Abstract

Metabolism is a complex process that transforms nutrients into energy, reducing power, and biosynthetic precursors, thereby enabling cellular functions such as mechanical work, signal transduction and macromolecule biosynthesis. Mammalian metabolism consists thousands of interconnected, tightly regulated biochemical reactions that take place in multiple cellular compartments. Understanding mammalian metabolism is particularly important within the context of cancer, given that in cancer cells metabolism is altered to support rapid cell growth. To gain a quantitative understanding of mammalian cell metabolism, we developed a comprehensive approach that integrates LC-MS-based isotope tracer studies with uptake/excretion measurements into metabolic flux models. We developed novel metabolic flux quantification methods in two ways: (1) We first applied oxygen uptake rate as a constraint and constructed a redox-balance model, (2) Based on traditional isotope tracers (e.g. $^{13}$C, $^{14}$C, $^{15}$N), we developed a new deuterium tracer approach that directly measures redox active hydrogen transfer, which enables quantifying reaction contribution in a cofactor specific manner. The potential pitfalls in isotope-based metabolic flux quantification that result from reaction reversibility have also been investigated using the specific example of isocitrate dehydrogenase, an enzyme of great interest in recent literature. We have further applied experimental-computational methods to quantitatively study the metabolism of cancer cells with particular emphasis on cofactor balance, which includes quantifying the contribution of various pathways in production and consumption of ATP (main currency of energy) and NADPH (main currency of reducing power). We found that glutamine-driven oxidative phosphorylation is a major means
of ATP production, even in hypoxic cancer cells. And we identified and confirmed that, beyond canonical pathways, in proliferating cells, the oxidation of serine-derived one-carbon units via folate-dependent pathway is a major NADPH source. Since metabolism is a dynamic process that responds to genetic and environmental conditions, we also investigated how oncogene activation and hypoxia influence cellular metabolism. Metabolism can also in turn regulate other cellular functions, here we demonstrated that human phosphoglycerate dehydrogenase, an enzyme amplified in tumors, produces the “oncometabolite” D-2-hydroxy-glutarate and influences histone methylation, providing an example in which the moonlighting activity of a metabolic enzyme has a potentially important role in epigenetic regulation.
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Chapter 1
General Introduction

1.1. Mammalian cell metabolism

1.1.1. Cofactor balance in metabolism

Metabolism is a complex process in which cells take up various nutrients (e.g. glucose, glutamine, oxygen) and convert them into biomass, waste products (e.g. lactate, CO₂) and resources that enable cellular functions such as mechanical work, signal transduction and macromolecule synthesis.

![Cofactor cycling in metabolism](image)

Mammalian metabolism consists thousands of interconnected, tightly regulated biochemical reactions that take place in multiple cellular compartments. This complex process can be
summarized as the production and consumption of three important elements during catabolism and anabolism. As shown Figure 1.1, these elements are: (1) **Biomass**, including all precursors and building blocks for cellular components, such as nucleotides for nucleic acids, amino acids for proteins, and acetyl-coA for lipids; (2) **Energy**, mainly in the form of ATP, which enables mechanical and electrical work (e.g., muscle contraction[1], neuronal firing [2]); and (3) **Reducing power**, mainly in the form of NADPH, which is needed for chemical work, powering redox defense and reductive biosynthesis of amino acids, deoxyribonucleotides, and lipids.

In this process, cofactors such as ATP, NADH, and NADPH, are the key nodes that connect the whole metabolic network in a balanced manner and play important roles in regulation. However, quantitative understanding of cofactor metabolism is still lacking. Cellular energy and reducing power can be generated via many pathways and from various nutrients. Their relative contributions, for example, the contribution of aerobic glycolysis versus oxidative phosphorylation to total ATP production; or the relative contribution of glucose, and glutamine versus other nutrients in generating reducing power that drives oxidative phosphorylation, needs to be quantified in different conditions. Also, evidence indicates that the currently known pathways may not be capable of supplying the metabolic demand for cofactor production and additional pathways must be involved. For example, the oxidative pentose phosphate pathway (oxPPP) is believed to be the main route for producing NADPH but individuals with a genetic deficiency of this pathway develop normally[3, 4]. In the work presented in this thesis, we have applied a holistic approach for the quantitative understanding of cofactor production and utilization (NADPH, ATP, and NADH) in mammalian systems, with an emphasis on cancer cell metabolism.
1.1.2 Metabolism and cancer

Like many other complex diseases, cancer has distinct metabolic features. Specifically, metabolic reprogramming is required to meet the high metabolic demands driven by rapid growth of cancer cells. Since Warburg, it has been known that cancer cells have altered metabolic fluxes, most famously “anaerobic glycolysis”[5]. Understanding metabolic features of cancer has facilitated the cancer diagnosis. For instance, tumors can be revealed by PET scan as glycolysis rates are abnormally high in many tumors, and 2-hydroxy-glurate is used as a biomarker in screening of glioma[6], as recent studies show that high percentages of glioma harbor a mutant isocitrate dehydrogenase that overproduces 2-hydroxy-glutarate[7-9]. Distinct metabolic features in cancer cells can also suggest targets for therapy. For example, anti-folate drugs are effective against cancer partly because they inhibit nucleotide synthesis, which is required for cancer growth but not very active in normal, non-proliferating cells.

Another important aspect of cancer metabolism is associated with its unique environment: unlike normal tissue surrounded by blood vesicles which supply sufficient nutrients and oxygen, in the inside of a solid tumor there is usually a poor-nutrient, hypoxic environment. Thus cancer cells need particular strategies to survive such metabolic stress. These distinct metabolic strategies may include increased dependence on autophagy to supply substrates for respiration when free glucose and amino acid levels are low, or increased metabolic flux via the oxidative pentose phosphate pathway to improve defenses against oxidative stress.

This thesis focuses particularly on the metabolism of cancer cells. By employing a holistic and quantitative approach, our studies on cancer cell metabolism provides insights into the following open questions:
As cancer cells take up nutrients more rapidly, how do they use them? How is the metabolic requirement for energy and reducing power associated with growth and what pathways do cancer cells use to meet this demand? What metabolic activities are cancer cells particularly dependent on and what are the mechanism behind this?

Beyond the fact that metabolism can be altered to meet the requirements for rapid cancer growth and to enhance survival within a high-stress tumor enviroment, recent discoveries suggest that metabolic enzymes can behave like oncogenes[10-12]. Among them, isocitrate dehydrogenase (IDH) is one of the most well studied cases. Mutations of IDH1 and IDH2 are associated with many brain cancers and leukemia [8, 13]. The common feature of these mutations is the production of D-2-hydroxyglutarate [9, 14], which influences epigenetic regulation, including histone demethylation and 5’-methylcytosine hydroxylation[15, 16], and alone is sufficient to promote leukemogenesis [17]. Another example is phosphoglycerate dehydrogenase (PHGDH), the first enzyme in de novo serine biosynthetic pathway, which has recently been found genomically amplified in a wide range of tumors[18, 19]. Suppression of PHGDH inhibits growth of PHGDH amplified cells both in vitro and in vivo [18, 20]. However, the mechanism by which PHGDH amplification supports cancer growth is not yet clear. In this thesis we provide insights into this mechanism.
1.2 Methods towards the quantitative understanding of mammalian metabolism

To gain a deeper understanding of mammalian cell metabolism, particularly cofactor metabolism, we face a fundamental and broad-scope challenge: how to better quantify metabolic fluxes? This is not a trivial challenge since mammalian cell metabolism is a highly complex system that involves many coupled reactions. Moreover, unlike concentrations, fluxes are dynamic features that cannot be measured directly.

Significant advances have been made in this area. Among the methods developed so far, the most powerful techniques include constraint-based flux balance analysis (FBA) and isotope-labeling based methods. Flux balance analysis describes the system as a set of equations and inequalities, and investigates the properties suggested by the structure of this network. Isotope-labeling based methods, on the other hand, probe metabolic fluxes by applying isotopically labeled nutrients to cultured cells and following the incorporation of labeled nutrients into downstream metabolites. The labeled fraction and labeling rate of a particular metabolite reflect the relative and absolute flux into this metabolite via different pathways. Both constraint-based and isotope tracing based methods have been applied to various systems and proven to be valuable [21] [22]. However, they both have shortcomings and it would be useful to combine them for a thorough study of cancer metabolism.

As it requires only measurements of boundary fluxes and does not dependent on intensive and localized measurements of intracellular metabolites, flux balance analysis has the ability to determine possible flux distribution at a large scale. It is mainly based on the principle that at steady state, influx is equal to outflux for each metabolite. Also, other reasonable biological
constraints can then be applied to construct the model. These constraints include enzyme activity, thermodynamic feasibility, and the correlation between the biosynthesis rates of different biomass building blocks with cell composition. Based on these, one can investigate properties such as allowable solution space [23], system robustness [24], and possible response under a perturbation [25], etc. The largest shortcoming of this method is that the model is usually not well constrained, so the real metabolic state under certain conditions cannot be well determined.

Isotopic tracing approaches traditionally analyze metabolic fluxes in cells using $^{13}$C and $^{14}$C isotope tracers[26-29]. It requires large amounts of labeling data from different metabolites to map the reaction rates around them, which give it the power of identifying fluxes more reliably and precisely. When cells are fed with a certain labeled nutrient, the steady state labeling pattern of a given metabolite that can be produced by multiple pathways for different sources, reflecting the relative contribution of these pathway fluxes. Mathematically, the labeling pattern of a metabolite can be described as a linear combination of all expected labeling patterns from every pathway that produces this metabolite that is measured, with their relative fluxes as coefficients. Metabolic fluxes through the whole network can be solved by combining the boundary flux measurements with convergent flux ratios obtained from labeling experiments[30]. Alternatively, fluxes can be measured by following the labeling kinetics of incorporation of label from a labeled precursor into downstream metabolites, i.e., kinetic flux profiling (KFP). Computationally, a KFP model describes the biological system as a set of differential equations where reaction rates correspond to parameters in the model. Searching for the parameters that make the simulated behavior consistent with observed data can identify the metabolic fluxes.
There is, however, a potential pitfall when using this type of labeling approach to investigate pathway activity and nutrient utilization: the metabolite labeling patterns are determined by gross reaction rates, not net pathway fluxes. A large number reactions in the human metabolic network are highly reversible. This high reversibility may cause two metabolites near equilibrium to have very similar labeling patterns, however, the net reaction flux between them, which is biologically meaningful in terms of nutrient utilization and material supply, cannot be effectively quantified by labeling. One such example is feeding cells with $^{13}$C-labeled glutamine and measuring the resulting labeling of citrate and fatty acids as a way to investigate how acetyl-CoA, a precursor for fatty acid synthesis, is produced. Experiments in cancer cells grown under hypoxia and in cells with defective mitochondria showed that fatty acids are labeled from glutamine via $\alpha$-ketoglutarate through reductive carboxylation of isocitrate dehydrogenase (IDH). Thus, reductive IDH was claimed to be particularly important in hypoxia and pseudohypoxia, where a major fraction of fatty acid carbon units originates from glutamine [31]. We employed a quantitative flux analysis in these cells to examine oxidative and reductive IDH flux, and found evidence for oxidative net flux in pseudohypoxia and for modest or no net flux in either direction in hypoxia. Thus, reductive IDH flux is not a major net contributor to acetyl-CoA production. The methodology is discussed in this thesis.

In our efforts towards quantitative understanding of cancer metabolism, especially cofactor metabolism, in this thesis we combined LC-MS-based isotope tracer studies with constraint based approaches, and developed methods to integrate the tracer studies with oxygen uptake measurements to construct a redox-balanced metabolic flux model.
The study of cofactor metabolism is particularly challenging because cofactors have fast turnover rates, which are not directly associated with changes in carbon skeleton. Additionally, although NADH and NADPH have distinct cellular functions, many metabolic reactions which produce high-energy electrons are catalyzed by isoenzymes that have different cofactor specificity, making it hard to investigate cofactor specific activities. To address these challenges, we developed a deuterium tracer approach that directly measures redox active hydrogen transfering, which enables determination of contribution of different pathways in NADPH production. We applied this method in combination with other tracer studies and genetic approaches to gain a quantitative understanding of overall cellular NADPH metabolism.

1.3 Metabolic regulation in mammal cells

Metabolism is a dynamic, tightly regulated process that adapts in response to environmental factors and cell fate. The regulation of mammalian metabolism occurs at many levels: cell signaling, gene expression, regulation of enzyme or transporter activity by covalent modifications or by small molecules.

Altered metabolism is now viewed as a hallmark of cancer; many metabolic changes have been shown to be induced by canonical oncogenes such as Ras, Myc, and Akt [32-34], and some of them have been found essential for rapid cancer growth [33]. These oncogenic signaling pathways are interconnected but their metabolic effects may not be necessarily addible. For example, the PI3K-Akt pathway, the natural effector pathway of insulin signaling that induces glucose uptake and lipogenesis (Elstrom et al, 2004; Robey & Hay, 2009), can be activated by Ras, whose mutation underlies most pancreatic cancers and many other cancers [35]. In addition to the PI3K-Akt pathway, Ras triggers several other pro-growth signaling cascades such as the
Raf-MAPK pathway. However, we found that the metabolic effects of Ras activation are not due to the combination of Raf activation and Akt activation.

