oligomycin, a specific ATP synthase inhibitor, and the ionophore carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), an uncoupler of the mitochondrial membrane potential. In normal respiration, electrons from NADH are transferred to the ETC and then ultimately to oxygen to generate an electrochemical proton gradient across the mitochondrial inner membrane, which can drive ATP synthesis. As an ATP synthase inhibitor oligomycin does not directly inhibit components of the ETC, but rather slows ETC electron transfer to oxygen by hyperpolarizing the mitochondrial membrane potential (Fig 3A) (Brand and Nicholls, 2011). Thus, treatment with oligomycin alone decreases oxygen consumption (Fig S2C). However, addition of the uncoupling agent FCCP can restore the ability to transfer electrons from the ETC to oxygen in the presence of oligomycin by relieving membrane hyperpolarization without reversing inhibition of ATP synthase function (Fig 3A). Therefore, if providing access to electron acceptors is the essential function of respiration independent of ATP production, restoring oxygen consumption with FCCP should restore proliferation of oligomycin treated cells. To test this, we compared the proliferation rate of 143B cells cultured in oligomycin in the absence or presence of FCCP. Consistent with the hypothesis, FCCP addition increased proliferation of oligomycin treated cells (Fig 3B). Also in agreement with the hypothesis, FCCP increased both oxygen consumption and the NAD+/NADH ratio (Fig 3C and Fig 3D). Since FCCP and oligomycin act independently, FCCP addition

does not restore mitochondrial ATP production. These data argue that providing access to electron acceptors, rather than supporting mitochondrial ATP production, is a limiting function of mitochondrial respiration for supporting proliferation.

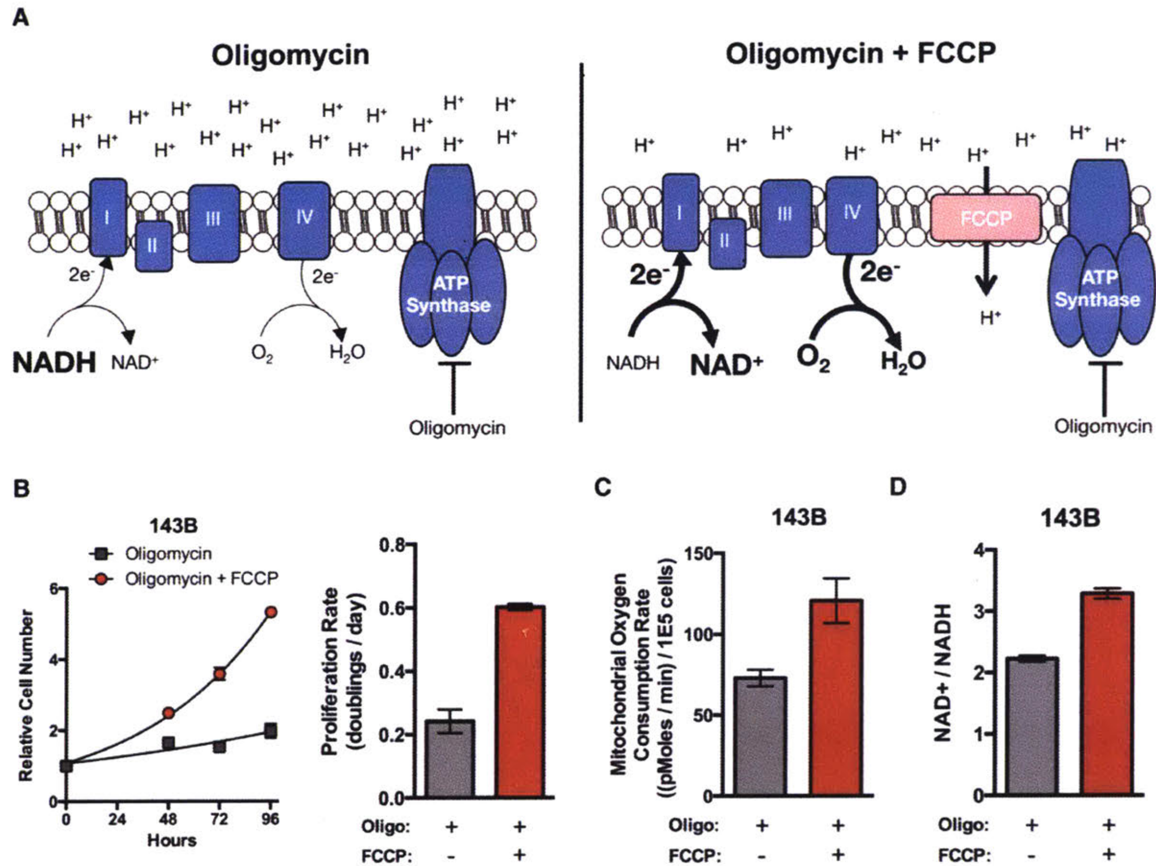


Figure 3. Oxygen Utilization in the Absence of Mitochondrial ATP Production is Sufficient for Cell Proliferation.

(A) Schematic illustrating the effects of oligomycin and FCCP on mitochondrial membrane potential and ATP synthesis. Oligomycin treatment inhibits ATP synthase, resulting in a hyperpolarized mitochondrial membrane. This hyperpolarization inhibits proton pumping and thereby inhibits ETC activity resulting in decreased NADH oxidation and O_2 consumption (left). Treatment with FCCP in addition to oligomycin relieves the hyperpolarization of the mitochondrial membrane allowing restoration of NADH oxidation and mitochondrial O_2 consumption without restoring ATP production. (right). (B) Proliferation rate of 143B cells treated with oligomycin in the presence or absence of FCCP treatment. (C) Mitochondrial oxygen consumption rate of 143B cells treated with oligomycin with or without FCCP. (D) Intracellular NAD+/NADH ratio in oligomycin treated 143B cells treated with oligomycin in the presence or absence of FCCP. Values in all figure panels denote mean ± standard error of the mean (SEM), n=3 (B, D), n=5 (C).

Electron acceptor insufficiency causes inhibition of nucleotide biosynthesis.

To gain mechanistic insight into why electron acceptors are required for proliferation, we characterized the phenotype of respiration incompetent cells under conditions where access to electron acceptors is limiting. Analysis of DNA content in non-proliferating 143B CytB cells without AKB suggested that these cells do not arrest at a specific stage of the cell cycle (Fig 4A). Surprisingly, despite complete cessation of proliferation for over 72 hours these cells remained viable and a substantial fraction of the cells had a DNA content between 2N and 4N suggesting that some cells were unable to progress through S-phase. Furthermore, compared to cells proliferating in the presence of AKB, the non-proliferating population showed a subtle accumulation of cells with a DNA content less than 4N. An inability to generate sufficient nucleotides to support DNA synthesis can prevent progression through S-phase (Lunt et al., 2015). To determine if a deficiency in nucleotide synthesis contributes to the inability to proliferate in the absence of AKB, we quantified nucleotide pools in 143B CytB cells in the presence and absence of AKB using liquid chromatography mass spectrometry (LCMS). Because pyrimidine nucleotide pools are confounded by excess uridine supplemented in the media, we focused analysis on purine nucleotides. Compared to AKB replete cells, cells cultured without AKB showed increased levels of the purine nucleotide inosine-5'-monophosphate (IMP) (Fig 4B). IMP is a precursor of both guanine-5'-monophosphate (GMP) and adenine-5'-monophosphate (AMP). Despite an increase in IMP, both GMP and AMP levels were decreased in cells without AKB supplementation (Fig 4B). To determine whether the deficiency of purine nucleotides is functionally significant, we supplemented 143B CytB cells with exogenous adenine, guanine, and hypoxanthine. Using nucleotide salvage pathways, these nucleobases are converted to AMP, GMP,

and IMP, respectively. Whereas hypoxanthine had no effect on proliferation and guanine supplementation was somewhat toxic, supplementation with adenine restored proliferation for one doubling, and alleviated the subtle accumulation of cells with DNA content less than 4N (Fig 4C, S3A). These data suggest that loss of respiration impairs adenine nucleotide synthesis, and that adenine nucleotide deficiency partially explains the inability of cells to proliferate in the absence of electron acceptors.

Both the AMP/IMP ratio and the GMP/IMP ratio dramatically decrease when AKB is withdrawn from cells cultured in the presence of this exogenous electron acceptor (Fig S3B). This decrease in the AMP/IMP and GMP/IMP ratios suggests an inability to convert IMP to either AMP or GMP. Conversion of IMP to GMP consumes an NAD+ by inosine 5'monophosphate dehydrogenase (IMPDH), and, thus, this reaction may be inhibited by decreased NAD+ availability (Fig 4D). In contrast, conversion of IMP to AMP does not directly require oxidation but instead consumes aspartate (Fig 4D). This suggests the possibility that decreased AMP production could be downstream of an aspartate deficiency. Importantly, an underlying aspartate deficiency could explain the transitory nature of adenine rescue; adenine can only restore proliferation to those cells in a phase of cell cycle where adenine is limiting and fails to compensate for other roles that aspartate might play in cell growth and division.

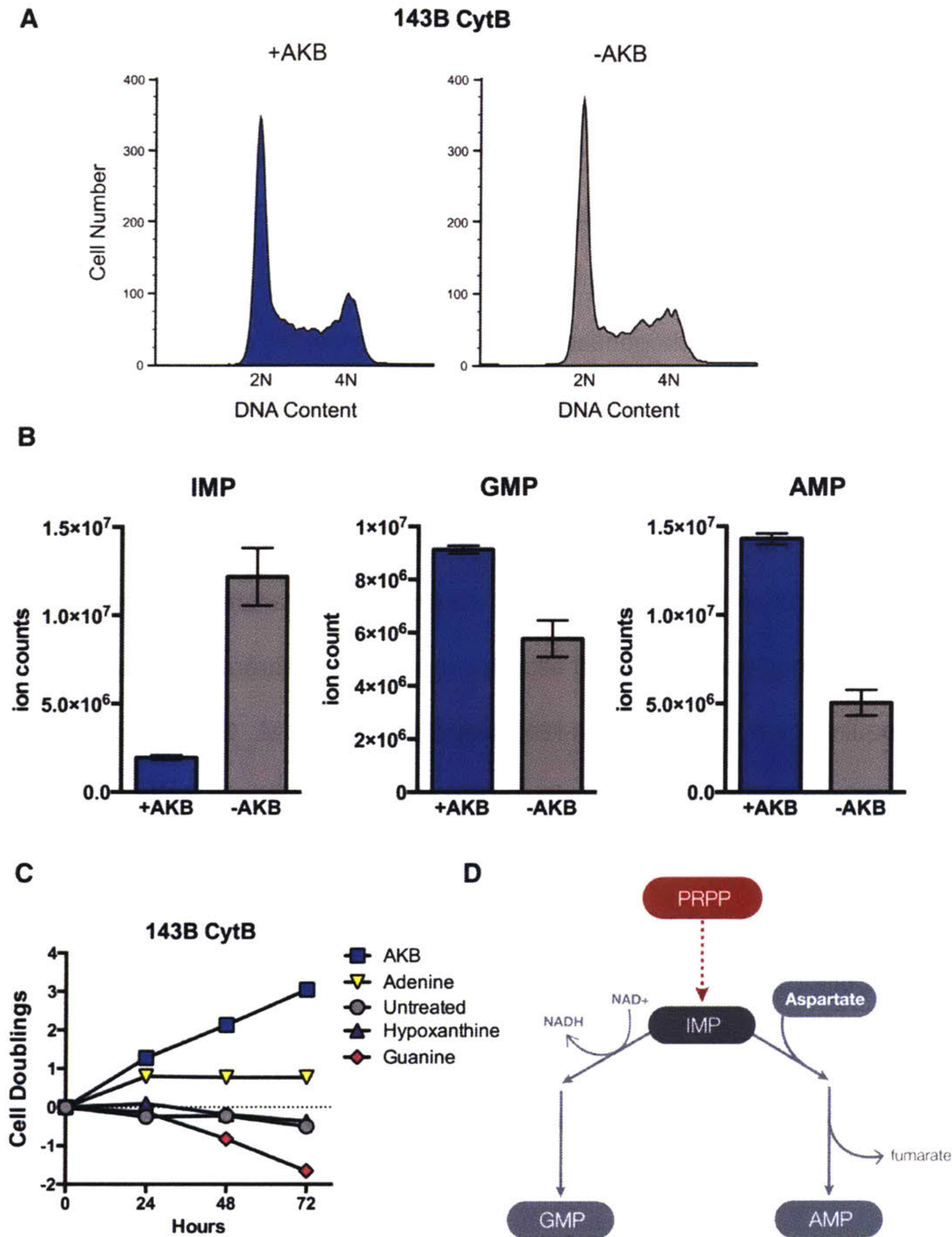


Figure 4. Electron Acceptor Insufficiency Affects Purine Nucleotide Levels.
(A) Analysis of DNA content by propidium iodide staining and flow cytometry of 143B CytB cells cultured with our without AKB supplementation. (B) LCMS quantification of purine nucleotide levels in 143B CytB cells cultured with or without AKB supplementation. (C) Cell doublings were measured over time of 143B Cytb cells cultured in media that was not or was supplemented with AKB, adenine, hypoxanthine, or guanine. (D) Schematic illustrating the use of IMP for GMP and AMP synthesis. Synthesis of GMP uses NAD+, whereas synthesis of AMP requires aspartate. Values in (B) and (C) denote mean ± standard error of the mean (SEM), n=3. See also Figure S3.

Aspartate synthesis is inhibited by electron acceptor insufficiency.

In most tumor cells in culture, carbon for *de novo* aspartate synthesis is supplied by anaplerotic glutamine (DeBerardinis et al., 2007; Hensley et al., 2013). Glutamine is converted to glutamate, which enters the TCA cycle upon conversion to alpha-ketoglutarate by glutamate dehydrogenase (GDH) or transamination (Fig 5A). While GDH uses NAD+ as a co-substrate, the glutamate transaminases utilize alpha-ketoacids, such as pyruvate, as co-substrates. Regardless of which pathway produces alpha-ketoglutarate from glutamate, conversion of a carbon-nitrogen single bond to a carbon-oxygen double bond necessitates that an electron pair is transferred to an electron acceptor. Reductive synthesis of aspartate by reductive carboxylation of alpha-ketoglutarate requires high levels of alpha-ketoglutarate, (Fendt et al., 2013), and electron acceptors are needed to maintain increased alpha-ketoglutarate pools. Oxidative synthesis of aspartate from alpha-ketoglutarate requires three additional oxidation reactions requiring net transfer of two electron pairs, thus imposing a further requirement for additional electron acceptors to produce aspartate from alpha-ketoglutarate (Fig 5A). Given that both aspartate synthesis pathways utilize electron acceptors, we reasoned that electron acceptor deficiency in respiration-inhibited cells could limit aspartate production and result in aspartate deficiency.

To test this possibility, we used gas chromatography mass spectrometry (GCMS) to measure the electron acceptor dependency of glutamate, aspartate, and TCA cycle metabolite levels in cells. In 143B CytB cells, which metabolize glutamine by reductive carboxylation (Fig S4)(Mullen et al., 2014; Mullen et al., 2012) glutamate levels are only modestly lower upon AKB withdrawal, whereas pool sizes of TCA cycle metabolites are

drastically decreased (Fig 5B). Aspartate was also dramatically decreased in these cells, supporting the hypothesis that aspartate limitation may explain the observed AMP/IMP imbalance in cells with electron acceptor insufficiency. In wild-type 143B cells, which normally produce aspartate by oxidative TCA cycle metabolism (Fig S4), treatment with the respiration inhibitors rotenone, antimycin, and oligomycin yielded similar results, with decreases in both TCA metabolites and aspartate, but not glutamate (Fig 5C). In all cases, treatment with AKB restored aspartate to levels comparable to, or higher than, untreated cells. These data demonstrate that lack of available electron acceptors restricts aspartate biosynthesis.

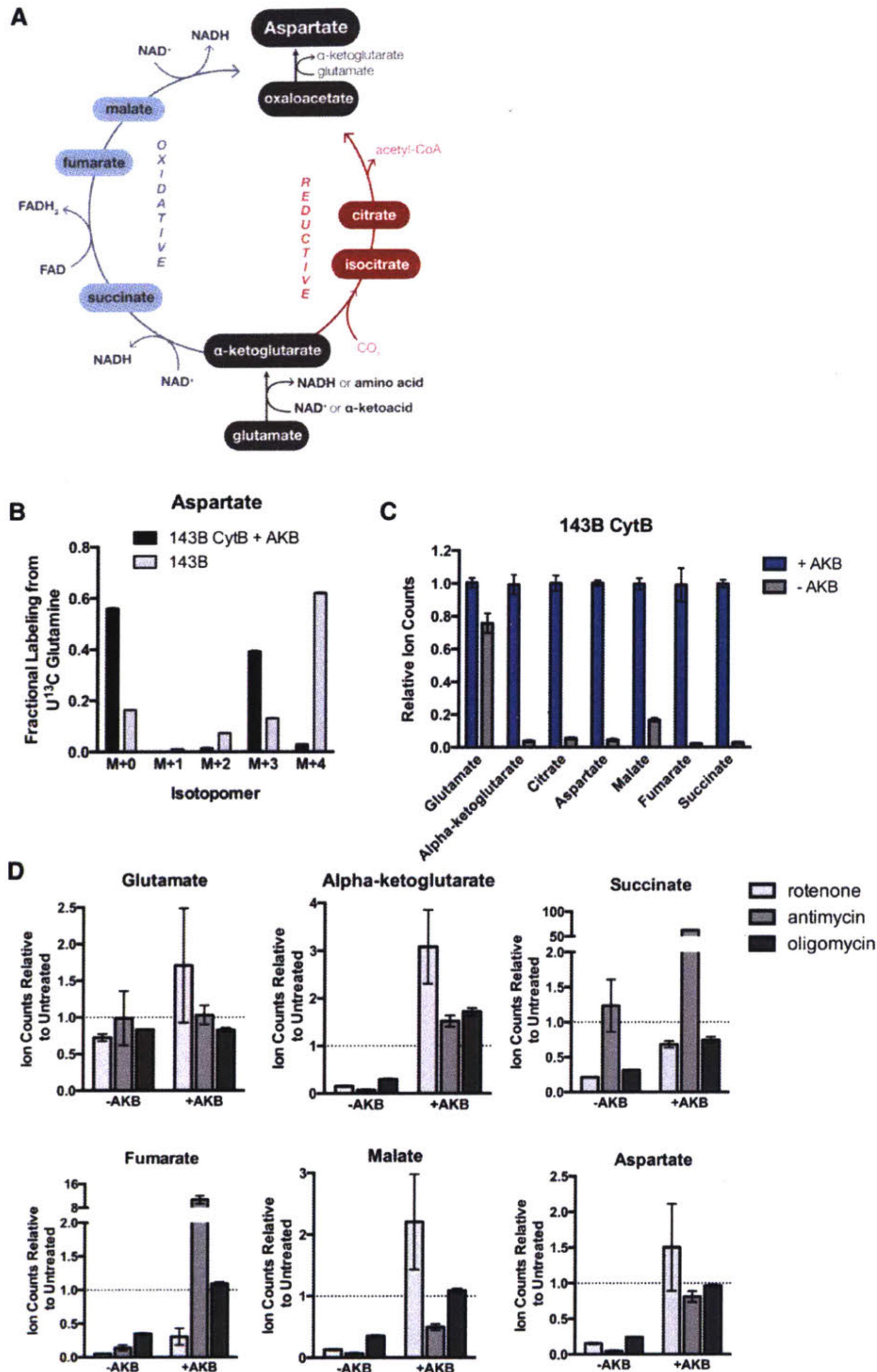


Figure 5. Electron Acceptor Insufficiency Suppresses TCA Metabolite and Aspartate Levels.
(A) Schematic detailing the TCA cycle reaction routes for the biosynthesis of aspartate from glutamine. (B) GCMS quantification of TCA cycle metabolites, glutamate, and aspartate from 143B CytB cells cultured with or without AKB. (C) GCMS quantification of TCA metabolites, glutamate, and aspartate from 143B cells cultured with or without AKB in the presence or absence of the indicated mitochondrial inhibitors. Ion counts are relative to untreated 143B cells, which is denoted by the dashed grey line in each panel. Values in (B) and (C) denote mean ± standard error of the mean (SEM), n=3. See also Figure S4.