In this thesis, we quantitatively measured the metabolic changes induced by different oncogenes, and developed insights into why and how these changes take place. Our results revealed key steps where metabolic fluxes are significantly changed by oncogenes, for example, flux from glucose into the TCA cycle is greatly reduced by oncogene Ras-activation, and the reduction is mainly via decreased pyruvate dehydrogenase flux. We also mapped the key regulatory points controlled by different oncogenes and the mechanisms by which this takes place by measuring expression and protein levels of the metabolic enzymes in parallel with metabolic studies (unpublished data). Beyond oncogenes, we also investigated how cellular metabolism changes in different conditions. Particularly we were interested in investigating oxidative stress and hypoxic conditions.

1.4 Organization of the thesis

We have applied integrated experimental-computational approaches to quantify metabolic fluxes, including co-factor fluxes, in cultured mammalian cells. In chapter 1, we developed a redox-balanced model, and applied it to evaluate the contribution of different pathways and nutrients in ATP production and NADH generation. We then further assessed the impact of Ras and Akt activation and hypoxia on energy metabolism. In chapter 2, we quantified reductive and oxidative flux via isocitrate dehydrogenase in multiple cancer cell lines, clarified the misinterpretation of carbon donor for lipogenesis in hypoxia and pseudohypoxia in recent
literature, and with this example, discussed the limitation and pitfalls of isotope tracing approaches. In chapter 3, we focused on investigating the metabolism of NADPH, the main reducing power in cells, quantified contribution and flux of main NADPH producing pathways, and evaluated the function of NADPH in cytosol and mitochondrial in biomass production and redox defense. Particularly, we identified serine-supported folate-dependent one carbon metabolism as a major NADPH source in proliferating cells, which provided a new function that explains the importance of folate metabolism and serine supply for proliferating cells. Though this is consistent with the recent finding that the first enzyme in \textit{de novo} serine synthesis pathway, 3-phosphoglycerate dehydrogenase (PHGDH), is amplified in cancer cells, it is not sufficient to fully explain the reason that cancer growth is dependent on this enzyme. We further investigated the biochemistry of PHGDH in chapter 4, and revealed that beyond synthesizing a serine precursor, it has a side-activity that produces the “oncometabolite” 2-hydroxy-glutarate and influences histone methylation.
Chapter 2

Glutamine-driven oxidative phosphorylation is a major ATP source in transformed mammalian cells in both normoxia and hypoxia

2.1. Abstract

Mammalian cells can generate ATP via glycolysis or mitochondrial respiration. Oncogene activation and hypoxia promote glycolysis and lactate secretion. The significance of these metabolic changes to ATP production remains however ill defined. Here, we integrate LC-MS-based isotope tracer studies with oxygen uptake measurements in a quantitative redox-balanced metabolic flux model of mammalian cellular metabolism. We then apply this approach to assess the impact of Ras and Akt activation and hypoxia on energy metabolism. Both oncogene activation and hypoxia induce roughly a two-fold increase in glycolytic flux. Ras activation and hypoxia also strongly decrease glucose oxidation. Oxidative phosphorylation, powered substantially by glutamine-driven TCA turning, however, persists and accounts for the majority of ATP production. Consistent with this, in all cases, pharmacological inhibition of oxidative phosphorylation dramatically reduces energy charge, and glutamine but not glucose removal markedly lowers oxygen uptake. Thus, glutamine-driven oxidative phosphorylation is a major means of ATP production even in hypoxic cancer cells.

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2.2 Introduction

The seminal work of Otto Warburg revealed that cancer cells avidly ferment glucose even in the presence of oxygen, a phenomenon called aerobic glycolysis or the Warburg effect [5]. This fundamental effect has been repeatedly confirmed in vitro, and also in vivo where it accounts for the effectiveness of fluorodeoxyglucose PET imaging. Warburg originally attributed aerobic glycolysis to impaired mitochondrial function [5], however, it subsequently became clear that most cancers display the Warburg effect despite intact mitochondrial respiration capacity. In fact, the contribution of oxidative phosphorylation to total ATP production was recently claimed by Zu and Guppy to exceed that of aerobic glycolysis in many cancer cells [36]. In light of this, it has been suggested that the shift to aerobic glycolysis serves to maximize ATP production per unit of enzyme synthesized (at the expense of ATP per glucose) or to increase total ATP production without requiring increased mitochondrial capacity [37]. Alternatively, instead of functioning to increase ATP production, aerobic glycolysis may promote tumor growth by increasing the concentration of central carbon metabolites available to drive biosynthesis [38].

Whatever its function, the occurrence of the Warburg effect reflects the activation of oncogenic signaling pathways whose physiological function is to promote glucose uptake and anabolic metabolism. These include the PI3K-Akt pathway, the natural effector pathway of insulin signaling, which induces glucose uptake and lipogenesis (Elstrom et al, 2004; Robey & Hay, 2009). The PI3K-Akt pathway is frequently mutated in cancer. In addition, it can be activated by Ras, whose mutation underlies most pancreatic cancer and many other lethal cancers [35]. In addition to the PI3K-Akt pathway, Ras triggers several other pro-growth signaling cascades such
as the MAPK pathway. Moreover, it has multifarious metabolic effects including induction of autophagy and macropinocytosis, and inhibition of oxidative phosphorylation [34, 39-42].

Like oncogenes, hypoxia promotes glycolytic flux, in part due to the activation of hypoxia induced factor (HIF) and its downstream target genes which include many glycolytic enzymes [43, 44]. Both Ras and hypoxia decrease flux of glucose through pyruvate dehydrogenase (PDH) into the TCA cycle, in part through activation of pyruvate dehydrogenase kinase (PDK). In such cases, the TCA cycle can be fed by alternative substrates including glutamine, whose importance for cell growth and survival is increased by both Ras-activation and hypoxia. This may reflect enhanced reliance on glutamine as a bioenergetic substrate [45], or as an anabolic precursor to amino acids or acetyl-CoA/ lipids [31, 39].

Here, we study how oncogene activation and hypoxia affect energy metabolism, specifically: (i) the contribution of aerobic glycolysis versus oxidative phosphorylation to total ATP production; and (ii) the relative contribution of glucose, glutamine versus other nutrients to making the reducing power that drives oxidative phosphorylation. Towards this end, we combine LC-MS-based isotope tracer data with oxygen consumption measurements in a quantitative redox-balanced metabolic flux model. Notably, while oxygen consumption rate measurements were previously shown to be valuable for metabolic flux analysis in microbes [46], oxygen uptake has not been used together with isotope tracer data to facilitate flux inference in mammalian cells. We apply this approach to study the effect of Ras and Akt activation and hypoxia on fluxes, providing a comprehensive and quantitative view of the impact of these factors on ATP production routes. Through this approach, we find that glutamine-driven oxidative phosphorylation is a major ATP source even in oncogene-expressing or hypoxic cells.
2.3 Results and Discussion

2.3.1 Quantifying ATP production routes via a redox-balanced metabolic flux model

To study ATP production routes, we used Bax\(^{-/-}\), Bak\(^{-/-}\) murine renal epithelial cells immortalized by expression of adenovirus E1A and dominant-negative p53 [47] (iBMK cells). Isogenic cell lines were generated by transfecting cells with vector expressing either oncogenic H-Ras\(^{G12V}\) or myr-Akt [48, 49]. Note that activation of these specific genes may not result in identical metabolic consequences to that of other related family members that are frequently mutated in cancer (e.g., K-Ras, PI3KCA). Introduction of either oncogene did not substantially impact cellular growth in vitro (Appendix Table A1), but greatly enhances tumorigenicity in vivo compared to isogenic cells without Ras or Myr-Akt, as evident by faster allograft growth, with the effect of Ras yet stronger than that of Akt [48].

To quantify fluxes in central metabolism, we combined three types of measurements (Figure 2.1, see also 2.4 Methods): (i) uptake and excretion rates of major nutrients (glucose, glutamine, and oxygen, including the fraction of oxygen consumed by oxidative phosphorylation as measured by respiratory chain inhibition) and waste products (lactate, glutamate, pyruvate, and alanine); (ii) cellular DNA, RNA, protein, and fatty acid content (Appendix Table A2) together with cellular growth rate to determine the flux of metabolic building blocks into biomass; (iii) steady-state labeling of intracellular metabolites determined by LC-MS when cells are fed media with [U-\(^{13}\)C]-glucose or [U-\(^{13}\)C]-glutamine (Appendix Figure A1-4).
Figure 2.1 Glutamine-driven oxidative phosphorylation supports ATP production in iBMK-parental cells.

(A) Redox-balanced metabolic flux analysis scheme. Experimental measurements including uptake/excretion rates and steady state intracellular labeling patterns were input into a redox-balanced metabolic flux analysis model. Fluxes with confidence intervals were obtained by optimizing the simulation to fit the steady-state experimental observations. The resulting fluxes also fit kinetic labeling data well. (B) Metabolic fluxes in the parental iBMK cell line. Numbers indicate metabolic flux in nmole/h/µl cells. Colors indicate the contribution of glucose (yellow) or glutamine (blue) in driving each redox reaction. Font size reflects absolute metabolite concentrations. Abbreviations: FBP, fructose-1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; 3PG, 3-phosphoglycerate; PEP, phosphoenolpyruvate; Pyr, pyruvate; AKG, α-ketoglutarate; OAA, oxaloacetate. (C) Effect of oxidative phosphorylation inhibitors on the ATP/ADP ratio and NADH/NAD⁺ ratio. ATP, ADP, NADH, and NAD⁺ levels were measured 5 min after addition of vehicle (DMSO), the complex III inhibitor antimycin A (4 μg/ml) or the ATP synthase inhibitor oligomycin (8 μg/ml) (mean ± SD of N = 3). (D) Oxygen consumption rates and NADH/NAD⁺ ratio measured after switching cells to complete media or media lacking glucose or glutamine for 8 hours (mean ± SD of N = 3).
To infer intracellular metabolic fluxes, we constructed a metabolic network model of glycolysis and TCA cycle (Appendix Table A3), and applied Metabolic Flux Analysis (MFA) to identify a flux distribution that would optimally fit the experimental datasets. The computational flux model is redox balanced, i.e., the high energy electron (NADH or FADH₂) production rate matches the consumption rate by oxidative phosphorylation. Flux confidence intervals were derived by directly computing the range of possible fluxes for each reaction that enable close-to-optimal fit with the experimental data, by iteratively running the MFA while constraining the flux through each reaction to increasing (and then decreasing) values (Methods; note that reactions not included in the metabolic network model may introduce additional error beyond that reflected in the computed confidence intervals). In all cases, we obtained fluxes that resulted in good agreement with the steady-state labeling data (Appendix Figures A1-4). As a further validation of the inferred fluxes, we measured the kinetic labeling of intracellular metabolites (for 72 hours) as well as their absolute concentrations (Appendix Table A4). The fluxes obtained solely by fitting the steady-state data resulted also in good agreement with the experimentally observed kinetic labeling patterns (Methods; Appendix Figures A1-4).

Predicted fluxes were used to calculate the contribution of glycolysis and oxidative phosphorylation to ATP production, considering that glycolysis makes 2 ATPs per glucose molecule and the oxidative phosphorylation makes on average 2.5 ATPs per NADH oxidation and 1.5 ATP per FADH₂ oxidation. To assess the contribution of glucose, glutamine, or acetyl-CoA (produced by the catabolism of fatty acids or amino acids) to generating the reducing power that drives oxidative phosphorylation, we calculated the rate of high energy electron donation from each of these nutrients. To this end, we computationally inferred for each NADH/FADH₂
producing reaction, the abundance of substrate carbons being oxidized that originate from glucose, glutamine, and other sources of acetyl-CoA (see Methods).

Our flux-based approach for inferring the contribution of specific nutrient oxidation to generating reducing power has much in common with a classical approach involving feeding cells with radioactive labeled nutrients and tracking the release of radioactive CO$_2$[50, 51]. However, these prior methods only tracked reactions that make CO$_2$, while many NADH and FADH$_2$-producing reactions do not. Moreover, these prior methods were blind to oxidation of alternative non-radioactive substrates, which are visible in our analysis as we track both labeled and non-labeled carbon atoms. Thus, our approach enables both the quantitation of the total generated reducing power and the contribution of individual reactions.

2.3.2 Glutamine-supported oxidative phosphorylation is a major source of ATP in the parental iBMK cell line

The total ATP production rate in the parental iBMK cell line was found to be 861 nmole/µL cells/h (where µL refers to the packed cell volume, with 1 µL equal to ~ 10$^6$ cells). The relative contribution of oxidative phosphorylation and aerobic glycolysis was ~ 80% and 20% respectively (Appendix Table A3), consistent with previous results[36]. The major contribution of oxidative phosphorylation to total ATP production in this cell line is evident when treating the cells with either the complex III inhibitor antimycin A or the ATP synthase inhibitor oligomycin, both of which lead to ~5-fold increase in the NADH/NAD$^+$ ratio and ~10-fold decrease in the ATP/ADP ratio (Figure 2.1C).

Tracking the source of reducing power, we found that oxidation of glutamine, of glucose, and of acetyl-CoA derived from other sources (such as unlabeled fatty acids or amino acids) contribute
60%, 30% and 10%, of the total NADH/FADH$_2$ production, respectively. Glutamine’s uptake rate is ~30% that of glucose and it directly feeds the TCA cycle through α-ketoglutarate. Glucose-derived two-carbon flux into the TCA cycle (via pyruvate dehydrogenase) is 60% lower than that of glutamine flux into the TCA cycle. Glucose-derived four-carbon flux into the TCA cycle (via pyruvate carboxylase) amounts to ~2% of glutamine flux into TCA cycle.