Aspartate restores proliferation in cells with electron acceptor insufficiency.

To determine whether the absence of electron acceptors affects cell proliferation because of an effect on aspartate biosynthesis, we tested whether exogenous aspartate could replace the requirement for exogenous electron acceptors. Strikingly, in the absence of exogenous electron acceptors, supplementation with supra-physiological levels of aspartate was capable of supporting exponential growth of 143B CytB cells (Fig 6A). Given the decrease in other TCA cycle intermediates observed following electron acceptor withdrawal, we also questioned whether other TCA cycle intermediates, or their cell-permeable derivatives, could restore proliferation of these cells. None were able to restore proliferation to a similar degree as aspartate (Fig S5A, B). Respiration-competent 143B WT cybrid cells proliferated at similar rates in the presence or absence of aspartate (Fig S5C). Importantly, aspartate does not function as an exogenous electron acceptor as it neither increases mitochondrial oxygen consumption, nor does it affect the NAD+/NADH ratio in 143B CytB cells (Fig S5D and E). These data imply that aspartate is not an electron acceptor, but rather is itself the biosynthetic demand that becomes limiting for proliferation in electron acceptor deficient cells.

Measurement of purine monophosphates shows that treatment of 143B CytB cells with aspartate relieves the accumulation of IMP and restores AMP pools (Fig S5F). This is consistent with the hypothesis that in cells with insufficient electron acceptors, aspartate is limiting for adenine nucleotide synthesis. Interestingly, GMP levels are not restored by aspartate treatment, consistent with the conversion of IMP to GMP being dependent on NAD+, which is not restored by aspartate (Fig S5E). Indeed, GMP levels were lower in aspartate treated cells, likely as a result of decreased IMP availability (Fig S5F).

To determine whether aspartate is sufficient to restore proliferation in wild-type cells where respiration is inhibited, wild-type 143B cells were treated with various respiration inhibitors with or without aspartate supplementation. Proliferation was restored in response to all ETC inhibitors when cells were supplemented with high levels of aspartate (Fig 6B). Aspartate supplementation also rescued ETC inhibitor-induced proliferation decreases in all other cell lines tested (Fig 6C). Taken together, these data support a model where a major metabolic requirement for proliferating cells fulfilled by respiration is providing access to electron acceptors in the form of oxygen. This access to electron acceptors is required to support *de novo* aspartate biosynthesis and in the absence of mitochondrial respiration, the demand for oxygen can be met through supplementation of other electron acceptors such as pyruvate or AKB. Alternatively, if the demand for aspartate can be met exogenously, electron acceptors become dispensable for proliferation. Surprisingly, this implies that the major function of respiration in proliferating cells is to support *de novo* aspartate synthesis (Fig 6D).

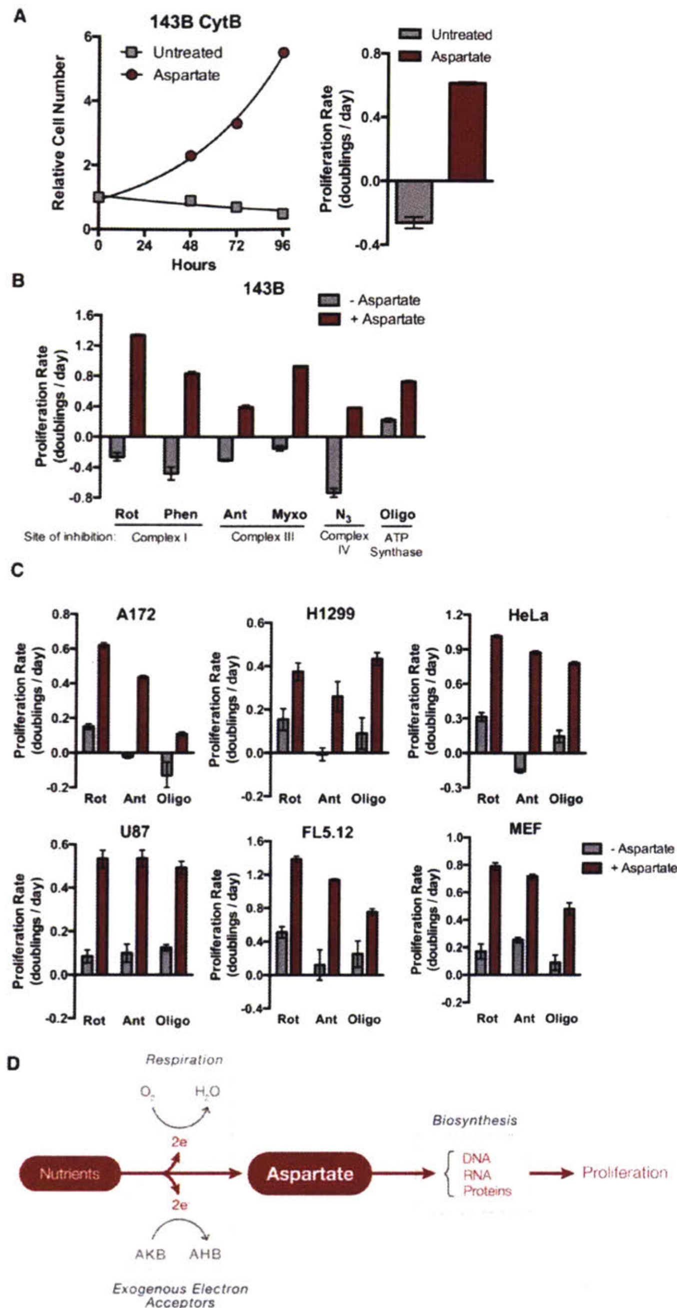


Figure 6. Aspartate is the Key Biosynthetic Precursor Provided by Respiration.
(A) Proliferation rate of 143B CytB cells determined in the presence or absence of aspartate. (B) The proliferation rate of 143B cells cultured with or without aspartate in the presence of the mitochondrial respiration inhibitors rotenone (Rot), phenformin (Phen), antimycin (Ant), myxothiazol (Myxo), azide (N_3), or oligomycin (Oligo). (C) The proliferation rate of A172, H1299, HeLa, U87, FL5.12, and MEF cells cultured with or without aspartate in the presence of rotenone, antimycin, or oligomycin. (D) Schematic detailing the role of respiration and exogenous electron acceptors in aspartate biosynthesis. The conversion of nutrients into aspartate requires the removal of electrons and therefore requires access to electron acceptors, which can be supplied by respiration (O_2) or exogenous electron acceptors such as AKB. Maintenance of aspartate pools supports nucleotide and protein biosynthesis. Values in all figure panels denote mean ± standard error of the mean (SEM), n=3. See also Figure S5.

Discussion

In this study we examine the role of mitochondrial respiration in proliferative metabolism. Whereas respiration is primarily an ATP-producing catabolic process in non-proliferating cells, in proliferating cells respiration serves a crucial anabolic role by providing access to electron acceptors to support aspartate synthesis. Furthermore, mitochondrial ATP production appears dispensable in proliferating cells with access to sufficient glucose. Proliferating cells have different metabolic requirements than non-proliferating cells (Lunt and Vander Heiden, 2011); yet, the components of the metabolic network in both proliferating and non-proliferating cells are largely the same (Hu et al., 2013). During cell proliferation these same network components must take on distinct roles to balance the contrasting anabolic and catabolic needs of the cell. While respiration likely supports ATP production in addition to aspartate biosynthesis in many contexts, our finding that respiration is specifically required for aspartate biosynthesis in proliferating cells highlights a distinct anabolic role for respiration.

All cells must perform thermodynamically unfavorable processes. One solution for accomplishing this is to harness the free-energy of nutrient oxidation. The source of electron donors for oxidation can vary widely across species from inorganic material for chemolithotrophic bacteria to reduced carbon for most heterotrophs including mammalian cells. In all cases, nutrient oxidation requires net transfer of electrons to a terminal electron acceptor. In mammalian cells, substrates for nutrient oxidation include carbohydrates, lipids, and amino acids. Various metabolic pathways, including the TCA cycle, are employed for nutrient oxidation with electrons transferred initially to electron accepting cofactors such as NAD+ or FAD. These reactions yield NADH or $FADH_2$,

respectively, and result in production of intermediates with more oxidized carbon. The electrons from the reduced cofactors are transferred to the ETC, which uses O_2 as a terminal electron acceptor to produce water and facilitate ATP production. Importantly, ATP production through this process intimately couples the oxidation of carbon substrates to O_2 consumption.

In contexts where mammalian cells cannot utilize O_2 as a terminal electron acceptor, cells are unable to regenerate oxidized cofactors via the ETC. Cells can ferment pyruvate to lactate to regenerate NAD+; however, because the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) step of glycolysis consumes NAD+, use of glucose-derived pyruvate for lactate fermentation does not net yield NAD+. Thus, in the absence of access to exogenous electron acceptors, in order to maintain redox balance and sustain glycolytic ATP production all glucose carbon metabolized via glycolysis past the GAPDH step must be excreted as lactate. While fermentation can produce sufficient ATP for proliferation, it is a redox neutral pathway. Consequently, in the absence of exogenous electron acceptors cells cannot net synthesize molecules that are more oxidized than the nutrients consumed. Thus, losing oxygen as an electron acceptor results in the inability to remove electrons from consumed carbon substrates.

To divide, proliferating cells must duplicate all cell components, some of which are more oxidized than the nutrients consumed. This suggests that net removal of electrons from carbon is an intrinsic anabolic requirement to support production of oxidized molecules, such as nucleotides, for biomass accumulation. This need to oxidize nutrients to generate biomass may result in electron acceptor insufficiency and limit proliferation even in the presence of oxygen. Notably, the NAD+/NADH ratio in normoxic cancer cells

is more reduced than previously assumed (Hung et al., 2011; Zhao et al., 2015), consistent with oxidation capacity being constrained. Because the NAD+/NADH ratio and the pyruvate/lactate ratio are tightly coupled in cells (Williamson et al., 1967), a low NAD+/NADH ratio is expected to drive increased net pyruvate to lactate conversion. This raises the possibility that the Warburg effect is a reflection of electron acceptor insufficiency.

In our study, we find that electron acceptors are most limiting for *de novo* aspartate synthesis. While cells in culture are exposed to atmospheric oxygen, oxygen availability in animal tissues is much lower (Bertout et al., 2008). Thus, it may be advantageous for cells to utilize less oxidative pathways when electron acceptors become more limiting. The need for electron acceptors is decreased when metabolic pathways that fix CO_2 are used for *de novo* aspartate synthesis. For instance, pyruvate carboxylation produces oxaloacetate that can be transaminated to form aspartate. Alternatively, reductive alpha-ketoglutarate carboxylation can generate isocitrate, which can be isomerized to citrate, cleaved to form oxaloacetate, and transaminated to produce aspartate. In both cases, fully oxidized carbon is incorporated to produce aspartate without directly requiring net removal of electrons. However, pyruvate and alpha-ketoglutarate are themselves oxidized relative to the major nutrients glucose and glutamine, imposing a need for electron acceptors to produce these carboxylation pathway substrates. Furthermore, the ability to maintain increased levels of pyruvate or alpha-ketoglutarate to support pyruvate carboxylation or reductive isocitrate dehydrogenase metabolism is compromised in electron acceptor deficient cells as illustrated by the dramatic fall in alpha-ketoglutarate levels when respiration is inhibited (Fig 5B, 5C). Therefore, regardless of the biosynthesis pathway used, electron acceptor insufficiency will limit aspartate synthesis.

Beyond its role as an amino acid in proteins, aspartate is required for conversion of IMP to AMP in *de novo* purine synthesis and provides the carbon backbone for *de novo* pyrimidines synthesis. Thus, aspartate deficiency will impair protein, purine nucleotide, and pyrimidine nucleotide synthesis. Notably, aspartate is inefficiently transported into most mammalian cells (Birsoy, et al., cosubmitted with this manuscript), with supra-physiological concentrations required to restore growth in electron acceptor deficient cells. The concentration of aspartate in blood is around 10 mM, among the lowest levels of all circulating amino acids (Mayers and Vander Heiden, 2015). Additionally, aspartate aminotransferase (AST) is among the most abundant enzymes in the liver, suggesting that circulating aspartate levels may be actively maintained at low levels. Given the critical role of aspartate in biosynthesis and the challenges associated with obtaining aspartate from the blood, access to electron acceptors for aspartate synthesis may be a requirement for proliferation in many tumors. Consistent with this idea, pharmacologic inhibition of ETC activity inhibits tumor growth in several cancer models (Shackelford et al., 2013; Wheaton et al., 2014; Zhang et al., 2014). Additionally, while ρ^0 cells are unable to form xenografts, reconstituting mtDNA in these cells restores tumorigenicity (Hayashi et al., 1992). This requirement for mtDNA appears so stringent that, in one study, injected ρ^0 cells formed tumors only after acquiring mtDNA from the host (Tan et al., 2015). While ρ^0 cells are also expected to be pyrimidine-limited due to loss of DHODH activity, genetic loss of complex I, which blocks respiration but not DHODH, also impairs tumorigenesis (Park et al., 2009).

Beyond loss of aspartate biosynthesis, we find loss of ETC activity affects the NAD+/NADH ratio. This redox change will also influence other pathways. For example,

NAD+ is integral to sirtuin and PARP activity, and thus, loss of ETC activity may perturb signaling pathways that involve these enzymes (Canto and Auwerx, 2011). Additionally, mitochondrial ETC activity impacts apoptosis (Newmeyer and Ferguson-Miller, 2003; Wallace, 2012), processes that depend on the mitochondrial membrane potential (Chen et al., 2014; Geissler et al., 2000), and levels of reactive oxygen species in cells (Schieber and Chandel, 2014). Nevertheless, exogenous aspartate addition is sufficient to restore proliferation of cells that otherwise stop proliferating or die when ETC activity is impaired. Thus, a primary role for mitochondrial respiration in cell proliferation must be to provide access to electron acceptors in support of aspartate synthesis.

Experimental Procedures

Cell Culture

143B, A172, H1299, HeLa, U87 and immortalized MEF cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin. FL5.12 cells were cultured in the same medium and supplemented with 5 µg/mL recombinant mouse IL-3 (R&D Systems). 143B cybrid cell lines (WT Cybrid and CytB) were cultured in DMEM with 10% FBS, penicillin-streptomycin, and 0.1 mg/mL uridine. All cells were incubated at 37°C with 5% CO_2.

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 FBS, 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 to basal measurements or 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, and the mitochondrial oxygen consumption rates 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.

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 106, 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.

Amino Acid and TCA cycle Metabolite Extraction and GCMS Analysis

143B and 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 8 hours, polar metabolites were extracted using 80% methanol in water with 1 μg norvaline standard added per sample. Soluble content was then dried under nitrogen gas. Polar samples were derivatized and measured as detailed in (Lewis et al., 2014). Relative metabolite abundances were determined by integrating ion peak area (Metran) and normalized to norvaline internal extraction standard.

Cell Cycle Distribution Measurements

143B CytB cells were incubated in untreated media or media containing AKB or 100 μM adenine for 78 hours before being washed with PBS, trypsinized, pelleted, and resuspended in 500 μL PBS. Cells were fixed by adding 4.5 mL 70% ethanol and incubated at 4°C overnight. The following day cells were pelleted and resuspended in 1 mL PBS + 0.1% (v/v) Triton X-100. RNase A and then propidium iodide (PI) were added to 0.2 mg/mL and 20 μg/mL, respectively. Samples were incubated at 37°C for 15 minutes and filtered into a flow cytometry tube. DNA content was measured by flow cytometry (BD FACS Canto II) and analyzed (FACS Diva Software).

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 $p \leq 0.05$.

Supplemental Figures

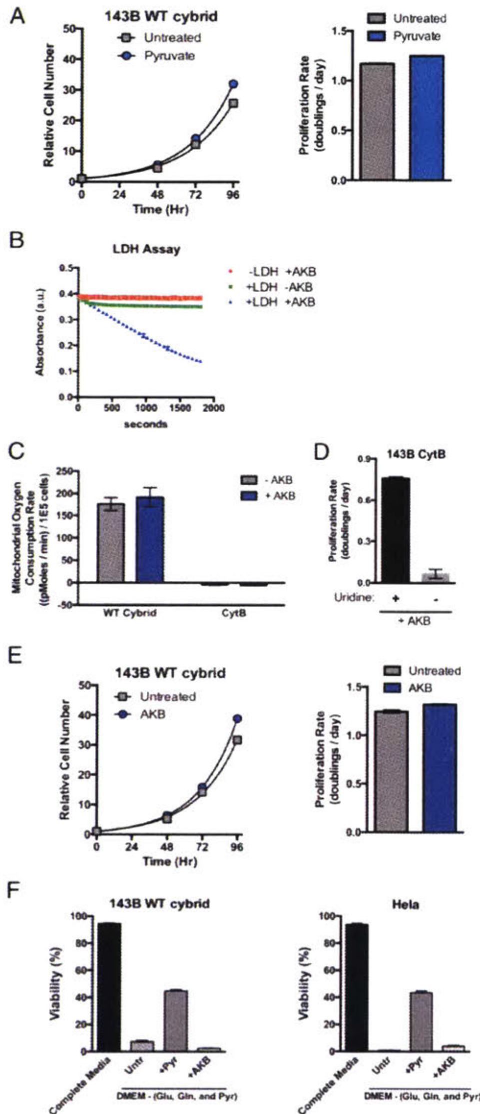


Figure S1. AKB can Function as a Substrate for LDH and does not Restore Oxygen Consumption or Replace Uridine Auxotrophy in Respiration Incompetent Cells, Related to Figure 1.

(A) Proliferation of 143B WT cybrid cells in the presence or absence of pyruvate. (B) The ability of lactate dehydrogenase (LDH) to use AKB as a substrate was assessed by changes in NADH absorbance (at 340 nm) in the presence or absence of AKB and LDH as indicated. (C) Mitochondrial oxygen consumption rate of 143B WT cybrid and 143B CytB cells in the presence or absence of AKB. (D) Proliferation rate of 143B CytB cells cultured in the presence of AKB was determined with or without uridine supplementation. (E) Proliferation of 143B WT cybrid cells was determined in the presence or absence of AKB. (F) Viability was determined by propidium iodide exclusion for 143B WT cybrid and HeLa cells cultured in complete DMEM or DMEM without glucose, glutamine, or pyruvate, in the presence or absence of pyruvate or AKB supplementation as indicated. Values in all figures denote mean ± standard error of the mean (SEM), n=3 (A, B, D-F), n=5 (C).

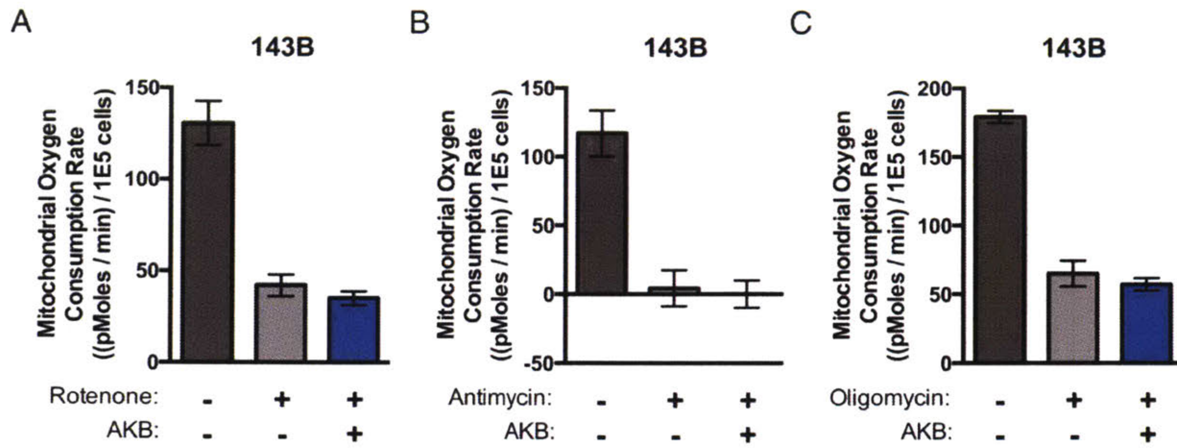


Figure S2. Mitochondrial Oxygen Consumption is Inhibited by Respiration Inhibitors and is not Restored by AKB, Related to Figure 2.
Mitochondrial oxygen consumption rate of untreated 143B cells or 143B cells treated with rotenone (A), antimycin (B), or oligomycin (C), with or without AKB treatment. Values in all figures denote mean ± SEM, n=5.

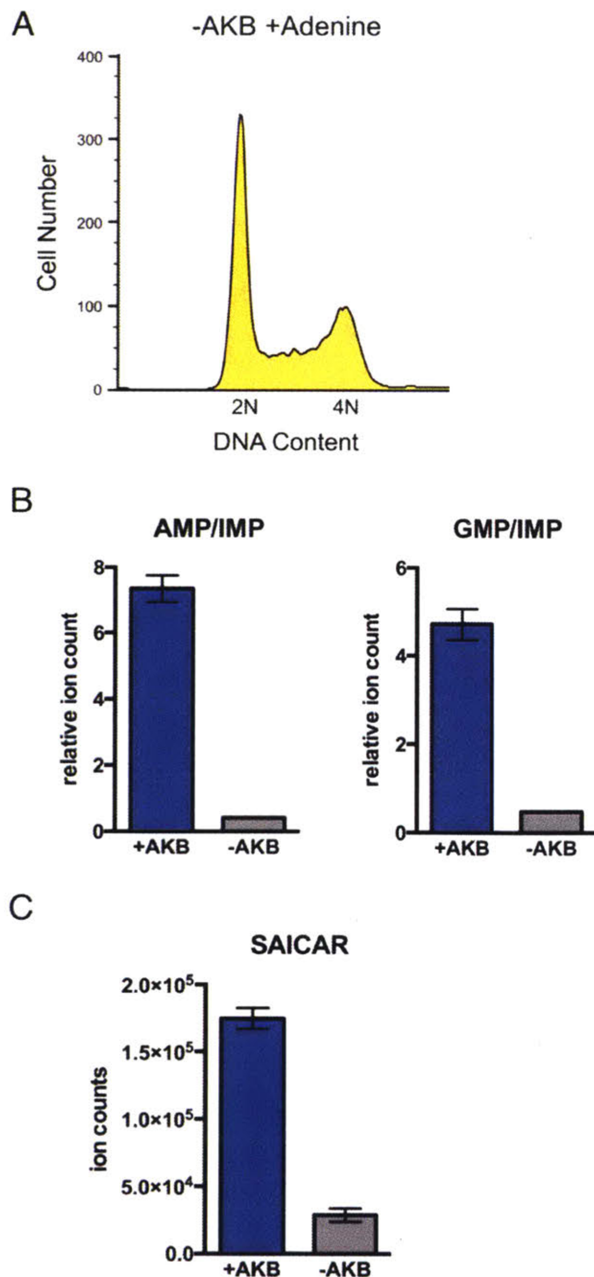
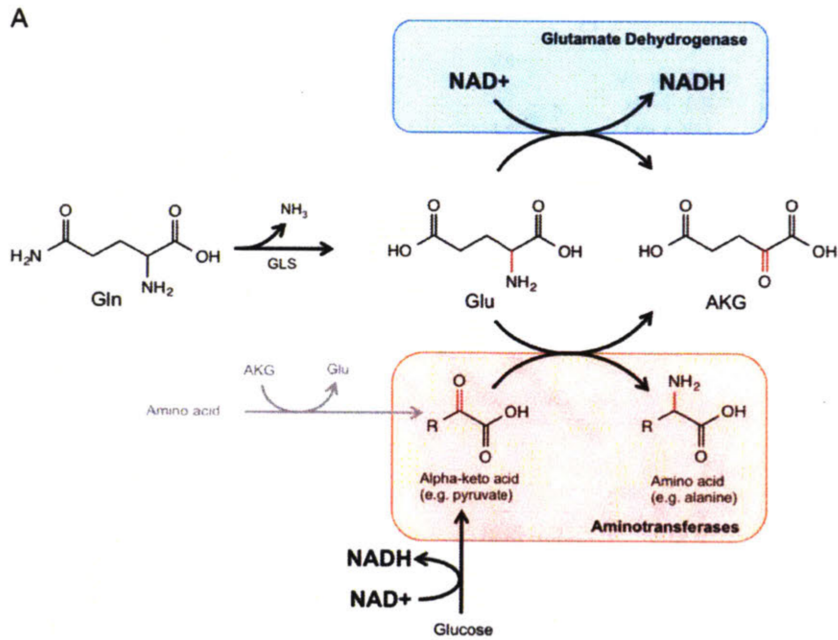


Figure S3. The Effect of Adenine on DNA Content and the Effect of AKB on Purine Nucleotide Ratios, Related to Figure 4.
(A) Analysis of DNA content by propidium iodide staining and flow cytometry of 143B CytB cells without AKB supplementation and with adenine supplementation. (B) Relative ion count ratios of AMP to IMP and GMP to IMP in 143B CytB cells with or without AKB supplementation. (C) LCMS quantification of SAICAR levels in 143B CytB cells cultured with or without AKB supplementation. Values denote mean ± SEM, n=3.

A



B

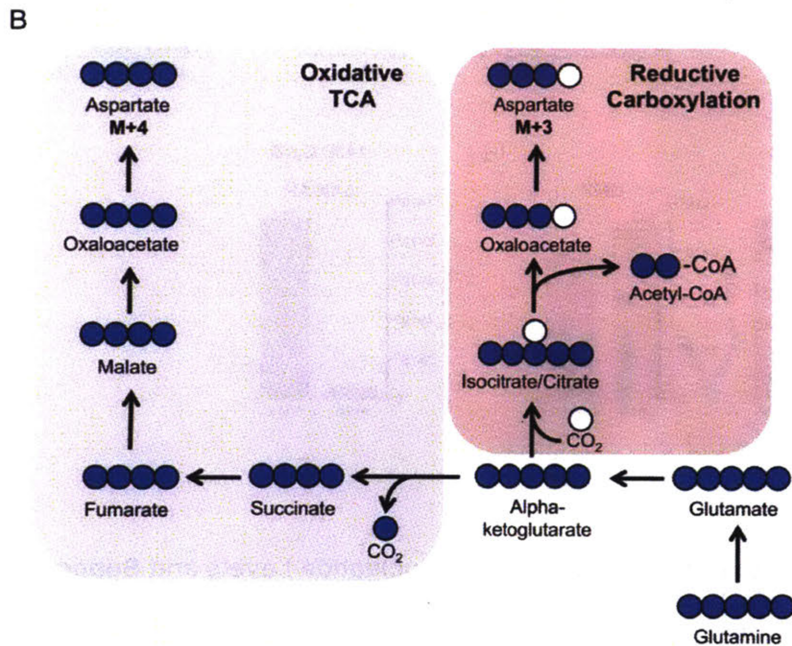


Figure S4. Aspartate is Derived from Glutamine by Reductive Carboxylation in 143B CytB cells and Oxidative TCA Metabolism in 143B cells, Related to Figure 5.
(A) Schematic detailing the pathways used to produce alpha-ketoglutarate from glutamine. Abbreviations: Gln, glutamine; Glu, glutamic acid; AKG, alpha-ketoglutaric acid. (B) Schematic showing expected aspartate labeling patterns if synthesized from glutamine using oxidative TCA metabolism or reductive carboxylation as indicated.

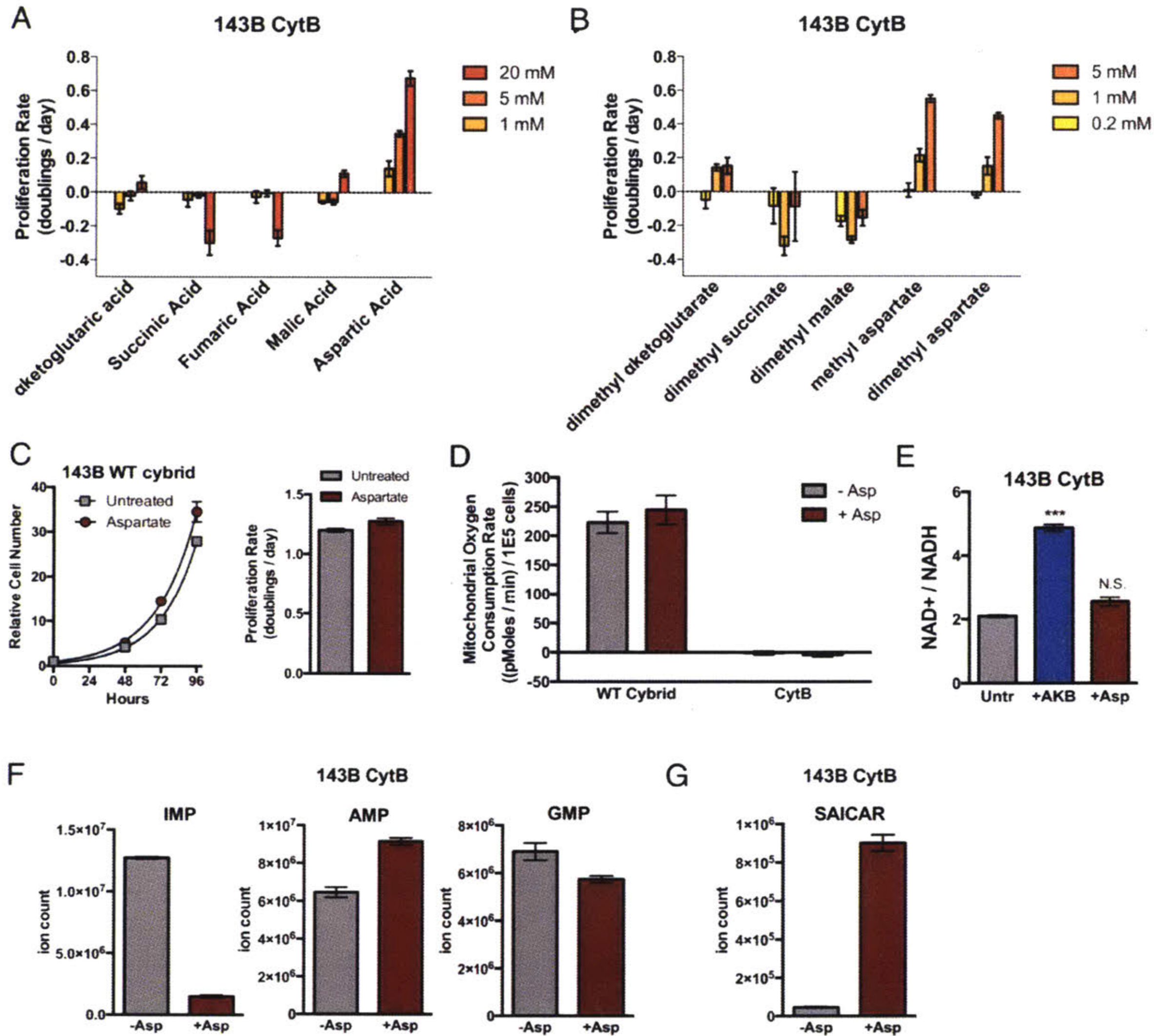


Figure S5. Aspartate Supplementation Restores Adenine Nucleotide Levels and Supports Proliferation Without Affecting NAD+/NADH or Oxygen Consumption, Related to Figure 6. (A) Proliferation of 143B CytB cells determined after supplementation with the indicated concentrations of TCA cycle intermediates or aspartic acid. (B) Proliferation of 143B CytB cells was determined after supplementing with the indicted concentrations of methyl ester derivatives of TCA cycle intermediates or aspartate. (C) Proliferation of 143B WT cybrid cells was determined in the presence or absence of aspartate. (D) Mitochondrial oxygen consumption rate was determined in 143B WT Cybrid and 143B CytB cells in the presence or absence of aspartate. (E) Intracellular ratio of NAD+/NADH measured in 143B CytB cells cultured in standard media (Untr) or in media supplemented with AKB or aspartate (Asp). (F) LCMS quantification of purine nucleotide levels in 143B CytB cells without or with aspartate supplementation. (G) LCMS quantification of SAICAR levels in 143B CytB cells cultured without or with aspartate supplementation. Values in all figures denote mean ± SEM, n=3 (A-C, E-G), n=5 (D). *** represents $p \leq 0.001$, N.S. indicates the measured values were not statistically different ($p > 0.05$).

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Chapter 3: Environment Dictates Dependence on Mitochondrial Complex I for NAD+ and Aspartate Production and Determines Cancer Cell Sensitivity to Metformin

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D.Y.G. and L.B.S. performed experiments to determine proliferation rates, oxygen consumption measurements, NAD+/NADH ratio measurements, and metabolite measurements. A.L. performed xenograft experiments and assisted with writing the manuscript. A.M.H. performed immunoblotting experiments. L.N.B. and N.G. performed proliferation rate experiments. S.M.D. provided preliminary data and technical expertise. E.F. performed LCMS experiments. C.J.T. provided critical supplies and experimental design. D.Y.G., L.B.S., and M.G.V.H. designed the study and wrote the manuscript.