In addition to glucose-derived carbon atoms being a contributor to TCA turning, glucose-driven glycolysis and serine synthesis in the cytosol can both produce high-energy electrons in the form of cytosolic NADH. To evaluate the potential contribution of the serine pathway to high-energy electron generation, we conducted studies with U-$^{13}$C-serine tracer, which revealed that NADH production via this pathway in the tested cell lines is ~ 3% of glycolytic flux (see Methods). Moreover, we observed that of total NADH generated in the cytosol, 84% is consumed to reduce pyruvate to lactate. For the remaining 16% of cytosolic high-energy electrons to contribute to ATP production, they must be imported into the mitochondrion via the malate-aspartate shuttle, which produces mitochondrial NADH, or the glycerol phosphate shuttle, which converts cytosolic NADH into mitochondrial FADH$_2$. For simplicity we assume exclusive use of the malate-aspartate shuttle. If cells were instead to exclusively use the glycerol-phosphate shuttle, oxidative ATP production would decrease by 3% (Appendix Figure A5).

To validate the inferred greater contribution of glutamine than glucose to oxidative metabolism, we measured O$_2$ uptake in cells deprived of either glucose or glutamine (Figure 2.1D). We find that deprivation of either glucose or glutamine decreases the whole cell NADH/NAD$^+$ ratio, with the effect of glutamine removal being greater ($p = 0.02$, Methods). Note that the observed change in the NADH/NAD$^+$ ratio reflects the combination of cytosolic and mitochondrial pools. The
effect on different compartments may vary by nutrient, a topic that merits further investigation using compartment-specific measurement methods [52, 53]. Similar to the greater effect of glutamine on the NADH/NAD\(^+\) ratio, glutamine removal was also found to more strongly decrease oxygen uptake \((p < 0.001)\). These results are in agreement with those of Le et al [45] showing a significant drop in ATP level when cells are treated with a glutaminase inhibitor.

### 2.3.3 Ras induces aerobic glycolysis without increasing the total ATP production rate

Activation of either Ras or Akt induces aerobic glycolysis, as evident by a roughly two-fold increase in glucose uptake and lactate secretion (Figure 2.2A). However, following the activation of either oncogene, most cellular ATP is still produced oxidatively, with Ras but not Akt activation causing a decrease in oxidative ATP production (Figure 2.2B). Oxygen consumption was significantly decreased by Ras but not Akt activation \((p = 0.01\) and \(p = 0.97\), respectively) with the Ras versus Akt difference also significant \((p = 0.02)\). Consistent with this, treating the oncogene-activated cells with mitochondrial inhibitors leads to a rise in the NADH/NAD\(^+\) ratio of 6.0 and a drop in ATP/ADP ratio of \(~88\%\) following Akt activation and to a lesser rise in NADH/NAD\(^+\) of 3.9 and smaller drop of ATP/ADP \(~76\%\) for Ras \((p = 0.05\) for greater fractional drop in ATP/ADP ratio in Akt-driven than Ras-driven cells for antimycin, \(p = 0.03\) for oligomycin) (Appendix Figure A6, Figure 2.2C). Our finding of Ras activation decreasing oxidative ATP production is in agreement with previous reports of Ras inhibiting both oxygen consumption and glycolytic two-carbon flux entering TCA cycle [34, 39, 41].
Figure 2.2 Energy metabolism in oncogene-driven cells.
(A) Metabolic fluxes in iBMK cells expressing H-Ras^{V12G} of myr-Akt. Colors indicate flux changes in the oncogene-driven cell lines compared to parental cells, showing only reactions whose flux confidence interval in the oncogene-activated cells does not overlap the corresponding
confidence interval in parental cell line. (B) ATP production rates from oxidative phosphorylation and glycolysis in parental, Ras and Akt iBMK cells. (C) Effect of oxidative phosphorylation inhibitors on the ATP/ADP ratio. ATP and ADP were measured 5 min after addition of vehicle (DMSO), the complex III inhibitor antimycin A (4 μg/ml) or the ATP synthase inhibitor oligomycin (8 μg/ml) (mean ± SD of N = 3). (D) Contribution of various nutrients to driving cofactor reduction (sum of NAD⁺ and FAD). (E) Oxygen consumption rates measured after switching cells to complete media or media lacking glucose or glutamine for 8 hours (mean ± SD of N = 3).

Glutamine oxidation remains the main source of reducing power following the activation of both Ras and Akt (Figure 2.2D). Similar to in the parental cell line, removal of glutamine decreases the consumption of oxygen (Figure 2.2E) and the NADH/NAD⁺ ratio (Appendix Figure A6) to a greater extent than glucose removal ($p = 0.01$ for oxygen uptake measurements in Ras and $p = 0.003$ in Akt). Moreover, for the Ras cell line, the importance of glutamine as a TCA substrate is increased, with the relative contribution of glutamine oxidation to reducing power rising from 62% to 75%. The increased reliance on glutamine results from unchanged glutamine flux into the TCA cycle via α-ketoglutarate, paired with decreased influx of other substrates, most importantly decreased glycolytic two carbon flux (Figure 2.2D).

Interestingly, while Ras activation induces aerobic glycolysis, it proportionally reduces oxidative phosphorylation, such that the total ATP production rate remains the same. This suggests that the induction of aerobic glycolysis by Ras, at least in normoxia, is not for the purpose of increasing ATP production, arguing against claims that Warburg effect promotes tumorigenesis by accelerating the production of usable energy [54, 55].
2.3.4 Glutamine-supported oxidative phosphorylation is a major source of ATP also in hypoxia

To quantify metabolic flux in hypoxia, we repeated the analysis described above, examining the parental iBMK cells grown in a hypoxic chamber with 1% oxygen. Similarly to oncogene activation, hypoxia induces aerobic glycolysis (Figure 2.3A). More interestingly, in hypoxia, the oxygen consumption rate is reduced by only 30% compared to normoxia. Our observation of persistent oxygen uptake in hypoxia is consistent with Frezza et al., who reported an oxygen consumption rate of the HCT116 cell line in hypoxia that is 50% of the normoxic rate [56]. Hence, we find that 60% of cellular ATP is still made oxidatively in hypoxia (Figure 2.3B), which is confirmed by profound ATP/ADP ratio drop and NADH/NAD\(^+\) ratio increase upon treatment with mitochondrial inhibitors (Figure 2.3C). As expected, the drop in ATP/ADP due to the oxidative phosphorylation inhibitors is less severe in hypoxia (\(p = 0.002\) for antimycin and \(p = 0.002\) for oligomycin).

In terms of reducing power, we find that also in hypoxia, more than 60% of NADH/FADH\(_2\) is produced by glutamine oxidation. The contribution of glucose oxidation decreases from 32% in normoxia to 17% in hypoxia (Figure 2.3D). The decreased contribution of glucose oxidation to making reductive power results from a 4-fold decrease in pyruvate dehydrogenase (PDH) flux in hypoxia, which is consistent with the known PDH inhibition by hypoxia [57]. On the other hand, the contribution of other sources increases in hypoxia. The role of glutamine-supported oxidative phosphorylation in hypoxia is further demonstrated via a significant decrease in oxygen consumption and NADH/NAD\(^+\) ratio upon glutamine removal in hypoxia (\(p = 0.03\) for oxygen consumption and \(p = 0.01\) for NADH/NAD\(^+\) ratio; Figure 2.3E).
The changes in ATP production routes induced by hypoxia resemble those induced by Ras activation: i.e. the increased reliance on aerobic glycolysis for ATP production, as well as the decreased reliance on glucose oxidation for generating mitochondrial reducing power. These changes may provide Ras-driven tumor cells an advantage in hypoxia.

To examine the generality of our observation of persistent oxidative ATP production in hypoxia, we analyzed the impact of antimycin and oligomycin treatment of hypoxic Ras- and Akt-driven cells (Figure 2.3F), with both drugs decreasing the ATP/ADP ratio by ~50% in both cell lines, while also substantially increasing the NADH/NAD⁺ ratio. Consistent with these observations, we observed persistent oxygen uptake also in these cell lines in hypoxia. Similar to in the parental cells, in hypoxia, glutamine deprivation was found to significantly decrease oxygen consumption and the NADH/NAD⁺ ratio in these cell lines (Figure 2.3G), emphasizing the general dependence of both normoxic and hypoxic cultured murine renal epithelial cells on glutamine to power oxidative phosphorylation irrespective of oncogene activation.
Figure 2.3 Energy metabolism in iBMK parental cells in hypoxia (1% O₂).

(A) Metabolic fluxes in the parental iBMK cell line in hypoxia. Colors indicate flux changes in hypoxia compared to normoxia, showing only reactions whose flux confidence interval in hypoxia does not overlap the corresponding confidence interval in normoxia. (B) ATP production rates from oxidative phosphorylation and glycolysis in iBMK parental cells in normoxia versus hypoxia. (C) Effect of oxidative phosphorylation inhibitors on the ATP/ADP ratio and the NADH/NAD⁺ ratio in parental cells in hypoxia. ATP, ADP, NADH, and NAD⁺ levels were measured 5 min after addition of vehicle (DMSO), the complex III inhibitor antimycin A (4 μg/ml) or the ATP synthase inhibitor oligomycin (8 μg/ml). (D) Contribution of various nutrients to driving cofactor reduction (sum of NAD⁺ and FAD). (E) Oxygen consumption rates and NADH/NAD⁺ ratio in parental cells in hypoxia measured after switching cells to complete media or media lacking glucose or glutamine for 8 hours (mean ± SD of N = 3). (F) Effect of oxidative phosphorylation inhibitors on the ATP/ADP ratio and NADH/NAD⁺ ratio in Ras and Akt cells in hypoxia. (G) Oxygen consumption rates and NADH/NAD⁺ ratio in Ras and Akt cells in hypoxia measured after switching cells to complete media or media lacking glucose or glutamine for 8 hours (mean ± SD of N = 3).
2.3.5 ATP production routes in cancer cell lines in both normoxia and hypoxia

To study the importance of glutamine-supported oxidative phosphorylation in other cell lines, we extended our analysis to two common cancer cell lines: 4T1, Akt-driven mouse mammary tumor cell line and ASPC1, a K-Ras-driven human pancreatic cancer cell line, in both normoxia and hypoxia. Similar to the Akt and Ras transformed iBMK cell line, in both 4T1 and ASPC, the majority of ATP is made oxidatively. Hypoxia decreases the oxidative phosphorylation rate by 60% in 4T1 and 40% in ASPC1, while increasing the glycolytic rate in both cell lines, and increases the fraction of glucose excreted as lactate instead of entering TCA cycle. However, in both cases, oxidative phosphorylation continues to account for more than 50% of total energy production (Figure 2.4A). To assess the importance of glucose and glutamine oxidation, we measured the oxygen consumption rate after glucose or glutamine removal (Figure 2.4B). In both cell lines, in either hypoxia or normoxia, glutamine removal more strongly reduced oxygen consumption than glucose removal, confirming an essential role for glutamine in supporting oxidative phosphorylation. Interestingly, in ASPC1 cells, especially in hypoxia, when glucose is removed from media, oxygen consumption increases. This suggests that in glucose starvation, cells can up-regulate oxidative phosphorylation, driven by glutamine and other sources, to compensate for the decreased glycolytic ATP production.

To analyze the role of oxidative phosphorylation in a broader set of cell lines, we applied flux balance analysis (FBA) to predict metabolic flux rates through glycolysis and oxidative phosphorylation in each of the NCI-60 cell lines based on available metabolite uptake and secretion rates from Jain et al [58] (see Methods), which did not include the oxygen uptake rate (preventing redox balancing in this analysis). Utilizing the same metabolic network model of
glycolysis and TCA cycle as for the $^{13}$C-based flux analysis (Appendix Table A3), FBA was applied to search for flux rates that maintain stoichiometric mass balance, optimally match the measured uptake and secretion rates (of glucose, lactate, glutamine, and glutamate), and have no thermodynamically infeasible cycles (Methods). Our FBA analysis shows that oxidative phosphorylation contributes 88% of the total ATP production on average across the 60 cell-lines (Figure 2.4C). Notably, the predicted mitochondrial ATP production rates for all 60 cell lines analyzed here are consistent with cellular solvent capacity constraint, which gives rise to an upper threshold of 8.4 umole/uL-cells/h (Appendix Figure A7) [59]. Reassuringly, applying the same FBA approach to predict ATP production routes in the iBMK cell lines (using just corresponding uptake and secretion measurements) resulted in qualitatively similar results to those obtained with the isotope tracer data (with an average error of 5% in the prediction of glycolytic versus oxidative phosphorylation ATP production).

To further validate the predicted contribution of oxidative phosphorylation to ATP production, we repeated the flux analysis in the NCI60 cell lines using a genome-scale human metabolic network model [60]. We constrained metabolite uptake and secretion rates using data from Jain et al. (as in the above analysis with the reduced network model of Appendix Table A3) and further included a biomass reaction constraint (Methods) [61]. Applying FBA to predict fluxes that maximize the ATP production rate resulted in an average contribution of oxidative phosphorylation to total ATP production of 84% across cell lines. Alternatively, constraining oxygen consumption rate to a range of likely rates (around the measured oxygen consumption in the iBMK cell lines) without maximizing ATP production predicted an average contribution of 70% (Appendix Figure A8). In both cases, the predictions obtained with the genome-scale
metabolic network model support the major contribution of oxidative phosphorylation to ATP production.