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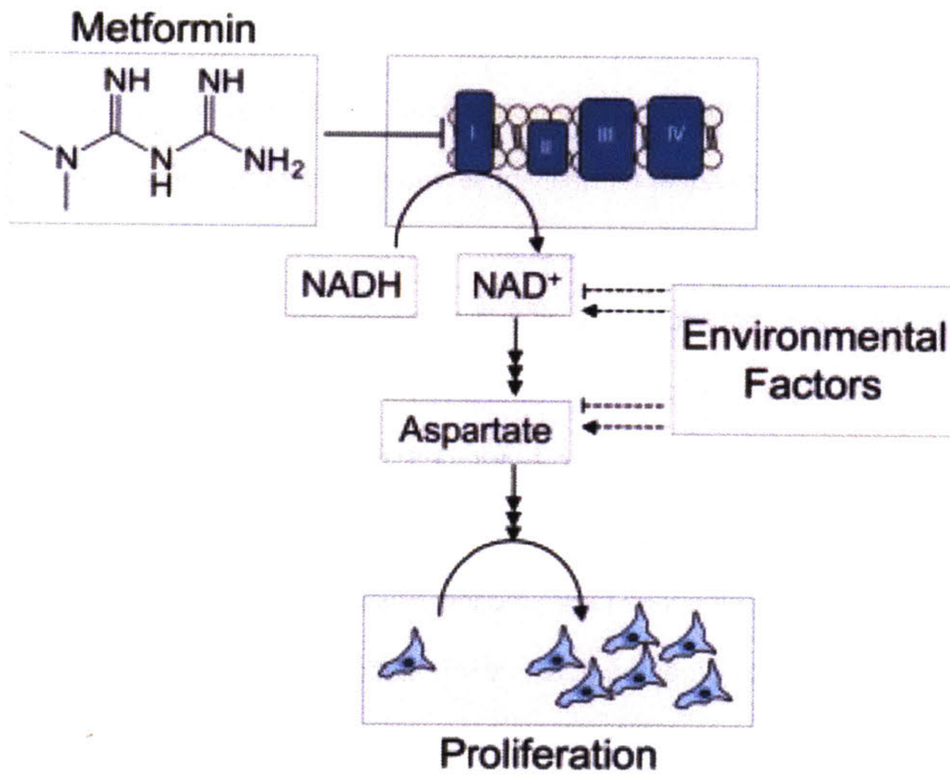
Summary

Metformin use is associated with reduced cancer mortality, but how metformin impacts cancer outcomes is controversial. While metformin can act cell autonomously to inhibit tumor growth, the doses of metformin that inhibit proliferation in tissue culture are much higher than what has been described in vivo. Here, we show that environment drastically alters sensitivity to metformin and other complex I inhibitors. We find that complex I supports proliferation by regenerating NAD+, and metformin's anti-proliferative effect is due to loss of NAD+/NADH homeostasis and inhibition of aspartate biosynthesis. However, complex I is only one of many inputs that determine cellular NAD+/NADH ratio, and dependency on complex I is dictated by the activity of other pathways that affect NAD+ regeneration and aspartate levels. This suggests that cancer drug sensitivity and resistance are not intrinsic properties of cancer cells, and demonstrates that environment can dictate sensitivity to therapies that impact cell metabolism.

Graphical Summary



Introduction

Metformin is a safe and effective anti-hyperglycemic agent that is commonly used by hundreds of millions of people worldwide to treat type 2 diabetes. Because of its widespread use, an abundance of epidemiological data is available for how metformin might influence other disease states. Retrospective studies have found that taking metformin is associated with improved cancer outcomes, with reductions in cancer incidence and with decreased cancer mortality observed across many tumor types (Evans et al., 2005; Gandini et al., 2014; He et al., 2012; Lee et al., 2012; Zhu et al., 2015). These findings have resulted in studies examining the anti-tumorigenic properties of metformin and other biguanides on cancer cell lines and in mouse models of cancer, as well as clinical trials exploring potential roles for metformin in cancer therapy. Systemic treatment with the biguanides metformin and phenformin can suppress tumor growth in xenograft and autochthonous tumor models (Buzzai et al., 2007; Huang et al., 2008; Shackelford et al., 2013; Wheaton et al., 2014). Metformin may also increase the pathological complete response rate in breast cancer patients receiving neoadjuvant chemotherapy (Jiralerspong et al., 2009). Whether the benefit of metformin in these different settings is attributable to direct action on the tumor is controversial (Birsoy et al., 2012; Foretz et al., 2014). Further, in a recent trial, metformin failed to improve outcomes in patients when added to standard pancreatic cancer therapy (Kordes et al., 2015), highlighting that the factors that determine which tumors are likely to respond to metformin are not known.

The molecular targets of metformin in cells and tissues have only recently come into focus (Luengo et al., 2014; Pernicova and Korbonits, 2014). Metformin can impair

respiration by inhibition of the glycerol-phosphate shuttle (Madiraju et al., 2014), and *in vitro* biochemistry studies have shown that metformin directly inhibits mitochondrial complex I (NADH dehydrogenase), also resulting in decreased mitochondrial respiration (Bridges et al., 2014; El-Mir et al., 2000; Owen et al., 2000; Wheaton et al., 2014). Consistent with these molecular targets, cell culture studies have found that metformin causes increased glucose consumption, increased lactate production, and decreased mitochondrial glucose oxidation (Andrzejewski et al., 2014; Fendt et al., 2013). Supporting a role for complex I as an important target of metformin in cancer, expression of a metformin resistant yeast analog of complex I (NDI1) makes cells insensitive to metformin in culture and resistant to the anti-tumor growth effects of metformin in xenograft models (Birsoy et al., 2014; Wheaton et al., 2014). Additionally, other complex I inhibitors have shown efficacy as anti-tumor agents (Schockel et al., 2015; Zhang et al., 2014). Together these data support the hypothesis that tumor autonomous inhibition of complex I and respiration play an important role in the anti-tumorigenic effect of metformin.

A major barrier for translating findings pertaining to metformin action in cultured cells to the clinic is a substantial inconsistency in the doses of metformin required to inhibit proliferation *in vivo* versus *in vitro*. Inhibition of cancer cell proliferation *in vitro* generally requires metformin doses that vastly exceed those achievable *in vivo* (Birsoy et al., 2012; Foretz et al., 2014; Pollak, 2014). Metformin concentrations required to inhibit cancer cell proliferation *in vitro* range from 1 to 50 mM, while plasma metformin levels in patients and in mice are in the micromolar range (Graham et al., 2011; He and Wondisford, 2015; Memmott et al., 2010). Indeed, even the maximum tolerable plasma

concentration of metformin reported for humans, around 400 μM (Dell'Aglio et al., 2009) is still well below the doses used in most studies to decrease proliferation in culture.

In this study, we provide an explanation for the substantial discrepancy between the effective metformin concentration *in vivo* and *in vitro*. We find that metabolic environment is a major determinant of complex I dependency, and hence, differences in environment alone can dramatically alter sensitivity to metformin and other complex I inhibitors. Complex I supports proliferation through NAD+ regeneration to maintain cellular NAD+/NADH balance and to allow aspartate synthesis. However, dependency on complex I is directly dictated by the activity of alternative pathways that produce and consume NAD+, which change based on environmental factors. We show that alternative NAD+ utilizing pathways modulates complex I dependency and metformin sensitivity in a predictable manner. Further, we show that perturbing NAD+/NADH balance by modulating complex I activity modulates aspartate levels in a titratable manner both *in vitro* and *in vivo*. These data indicate that the anti-proliferative effects of metformin are caused by a decrease in the intracellular NAD+/NADH ratio and aspartate levels and suggest that environment can alter dependency on metabolic drug targets.

Results

Pyruvate suppresses the anti-proliferative effects of complex I inhibition

Mirroring the discrepancy between metformin effective concentration *in vivo* and *in vitro*, we observed that for all cell lines tested, the choice of culture media dramatically alters metformin sensitivity. We cultured a diverse group of cancer cell lines in two different standard cell culture media, Dulbecco's Modified Eagle's Medium (DMEM) and Roswell Park Memorial Institute 1640 medium (RPMI), which both fully support proliferation of the cells examined (Figure 1A, B). Consistent with previous reports, cells cultured in DMEM require up to 10 mM metformin to partially inhibit proliferation (Figure 1A) (Foretz et al., 2014). In contrast, cells cultured in RPMI are much more sensitive to metformin, with lower metformin doses inhibiting proliferation (Figure 1B). While the doses of metformin tested were still higher than what is generally achievable in vivo, these data directly suggest that metabolic environment alone is sufficient to dictate metformin sensitivity.

To better understand the differential effects of metformin in the different culture media, we examined how the media compositions differ. A binary difference is the presence of 1 mM pyruvate in DMEM and the absence of pyruvate in RPMI. To test whether pyruvate concentration could be a determinant of metformin sensitivity, we examined the anti-proliferative effect of metformin on cells cultured in DMEM without pyruvate. Strikingly, cells cultured in DMEM without pyruvate exhibit increased sensitivity to metformin, suggesting that pyruvate suppresses the anti-proliferative effect of metformin (Figure 1C). Consistent with this hypothesis, cells cultured in RPMI supplemented with 1 mM pyruvate (RPMI + Pyruvate) are less sensitive to metformin (Figure 1D). These data indicate that pyruvate availability is a crucial environmental variable that can alter sensitivity to the anti-proliferative effects of metformin.

Interestingly, addition of 1 mM pyruvate to RPMI made cells more resistant to metformin than cells cultured in DMEM with 1 mM pyruvate, further highlighting that environment can influence drug sensitivity. To better understand environmental determinants other than pyruvate that impact metformin sensitivity, we decreased the glucose concentration of DMEM to match RPMI (11 mM), or added aspartate to DMEM at the same concentration as that found in RPMI (150 μM) (Figure S1A, B). Lowering glucose, or addition of aspartate both affected metformin sensitivity to a degree, although the magnitude of effect was much smaller than that observed with pyruvate and was variable across cell lines. Thus, further experiments focused on understanding the influence of pyruvate on metformin sensitivity were performed using DMEM without pyruvate, unless otherwise noted.

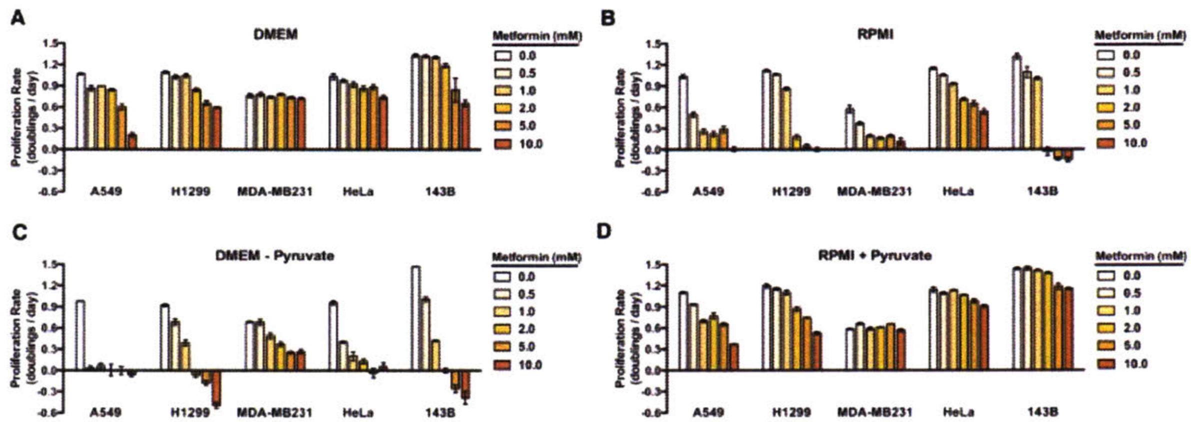


Figure 1. Pyruvate suppresses the antiproliferative effects of metformin.
Proliferation rates for A549, H1299, MDA-MB231, HeLa, and 143B cells in media treated with the indicated concentrations of metformin. Cells were cultured in DMEM (A), RPMI 1640 (B), DMEM without pyruvate (C), and RPMI 1640 supplemented with 1 mM pyruvate (D). Values denote mean ± standard error of the mean (SEM). *n*=3. See also Figure S1.

Pyruvate modulates complex I dependency by providing an alternative pathway for NAD+ regeneration

It has been classically described that respiration-deficient cells are pyruvate auxotrophs (King and Attardi, 1989). In the absence of respiration, pyruvate can support proliferation by acting as an electron acceptor to regenerate NAD+ and allow aspartate synthesis (Birsoy et al., 2015; Sullivan et al., 2015). We hypothesized that a similar model could explain why pyruvate blocks the anti-proliferative effects of metformin.

While it is generally accepted that metformin is a mitochondrial inhibitor; the mechanism by which it inhibits mitochondrial activity is controversial. Some reports suggest that metformin acts primarily as an inhibitor of complex I, while recent work has implicated metformin as a mitochondrial glycerol phosphate dehydrogenase (mGPD) inhibitor that disrupts the glycerol phosphate shuttle in the liver (Madiraju et al., 2014). Since both complex I inhibition and mGPD inhibition could lead to NAD+ deficiency, both mechanisms are consistent with pyruvate restoring NAD+ to block metformin's anti-proliferative effect. Nevertheless, to distinguish between these two mechanisms in the cancer cells studied, we assessed complex I-mediated or glycerol-3-phosphate shuttle-mediated oxygen consumption in saponin-permeabilized cells. Whereas metformin dramatically inhibits oxygen consumption when permeabilized cells are supplied with pyruvate and malate as substrates for complex I, oxygen consumption from glycerol-3-phosphate is not disrupted by metformin (Fig 2A and S2A). These data argue that metformin inhibits electron transport chain from mitochondrial complex I but not from the glycerol-3-phosphate shuttle in these cells.

Previous studies have shown that compounds that inhibit respiration by hyperpolarizing the mitochondrial membrane potential, such as the ATP synthase inhibitor oligomycin, also decrease the NAD+/NADH ratio (Sullivan et al., 2015). In contrast to direct electron transport chain inhibition, under these circumstances, proliferation defects caused by these inhibitors can be restored by adding a mitochondrial uncoupler such as the ionophore carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) (Sullivan et al., 2015). While proliferation inhibition by oligomycin is reversed by FCCP, proliferation inhibition due to metformin is not reversed by FCCP, further supporting that metformin acts as an inhibitor of electron transport and not by causing mitochondrial hyperpolarization (Figure S2B).

To determine if the ability of pyruvate to restore proliferation is specific to metformin or generalizable to other complex I inhibitors, we treated cells in the presence or absence of pyruvate with the complex I inhibitors rotenone, phenformin, and piericidin A. While cells were generally resistant to these inhibitors in the presence of pyruvate, in the absence of pyruvate, cells exhibited increased sensitivity to the anti-proliferative effects of complex I inhibition (Figure 2B-C, S2C). Importantly, the presence or absence of pyruvate minimally alters proliferation rate in the absence of complex I inhibitors. Taken together, these data suggest that exogenous pyruvate does not alter the intrinsic proliferation rate of cells in culture, but rather, pyruvate decreases cellular dependence on complex I activity to support proliferation.

When complex I is inhibited, electrons from NADH cannot be transferred to molecular oxygen as an electron acceptor to form water and regenerate NAD+. However, in the presence of exogenous pyruvate, cells can regenerate NAD+ via conversion of pyruvate

to lactate (Figure 2D). Thus, when excess pyruvate is available in the environment, cells can utilize this orthogonal pathway to maintain the NAD+/NADH ratio in the absence of complex I activity. Consistent with this idea, addition of the lactate dehydrogenase (LDH) inhibitor GSK2837808A, which partially inhibits pyruvate to lactate conversion, restores metformin sensitivity to cells cultured in pyruvate containing media (Figure S2D). Additionally, we cultured cells in media with a range of lactate to pyruvate ratios. In the absence of metformin, cells grew at a similar rate regardless of the lactate to pyruvate ratios supplied. Conversely, in the presence of metformin, increasing the lactate to pyruvate ratio significantly decreased proliferation rate (Figure S2E). These data support the hypothesis that net pyruvate to lactate conversion is required to suppress metformin effects on proliferation.

To further test this hypothesis, we treated cells with metformin and other complex I inhibitors at doses that inhibited proliferation in pyruvate-free media but did not markedly affect proliferation in pyruvate containing media. Under these conditions, we measured mitochondrial oxygen consumption and found that exposure to metformin decreases mitochondrial oxygen consumption (Figure 2E-F). Importantly, while the presence of pyruvate suppresses the anti-proliferative effect of these drugs, it does not restore mitochondrial oxygen consumption (Figure 2E-F). In contrast, while treatment with metformin decreases NAD+/NADH ratio in the absence of pyruvate, supplementation with pyruvate restores the NAD+/NADH ratio under these conditions (Figure 2G-H). We repeated these experiments with other complex I inhibitors including rotenone and piercidin A, and obtained similar results (Fig 2I-L, S2F-G). These results are consistent with a model where complex I activity is dispensable in environmental contexts in which NAD+ can be regenerated through orthogonal pathways. Of note, proliferation rate in

such contexts is not correlated with oxygen consumption or, by extension, with mitochondrial ATP production.

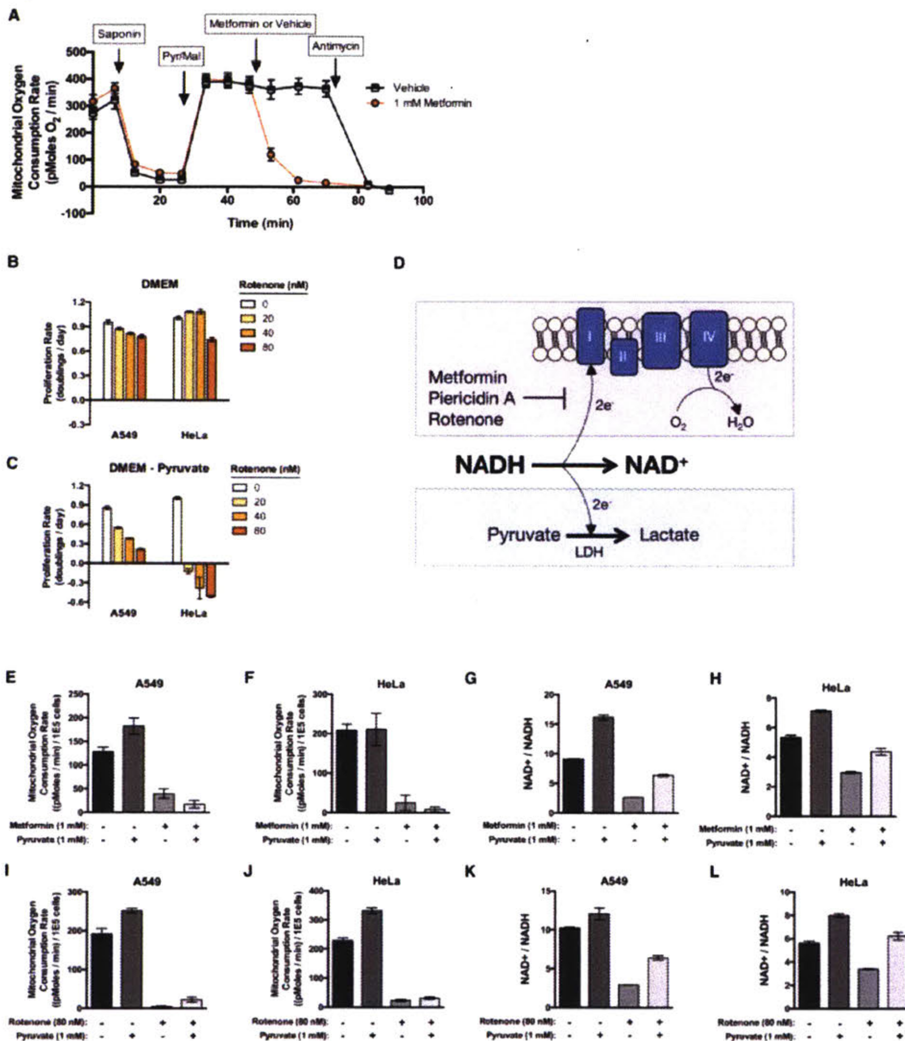


Figure 2. Complex I activity is dispensable when exogenous pyruvate is available to act as an electron acceptor.