**Figure 2.4 Glutamine-driven oxidative ATP production in normoxic and hypoxic cancer cells.**

(A) ATP production rates from oxidative phosphorylation and glycolysis in 4T1 and ASPC1 cancer cells. (B) Oxygen consumption rates in hypoxia measured after switching cells to complete media or media lacking glucose or glutamine for 8 hours (mean ± SD of N = 3, significant differences (p < 0.05) by T-test marked with *). (C) Contribution of oxidative phosphorylation to ATP production in NCI-60 cell lines as predicted by nutrient uptake rate-constrained flux balance analysis.

In summary, we have developed an approach to analyzing cellular metabolic fluxes including ATP production routes which combines quantitatively $^{13}$C-tracer and oxygen uptake measurements. The results contribute to growing literature on the importance of glutamine in driving TCA cycle flux, especially in cells with activated Ras or grown in a hypoxic environment [31, 39, 45, 56]. Importantly, even in such circumstances, we show that oxidative phosphorylation remains the largest quantitative contributor to ATP production. Moreover, we find that Ras has no net effect on ATP production, as the increase in glycolysis is offset by decreased oxidative phosphorylation. Taken in totality, these observations argue for a primary role of oxidative metabolism in most cancers.
2.4 Materials and Methods

2.4.1 Cell lines and culture conditions

Immortalized baby mouse kidney (iBMK) epithelial cells were generated as described previously[47]. Briefly, primary kidney epithelial cells from mice double deficient for Bax and Bak (Bax^{-/-}/Bak^{-/-}) were immortalized by E1A and dominant-negative p53 expression [47, 62]. iBMK cells expressing human oncogenic H-Ras^{G12V} or myr-Akt were derived by electroporation with pcDNA1.H-Ras^{G12V} [63] or pcDNA3. myr-Akt [64] respectively, followed by zeocin selection. The resulting cell lines were grown in Dulbecco’s modified eagle media (DMEM) without pyruvate (Cellgro), supplemented with 10% dialyzed fetal bovine serum (HyClone) in all metabolomics experiments. For normoxia experiments, cells are grown in an incubator containing 5% CO₂ and ambient oxygen at 37°C. For hypoxia experiments, cells are grown and all experiments are completed inside a hypoxia glove box (Coy Lab) containing 1% oxygen and 5% CO₂ at 37°C. For labeling experiments, medium was prepared from DMEM without glucose or glutamine (Cellgro), with the desired isotopic form of glucose and/or glutamine added to a final concentration of 4.5 g/l glucose and 0.584 g/l glutamine. Short-term experiments (e.g. nutrient uptake and kinetic flux profiling) were conducted at 70%-80% confluency; for longer-term labeling experiments, confluency varied as the cells multiplied.

2.4.2 Exchange rate measurements

Media samples were collected various time points. Glucose, glutamine, and lactate were measured by enzymatic assay with electrochemical detection on a YSI7200 instrument (YSI, Yellow Springs, OH). Alanine and pyruvate were measured by LC-MS. Oxygen consumption was measured by a Seahorse XF24 flux analyzer (Seahorse Bioscience, North Billerica, MA). To
measure oxygen uptake in hypoxia, the Seahorse instrument was placed in the hypoxia chamber with 1% oxygen. To probe the fraction of oxygen consumption which is effectively coupled with electron transport chain, we measured the oxygen consumption rate when cells were treated with electron transport chain inhibitor, antimycin A. We observed that ~80% of oxygen consumption across the studied cell lines and growth conditions was used for oxidative phosphorylation.

2.4.3 Metabolomic experiments and LC-MS analysis

For all metabolomic and isotope-tracer experiments, metabolism was quenched and metabolites extracted by quickly aspirating media and immediately adding -80°C 80:20 methanol: water extraction solution.

Samples were analyzed by multiple LC-MS systems (each from Thermo Scientific and fed by electrospray ionization), as described previously [21, 65, 66]. Briefly, a stand-alone orbitrap mass spectrometer (Exactive) operating in negative ion mode was coupled to reversed-phase ion-pairing chromatography and used to scan from m/z 85-1000 at 1 Hz and 100,000 resolution; a TSQ Quantum Ultra triple-quadrupole mass spectrometer operating in positive ion mode was coupled to hydrophilic interaction chromatography on an aminopropyl column and used to analyze selected compounds by multiple reaction monitoring; and a TSQ Quantum Discovery triple-quadrupole mass spectrometer operating in negative ion mode was coupled to reverse-phase ion-pairing chromatography and used to analyze selected compounds by multiple reaction monitoring. Data were analyzed using the MAVEN software suite [67]. The results are adjusted for natural $^{13}$C abundance and enrichment impurity of labeled substrate supplied to cells.
Absolute metabolite levels were quantified as previously described [68] and normalized by packed cell volume.

### 2.4.4 De novo serine synthesis rate

To quantify the rate of serine synthesis, cells were cultured in DMEM media containing U-\(^{13}\)C-serine. Steady state labeling pattern of intracellular 3-phosphoglycerate, serine and glycine were measured by extracting metabolites after washing 3-times with ice-cold PBS. Labeled 3-phosphoglycerate was never observed, confirming that reverse serine synthesis pathway flux is negligible. Glycine was observed in two forms: unlabeled and M+2 (whose relative fractions are referred to as \(G_0\) and \(G_2\) below). Serine was observed in four forms: unlabeled, M+1, M+2, and M+3, with fractions \(S_0\), \(S_1\), \(S_2\), \(S_3\), respectively. These forms arise via \textit{de novo} synthesis from 3-phosphoglycerate (making unlabeled via F1, see Appendix Figure A9), uptake from media (making M+3 via F2), and reverse SHMT flux from glycine (making all possible forms, depending on the labeling of glycine and the methylene-THF methyl group, via F3). The fraction of methylene-THF with the reactive one-carbon unit unlabeled is denoted \(T_0\), and that labeled on the one-carbon unit is denoted \(T_1\). We also measured net serine uptake flux F2 by monitoring the change of serine concentration in the media.

Under isotopic steady-state, the balance equations for the four labeling forms of serine can be formulated as following:

\[
S_0(F1 + F2 + F3) = F1 + F3 \times T_0 \times G_0 \\
S_i(F1 + F2 + F3) = F3 \times T_i \times G_0
\]
The de novo serine biosynthesis flux is derived by solving the above equations for F1:

\[
F1 = F2 \times \frac{G_2}{S_3 - \frac{G_2}{G_0} S_2}
\]  

2.4.5 Redox-balanced metabolic flux analysis (MFA)

To infer intracellular metabolic fluxes, we constructed a metabolic network model of glycolysis and TCA cycle (Appendix Table A3), and applied Metabolic Flux Analysis (MFA) to identify a flux distribution that optimally fits the following experimental datasets (Appendix Figures A1-4 and Appendix Table A5): (i) Measured uptake and secretion rates of glucose, glutamine, lactate, pyruvate, alanine; (ii) Measured oxygen consumption rate used by oxidative phosphorylation (iii) Steady-state isotopic labeling pattern of pyruvate, α-ketoglutarate, citrate, malate, and cytosolic acetyl-CoA, where the labeling pattern of cytosolic acetyl-CoA was inferred based on the steady-state labeling pattern of fatty acids via Isotopomer Spectral Analysis (ISA)[69]; (iv) Consumption of metabolic intermediates for biomass production based on measured growth rate and biomass component contents. Specifically, we measured the DNA, RNA, and protein contents to be equal to 11, 14, and 88, µg/µl cells, respectively in iBMK cells. Cellular demands for acetyl-CoA based on steady-state fatty acid concentrations, cellular growth rate, and fatty
acid uptake rates are shown in Appendix Table A5; (v) Measured flux in the serine biosynthesis pathway.

The MFA method was formulated as an optimization problem aiming to select a vector of fluxes \( \nu \) that maximizes the log-likelihood of measured mass-isotopomer distributions in both \(^{13}\text{C}\)-glucose and \(^{13}\text{C}\)-glutamine experiments, denoted \( X_j^{\text{glc}} \) and \( X_j^{\text{gln}} \). The mass-isotopomer distributions produced by the set of fluxes \( \nu \) given the metabolic network in Appendix Table B 3 are denoted \( Y_j^{\text{glc}}(\nu) \) and \( Y_j^{\text{glc}}(\nu) \). Assuming a Gaussian error model for measured isotope labeling data, maximum log-likelihood is obtained by minimizing the variance-weighted sum of squared residuals between measured and computed mass-isotopomer distributions[70, 71], where \( V_j^{\text{glc}} \) and \( V_j^{\text{gln}} \) are diagonal matrices with the inverse of experimental variance for the labeling pattern of metabolite \( j \):

\[
\min_{\nu} \sum_j \left( (X_j^{\text{glc}} - Y_j^{\text{glc}}(\nu))^T \cdot V_j^{\text{glc}} \cdot (X_j^{\text{glc}} - Y_j^{\text{glc}}(\nu)) + (X_j^{\text{gln}} - Y_j^{\text{gln}}(\nu))^T \cdot V_j^{\text{gln}} \cdot (X_j^{\text{gln}} - Y_j^{\text{gln}}(\nu)) \right)
\]

s. t.

\[
S \cdot \nu = 0
\]

\[
f_i - 2\sigma_i \leq \nu_i \leq f_i + 2\sigma_i \quad \text{for reactions } i \text{ with directly measured rate}
\]

\[
\nu_i \geq 0 \quad \text{for reactions } i \text{ that are irreversible}
\]
where $S$ represents a stoichiometric matrix (with $S_{ji}$ representing the stoichiometric coefficient of the $j$'th metabolite in the $i$'th reaction), and $f_i$ and $\sigma_i$ represent the measured flux through reaction $i$ and standard deviation, respectively. Stoichiometric mass and redox-balance constraint is enforced by Eq. 6. Metabolite uptake and secretion rates, biomass demand fluxes, serine biosynthesis rate, and oxidative phosphorylation rate (based on oxygen consumption rate) were constrained to experimental measurements, allowing them to vary by no more than two experimental standard deviations from the mean measured fluxes (Eq. 7; Appendix Table A3, 5). Irreversible reactions are constrained to have positive flux (Eq. 8). To efficiently compute the mass-isotopomer distributions for both the glucose and glutamine labeling experiments ($Y_{j}^{\text{glc}}(v)$ and $Y_{j}^{\text{gin}}(v)$) per each candidate flux vector ($v$) as part of the optimization, we employed Elementary Metabolite Units (EMU)[71]. The non-convex optimization problem was solved using Matlab’s Sequential Quadratic Optimization (SQP). To overcome potential local minima in SQP algorithm, the optimization problem was solved several times, starting from different sets of random fluxes.

To compute flux confidence intervals, we used the likelihood ratio test to compare the maximum log-likelihood estimation, computed by the above SQP optimization, to that obtained when constraining the flux to higher or lower values. Specifically, we iteratively ran the SQP optimization to compute the maximum log-likelihood estimation while constraining the flux to increasing (and then decreasing) values (with a step size equal to 5% of the flux predicted in the initial maximum log-likelihood estimation). The confidence interval bounds were determined based on the 95% quantile of $\chi^2$-distribution with one degree of freedom [70]. The results of this iterative process (i.e. the probability of a reaction having a range of possible flux rates) were also
used to calculate the standard deviation of flux estimates. This computationally intensive computation of accurate flux confidence intervals took a total of ~48 hours on a standard laptop computer using Matlab.

To compute metabolite labeling kinetics given a flux vector \( (v) \) derived by the above optimization problem, we considered a system of ordinary differential equations that describe the abundance of isotopomer \( k \) of metabolite \( j \) at time \( t \) (denoted \( U_{j,k}(t) \)) as following[72]:

\[
\frac{dU_{j,k}(t)}{dt} = \sum_{i:S_{j,i}>0} v_i \alpha(U(t), i, k) \beta(U(t), i, k) - \frac{U_{j,k}(t)}{C_j} \sum_{i:S_{j,i}<0} v_i - \mu C_j \tag{9}
\]

where the \( \alpha(U(t), i, j, k) \) and \( \beta(U(t), i, j, k) \) are defined as following, assuming that reaction \( i \) that makes metabolite \( j \) has two substrates \( s1 \) and \( s2 \): \( \alpha(U(t), i, j, k) \) is equal to the sum of abundances of isotopomers of \( s1 \) that produce isotopomer \( k \) of metabolite \( j \) via reaction \( i \), normalized by the steady-state concentration of metabolite \( j \) (denoted \( C_j \)). \( \beta(U(t), i, j, k) \) is defined analogously based on the abundance of isotopomers of \( s2 \). For reaction \( i \) that has one substrate, \( \alpha(U(t), i, j, k) \) is defined in the same way, while \( \beta(U(t), i, j, k) \) is equal to 1. Growth rate is denoted \( \mu \). The three terms on the right hand side of the equation (from left to right) represent the sum of production rate of isotopomer \( k \) of metabolite \( j \) by metabolic reactions, the sum of consumption rate of the isotopomer by metabolic reactions, and the consumption of the isotopomer due to cellular growth. Metabolite labeling kinetics in terms of relative abundance of mass-isotopomers was calculated by summing the computed abundance of all isotopomer with a given mass in each time point. Calculated metabolite labeling kinetics for each cell line in normoxia for both glucose and glutamine labeling were compared with experimental measurements Appendix Figure A1-4).
2.4.6 Flux balance analysis (FBA) in NCI-60 cell lines

To predict the most likely fluxes for NCI-60 cell lines, we employed the same metabolic network model used in the MFA analysis described above (Appendix Table A3) and applied the following FBA analysis to search for a steady-state flux distribution (denoted \( v \)) that matches metabolite uptake and secretion rates measured by Jain et al [58]:

\[
\min_v \sum_{i \in E} (f_i - v_i)^2 + \varepsilon \sum_i v_i^2
\]

s. t.

\[
S \cdot v = 0
\]

\[
v_i \geq 0, \quad \text{for reactions } i \text{ that are irreversible}
\]

where \( f_i \) denotes the measured uptake or secretion rate through reaction \( i \) (for every reaction \( i \) in reactions’ set \( E \)). Specifically, we used measured reactions for glucose uptake (F1) and lactate secretion (F12) taken from Jain et al [58]. Glutamine flux into TCA cycle (F11) was defined as glutamine uptake minus glutamate secretion based on measurements from Jain et al. An upper bound on acetyl-CoA demand for fatty acid biosynthesis (F14) was calculated based on the cell line specific growth rate (NCI’s DTP database http://dtp.nci.nih.gov/docs/misc/common_files/cell_list.html), assuming that fatty acids comprise less than 20% of cellular dry weight of 200 µg per 10^6 cells. \( S \) is the same stoichiometric matrix as in the MFA analysis (see Appendix Table A3). The first term of the optimization function aims to minimize the difference between measured and predicted metabolite uptake and secretion.
rates, while the second term aims to minimize the total sum of square of flux [73]. $\varepsilon$ was set to 0.001. Similar results were obtained also for lower values of $\varepsilon$.

We used a similar approach to predict likely fluxes for NCI60 cell lines using a genome-scale metabolic network model[60]. Here, we explicitly constrained the flux through nutrient uptake and byproduct secretion reactions (for F1, F11 and F12) based on the experimental measurements. We further defined a growth reaction based on cellular biomass being 60% protein, 10% DNA and RNA, and 10% lipids, and constrained cellular growth rate based on experimental data (from NCI’s DTP database). We then applied FBA in two way: (i) Optimizing for maximal ATP production rate (and then minimizing the total sum of fluxes squared as in the above analysis); (ii) Constraining oxygen consumption to be between 75-225 nmol/uL-cells/h (where the lower bound is 50% lower and the upper bound is 50% higher than the average oxygen consumption by oxidative phosphorylation measured in the iBMK cell lines), and optimizing for minimal sum of fluxes squared. Varying protein mass between 50%-90% and DNA/RNA and lipid mass between 3% and 20% changed the predicted contribution of oxidative phosphorylation by less than 1%.

2.5 Acknowledgement

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Chapter 3

Fatty Acid Labeling from Glutamine in Hypoxia Can Be Explained by Isotope Exchange without Net Reductive Isocitrate Dehydrogenase (IDH) Flux

3.1. Abstract

Acetyl-CoA is an important anabolic precursor for lipid biosynthesis. In the conventional view of mammalian metabolism, acetyl-CoA is primarily derived by the oxidation of glucose-derived pyruvate in mitochondria. Recent studies have employed isotope tracers to show that in cancer cells grown in hypoxia or with defective mitochondria, a major fraction of acetyl-CoA is produced via another route, reductive carboxylation of glutamine-derived $\alpha$-ketoglutarate (catalyzed by reverse flux through isocitrate dehydrogenase, IDH). Here, we employ a quantitative flux model to show that in hypoxia and in cells with defective mitochondria, oxidative IDH flux persists and may exceed the reductive flux. Therefore, IDH flux may not be a net contributor to acetyl-CoA production, although we cannot rule out net reductive IDH flux in some compartments. Instead of producing large amounts of net acetyl-CoA reductively, the cells adapt by reducing their demand for acetyl-CoA by importing rather than synthesizing fatty acids. Thus, fatty acid labeling from glutamine in hypoxia can be explained by spreading of label without net reductive IDH flux.

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3.2. Introduction

Oxygen limitation leads to genetic and biochemical reprogramming of central metabolism, which increases reliance on glycolysis for energy production [74, 75]. The transcription factor hypoxia inducible factor HIF1 plays a central role in cellular adaptation to hypoxia, upregulating glucose intake, glycolytic enzymes, and lactate secretion. HIF further suppresses glycolytic flux entering TCA cycle through pyruvate dehydrogenase (PDH) by inducing transcription of pyruvate dehydrogenase kinase 1 (PDK1) which is an inhibitor of PDH[76]. In addition to inhibiting glycolytic flux into TCA cycle, HIF activates c-MYC[77], which induces glutamine uptake and catabolism, generating TCA cycle intermediates[32].

Even in the presence of adequate oxygen, tumor cells manifest upregulated glycolysis relative to oxidative phosphorylation (Warburg effect)[5]. In most cases this is due to increased glycolysis that is induced by ongogene signaling rather than impairment of mitochondrial function [36]. In some tumors, however, mitochondrial function is impaired by mutated mitochondrial proteins. For example, somatic mutations in the TCA cycle genes fumarate hydratase (FH) and succinate dehydrogenase (SDH) are tumorigenic [78]. In both cases, the mutations lead to the activation of HIF, which causes a pseudohypoxic state, resulting in similar phenotypes to those of hypoxic cells even in the presence of oxygen.

An important intermediate of oxidative mitochondrial metabolism is acetyl-CoA. Cytosolic acetyl-CoA is the main precursor for de novo fatty acid biosynthesis. The canonical pathway for production of cytosolic acetyl-CoA begins with the oxidation of glucose-derived pyruvate in mitochondria (Figure 1). The resulting mitochondrial acetyl-CoA is consumed by citrate synthase to convert oxaloacetate into citrate. Citrate may then either be oxidized in TCA cycle or
shuttled to cytoplasm, where its cleavage by ATP citrate lyase produces cytosolic acetyl-CoA[79].

Recent studies have employed isotope tracing to study how acetyl-CoA is produced in mammalian cell by feeding with \[^{13}\text{C}\]-labeled glutamine, glutamate or succinate and measuring the resulting labeling of citrate and fatty acids [31, 80-85]. Experiments in liver and cardiac cells established that a fraction of citrate and fatty acid 2-carbon units originates from \(\alpha\)-ketoglutarate through reductive carboxylation of isocitrate dehydrogenase (IDH)[83-85]. More recently, reductive IDH was claimed to be particularly important in hypoxia and pseudohypoxia, where a major fraction fatty acid carbon units originates from glutamine [31, 80-82].

While these isotope tracer experiments unambiguously demonstrate reverse IDH flux, they do not address the question of whether there is actually net flux in the reductive direction. In general, isotope labeling patterns reflect gross (i.e., total) flux in a given direction, which may be offset by yet greater flux in the opposite direction and not necessarily net conversion. This key principle was elucidated more than half a century ago to refute claims that liver synthesize glucose from fatty acids based on the experimental observation that feeding cells with labeled fatty acids result in glucose labeling [86, 87]. More recently, Landau and Wahren have emphasized the difference between label incorporation and net flux [88] in the context of identification of pseudoketogenesis which can be misinterpreted to grossly overestimate ketogenesis [89-91].

In this work, we employ a quantitative flux model to examine oxidative and reductive IDH flux in cancer cells grown in hypoxia and in cells with defective mitochondria. We analyze the lung cancer cell line A549 grown in hypoxia and the osteosarcoma cell line 143B-CYTB with a
defective electron transport chain. In both of cell types reductive IDH flux was recently claimed to have a central role in lipogenesis [31, 80]. We show that the observed fatty acid labeling from glutamine does not necessarily imply net reductive IDH flux. Indeed, by placing analytical bounds on the oxidative and reductive IDH fluxes based on metabolite isotope labeling, we find evidence for oxidative net flux in pseudohypoxia and for modest or no net flux in either direction in hypoxia. Thus, reductive IDH flux is not a major net contributor to acetyl-CoA production. Instead, cells cope with limited oxidative acetyl-CoA production by reducing the biosynthetic utilization of acetyl-CoA for fatty acid synthesis [92].

3.3. Experimental Procedures

Cell lines were grown in Dulbecco’s modified Eagle media (DMEM) without pyruvate (Cellgro), supplemented with 10% dialyzed fetal bovine serum (HyClone). Isogenic 143B human osteosarcoma cells that contained (143B-CYTB) or lacked (143B-WT) a loss-of-function mutation in mitochondrial complex III were grown in an incubator containing 5% CO₂ and ambient oxygen at 37℃. For hypoxia experiments, A549 cells were grown inside a hypoxic chamber (Coy Lab) containing 1% oxygen and 5% CO₂ at 37℃. For labeling experiments, medium was prepared from DMEM without glutamine (Cellgro), with the desired isotopic form of glutamine added to a final concentration 0.584 g/l. Metabolite extractions were conducted at 70%-80% confluency.

For all metabolomic and isotope-tracer experiments, metabolism was quenched and metabolites extracted by quickly aspirating media and immediately adding -80℃ 80:20 methanol: water
extraction solution. Samples were analyzed for water soluble metabolites and saponified fatty acids by a stand-alone orbitrap mass spectrometer (Exactive) operating in negative ion mode was coupled to reversed-phase ion-pairing chromatography as previously described [66, 92]. In addition, confirmatory measurements of water soluble metabolites were acquired with a TSQ Quantum Discovery triple-quadrupole mass spectrometer operating in negative ion, multiple-reaction monitoring mode coupled to reverse-phase ion-pairing chromatography as described[93]. Data were analyzed using the MAVEN software suite [67]. Metabolite labeling patterns were adjusted for natural $^{13}$C abundance and for enrichment impurity of labeled substrate. Absolute metabolite levels were quantified as previously described [68], and normalized by packed cell volume. Acetate secretion rate was measured using the K-ACET kit (Megazyme) according to manufacturer’s instructions. Oxygen consumption was measured by a Seahorse XF24 flux analyzer (Seahorse Bioscience, North Billerica, MA). To measure oxygen uptake in hypoxia, the Seahorse instrument was placed in the hypoxia chamber with 1% oxygen.

3.4. Results

3.4.1 Analysis of feasible IDH fluxes based on the experimentally observed acetyl-CoA labeling from $[^{13}$C]-glutamine

While previous studies have shown that a major fraction of cytosolic acetyl-CoA gets labeled from $[^{1}$U-$^{13}$C]-glutamine in hypoxia and pseudohypoxia, it is possible that oxidative IDH persists and exceeds the reductive flux. Here, we employ a simple isotopomer model to probe the feasible net IDH fluxes based on experimental measurements of TCA cycle influxes and effluxes
(of α-ketoglutarate from glutamine and of 2-carbon units) and based on acetyl-CoA labeling from [U-\(^{13}\text{C}\)]-glutamine.

**Figure 3.1 Network diagram**

Assuming pseudo steady-state for metabolite labeling, the isotopomer balance model shown in Figure 3.1 results in the following balance equation for citrate m+5:

\[
v_3X_{akg5} = (v_2 + v_5)X_{cit5} \quad \text{(Eq. I)}
\]

and α-ketoglutarate m+5:

\[
v_2X_{cit5} + v_4 = (v_3 + v_6)X_{akg5} = (v_2 + v_4)X_{akg5} \quad \text{(Eq. II)}
\]
where $X_{\alpha\text{KG}5}$ and $X_{\text{cit}5}$ represent the fractional abundance of $\alpha$-ketoglutarate $m+5$ and citrate $m+5$, respectively. The left hand side in both equations represents biosynthetic routes, while the right hand side represents consumption. Given experimental measurements of glutamine flux into TCA cycle via $\alpha$KG (v4), acetyl-CoA demand for fatty acid biosynthesis (v5), labeling of citrate $m+5$, and unidirectional reductive IDH flux (v3), Equations I and II enable computation of net oxidative IDH flux (v2-v3). For simplicity, in this section, we assume zero labeling of mitochondrial acetyl-CoA from glutamine (i.e. negligible mitochondrial malic enzyme flux). This assumption tends to favor net flux in the reductive direction; hence, given our conclusions nevertheless show a propensity for net oxidative flux, it only strengthens them. Under this assumption, there is no contribution of citrate synthase to making citrate $m+5$ and the abundance of cytosolic acetyl-CoA $m+2$ equals that of citrate $m+5$.

**Figure 3.2 Glutamine intake to TCA cycle and acetyl-CoA demand.**
(a) Glutamine influx into TCA cycle inferred based on measurement of total glutamine uptake from media, minus glutamate and proline secretion, glutamine demand for protein biosynthesis, and dilution of glutamine and glutamate pools. (b) Labeling pattern of palmitate in 143B-WT and 143B-CYTB when fed with both [U-\(^{13}\text{C}\)]-glucose and [U-\(^{13}\text{C}\)]-glutamine. (c) Acetyl-CoA demand for fatty acid biosynthesis and protein acetylation.