(A) Mitochondrial oxygen consumption of A549 cells following permeabilization by saponin, addition of pyruvate and malate, addition of either metformin or vehicle, and addition of antimycin as indicated. Proliferation rates for A549 and HeLa cells treated with indicated dose of complex I inhibitor rotenone in DMEM (B) or DMEM without pyruvate (C). (D) Schematic illustrating how oxygen allows cells to regenerate NAD+ from NADH via complex I activity and the site of action of complex I inhibitors. Exogenous pyruvate provides an alternate way to regenerate NAD+ through lactate dehydrogenase (LDH) activity. Mitochondrial oxygen consumption rates of A549 (E) and HeLa (F) cells treated with the indicated concentrations of metformin and pyruvate. Intracellular NAD+/NADH ratios of A549 (G) and HeLa (H) cells treated with the indicated concentrations of metformin and pyruvate. Mitochondrial oxygen consumption rates of A549 (I) and HeLa (J) cells treated with the indicated concentrations of rotenone and pyruvate. Intracellular NAD+/NADH ratios of A549 (K) and HeLa (L) cells treated with the indicated concentrations of rotenone and pyruvate. Values denote mean ± SEM. *n*=5 (A, E-F, I-J) *n*=3 (B, G-H, K-L). See also Figure S2.

Modulating pathways that alter NAD+/NADH homeostasis changes complex I dependency

The observation that pyruvate can maintain the NAD+/NADH ratio despite complex I inhibition demonstrates that orthogonal pathways can influence the NAD+/NADH ratio. Further, the activity of orthogonal NAD+ modulating pathways should alter complex I dependency and, thus, modify metformin sensitivity in a titratable way. Consistent with this notion, we show that increasing pyruvate to lactate conversion by titrating the concentration of exogenous pyruvate in the environment decreases metformin sensitivity in a dose dependent manner (Figure 3A). To generalize this finding, we tested if changing the activity of other orthogonal NAD+/NADH modulating pathways could also alter complex I dependence and metformin sensitivity. Poly(ADP-ribose) polymerases (PARPs) are a family of proteins that use NAD+ to transfer ADP-ribose groups onto proteins (Figure 3B), and PARP activity is reported to be a major consumption pathway for NAD+ (Pillai et al., 2005). Blocking PARP activity increases intracellular NAD+ (Bai et al., 2011; Pirinen et al., 2014); thus, we tested if altering PARP activity could change complex I dependence. A549 and HeLa cells were treated with increasing amounts of the PARP inhibitor 3-aminobenzamide (Fang et al., 2014) in the presence of metformin. Metformin was added at approximately the IC_{50} dose for each cell line in DMEM in the absence of pyruvate. Since blocking NAD+ consumption will decrease the need for NAD+ regeneration, a PARP inhibitor is expected to decrease complex I dependency, and as predicted, addition of 3-aminobenzamide decreased metformin sensitivity (Figure 3C).

Another input into cellular NAD+/NADH homeostasis is NAD+ synthesis. Recent data have shown that the NAD+ salvage pathway is frequently upregulated in cancer and providing nicotinamide mononucleotide (NMN) increases NAD+ biosynthesis (Canto et al., 2012; Gomes et al., 2013; Wang et al., 2011) (Figure 3D). This predicts that NMN treatment may decrease complex I dependency, and indeed, we found that treating cells with NMN also decreased metformin sensitivity (Figure 3E).

Next, we reasoned that other redox reaction pairs that use NAD+/NADH as cofactors should also modulate cellular NAD+/NADH balance. When cells are exposed to duroquinone, the intracellular reductase NQO1 converts duroquinone to reduced durohydroquinone and, in the process, consumes NADH to produce NAD+ (Merker et al., 2006) (Figure 3F). Thus, providing cells with duroquinone allows for an orthogonal source of NAD+ regeneration and is predicted to decrease complex I dependency. Consistent with this, we found that addition of duroquinone dose-dependently decreases metformin sensitivity (Figure 3G).

To verify that these orthogonal pathways modulate NAD+/NADH in the expected manner, NAD+/NADH was measured after the respective treatments, and indeed, all treatments restored the NAD+/NADH ratio as predicted (Figure S3A). Next, we investigated whether the orthogonal NAD+/NADH modulators affected NAD+ and NADH levels as would be predicted by their mechanism of action. We found that, as expected, 3-aminobenzamide, NMN, and duroquinone all raised NAD+ levels (Figure S3B). Additionally, in HeLa cells, consistent with the expected effects of modulating enzymes that influence the production or consumption of NAD+ but not net cycling NADH back to NAD+, neither 3-aminobenzamide nor NMN significantly changed NADH levels, while

duroquinone, which can regenerate NAD+ from NADH, decreased NADH levels (Figure S3C). Similar results were observed in A549 cells, although there were small decreases in NADH in NMN treated cells (Figure S3C). These data are consistent with NMN having an outsized effect on NAD+/NADH ratio relative to its effect on proliferation. How NMN causes decreased NADH levels in A549 cells is unknown, however it suggests that other feedback regulation of NAD+ synthesis may exist in cells.

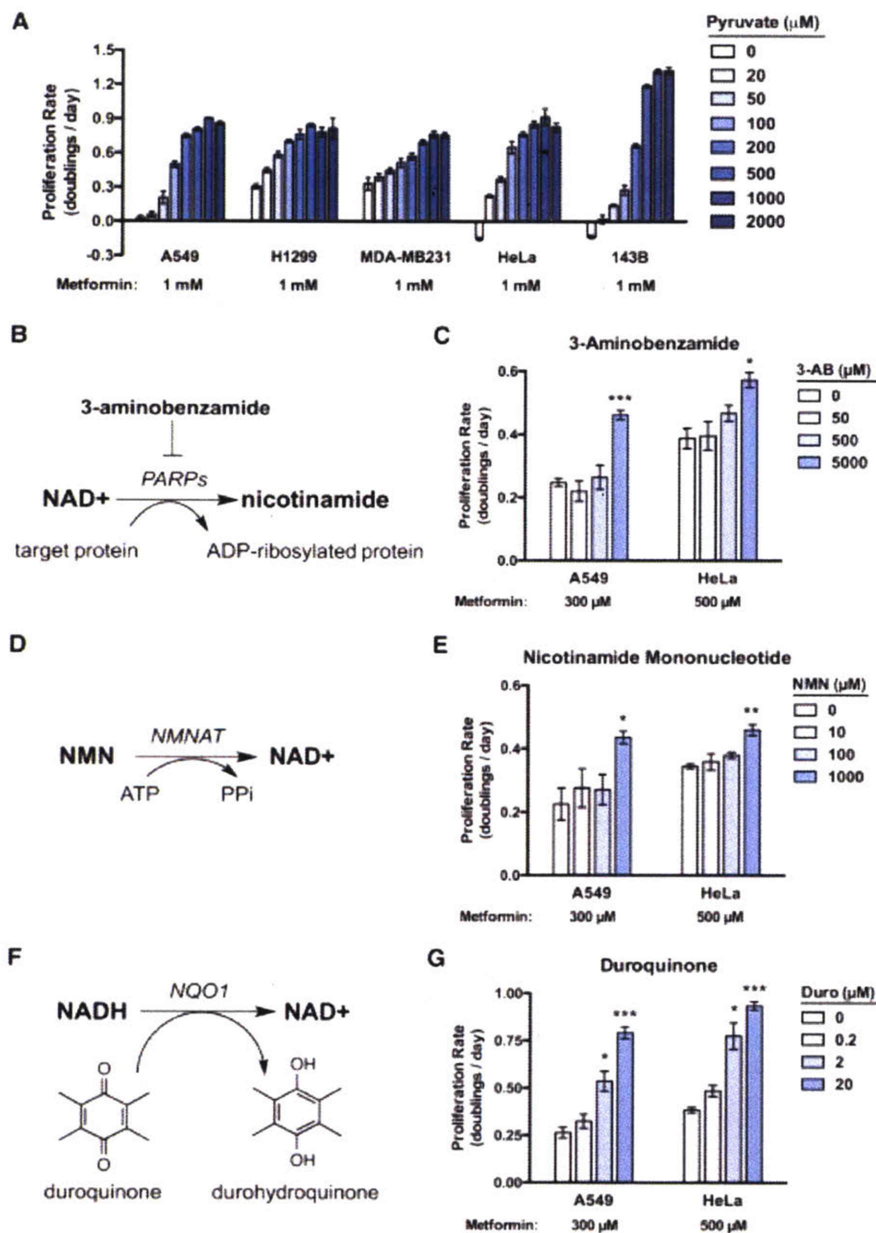


Figure 3. Altering cellular NAD+/NADH ratio titrates dependency on complex I activity. (A) Proliferation rates for A549, H1299, MDA-MB231, HeLa, and 143B cells treated with 1 mM metformin and supplemented with the indicated amounts of pyruvate. (B) Schematic illustrating how poly(ADP-ribose) polymerases (PARPs) consume NAD+. (C) Proliferation rates of A549 and HeLa cells treated with metformin at the indicated concentration in media supplemented with the indicated concentration PARP inhibitor 3-aminobenzamide (3-AB). (D) Schematic illustrating how nicotinamide mononucleotide (NMN) supplementation can increase NAD+ synthesis by nicotinamide mononucleotide adenylyltransferase (NMNAT) activity. (E) Proliferation rates of metformin treated A549 and HeLa cells supplemented with the indicated concentration of NMN. (F) Schematic illustrating how duroquinone can oxidize NADH to yield NAD+ and durohydroquinone by the activity of NAD(P)H dehydrogenase, quinone 1 (NQO1). (G) Proliferation rates of metformin treated A549 and HeLa cells supplemented with the indicated concentration of duroquinone. Values denote mean ± SEM. *n*=3. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. See also Figure S3.

Changes in NAD+/NADH ratio correlate with the anti-proliferative effects of metformin

Our data indicate that while complex I activity is dispensable in the presence of pyruvate, complex I is important for maintenance of cellular NAD+/NADH ratio in the absence of pyruvate. We treated cells with a large range of metformin doses and found that in pyruvate-free media, decreasing complex I activity by increasing metformin concentration decreases both the NAD+/NADH ratio (Figure 4A) and proliferation rate (Figure 4B) in a dose dependent manner. Plotting the effects of metformin on NAD+/NADH ratio and proliferation rate reveals a striking correlation between the two phenotypes (Figure 4C). In metformin-treated A549 cells, an NAD+/NADH ratio less than three is associated with a dramatic decrease in cell proliferation, whereas an NAD+/NADH ratio greater than six is associated with maximal rates of cell proliferation. Interestingly, this relationship between the NAD+/NADH ratio and proliferation rate is also observed in A549 cells treated with other complex I inhibitors either in the presence or absence of pyruvate. In HeLa cells, although the absolute NAD+/NADH ratio that supports proliferation differs from that of A549 cells, a similar correlation between NAD+/NADH ratio and proliferation is observed that is also generalizable to other complex I inhibitors.

We next investigated the effect of metformin on the ATP/AMP ratio as this has also been proposed to be a mechanism by which metformin affects cell proliferation. Again, we treated cells with a large range of metformin doses and found that in pyruvate-free media, decreasing complex I activity also decreases the ATP/AMP ratio (Figure S4A). Interestingly, this decrease in the ATP/AMP ratio is also reversed by pyruvate (Figure

S4B). Previous work has shown that maintaining NAD+/NADH balance is required for proliferation in order to support *de novo* aspartate biosynthesis (Birsoy et al., 2015; Sullivan et al., 2015); however, an abundance of literature implicates activation of AMP-activated protein kinase (AMPK) from a decreased ATP/AMP ratio as being a key modulator of metformin's anti-proliferative effect (Foretz et al., 2014; Hardie, 2015; Li et al., 2015; Pernicova and Korbonits, 2014). Thus, we sought to distinguish whether the NAD+/NADH correlation with proliferation inhibition under metformin treatment was downstream of effects on aspartate or the ATP/AMP ratio.

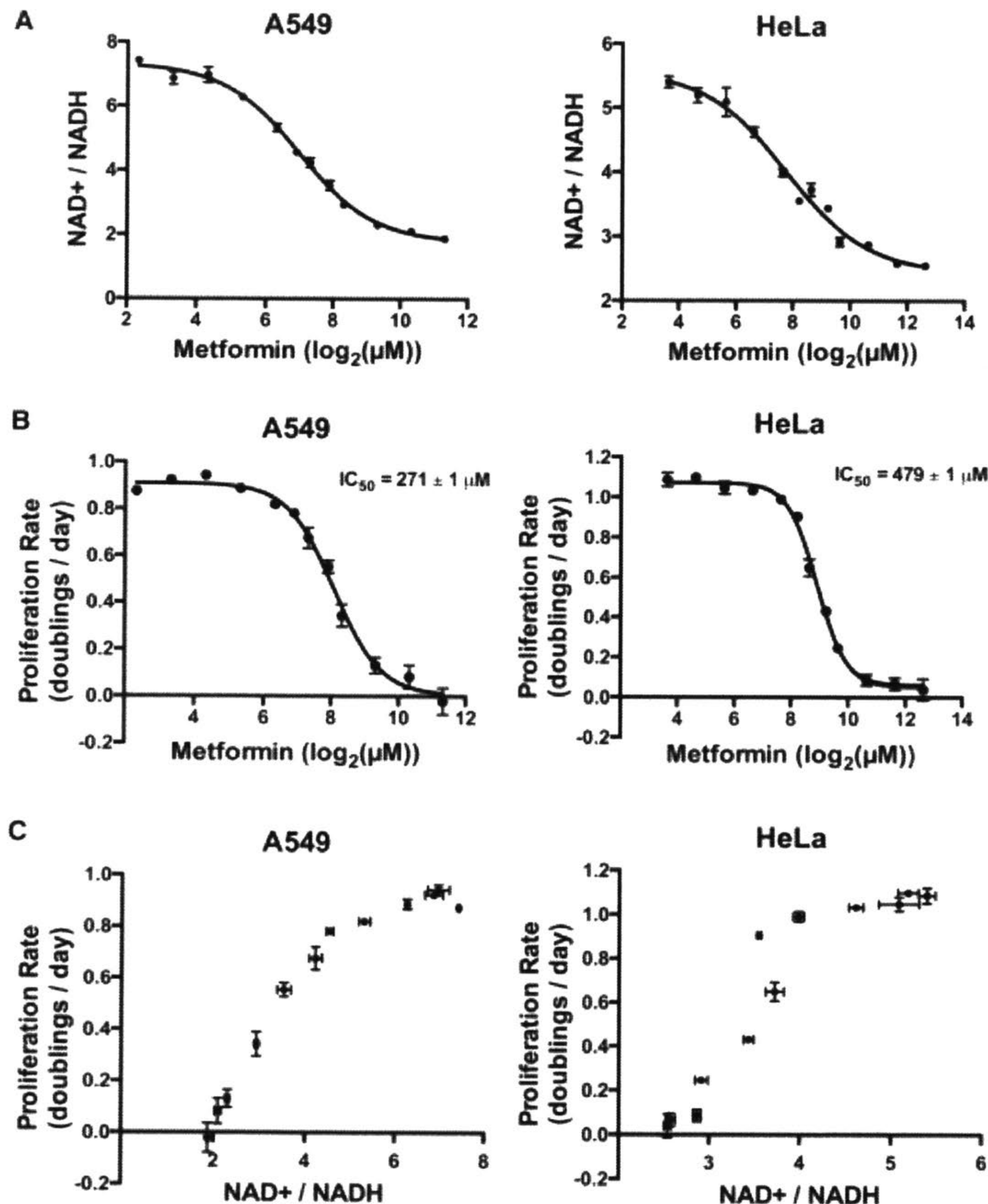


Figure 4. NAD+/NADH ratio tracks with proliferation rate in metformin treated cells. Intracellular NAD+/NADH ratios (A) and proliferation rates (B) were measured after treatment with metformin at the indicated doses in A549 cells and HeLa cells. Proliferation rates and NAD+/NADH ratios were plotted independent of metformin concentrations to determine the relationship between NAD+/NADH ratio and proliferation rate in A549 cells and HeLa cells, respectively (C). Values denote mean ± SEM. n=*3*. See also Figure S4.

Treating cells with a titration of metformin showed that metformin dose-dependently decreases intracellular aspartate levels (Figure 5A). The decrease in aspartate levels strongly correlates with metformin's effect on cellular NAD+/NADH ratio (Figure 5B). Addition of pyruvate, which increases cellular NAD+/NADH ratio, restores cellular aspartate levels, and addition of aspartate alone is sufficient to reverse the anti-proliferative effects of metformin (Figure 5C and D). An important anabolic role of aspartate is to support synthesis of purines, including the conversion of IMP to AMP. Consistent with metformin causing nucleotide insufficiency downstream of decreased aspartate levels, we found that in the presence of metformin the IMP/AMP ratio is dramatically elevated and that this elevation is reversed by addition of aspartate (Figure 5E).

To show that the ability of aspartate to rescue metformin toxicity is downstream of the effects of metformin on NAD+/NADH, we verified that addition of aspartate does not change the cellular NAD+/NADH ratio (Figure S5A). Interestingly, we observed that ATP/AMP levels are also partially restored by aspartate (Figure S5B). However, comparing the proliferation rate with ATP/AMP ratio we observe that the degree to which aspartate restores proliferation rate is outsized relative to its effect on ATP/AMP ratio, suggesting that the effect of metformin on aspartate via changing NAD+/NADH is at least, in part, orthogonal to its effect on ATP/AMP ratio (Figure S5C). Indeed, given that there are no known pathways to generate ATP from aspartate in the absence of complex I activity, it is mechanistically unclear how aspartate can directly alter the ATP/AMP ratio, suggesting that perhaps ATP/AMP ratio changes are downstream of changes in proliferation rate, rather than a direct effect of changes in NAD+/NADH.

We next explored the relationship between the anti-proliferative effects of metformin, and AMPK signaling and mechanistic target of rapamycin complex I (mTORC1) signaling. We studied a panel of cell lines including A549 cells that are null for the upstream AMPK activator liver kinase B1 (LKB1). In H1299 and MDA-MB231 with intact LKB1, an increase in phosphorylation of the AMPK substrate acetyl-CoA carboxylase (ACC) upon metformin treatment is not reversed with pyruvate or aspartate supplementation (Figure S5D), further suggesting that changes in AMPK signaling and ATP/AMP ratio cannot fully explain the growth inhibitory effects of metformin (Griss et al., 2015). We did not observe any consistent trends in AMPK or mTORC1 signaling across cell lines with respect to metformin treatment, and pyruvate or aspartate restoration of proliferation in the presence of metformin (Figure S5D). These data suggest that effects on these signaling pathways cannot account for all of the anti-proliferative effects of metformin in these cells.

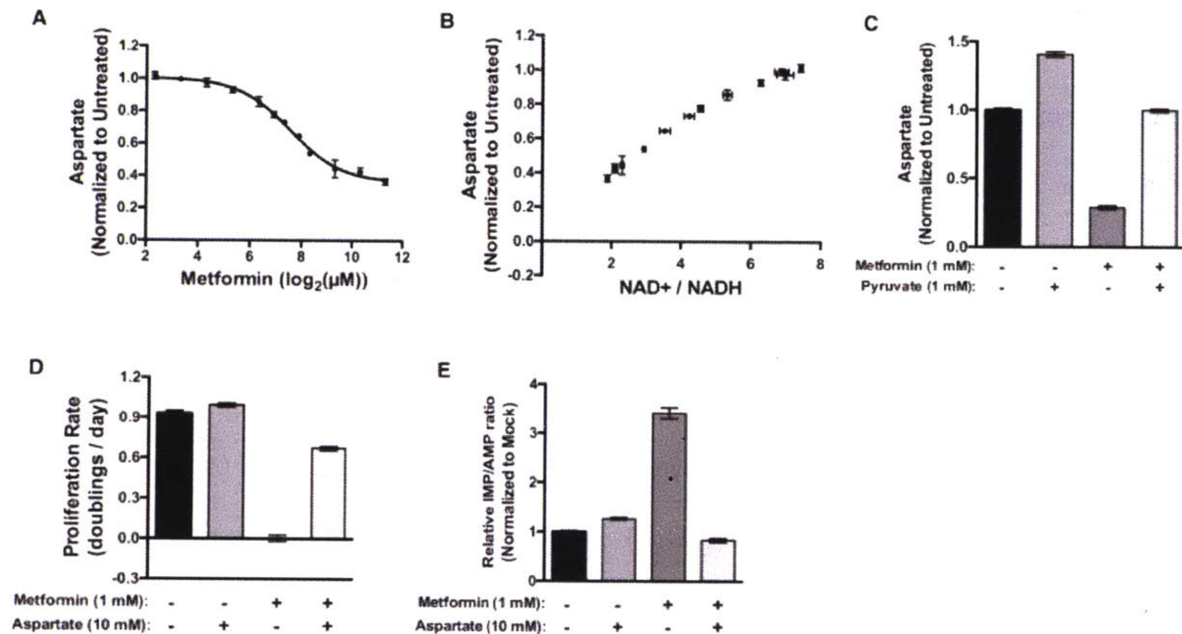


Figure 5. The antiproliferative effects of metformin are suppressed by exogenous aspartate.
(A) Intracellular aspartate levels were measured after treatment with metformin at the indicated doses in A549 cells. (B) Aspartate levels and NAD+/NADH ratios were plotted independent of metformin concentrations to determine the relationship between NAD+/NADH ratio and aspartate levels in A549 cells. (C) Intracellular aspartate levels in A549 cells treated with the indicated doses of metformin and pyruvate. (D) Proliferation rates of A549 treated with the indicated doses of metformin and exogenous aspartate. (E) Normalized IMP/AMP ratio in A549 treated with the indicated doses of metformin and exogenous aspartate. Values denote mean ± SEM. n=*3*. See also Figure S5.

Metformin treatment decreases NAD+ and aspartate levels in tumors and slows tumor growth

Finally, we sought to explore whether our findings regarding the anti-proliferative effects of metformin were relevant *in vivo*. For these studies A549 cells were xenografted into nude mice. When the tumors reached 50 mm^3 the mice were randomized into three groups and treated with once a day oral gavage with vehicle, metformin at 500 mg/kg, or metformin at 1500 mg/kg. Increasing dosages of metformin dose-dependently inhibited tumor growth (Figure 6A). Measurement of plasma metformin concentration confirmed that the levels achieved with these doses are therapeutically relevant and expected to be tolerable in humans (Dell'Aglio et al., 2009) (Figure 6B). Further, we showed that escalating doses of metformin resulted in an increase in the tumor concentration of metformin (Figure 6C). NADH levels were too low to detect in tumors; however, NAD+ levels dose-dependently decreased in tumors with increasing doses of metformin (Figure 6D). We also assessed whether intratumoral ATP/AMP ratios were effected by metformin and found that while metformin appeared to decrease the ATP/AMP ratio in some tumors, the effect was neither statistically significant nor dose dependent. (Figure S6A). Finally, intratumoral aspartate levels decreased with increasing doses of metformin (Figure 6E). Taken together these data suggest that there is a correlation between metformin treatment at therapeutically relevant doses and tumor growth inhibition. In addition, the degree of tumor growth inhibition correlates with both intratumoral NAD+ and aspartate levels.

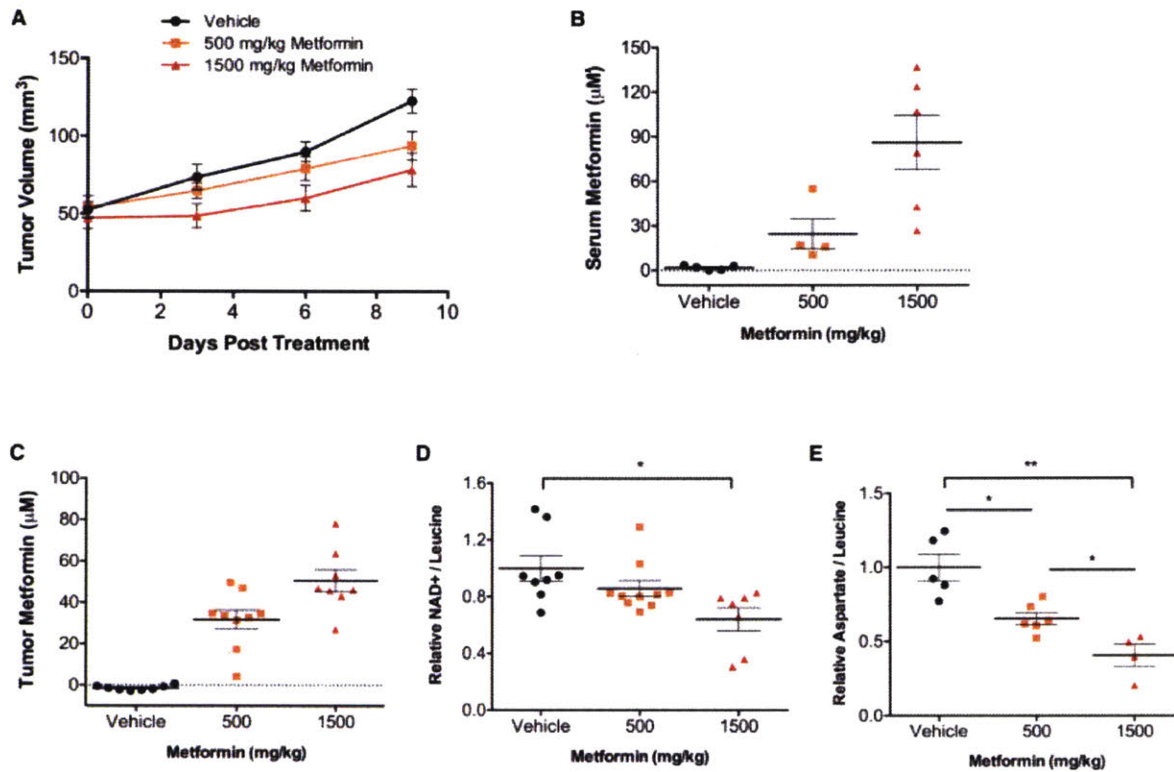


Figure 6. Metformin slows tumor growth and decreases intratumoral NAD+ and aspartate levels. (A) A549 xenografts in nude mice were treated with vehicle or the indicated doses of metformin, once a day by oral gavage. (B) Serum and (C) tumor metformin concentrations measured in material from mice treated for 10 days at the indicated dose of metformin, with tissue harvested 2 hours after the last dose. Relative intratumoral (D) NAD+ and (E) aspartate levels after 10 days of vehicle or metformin treatment at the indicated dose. Relative NAD+ and aspartate levels are shown as NAD+ and aspartate total ion counts (TIC) normalized to leucine TIC. Values denote mean ± SEM. *$p < 0.05$, **$p < 0.01$. Related to Figure S6.

Discussion

Recent data suggests that a primary function of respiration in support of proliferation is to provide electron acceptors and regenerate oxidized cofactors (Birsoy et al., 2015; Sullivan et al., 2015). Extending this idea, we show that the anti-proliferative effect of metformin tracks with its effect on cellular NAD+/NADH ratio. Further, while there are correlations between ATP/AMP ratio and the cellular NAD+/NADH ratio, we find the downstream anti-proliferative effects of metformin track more consistently with its effect on NAD+/NADH ratio and intracellular aspartate. Importantly, we find that altering the metabolic environment in a way that influences NAD+/NADH homeostasis is sufficient to change cellular dependency on complex I and, by extension, alter metformin sensitivity. In contexts where cells are allowed to regenerate NAD+ by alternative pathways, complex I dependency is low. However, in other environmental contexts, including those found *in vivo* with A549 xenografts, complex I activity appears to be important for maintaining cellular NAD+/NADH homeostasis. Treatments with metformin at therapeutically relevant doses are sufficient to alter intratumoral NAD+ levels and intratumoral aspartate levels. While our data does not rule out the possibility of other whole body metabolic anti-tumor effects of metformin, our findings show that the cell autonomous anti-proliferative effects of metformin are, at least in part, mediated by its effect on NAD+/NADH homeostasis and aspartate levels and further support that complex I itself can be a cancer target.

The sensitivity of cancer cells to a drug is typically considered to be a cell intrinsic property, with efficacy matched directly to ability to inhibit a protein target in a specific

genetic context. In many cases, the degree to which the direct drug target is inhibited is an effective proxy and predictor for downstream effects on proliferation. In contrast to this paradigm, we find that for metformin and other complex I inhibitors, the environmental context can decouple the biological effect of decreased proliferation from inhibition of the drug target. We show that inhibition of complex I activity, as measured by oxygen consumption, is a poor predictor of the anti-proliferative effects of metformin. Indeed, while media supplementation of pyruvate does not change metformin action as a complex I inhibitor, it is sufficient to alter the effective anti-proliferative dose of metformin by approximately two orders of magnitude. While variable sensitivity to biguanides has been attributed to specific genetic events (Buzzai et al., 2007; Cuyas et al., 2015; Shackelford et al., 2013) or to factors that alter drug uptake (Chen et al., 2015; Chen et al., 2010; Goswami et al., 2014; Madera et al., 2015; Nies et al., 2011; Shu et al., 2007), our data suggest that the metabolic environment is an additional determinant of drug sensitivity independent of cell intrinsic factors.

These findings indicate that considering environmental context is critical for *in vitro* screening of drugs targeting metabolism. Indeed, had metformin not been identified to promote cancer survival in patients taking this drug for diabetes, *in vitro* studies in DMEM would have concluded that clinically unachievable doses are required for metformin to have an effect on cancer cells. These results highlight the importance of considering environmental context for determining the potential efficacy of newly developed drugs.

A major barrier to extrapolating preclinical data on the anti-tumorigenic effects of metformin has been the large inconsistency in dosing between tissue culture and tumor

models. Showing that metabolic environment is a crucial determinant of complex I dependency begins to explain this discrepancy. Indeed, cellular sensitivity to metformin in DMEM without pyruvate is within a few fold of what has been observed to have clinical benefit in patients. Additionally, due to dose limiting toxicity in animals, it is impossible to achieve high enough doses to determine the *in vivo* tumor autonomous IC50 and because other environmental factors also influence complex I sensitivity, comparing the effects of metformin in DMEM without pyruvate to the effects in tumors may not be the best comparison.

Standard *in vitro* tissue culture is a poor mimic for the metabolic environment of tumors (Davidson et al., 2016; Mayers and Vander Heiden, 2015). Given the dramatic differences in oxygen and nutrient availability in culture versus tumors, electron acceptors are likely to be more limited *in vivo*, thus increasing dependence on pathways that regenerate NAD+ (Sullivan et al., 2015). One notable example regarding metformin and nutrient availability involves recent work showing that serine limitation increases *in vivo* metformin sensitivity (Gravel et al., 2014). Serine levels *in vivo* are much lower than what is found *in vitro* and *de novo* serine biosynthesis requires multiple NAD+ consuming redox reactions, likely impinging on cellular NAD+/NADH homeostasis and potentially partially explaining its interaction with metformin sensitivity. Additionally, given other differences in waste disposal and carbon dioxide levels, there are other environmental influences on the cellular NAD+/NADH ratio that are not accounted for *in vitro*. A better understanding of NAD+/NADH homeostasis in tumors will be critical for identifying contexts in which metformin therapy may be most impactful and for developing combination therapies that increase the efficacy of metformin in cancer. Developing culture systems that better mimic the *in vivo* metabolic environment could

also be important for identifying cancer targets that might otherwise be missed. Regardless, our findings highlight that consideration of the metabolic environment is critical for the development of cancer therapies that target metabolism.

Experimental Procedures

Cell Culture

A549, H1299, MDA-MB231, HeLa, and 143B cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Corning) with or without pyruvate or in Roswell Park Memorial Institute 1640 Media (RPMI) (Corning) as indicated. In all cases media was supplemented with 10% dialyzed fetal bovine serum (Sigma) and penicillin-streptomycin (Corning). Cells were cultured at 37°C with 5% CO_2.

Proliferation Rates

Cell were plated in replicate in 6 well dishes (Corning), with an initial seeding density of 20,000 cells per well for A549, H1299, HeLa, and 143B and an initial seeding density of 40,000 cells for MDA-MB231. After seeding, cells were allowed to settle overnight and one 6 well dish was counted to determine starting cell number prior to treatment. For the remaining dishes, cells were washed twice in phosphate buffered saline (PBS), and 4 mL of media containing the indicated treatment was added. For pyruvate titration experiments, media was changed daily to maintain a constant pyruvate concentration.

Cell counts were determined four days after initial treatment using a Cellometer Auto T4 Plus Cell Counter (Nexcelom Bioscience) (Sullivan et al., 2015). Proliferation rate was calculated based on the following formula:

Proliferation Rate (Doublings per day) = (Log_2(Final cell count (day 5)/ Initial cell count (day 1))/ 4 (days)

Mitochondrial Oxygen Consumption

Oxygen consumption rates (OCR) were measured using a Seahorse Bioscience Extracellular Flux Analyzer (XF24). Cells were plated in Seahorse Bioscience 24-well plates at a seeding density of 50,000 cells per well in 100 μl of DMEM without pyruvate. After a 1 hour incubation, an additional 500 μl of DMEM was added with or without metformin and/or pyruvate before overnight incubation. The next day, cells were washed twice in DMEM without phenol red containing 0.5% dialyzed FBS at pH 7.4 containing treatments, and were then incubated in 500 μl of the same media with the corresponding treatments. OCR measurements were determined 24 hours after metformin and/or pyruvate treatment. Mitochondrial OCR was calculated by taking the total OCR and subtracting the residual OCR following addition of high dose rotenone and antimycin (2 μM each). Following the OCR measurements, cells from each well were counted using a Cellometer Auto T4 Plus Cell Counter (Nexcelom Bioscience) and used to normalized OCR per 100,000 cells. For determining complex I and mGPD dependent oxygen consumption, cells were seeded at 100,000 cells per well, incubated overnight, and prior to measurement media was changed to mitochondrial assay buffer (70 mM sucrose, 220 mM mannitol, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 2 mM HEPES, 1 mM EGTA, 0.2 % (w/v) fatty acid free BSA, pH 7.2) supplemented with 10 mM ADP. The first

injection contained saponin (50 μg/mL final) to permeabilize cells and OCR was monitored until intracellular metabolites diffused out and OCR stabilized. Respiration substrates of complex I (10 mM pyruvic acid and 2 mM malic acid) or mGPD (10 mM glycerol-3-phosphate) were then injected until respiration was induced and stabilized followed by injection of metformin (1 mM final) or vehicle. Mitochondrial OCR was determined by subtracting OCR after addition of 2 μM antimycin from the experimental OCR measurements.