Next, we applied this model to examine IDH fluxes in a lung cancer cell line A549 grown in hypoxia[31] and the osteosarcoma cell line 143B-CYTB having a defective electron transport chain and its isogenic wild-type cell line 143B (143B-WT)[80]. Glutamine flux into TCA cycle (v4), as measured based on glutamine uptake rate from medium, minus glutamate and proline secretion, glutamine demand for protein biosynthesis, and dilution of glutamine and glutamate pools due to cell growth, was found to be 26-35 nmol/uL-cells/h across the three cell lines (Figure 3.2a).

To quantify the rate of acetyl-CoA demand for de novo fatty acid biosynthesis, we measured the total cellular fatty acid concentration (free and lipid-incorporated), the fraction of fatty acid that is de novo synthesized (measured by monitoring fatty acid labeling in cells fed with both [U-\(^{13}\text{C}\)]-glucose and [U-\(^{13}\text{C}\)]-glutamine), and cellular growth rate [92] (Figure 3.2b). Overall, the acetyl-CoA demand for fatty acid biosynthesis is 0.55-2.24 nmol/uL-cells/h across the three studied cell lines (Figure 3.2c).

To derive an upper bound on the additional acetyl-CoA demand for protein acetylation, we computed the acetyl-CoA requirement for acetylating all proteomic lysine residues (assuming that protein amounts to 50% of cellular weight and that the frequency of lysine is 5%), which is 0.5-0.9 nmol/uL-cells/h (Figure 3.2c). To examine whether an additional demand for acetyl-CoA exists due to protein acetylation and deacetylation cycling, we measured acetate secretion to the media[94]. We find that in all three cell lines, acetate secretion is negligible (smaller than 0.02
nmol/uL-cells/h). Thus, to the extent that protein acetylation cycling occurs, it is not a major net consumer of 2C units.

Figure 3.3 Extensive labeling of acetyl-CoA from glutamine can occur without net reductive IDH flux.
(a) Fraction of cytosolic acetyl-CoA m+2 labeling from [U-\textsuperscript{13}C]-glutamine (y-axis) for various combinations of net IDH flux (represented by color) and unidirectional reductive IDH flux (x-axis) (fluxes shown in nmol/uL-cells/h). Oxidative IDH net flux is shown in blue, while reductive IDH net flux in green. The solid red line represents an upper bound on acetyl-CoA m+2 labeling when the net IDH flux is oxidative. The analysis was done based on measurements in 143B-CYTB. (b) Measured acetyl-CoA m+2 labeling (blue) versus the feasible upper bound (red) assuming oxidative IDH net flux.

Given these measurements of glutamine intake to TCA cycle (v4) and acetyl-CoA demand (v5) in 143B-CYTB, we applied Equations I and II to compute the IDH net flux, for various combinations of hypothetical cytosolic acetyl-CoA m+2 labeling and unidirectional reductive IDH flux (v3) (Figure 3.3a). We find that when feeding [U-\textsuperscript{13}C] glutamine, for a given IDH net flux (v2-v3; which determines citrate synthase rate due to mass-balance considerations, as
v1=v5-v2-v3), the fraction of acetyl-CoA m+2 increases as the reductive IDH flux (v3) increases, asymptotically reaching v4/(v4+v1) when the citrate and αKG pools are completely mixed. When IDH net flux is oxidative (v2>v3), mass-balance considerations entail that v1>v5, and hence the maximal fraction of acetyl-CoA m+2 is bounded by v4/(v4+v5), which is between 0.85 and 0.97 in the cell lines studied here (Figure 3.3b). To measure the actual cytosolic acetyl-CoA m+2 in these cell lines, we quantified fatty-acid labeling from [U-13C] glutamine by LC-MS[92] and used isotopomer spectral analysis[69]. We find that in all cases, the fraction of cytosolic acetyl-CoA m+2 is substantially lower than the feasible upper bound consistent with net oxidative IDH flux. Thus net IDH flux in the oxidative direction is possible in all three cell lines (Figure 3.3b).

3.4.2 Quantifying IDH oxidative and reductive flux via isotopic labeling of intracellular metabolites

To determine the actual direction of net IDH flux, we derive analytical bounds on oxidative and reductive IDH flux based on experimentally observed metabolite steady-state labeling patterns. For this analysis, we no longer rely on the simplifying assumption of negligible labeling of mitochondrial acetyl-CoA from glutamine. Thus, we must account for citrate synthase potentially making citrate m+5. The resulting balance equation for citrate m+5 is:

\[ v3X_{\text{akg}5} + v1X_{\text{mal}3}X_{\text{ac}2}^m = (v2 + v5)X_{\text{cit}5} \]  

(Eq. III)

where \(X_{\text{mal}3}\) and \(X_{\text{ac}2}^m\) represent the fractional abundance of malate m+3 and mitochondrial acetyl-CoA m+2, respectively. Here, we assume rapid exchange between oxaloacetate and malate (which is easier to detect via LC-MS analysis) and hence a similar labeling pattern of
both. Citrate m+5 whose only carbon #6 is unlabeled (denoted $X_{cit}$) is made through reductive carboxylation of $\alpha$-ketoglutarate m+5 and potentially through citrate synthase, depending on the positional labeling of malate m+3. Writing the balance equation for $X_{cit}$, while omitting the undetermined contribution of citrate synthase gives:

$$v3X_{akg5} \leq X_{cit}(v2 + v5) \quad \text{(Eq. IV)}$$

which combined with (III) (and assuming $v1=v5-v2-v3$ due to mass-balance considerations) gives the following lower bound on $X_{cit}$:

$$X_{cit} - X_{mat} \times X_{ac} \leq X_{cit} \quad \text{(Eq. V)}$$

The balance equation for $\alpha$-ketoglutarate m+5 is

$$v2(X_{cit} + X_{cit}e) + v4 = (v3 + v6)X_{akg5} = (v2 + v4)X_{akg5} \quad \text{(Eq. VI)}$$

which gives:

$$v2 = v4\frac{1-X_{akg5}}{(X_{akg5} - X_{cit} - X_{cit}e)} \quad \text{(Eq. VII)}$$

Taking together Eq. (V) and (VII) give a lower bound on oxidative IDH flux ($v2$):

$$v2 \geq v4\frac{1-X_{akg5}}{X_{akg5} - (X_{cit} - X_{mat} X_{ac2}) - X_{cit}e} \quad \text{(Eq. VIII)}$$

This derivation relies on the fact that the fraction of cytosolic acetyl-CoA that is m+2 ($X_{ac2}^c$) exceeds that in the mitochondrion because cytosolic citrate (the source of cytosolic acetyl-CoA, assuming negligible acetyl-CoA synthetase activity since there is no acetate in the media) has
only two sources: mitochondrial citrate (which will give equivalent labeling to the mitochondrion) or cytosolic α-ketoglutarate (which will give more labeling than the mitochondrion).

Since \( \text{cit5\_RC} \) is a specific isotopomer of citrate \( m+5 \), its abundance, \( X_{\text{cit5\_RC}} \), is no larger than \( X_{\text{cit5}} \). Hence, based on Equation VII, we get the following upper bound on \( v2 \):

\[
v2 \leq v4 \frac{1 - X_{\text{Akgs}}}{X_{\text{Akgs}} - X_{\text{cit5}} - X_{\text{cit6}}} \quad \text{(Eq. IX)}
\]

which combined with (III) gives an upper bound on reductive IDH flux (\( v3 \)):

\[
v3 \leq \left( v4 \frac{1 - X_{\text{Akgs}}}{X_{\text{Akgs}} - X_{\text{cit5}} - X_{\text{cit6}}} + v5 \right) \frac{X_{\text{cit5}}}{X_{\text{Akgs}}} \quad \text{(Eq. X)}
\]

To employ Equations VIII and X to derive bounds on the oxidative versus reductive IDH fluxes, we measured by LC-MS the labeling pattern of citrate, α-ketoglutarate, malate (Figure 3.4a). In both the wild-type and mitochondrial defective 143B cell lines, the oxidative IDH flux is significantly higher than the reductive flux, being at least 30-fold higher in 143B-WT and 2-fold higher in 143B-CYTB (Figure 3.4b). In A549 grown in hypoxia, the lower bound on oxidative IDH flux is roughly the same as the upper bound on the reductive flux, implying that there is at most modest net flux in the reductive direction (Figure 3.4b). Notably, considering the potential existence of other glutamine-consuming pathways not quantified here, glutamine flux into the TCA cycle may be overestimated in this study. However, as shown in Figure 3.4c, our results regarding no major IDH net flux still holds for substantially lower glutamine flux into TCA cycle via α-ketoglutarate.
Figure 3.4 Bounding oxidative and reductive IDH fluxes via steady-state metabolite labeling patterns.

(a) Measured fractional labeling of $\alpha$-ketoglutarate m+5, citrate m+5, and malate m+3 from [U-13C]-glutamine. (b) A lower bound on IDH oxidative flux (blue) versus upper bound on IDH reductive flux (red) calculated based on Equations VIII and X. (c) Lower bound on IDH net flux (calculated by subtracting the upper bound on IDH reductive flux from the lower bound on IDH oxidative flux), assuming potentially lower glutamine flux into TCA cycle via $\alpha$-ketoglutarate (v4). The lower bound on net oxidative IDH flux is marked with a straight line while the standard deviation is marked with a dashed line.

3.4.3 Mechanisms of redox and 2C-unit balancing in hypoxia and pseudohypoxia

To assess how redox balancing is achieved in hypoxia without reductive IDH net flux, we measured the oxygen consumption in A549 and its fraction used by oxidative phosphorylation (using respiratory chain inhibition). We find that the oxygen consumption rate is $\sim 60$ nmol/uL-
cells/h, which suffices to account for the observed oxidative TCA flux, given the that glutamine flux to α-ketoglutarate is ~35 nmol/uL-cells/h and that glutamine is only partially oxidized in TCA cycle.

In 143B-CYTB, we find that redox balance is maintained by a branched TCA cycle activity, in which succinate is made both through the oxidation of acetyl-CoA and through the reduction of oxaloacetate. Specifically, the succinate secretion rate increases from a non-detected level in 143B to ~11 nmol/uL-cells/h in 143B-CYTB. Consistent with reductive production of succinate from oxaloacetate, we observed higher pyruvate carboxylase activity in 143B-CYTB, with unlabeled malate (in cells fed [U-13C]-glutamine and unlabeled glucose) increasing from 35% in 143B to 75% in 143B-CYTB.

These observations left open the question of how cells compensate for reduced acetyl-CoA production by pyruvate dehydrogenase in hypoxia. Based on recent evidence for increased fatty acid scavenging in hypoxia[92], we considered the possibility that this might serve to reduce 2C-unit demand. Indeed, quantitative analysis reveals that A549 cells in hypoxia decrease total 2C-unit consumption for fatty acid biosynthesis by 73%. Moreover, reexamination of the fatty acid labeling patterns in Figure 3.2b revealed a large increase in the unlabeled fatty acid peak in cells fed with both [U-13C]-glucose and [U-13C]-glutamine in 143B-CYTB versus 143B-WT cells (i.e., in pseudohypoxia). Given that absolute total fatty acids concentrations are roughly the same in both cell lines (e.g., palmitate is 18.7±1.0 and 15.1±2.6 nmol/uL-cells in 143B-WT and 143B-CYTB, respectively), the higher fraction of palmitate m+0 in 143B-CYTB implies enhanced fatty acid scavenging. Integrating across all experimentally measured fatty acids and accounting for cellular doubling time, we obtain a decrease in the 2C-unit requirement of 75%.
3.5 Discussion

Several recent studies have employed isotopic tracers to investigate how acetyl-CoA is produced in cancer cell lines in hypoxia and with defective mitochondria[31, 80-82]. Their conclusion was that acetyl-CoA is primarily made through reductive carboxylation of glutamine-derived α-ketoglutarate, suggesting potential therapeutic targets along this pathway for inhibiting hypoxic tumor growth. These studies followed previous reports of IDH reductive carboxylation flux in normal liver and cardiac cells [83-85]. Here, we followed up on these studies and employed a quantitative flux model to analyze IDH flux in the same cancer cell lines. Our analysis shows that while reductive IDH flux indeed occurs in hypoxia and with mitochondrial deficiency, oxidative IDH flux persists, with net flux much less than flux in either direction.

A limitation of our analysis (as well as prior related analyses) is that it does not account for subcellular compartmentalization of most metabolites. For acetyl-CoA, we do account for the possibility of distinct labeling patterns in the mitochondria versus cytoplasm, using fatty acid labeling to infer cytosolic acetyl-CoA labeling. For other metabolites, the LC-MS approach employed here measures the isotopic labeling of the overall cellular pool, which may represent a mixture of different labeling patterns in distinct compartments (depending on the relative concentration of the metabolite in the various compartments and compartment volumes). Thus, we cannot rule out net reductive IDH flux in at least some compartments, e.g., if cytosolic and mitochondrial IDH are working in opposite directions, perhaps as a means for shuttling high energy electrons from mitochondria to cytoplasm [9, 65].

Nevertheless, a simple mechanistic explanation for the observed labeling patterns involves simultaneous oxidative and reductive IDH flux due to near equilibrium between the isocitrate
oxidation and α-ketoglutarate reductive carboxylation. Such bidirectional flux can result in extensive citrate and lipid labeling via reductive carboxylation without reductive IDH being a net contributor to citrate or acetyl-CoA production. In such cases net acetyl-CoA production may come from glucose or other carbon sources, with these influxes mixing with the larger TCA influx from glutamine via the reversible IDH reaction. The outcome is an apparent predominance of glutamine as the source of two-carbon units, despite net production coming from other sources.