Measurement of NAD+/NADH

Cells were plated in 6-well dishes at 20,000 cells per well, allowed to adhere overnight, washed twice in PBS, and cultured in 4 ml of the indicated treatment media for 24 hours. Cells were then washed three times in ice cold PBS and extracted in 100 μL of ice cold lysis buffer (1% Dodecyltrimethylammonium bromide (DTAB) in 0.2 N NaOH diluted 1:1 with PBS), and immediately frozen at -80°C. NAD+/NADH ratio was measured using a modified version of instructions provided with the NAD/NADH Glo Assay Kit (Promega). For NADH measurement, 20 μL of the freshly thawed lysate was transferred to PCR tubes and incubated at 75°C for 30 minutes which allows for base-mediated degradation of NAD+. For NAD+ measurement, 20 μL of the freshly thawed lysate was transferred to PCR tubes containing 20 μL lysis buffer and 20 μL 0.4 N HCl. NAD+ samples were incubated at 60°C for 15 minutes, where acidic conditions result in selective degradation of NADH. Following respective incubations, samples were allowed to equilibrate to room temperature for 8 minutes and then quenched with the respective neutralizing solution with 20 μL 0.25 M Tris in 0.2 N HCl (NADH) or 20 μL 0.5 M Tris base (NAD+). Following

sample prep, manufacturer instructions were used for enzyme-linked luminescence based NAD+ and NADH measurement (Sullivan et al., 2015).

Aspartate measurement by GCMS

Cells were seeded in 6 well dishes and incubated overnight to allow the cells to adhere. Following incubation cells were washed twice in PBS and media containing the indicated treatments was added. After overnight treatment, aspartate was extracted using 80% methanol in water with 1 µg norvaline standard added per sample. The extracted content was dried under nitrogen gas, derivatized, and measured as detailed in (Lewis et al., 2014).

Polar Metabolite Quantification by LCMS

Measurements were as described previously (Sullivan et al., 2015). Briefly, metabolites were first extracted using ice cold 80% methanol and chloroform. Relative metabolites abundances were measured using 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). To quantify metabolite abundance from resulting chromatogram XCalibur QuanBroswer 2.2 (Thermo Fisher Scientific) was used in conjunction with in-house retention time library of chemical standards.

Immunoblotting

Cells were treated with metformin, pyruvate, and aspartate for 24 hrs, washed with cold PBS, and lysed with cold RIPA buffer containing cOmplete Mini protease inhibitors (Roche) and phosSTOP phosphatase inibitors (Roche). Protein concentration was quantified by BCA Protein Assay (Pierce) using BSA as a standard. Samples were resolved by SDS-PAGE using standard techniques, and protein was detected with the following antibodies: 4EBP1 (total and S65), ACC (total and pS79), Raptor (pS792) and S6K (total and S6K-pT389) from Cell Signalling Technologies, and Raptor (total) from Millipore.

Xenografts

Two million A549 cells were injected into flanks of nu/nu mice (088; Charles River Laboratories). Tumor volume was measured by caliper in two dimensions and volumes were estimated using the equation $V = (\pi/6)(L*W^2)$. The tumors were permitted to grow to $50mm^3$, after which the animals were randomly assigned to a treatment or vehicle group. Vehicle, 500 mg/kg, or 1500 mg/kg metformin were dosed via oral gavage daily for 10 days. The vehicle consisted of 0.1% (w/v) Tween 80 and 0.5% (w/v) methylcellulose. The tumors and plasma samples were collected 2 hours after the final dosage on day 10, and metformin and metabolite concentrations were quantified by LCMS.

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

experiments were validated by independent repetitions. Statistical significance was determined using an unpaired two-tailed t test with Welch's correction where significance was determined as $p \leq 0.05$.

Supplemental Figures

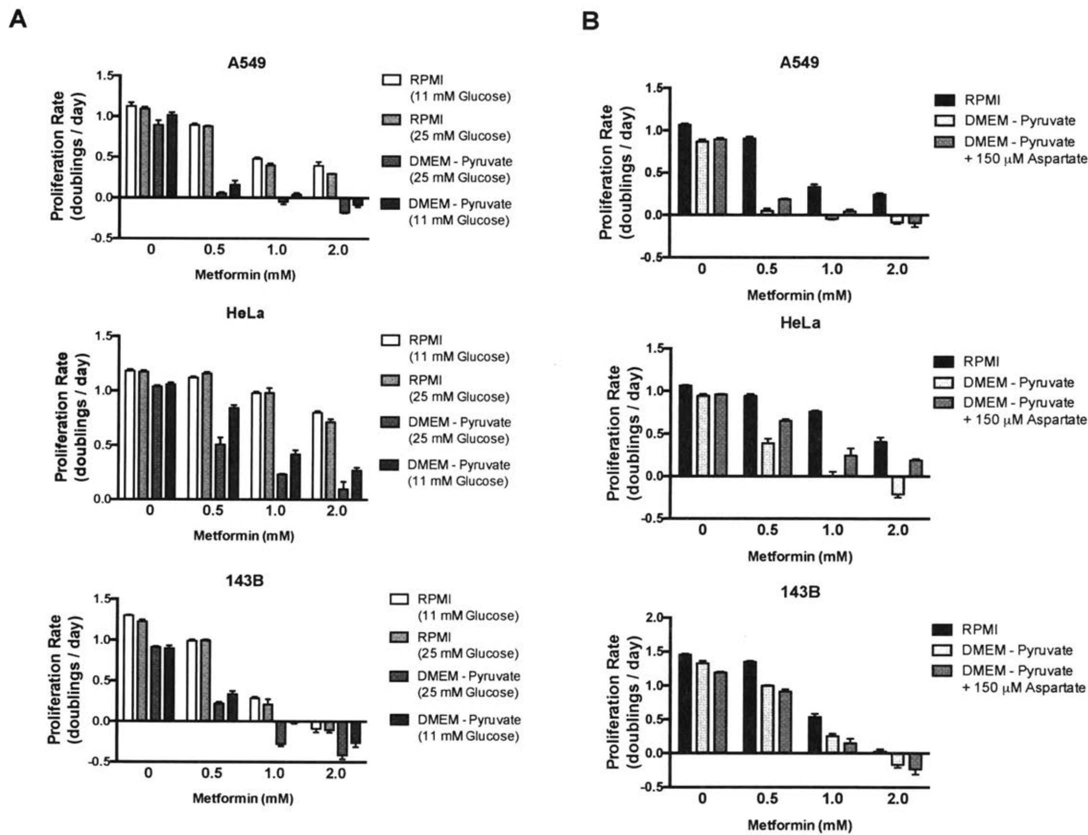


Figure S1. The antiproliferative effect of metformin is influenced by media composition, related to Figure 1. Proliferation rates for A549, HeLa, and 143B cells in media treated with the indicated concentrations of metformin. Cells were cultured in DMEM without pyruvate or RPMI 1640 with the indicated concentration of glucose (A) or with addition of RPMI levels of aspartate (150μM) (B). Values denote mean ± SEM. *n*=3.

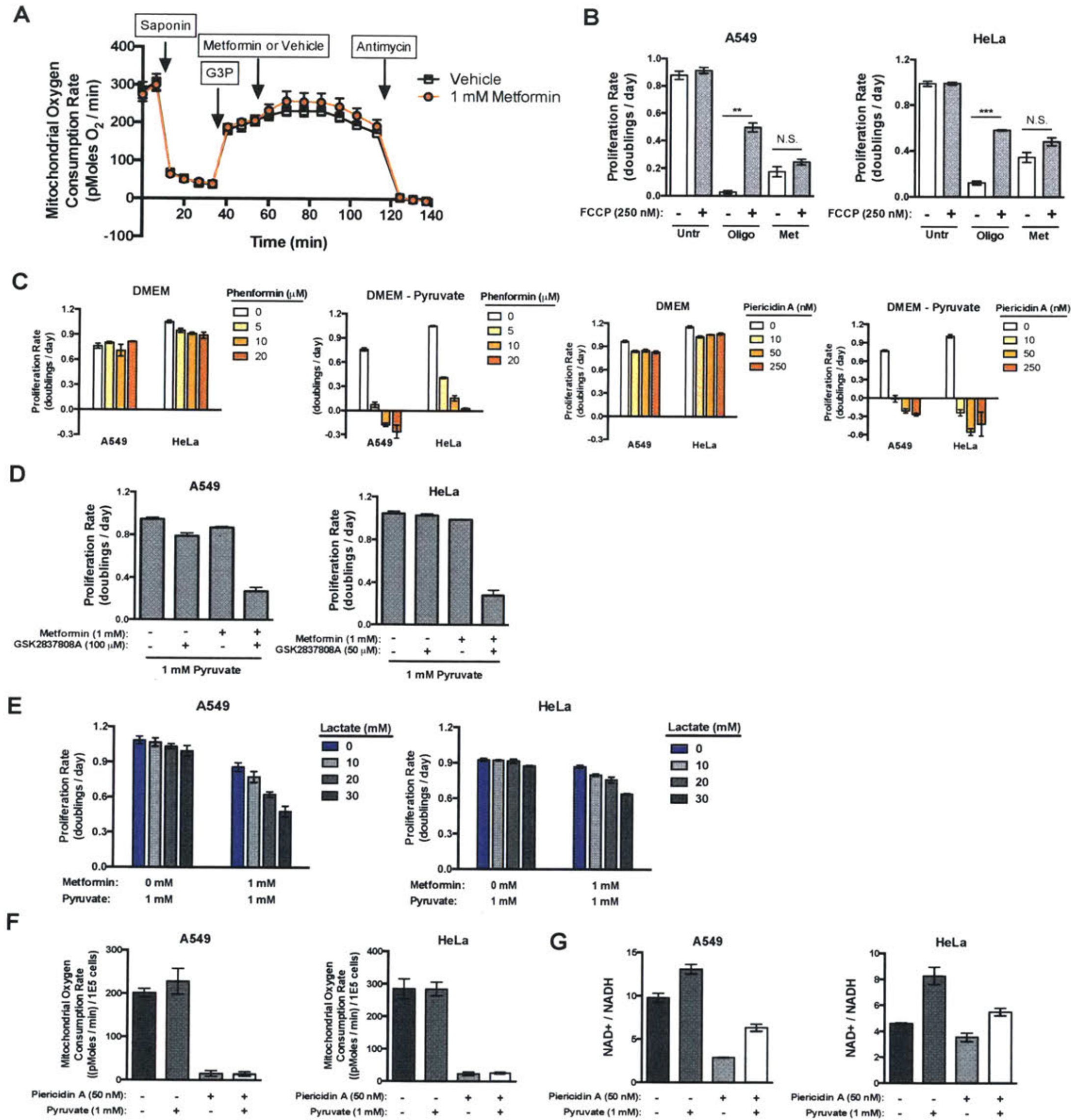
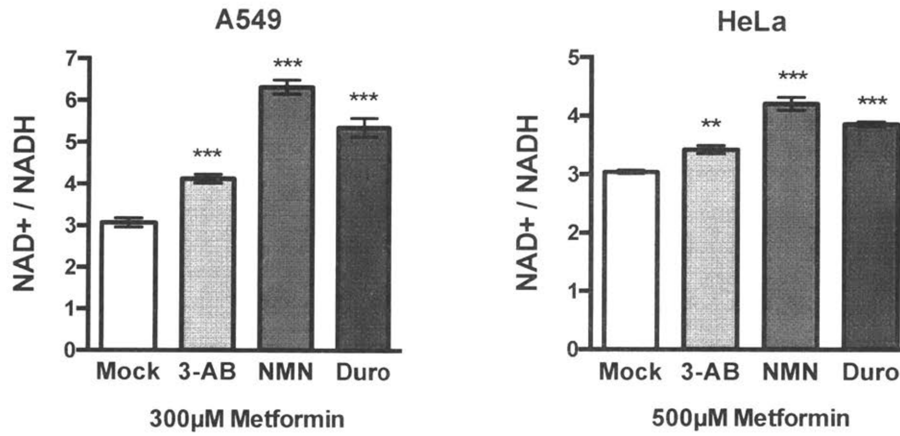


Figure S2. Conversion of pyruvate to lactate is required to restore proliferation upon complex I inhibition, related to Figure 2.(A) Mitochondrial oxygen consumption of A549 cells following permeabilization by saponin, addition of glycerol-3-phosphate (G3P), addition of either metformin or vehicle and addition of antimycin as indicated. (B) Proliferation rates for A549 and HeLa cells treated with oligomycin or metformin in the presence or absence of FCCP. (C) Proliferation rates for A549 and HeLA cells in DMEM or DMEM without pyruvate treated with the indicated doses of the complex I inhibitors phenformin or piericidin A. (D) Proliferation rates for A549 and HeLa cells cultured in DMEM with pyruvate treated as indicated with metformin and the LDH inhibitor GSK2837808A. (E) Proliferation rates for A549 and HeLa cells with and without metformin in the presence of the indicated amounts of pyruvate and lactate in the media. (F) Mitochondrial oxygen consumption rates for A549 and HeLa cells treated with the indicated concentrations of piericidin A and pyruvate. (G) Intracellular NAD+/NADH ratios of A549 and HeLa cells treated with the indicated concentrations of piericidin A and pyruvate. Values denote mean ± SEM. *n*=5 (A,F), *n*=3 (B-E, G). *p < 0.05, **p < 0.01.

A



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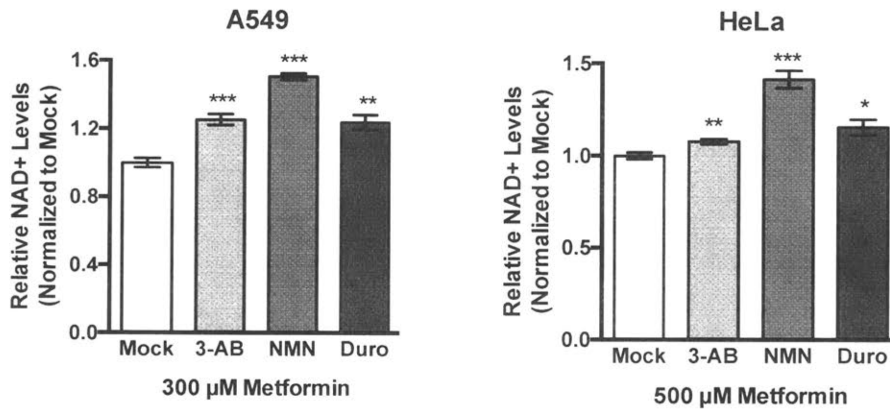

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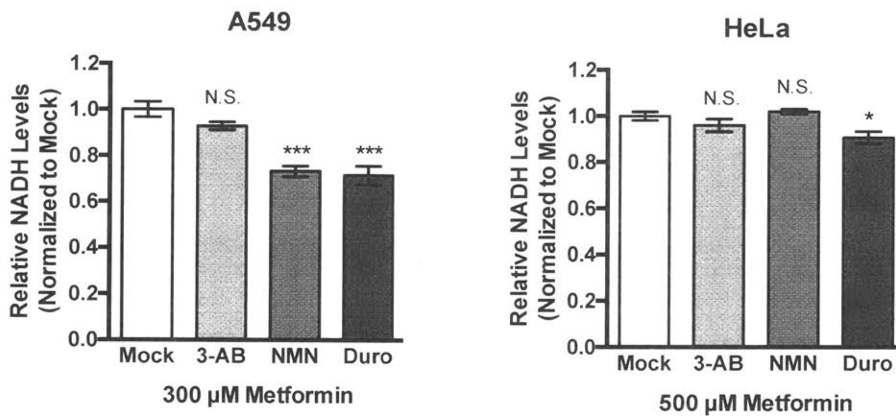
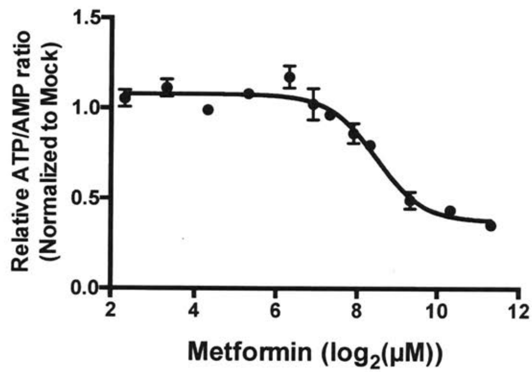


Figure S3. Treatment with compounds that alter NAD+/NADH metabolism result in altered NAD+/NADH ratio, related to Figure 3.(A) Intracellular NAD+/NADH ratios of A549 and HeLa cells treated with 3- aminobenzamide (3-AB), nicotinamide mononucleotide (NMN), and duroquinone (duro). Normalized intracellular levels of NAD+ (B) and NADH (C) in A549 and HeLa cells treated with 3-AB, NMN, and duro. Values denote mean ± SEM. *n*=3. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

A



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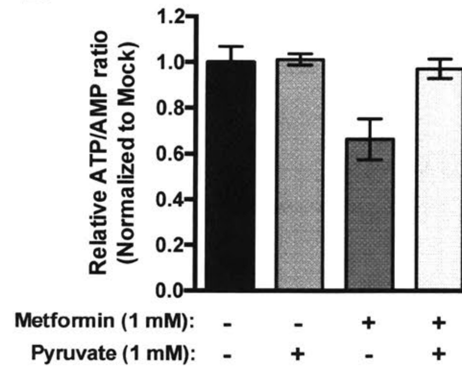

Figure S4. ATP/AMP ratio is affected by metformin treatment, related to Figure 4.(A) Intracellular ATP/AMP ratios were measured after treatment with metformin at the indicated doses in A549 cells. (B) Intracellular ATP/AMP ratios were measured in A549 cells with the indicated doses of metformin and pyruvate. Values denote mean ± SEM. n=3.