More complete examination of fatty acid labeling patterns in hypoxia and pseudohypoxia reveals that glutamine labeling occurs in parallel with a rise in fatty acids that do not label from glucose or from glutamine. Such fatty acids are scavenged from media, and their assimilation into lipids decreases cellular requirements for 2C-unit production, thereby mitigating the need for either pyruvate dehydrogenase flux or reductive carboxylation.

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Chapter 4
Quantitative flux analysis reveals folate-dependent NADPH production

4.1. Abstract

ATP is the dominant energy source in animals for mechanical and electrical work (e.g., muscle contraction[1], neuronal firing[2]). For chemical work, there is an equally important role for NADPH, which powers redox defense and reductive biosynthesis of amino acids, deoxyribonucleotides, and lipids [95, 96]. The oxidative pentose phosphate pathway (oxPPP) is the most direct route for producing NADPH from glucose. However, individuals with genetic deficiency of this pathway develop normally[3, 4, 97], indicating the functional sufficiency of alternatives (e.g., malic enzyme, isocitrate dehydrogenase). To date, methods have not allowed quantitative determination of the flux contribution of different NADPH production and consumption pathways. Here we combined carbon isotope labeling experiments with direct measurement of NADPH deuterium labeling by liquid chromatography-mass spectrometry to quantitate NADPH fluxes. Surprisingly we found that, in proliferating cells, the oxidation of serine-derived one-carbon units to make 10-formyl-tetrahydrofolate is a major NADPH source. Since folate metabolism has not previously been considered an NADPH producer, confirmation of its functional significance for NADPH homeostasis was undertaken through knockdown of methylenetetrahydrofolate dehydrogenase, which decreased the cellular NADPH/NADP⁺ and GSH/GSSG ratios and increased sensitivity to oxidative stress. Thus, while the importance of folate metabolism for proliferating cells has been long recognized and attributed to its function of
producing one carbon units for nucleic acid synthesis, another crucial function of this pathway is generating reducing power.

4.2. **Result and Discussion**

Past examination of NADPH production during cell growth has analyzed metabolic fluxes in cells using $^{13}$C and $^{14}$C isotope tracers[26-29]. For NADPH metabolism, however, carbon tracers alone are insufficient, because they cannot determine whether a particular redox reaction is making NADH versus NADPH or the reaction’s fractional contribution to total cellular NADPH production. To address these limitations, we developed a deuterium tracer approach that directly measures NADPH redox active hydrogen labeling. To probe the oxPPP, we shifted cells from unlabeled to 1-$^2$H-glucose or 3-$^2$H-glucose (Figure 4.1A) and measured the resulting NADP$^+$ and NADPH labeling by liquid chromatography-mass spectrometry[66], as shown in the mass spectrum in Figure 4.1B (for associated chromatogram, see Appendix Figure B1). The M+1 and M+2 peaks in NADP$^+$ are natural isotope abundance, primarily from 13C. The difference between NADP$^+$ and NADPH reflects the redox active hydrogen labeling. The labeling of NADPH’s redox-active hydrogen is fast (t$_{1/2}$ ~ 5 min) (Figure 4.1C; note: as opposed to relative mass intensities, all fractional labeling data are corrected for natural isotope abundance). We observed quantitatively similar labeling across the four transformed mammalian cell lines that we studied: human embryonic kidney 293 cells (HEK293T), the triple negative breast cancer line MDA-MB-468, and immortalized baby mouse kidney cells with or without expression of myr-Akt (iBMK-Parental and iBMK-Akt) (Figure1D). Knockdown of the committed enzyme of the oxPPP, glucose-6-phosphate dehydrogenase, eliminated most of the labeling, confirming that the NADPH-deuterium labeling reflects oxPPP flux.
Figure 4.1 Quantitation of NADPH labeling via oxPPP and of total NADPH production.
(A) OxPPP pathway schematic highlighting the carbon atom that is released as CO$_2$ (red) and the hydrogen atoms that are transferred to NADPH: H1 (blue) and H3 (green). (B) Mass spectra of NADPH and NADP$^+$ from cells labeled with 1-^2$H$-glucose (iBMK-parental cell, 20 min). (C) Kinetics of NADPH labeling from 1-^2$H$-glucose (iBMK parental cell line). (D) NADPH labeling from 1-^2$H$-glucose (20 min) across cell lines. (E) Similar labeling from 1-^2$H$-glucose and 3-^2$H$-glucose (20 min, iBMK-parental cell line). Substrate labeling is reported for the ^2$H-transfer step: glucose-6-phosphate at C1 position for 1-^2$H$-glucose and 6-phosphogluconate for 3-^2$H$-glucose. (F) Conceptual approach to measurement of total cytosolic NADP$^+$ reduction flux. Total flux is the absolute oxPPP flux (measured based on ^14$C$-CO$_2$ excretion) divided by the fractional oxPPP contribution (measured based on NADPH ^2$H-labeling). (G) Rate of CO$_2$ release from glucose C1 and glucose C6 in HEK293T cells. The difference between the two is the absolute oxPPP flux. Results are adjusted for intracellular glucose labeling fraction. (H) Labeling rate of the oxPPP intermediate 6-phosphogluconate is consistent with oxPPP flux measured by ^14$C$-CO$_2$ release. Fraction of unlabeled of 6-phosphogluconate was measured after switching cells into U-^13$C$-glucose media. Simulated curve based on the flux that best fits the labeling data is shown in blue, and that for the flux from ^14$C$-^14$C$-CO$_2$ release measurements in green. (I) Oxidative pentose phosphate flux in different cell lines. (J) Total cytosolic NADP$^+$ reduction flux (i.e., NADPH production rate). All results are mean ± SD, N ≥ 2 biological replicates from a single experiment; in addition, all results were confirmed in N ≥ 2 independent experiments.

Since most NADPH is cytosolic[98], the ^2$H-glucose labeling results can be used to quantitate the fractional contribution of the oxPPP to total cytosolic NADPH production: The fraction of cytosolic NADPH made by the oxPPP is given by isotope flux balance with each glucose producing two NADPH (one each from glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase):

$$\text{Fraction}_{\text{NADPH from oxPPP}} = 2 \times (\text{NADP}^2\text{H/Total NADPH}) \times (\text{^2H-G6P/Total G6P})^{-1} \times C_{\text{KIE}} \quad (\text{Eqn. 1})$$

The terms in parentheses are the fractional redox active hydrogen labeling of NADPH (Figure 4.1D) and of glucose-6-phosphate at targeted position (Appendix Figure B2). The term C$_{\text{KIE}}$ accounts for the deuterium kinetic isotope effect[99] [100], under the assumption that ^2$H-labeling does not alter total oxPPP flux (see Methods). This assumption is supported by the similar NADPH labeling from 1-^2$H$-glucose, where the kinetic isotope effect impacts the
committed oxPPP step, and 3-\textsuperscript{2}H-glucose, which impacts a downstream pathway step (Figure 4.1E), and the lack of change in oxPPP metabolite concentrations upon feeding \textsuperscript{2}H-labeled glucose (Appendix Figure B3). Using either form of \textsuperscript{2}H-glucose, we find that oxPPP accounts for 30-50\% of overall NADP\textsuperscript{+} reduction depending on the cell line (for results also including alternative ways of correcting of the deuterium kinetic isotope effect, see Appendix Figure B4).

The inferred fractional contribution of oxPPP to NADPH production can be used to deduce the total cytosolic NADPH production rate, which is equal to the absolute oxPPP flux divided by the fractional contribution of oxPPP to NADPH production (Figure 4.1F). To this end, we measured absolute oxPPP flux using two orthogonal approaches. The first approach measures \textsuperscript{14}C-CO\textsubscript{2} release from 1-\textsuperscript{14}C versus 6-\textsuperscript{14}C-glucose. It relies on the fact that the oxPPP selectively releases C1 of glucose as CO\textsubscript{2}, whereas all other CO\textsubscript{2}-releasing reactions are downstream of triose phosphate isomerase (TPI). As TPI renders C1 and C6 of glucose indistinguishable (both positions become C3 of glyceraldehyde-3-phosphate), the difference in CO\textsubscript{2} release from C1 versus C6, multiplied by two, gives the absolute rate of NADPH production via oxPPP (Appendix Figure B5). The second approach, which relies on this straightforward principle that higher flux gives faster labeling, is based on the kinetics of 6-phosphogluconate labeling from U-\textsuperscript{13}C-glucose. While both approaches gave consistent fluxes (Figure 4.1H, Appendix Figure B6, Appendix Table B 1), the radioactive measurement was more precise (Appendix Figure B6E) and therefore we rely on it in further calculations. As confirmation of its specificity, we knocked down glucose-6-phosphate dehydrogenase and observed markedly reduced \textsuperscript{14}C-CO\textsubscript{2} release from the oxPPP (Figure 4.1I). In the absence of such knockdown, depending on the cell line, the observed oxPPP flux ranges from 1 - 2.5 nmol uL\textsuperscript{-1} h\textsuperscript{-1} (where volume is the packed cell volume; Figure 4.1I). In combination with the fractional NADPH labeling, we deduced a total cytosolic
NADPH production rate of 10 nmol uL⁻¹ h⁻¹ (Figure 4.1J), which is 5 – 20% of the glucose uptake rate.

The above results argue that the oxPPP accounts for less than half of NADPH cytosolic production during cell growth. To examine other NADPH-producing pathways (Figure 4.2A), we conducted ²H-labeling experiments with 2,3,3,4,4-²H-glutamine and 2,3,3-²H-aspartate. Downstream products of glutamine can potentially transfer ²H to NADPH via glutamate dehydrogenase or malic enzyme, while downstream products of aspartate may do so via isocitrate dehydrogenase (Appendix Figure B 7 - 8). We observed identical mass spectra for NADP⁺ and NADPH after feeding the deuterium-labeled glutamine and aspartate (Figure 4.2B, C). While not ruling out the possibility that these enzymes may be acting on unlabeled substrates (e.g., due to enzyme-mediated hydrogen-deuterium exchange, see Appendix Figure B9), the simplest interpretation is a limited role, at least in these cell lines, for glutamate dehydrogenase, malic enzyme, and isocitrate dehydrogenase in cytosolic NADPH production. Given recent evidence that malic enzyme is particularly important in cancer[101, 102], we used an orthogonal approach based on feeding U-¹³C-glutamine and measuring labeling of pyruvate, lactate and citrate to further evaluate its activity (Appendix Figure B10). While such carbon tracer studies cannot distinguish between NADH-dependent and NADPH-dependent malic enzyme, they put an upper bound on their collective activities. This upper bound ranged from 15% to 50% of cytosolic NADPH production depending on the cell line.

As the above experiments raise the possibility that the studied enzymes collectively might not account for all NADPH production, we used a genome-scale human metabolic model[60] to
identify other potential NADPH producing pathways. We constrained the model based on the observed steady-state growth rate, biomass composition, and metabolite uptake and excretion rates of iBMK-parental cells, without enforcing any constraints on NADPH production routes. The model, assessed via flux balance analysis with an objective of minimizing total enzyme expression requirements and hence flux[61] (see Methods), predicted that both the oxPPP and malic enzyme contribute ~ 30% of NADPH (Figure 4.2D). Surprisingly, however, the greatest NADPH production was predicted to come from serine/glycine catabolism in a cycle mediated by the cofactor tetrahydrofolate (THF). The prediction of a major contribution of folate metabolism to NADPH production is robust to the specific choice of biomass composition (in terms of cellular mass taken by protein, DNA, RNA and lipids), as repeating the analysis for a range of physiologically possible biomass compositions predicts a median contribution of 24% (Methods). Considering an alternative objective function of maximizing growth rate further predicts a potentially major contribution of folate metabolism to NADPH production. Specifically, calculating the range of feasible flux through reactions in the model under the assumption of maximal growth rate, MTHFD is identified as having the highest possible flux among all NADPH producing reactions (Appendix Figure B11).

The main folate-dependent NADPH-producing pathway was predicted to involve transfer of a one carbon unit from serine to THF, followed by oxidation of the resulting product (methylene-THF) by the enzyme methylene tetrahydrofolate dehydrogenase to form the purine precursor formyl-THF with concomitant NADPH production. To assess whether this pathway indeed contributes to NADPH production, we fed cells 2,3,3-2H-serine and observed labeling of both NADP+ and NADPH. The NADP+ labeling results from incorporation of the serine-derived formyl-THF one carbon unit into NADP+’s adenine ring. Relative to NADP+, however, the
labeling pattern of NADPH was shifted towards more heavily labeled forms, indicating specific labeling of NADPH’s redox active hydrogen (Figure 4.2E, Appendix Figure B12). Thus, serine-driven folate metabolism contributes to NADPH production, with the extent of labeling consistent with the contribution of up to 40% of cytosolic NADPH (see Methods, Appendix Figure B12).