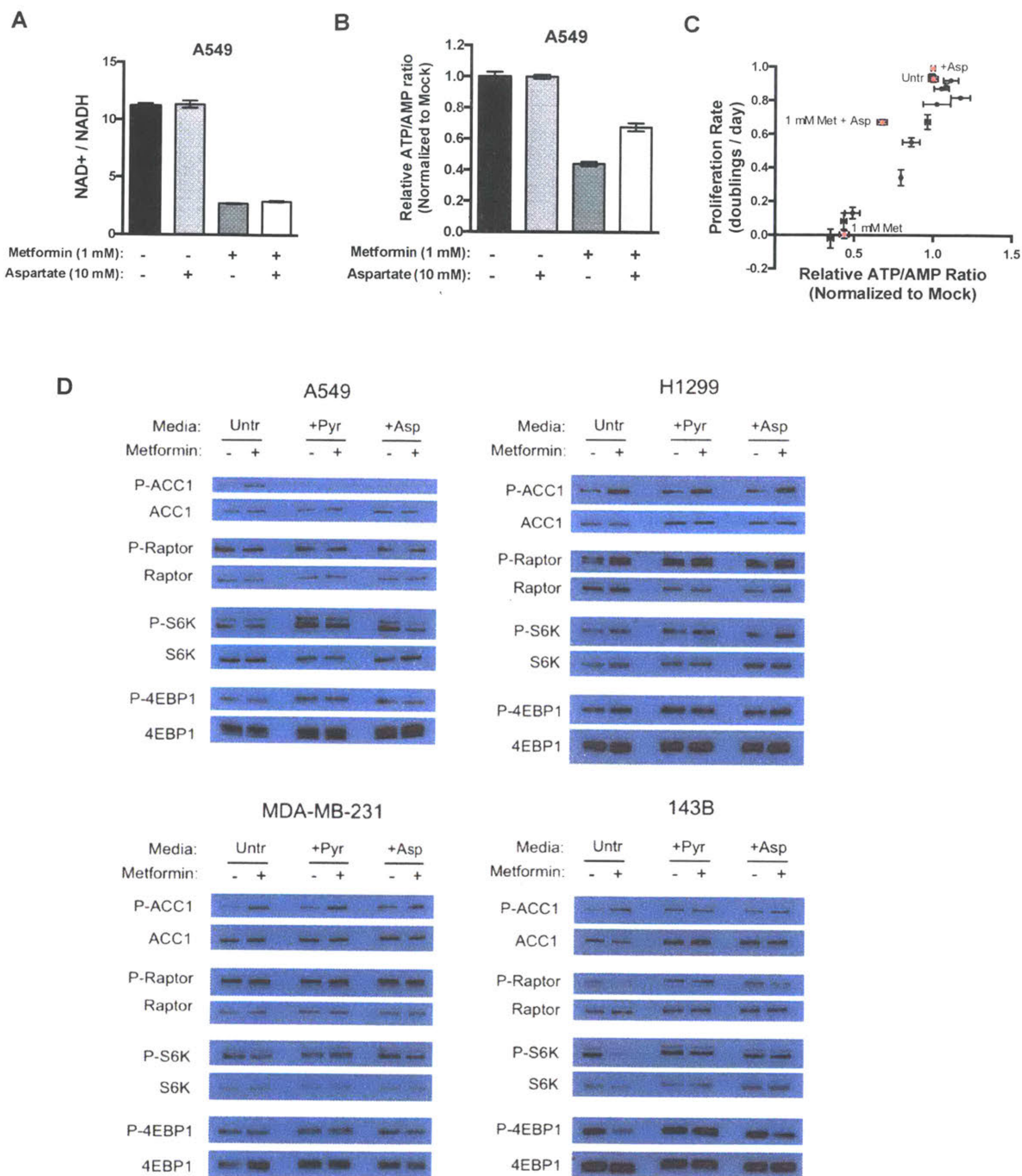

Figure S5. Aspartate suppression of the anti-proliferative effects of metformin is downstream of changes in cellular NAD+/NADH, and does not correlate with AMPK or mTORC1 signaling, related to Figure 5. Intracellular NAD+/NADH ratio (A) and ATP/AMP ratio (B) in A549 cells treated with the indicated doses of metformin and aspartate. (C) Proliferation rate and relative ATP/AMP ratios of A549 cells were plotted independent of treatment condition, cells treated with the indicated concentration of metformin and aspartate are highlighted in red. (D) Western blot for P-ACC, P-Raptor, P-S6K, P- 4EBP1 from cell lysates from A549, H1299, MDA-MB231, and 143B cells treated with metformin in the presence or absence of either pyruvate or exogenous aspartate. Values denote mean ± SEM. *n*=3 (A-C).

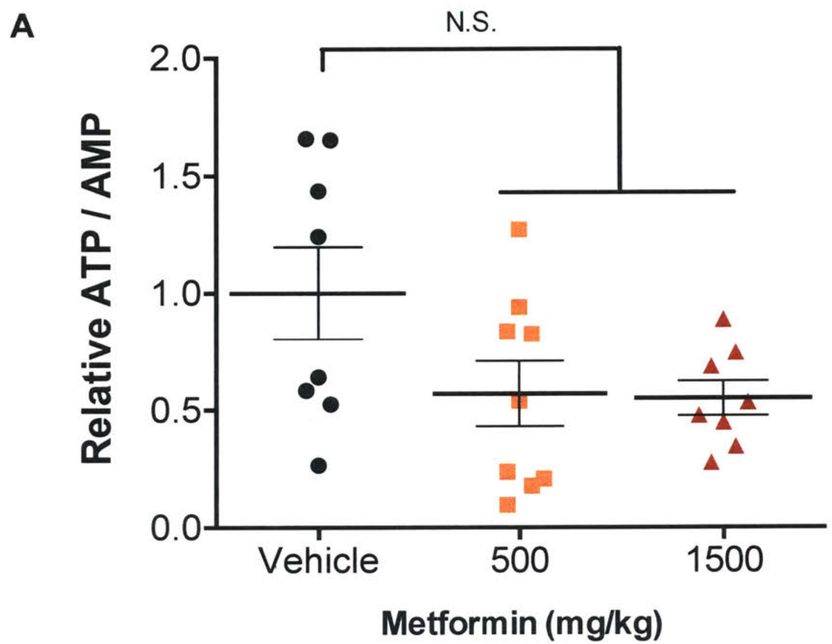

Figure S6. Intratumoral ATP/AMP ratios following metformin treatment, related to Figure 6. Relative intratumoral ATP/AMP ratio was measured following 10 days of vehicle or metformin treatment at the indicated dose with tissue harvested 2 hours after the last dose. N.S. indicates $p > 0.05$.

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Chapter 4: Discussion and Future Directions

Discussion

In order to satisfy the metabolic requirements of proliferation, cancer cells must simultaneously balance the catabolic and anabolic demands of maintaining homeostasis and acquiring biomass, respectively. Because proliferating cancer cells have this distinct additional anabolic burden compared to most other cells in normal tissues, this is a potential metabolic vulnerability that can be targeted for cancer therapy. In order to better understand how the mammalian metabolic network supports proliferative metabolism, we examined how mitochondrial respiration supports proliferation.

For non-proliferating cells, respiration is typically considered as an ATP-producing catabolic process that is important to maintain homeostasis. In contrast, we find that in proliferating cells, respiration serves a crucial anabolic role by providing access to electron acceptors to support aspartate synthesis (Sullivan et al., 2015). Indeed, we find that mitochondrial ATP production appears dispensable for supporting proliferation in cells with access to sufficient glucose. While in many proliferative contexts respiration likely supports both ATP production and aspartate biosynthesis, our finding that respiration is specifically required for aspartate biosynthesis for proliferation as opposed to ATP production highlights a distinct anabolic role for respiration.

Defining this anabolic role for respiration sheds some light on a potentially targetable vulnerability of proliferative metabolism. Proliferating cells require respiration for access

to electron acceptors to maintain a high NAD+/NADH ratio, as this ability to oxidize nutrients is needed by cells for aspartate synthesis. However, with respect to catabolism the NAD+/NADH ratio inversely correlates with energy charge, such that a low NAD+/NADH ratio favors ATP production. Thus, while respiration is seen as an essential process in most tissues, because the redox ratio needed for respiration to support ATP production is disparate for from what is needed for oxidative biosynthesis, there may exist a therapeutic window for respiration inhibitors in targeting cancer.

Consistent with this possibility, recently an abundance of clinical data has shown that the use of the mitochondrial complex I inhibitor, metformin, is associated with improved cancer outcomes, with reductions in cancer incidence and with decreased cancer mortality observed across many tumor types (Evans et al., 2005; Gandini et al., 2014; He et al., 2012; Lee et al., 2012; Zhu et al., 2015). We extend these findings by showing that the anti-proliferative effects of metformin tracks with its effect on cellular NAD+/NADH ratio (Gui et al., 2016). Furthermore, altering the metabolic environment to externally perturb the cellular NAD+/NADH ratio and aspartate levels, modulates the anti-proliferative efficacy of metformin in predictable ways. These findings highlight inhibiting mitochondrial respiration, NAD+/NADH homeostasis and/or decreasing aspartate availability as possible approaches to target cancer metabolism. Additionally, as these factors are related and will be affected by available nutrients and access to electron acceptors that keep the NAD+/NADH ratio in a range that allows proliferation, the metabolic environment can be an additional determinant of whether cells are sensitive to drugs that aim to target these pathways. Since some existing chemotherapies can target metabolism in ways that may affect these pathways, considering environment may inform how best to deploy existing therapies in patients.

Future Directions

The findings in this thesis offer some insight into how respiration and NAD+/NADH ratio support proliferative metabolism and suggest some considerations for how best to target metabolism for cancer therapy. However, major questions regarding the role of respiration and NAD+/NADH homeostasis in supporting proliferative metabolism remain, as discussed below.

Targeting respiration to limit electron acceptor and aspartate availability in vivo

Chapter 2 of this thesis, as well as corroborating work from others (Birsoy et al. 2015), offers evidence that when respiration is inhibited in tissue culture, electron acceptors availability and aspartate availability can be limiting for proliferation. However, as highlighted in Chapter 3 of this thesis, there is no one universal proliferative metabolism, and metabolic dependencies can be modulated by factors such as environment. Importantly, whether targeting respiration ever limits electron acceptor availability or aspartate availability for proliferation in tumor metabolic environments remains an open question.

Standard tissue culture for cancer cells is a poor mimic for the metabolic environment of tumors for a variety of reasons, most notably in supplying non-physiological levels of many nutrients to cells (Davidson et al., 2016; Mayers and Vander Heiden, 2015). With respect to oxygen availability, while cells in culture are exposed to atmospheric oxygen, oxygen availability in animal tissues is much lower (Bertout et al., 2008). The concentration of aspartate in blood is around 10 μM, among the lowest levels of all circulating amino acids (Mayers and Vander Heiden, 2015). Some tissue culture media

contains aspartate, but aspartate is not permeable in most cells, thus, it unlikely to be taken up from extracellular source in most tissues at physiological concentrations of this amino acid. Thus, it would be reasonable to predict that cells in tumors may be more limited for electron acceptors than cells in vitro.

While it is not known whether electron transport activity (ETC) is required in vivo to provide electron acceptor availability, it has been shown that pharmacologic inhibition of ETC activity inhibits tumor growth in several cancer models (Shackelford et al., 2013; Wheaton et al., 2014; Zhang et al., 2014). In Chapter 3 we show that decreased proliferation following metformin treatment correlated with decreased aspartate and NAD+ levels. However, whether the decrease in aspartate and NAD+ are causative for inhibiting proliferation as opposed to correlative is currently unknown. Adding another layer of complexity to the question is the observation that many cancers have decreased mitochondrial respiratory capacity compared to normal tissues. There are also familial cancer syndromes, where mitochondrial enzymes including fumarate hydratase and succinate dehydrogenase are deleted as tumor suppressors leading to decreased respiratory activity. The fact that tumors can proliferate with decreased or minimal mitochondrial respiratory capacity suggests that there may be contexts where electron acceptors and/or aspartate are not limiting. An alternative possibility is that in some tumor contexts, alternative metabolic pathways other than respiration exist for cells to access electron acceptors or aspartate. In either event, understanding how tumor cells either bypass or satisfy the requirement for electron acceptors and/or aspartate will lead to important insights into proliferative metabolism in general.

Interactions between de novo fatty acid synthesis, access to electron acceptors, and aspartate availability

The metabolic network is highly interconnected, and here we discuss the potentially underappreciated interplay between fatty acid synthesis and electron acceptor availability. When electron acceptors are limiting, such as when cells are exposed to decreased oxygen tension, it may be advantageous for cells to utilize less oxidative pathways for synthesizing aspartate. One important pathway for de novo aspartate synthesis with decreased electron acceptor demand is reductive aspartate synthesis. For reductive aspartate synthesis, alpha-ketoglutarate is reductively carboxylated at the isocitrate dehydrogenase (IDH) step to generate isocitrate. Isocitrate is subsequently isomerized to form citrate. Next, citrate is cleaved by the enzyme ACLY to form oxaloacetate and acetyl-coA, and oxaloacetate is subsequently transaminated to produce aspartate.

Consistent with the possibility that reductive aspartate synthesis is an important adaptive metabolic pathway, reductive carboxylation of alpha-ketoglutarate increases under hypoxia and in other contexts of electron acceptor insufficiency including as a result of genetic defects in mitochondrial respiration (Fendt et al., 2013; Metallo et al., 2011; Mullen et al., 2011; Wise et al., 2011). Activation of HIF, a physiological response to hypoxia, also drives reductive carboxylation of alpha-ketoglutarate (Gamiero et al., 2013). Additionally, recent work suggests that tumors may engage in increased reductive alpha-ketoglutarate carboxylation relative to the same cancer cells grown in culture, consistent with conditions of decreased oxygen availability favouring reductive carboxylation (Davidson et al., 2016).

Reductive carboxylation of alpha-ketoglutarate to produce fatty acids is accompanied by increased reductive aspartate synthesis (Mullen et al., 2014; Sullivan et al., 2015); however, the use of this pathway has been primarily investigated from the perspective of its role in de novo lipid synthesis. Whereas, in normoxia cells primarily use oxidized glucose carbons for *de novo* lipid synthesis, under hypoxia reductive carboxylation of alpha-ketoglutarate (from glutamine) accounts for upwards of 80% of the carbons in *de novo* synthesized lipids (Metallo et al., 2011). This dramatic change in the *de novo* lipid biosynthetic pathway under hypoxia suggests a possible adaptive role. Lipids are not necessarily intrinsically limiting during hypoxia for cells in culture, because most cells are capable of scavenging the lipids which are abundant in tissue culture media (Bensaad et al., 2014; Kamphorst et al., 2013). The fact that lipids accumulate during hypoxia is another long standing observation arguing against fatty acid limitation in standard culture conditions, even under hypoxia (Gordon et al., 1977).

An alternative possibility for explaining the change in lipid metabolism under hypoxia is that *de novo* lipid synthesis via reductive carboxylation, may be an important adaptation to electron acceptor insufficiency independent of the net production of lipids. Both reductive aspartate synthesis and lipid synthesis via reductive carboxylation requires the ACLY step where citrate is cleaved to form oxaloacetate and acetyl-CoA. Since, reductive carboxylation flux through IDH is governed by the alpha-ketoglutarate to citrate ratio (Fendt et al., 2013), it is possible that lipid production may drive citrate cleavage by consuming acetyl-CoA. In this scenario, lipid production would increase reductive flux through IDH in order to drive reductive aspartate synthesis. In this model, lipid synthesis would have an adaptive role in by favoring a more electron acceptor efficient pathway to

acquire aspartate. Understanding the extent by which *de novo* lipid synthesis is influenced by electron acceptor insufficiency and uncovering other unrecognized connections between disparate metabolic pathways may reveal insight into how to better therapeutically target the metabolism of proliferating cancer cells.

Revisiting the Warburg Effect

Warburg's observation that cancer cells have dramatically increased glucose consumption and lactate production, even in the presence of oxygen was one of the first cancer phenotypes identified (Warburg et al., 1924). Since Warburg's observation there have been countless hypotheses regarding the potential functions of the Warburg effect ranging from supporting rapid ATP synthesis to supporting cellular signalling. However, almost a hundred years later, the function of the Warburg effect is still an open question (Liberti and Locasale, 2016).

One of the key mysteries regarding the Warburg effect is the paradox of why cells continue lactate fermentation of glucose even in the presence of oxygen. Oxidative metabolism of glucose yields far more ATP per molecule of glucose than lactic acid fermentation. Thus, at first glance aerobic glycolysis appears to be a highly inefficient pathway for glucose consumption. However, when considering the question of metabolic efficiency it is important to define both what nutrient substrates are available and/or limiting as well as define which products require optimization to produce.

While all cells must satisfy ATP demands for both survival and proliferation, ATP may not be limiting for proliferation. Thus, cancer cell metabolism is not necessarily optimized

for ATP yield per glucose consumed. Furthermore, among nutrients, glucose may also not be limiting, and in an environment where glucose is not limiting, glucose utilization would not have to be "efficient."

In this thesis we identify that solving the bioenergetics problem of balancing NAD+/NADH homeostasis is required for cellular proliferation independent of ATP generation. Indeed, in Chapter 3 of this thesis we show that under certain environmental conditions cellular NAD+/NADH ratio directly correlates with proliferation rate, while neither ATP levels nor energy charge (ATP/AMP ratio) correlate with proliferation rate. This suggests that there are at least some contexts where the NAD+/NADH ratio is more limiting for proliferation than ATP levels. Under these conditions, to support continued proliferation, cellular metabolism would need to maximize the NAD+/NADH ratio even at the expense of ATP production.

Interestingly, the Warburg effect allows cells to maintain a high NAD+/NADH ratio by decreasing NADH production from pyruvate oxidation and increasing NAD+ production by lactic acid fermentation. Thus, if proliferating cells were more limited for NAD+ regeneration than ATP production, aerobic glycolysis would be the more "efficient" pathway for supporting proliferative metabolism. Importantly, while we have defined some contexts where the NAD+/NADH ratio is more limiting for proliferation than ATP, as previously discussed, it remains an open question the extent to which electron acceptor availability for maintaining NAD+/NADH ratio is ever limiting in tumors. Answering this question may be the key to finally unraveling one of the primary mysteries surrounding the Warburg effect.

Closing thoughts

Understanding how metabolic reprogramming contributes to tumor development and how metabolic liabilities can be exploited to treat cancer is far from complete. Nevertheless, chemotherapies targeting metabolism have been effective cancer treatments for decades, and the success of these therapies provides evidence that a therapeutic window to target malignant metabolism exists. New insights into the differential metabolic dependencies of tumors have suggested new therapeutic strategies to exploit altered metabolism, and many are being evaluated in pre-clinical models or clinical trials. A better understanding of cancer metabolism will inform future strategies for targeting the metabolic requirements of cancers.

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