**Figure 4.2 Potential NADPH production routes.**
(A) Canonical NADPH production pathways. (B) NADPH and NADP⁺ isotopic distribution (without correction for natural isotope abundances) after 48 h incubation of HEK293T cells with 2,3,3,4,4-²H-glutamine to probe NADPH production via glutamate dehydrogenase and malic enzyme. For pathway schematic and data on other cell lines, see Appendix Figure B 8 and 9. The indistinguishable labeling of NADPH and NADP⁺ implies lack of NADPH redox active hydrogen labeling. (C) NADPH and NADP⁺ isotopic distribution after 48 h incubation HEK293T cells with 2,3,3-²H-aspartate to probe NADPH production via IDH. See also Appendix Figure B 7. The indistinguishable labeling of NADPH and NADP⁺ implies lack of redox active hydrogen labeling. (D) NADPH production routes predicted by experimentally-constrained genome-scale flux balance analysis. (E) NADPH and NADP⁺ isotopic distribution after 48 h incubation of HEK293T cells with 2,3,3-²H-serine (no glycine present in the media) to probe NADPH production via 10-formyl-THF pathway. The greater abundance of more heavily labeled forms of NADPH relative to NADP⁺ indicates redox active hydrogen labeling. For pathway schematic and data on other cell lines, see Appendix Figure B 12. (F) Relative NADPH/ NADP⁺ ratio in HEK293T cells with knockdown of various potential NADPH producing enzymes: glucose-6-phosphate dehydrogenase (G6PD), cytosolic malic enzyme (ME1), cytosolic and mitochondrial
isocitrate dehydrogenase (IDH1 and IDH2), transhydrogenase (NNT), and cytosolic and mitochondrial methylene tetrahydrofolate dehydrogenase (MTHFD1 and MTHFD2). Plotted ratios are relative to vector control knockdown. Results are mean ± SD, N ≥ 2 biological replicates from a single experiment; in addition, all results were confirmed in N ≥ 2 independent experiments.

As an orthogonal approach to assessing the contribution of different pathways to NADPH production, we knocked down in HEK293T cells a variety of potential NADPH-producing enzymes and measured the cellular NADPH/NADP⁺ ratio (Figure 4.2F). While knockdown of malic enzyme 1 (ME1), cytosolic or mitochondrial NADP-dependent isocitrate dehydrogenase (IDH1 and IDH2) and transhydrogenase did not significantly impact NADPH/NADP⁺, knockdown of glucose-6-phosphate dehydrogenase or either isozyme of methylene tetrahydrofolate dehydrogenase (MTHFD1, cytosolic, makes NADPH; MTHFD2, mitochondrial, can make NADH or NADPH) substantially decreased it. These observations further support the primacy, at least in this growing cell line, of the pentose phosphate pathway and folate pathway in NADPH production.

The importance of both isozymes of methylene tetrahydrofolate dehydrogenase suggests that cytosolic and mitochondrial folate metabolism (Figure 4.3A) both contribute to NADPH homeostasis. In the cytosol, purine synthesis is a major consumer of 10-formyl-THF. Each purine ring contains two formyl groups; thus, the cytosolic 10-formyl-THF production rate must be at least twice the purine biosynthetic flux, which can be calculated from the measured cellular purine content and growth rate. The most direct path to cytosolic 10-formyl-THF is via MTHFD1 with concomitant NADPH production (Figure 4.3A, solid blue lines). Alternatively, 10-formyl-THF could potentially be made from formate initially generated in the mitochondrion (Figure 4.3A, dashed lines). To distinguish between these possibilities, we fed U-¹³C-glycine,
which contributes selectively to mitochondrial one-carbon pools (Figure 4.3A, green lines). Glycine is assimilated intact into purines, resulting in M+2 labeling of ATP (Figure 4.3B); however, we did not observe any M+1, M+3, or M+4 labeling of ATP, indicating that mitochondrial-derived one-carbon units do not contribute to purine biosynthesis. In contrast, feeding of U-13C-serine resulted in extensive purine labeling (Figure 4.3C), consistent with serine contributing to purines both via glycine and via one carbon units derived from serine C3. Quantitative analysis of the ATP and serine labeling patterns after feeding U-13C-serine implies that most one-carbon units assimilated into purines come from serine (Appendix Figure B13). Consistent with cytosolic oxidation of serine-derived one-carbon units being coupled to NADPH production, knockdown of MTHFD1 nearly eliminated NADPH redox-active hydrogen labeling from 2,3,3-2H-serine (Figure 4.3D). Assuming that all 10-formyl-THF production for purine synthesis is coupled via MTHFD1 to NADP⁺ reduction, the total NADPH production rate is ~ 2 nmol uL⁻¹ h⁻¹ (Figure 4.3E) or ~ 25% of total cytosolic NADPH flux. To probe potential further oxidation of serine or glycine-derived one-carbon units to CO₂, we fed 3-14C-serine and 2-14C-glycine and observed 14C-CO₂ release, implying that the THF pathway is runs in excess of one-carbon demand yielding additional NADPH at the rate of 2 NADPH/CO₂ (Figure 4.3E).

Given the importance of cytosolic folate metabolism as a source of NADPH, we investigated the consequences of removal of serine, a non-essential amino acid, from the media. As is typical for cell lines lacking amplification of the committed serine biosynthetic enzyme 3-phosphoglycerate dehydrogenase[18], serine removal impaired cell growth (Figure 4.3F). Moreover, consistent with one important function of serine being NADPH production, serine removal decreased NADPH/NADP⁺ (Figure 4.3G). Glycine is both a product of serine metabolism, and itself a potential source of one-carbon units via the mitochondrial glycine cleavage system, whose
expression has been linked to oncogenic transformation\[103\]. We accordingly tested the impact of both removing serine and increasing glycine in the culture media. Interestingly, we found that increased glycine further impaired cell growth and further decreased the NADPH/NADP\(^+\) ratio (Figure 4.3F, G). These results are consistent with increased glycine impairing cytosolic methylene-THF production by serine hydroxymethyl transferase, via some combination of product inhibition or reverse flux through this near-equilibrium reaction (Appendix Figure B14).

The above results establish a major contribution of serine-driven cytosolic one-carbon metabolism in NADPH homeostasis. The observation that knockdown of MTHFD2 also alters NADPH/NADP\(^+\) suggests a role also for mitochondrial one-carbon metabolism. To probe specifically mitochondrial folate metabolism, we fed \(^{14}\)C-labeled glycine and monitored radioactive CO\(_2\) release (Figure 4.3H, Appendix Figure B15). The glycine cleavage system releases glycine C1 as CO\(_2\), while transferring glycine C2 to THF, making methylene-THF. There was no conversion of glycine into pyruvate, ruling out CO\(_2\) release via TCA cycle (Appendix Figure B16). Notably, almost as much radioactive CO\(_2\) was released from 2-\(^{14}\)C-glycine as from 1-\(^{14}\)C-glycine (Figure 4.3H), indicating that most mitochondrial methylene-THF is fully oxidized to CO\(_2\). As expected based on the pathway, this release was blocked by knockdown of THFD2 (Appendix Figure B17). Such complete one-carbon unit oxidation may be beneficial for reducing the cellular glycine concentration. In addition, it produces mitochondrial NADPH. Thus, two likely major functions of mitochondrial folate metabolism are glycine detoxification and NADPH production.
Figure 4.3 Quantitation of NADPH production by 10-formyl-THF pathway.
(A) Pathway schematic showing relevant reactions and their compartmentation. Serine C3 and resulting one-carbon units are shown in blue, glycine C1 in red, and glycine C2 in green. (B) Glycine and ATP labeling pattern after 24 h incubation of HEK293T cells with U-13C-glycine. The lack of M+3 and M+4 ATP indicates that no glycine-derived one-carbon units contribute to purine synthesis. (C) Serine and ATP labeling pattern after 24 h incubation of HEK293T cells with U-13C-serine. The presence of M+1 to M+4 ATP indicates that serine contributes carbon to purines through both glycine and one-carbon units. (D) Fraction of NADPH labeled at the redox active hydrogen after 24 h incubation with 2,3,3-2H-serine in cells with stable MTHFD1 or MTHFD2 knockdown. (E) NADPH production from the 10-formyl-THF pathway. Error bars show the feasible range of NADPH production depending on the cofactor preference of MTHFD2. (F) Relative cell number after culturing HEK293T cells for 3 days in regular DMEM, DMEM with no serine, and DMEM with no serine with 12.5-times the normal concentration of glycine (5 mM instead of 0.4 mM). (G) Relative NADPH/NADP⁺ ratio after culturing HEK293T cell for 3 days in regular DMEM, DMEM with no serine, and DMEM with no serine and 12.5-times the normal concentration of glycine, normalized to cells grown in DMEM. (H) CO₂ release rate from glycine C1 and glycine C2, measured by incubating cells in media containing trace amount of 1-¹⁴C-glycine or 2-¹⁴C-glycine. (I) GSH/GSSG ratio in HEK293T cells with stable MTHFD1 or MTHFD2 knockdown (normalized to ratio in cells with non-targeting shRNA, shNT). (J) Relative growth of shNT, shMTHFD1 and shMTHFD2 cells during 48 h exposure to H₂O₂. Cell numbers are normalized to untreated samples. (K) Fractional death as measured by trypan blue staining of shNT, shMTHFD1 and shMTHFD2 cells after 24 h exposure to 250 µM H₂O₂. (L) Fractional death after 24 h exposure to 300µM diamide. (M) Relative ROS levels in shNT, shMTHFD1 and shMTHFD2 cells, measured using DCFH assay. The results were normalized to shNT cells. Results are mean ± SD, N=3.

One important role of NADPH is antioxidant defense. Consistent with folate metabolism being a significant NADPH producer, antifolates have been found to induce oxidative stress[104]. To more directly link folate-mediated NADPH production with cellular redox defenses, we measured glutathione, reactive oxygen species, and hydrogen peroxide sensitivity of MTHFD1 and MTHFD2 knockdown cells. Knockdown of either isozyme decreased the ratio of reduced to oxidized glutathione (Figure 4.3I) and impaired resistance to oxidative stress induced by hydrogen peroxide (Figure 4.3J, K) or diamide (Figure 4.3L). Moreover, consistent with a particular importance of mitochondrial NADPH in controlling free radicals, MTHFD2 knockout specifically increased reactive oxygen species (Figure 4.3M).
A major open question regards the relative use of NADPH for biosynthesis versus redox defense. To address this, we compared total cytosolic NADPH production (as measured above) to consumption for biosynthesis (Figure 4.4A, Methods) based on the measured cellular content of DNA, amino acids, and lipids (Appendix Figure B18-19, Appendix Table B2); their production routes (measured by $^{13}$C tracer experiment, see Methods, Appendix Figure B20-21); and cellular growth rate (Appendix Table B3). In DNA biosynthesis, ribonucleotide reductase consumes one NADPH per nucleotide, and methylation of dUMP to dTMP consumes one NADPH. The associated average cellular NADPH consumption rate for deoxyribonucleotide synthesis is $\sim$1.2 nmol $\mu$L$^{-1}$ h$^{-1}$ (Appendix Figure B22). In amino acid synthesis, NADPH is required to make arginine, which is included in the medium, and proline, which is not; the associated NADPH consumption ranges from 0.6 to 1.1 nmol $\mu$L$^{-1}$ h$^{-1}$ across the tested cell lines. In fatty acid synthesis, NADPH is consumed in reducing acetyl groups, with the NADPH requirement depending on the extent to which fatty acids are synthesized de novo versus imported from serum. To evaluate this, cells were cultured in a combination of both U-$^{13}$C-glucose and U-$^{13}$C-glutamine and the extent of de novo lipid synthesis determined by LCMS (Appendix Figure B18, 19). The associated NADPH consumption was about $\sim$7 nmol $\mu$L$^{-1}$ h$^{-1}$, a majority of total cytosolic NAPDH. The overall demand for NADPH for biosynthesis is $>80\%$ of total cytosolic NADPH production (Figure 4.4B), suggesting that at least in transformed cells growing under aerobic conditions, a large majority of cytosolic NADPH is devoted to biosynthesis, not redox defense.
To evaluate NADPH consumption for redox defense under overt redox stress, we treated HEK293T cells with hydrogen peroxide at a concentration that blocks growth without causing substantial cell death. Using the methods described above, the total cytosolic NADPH production rate was quantified as 5.5 nmol µL⁻¹ h⁻¹ (Appendix Figure B23), about half the rate in freely growing cells. Thus, consistent with most cytosolic NADPH in growing cells being used for biosynthesis, growth-inhibiting oxidative stress decreases rather than increases cytosolic NADPH production.

The production of NADPH by the oxidative pentose phosphate pathway, which makes the nucleotide building block ribose, and by the 10-formyl-THF pathway, which contributes to purine synthesis, leads to an inherent coupling of nucleotide synthesis with NADPH production. These reactions coupled to nucleotide synthesis together produce in growing cells roughly the
amount of NADPH required for replication of cellular lipids (Figure 4.4B). Interruption of this intrinsic coordination by feeding of purines can impair cell growth[105]. In non-growing cells, or other cases where NADPH needs outstrip production coupled to nucleotide synthesis, it is likely that other pathways, e.g., malic enzyme and IDH, will be of greater importance than observed here.

The contribution of the 10-formyl-THF pathway to NADPH production is particularly interesting in light of the importance of metabolism of serine and glycine, the major carbon sources of this pathway, to cancer growth[106]. Serine synthesis is promoted by the cancer-associated M2 isozyme of pyruvate kinase (PKM2) and yet more directly by amplification of 3-phosphoglycerate dehydrogenase, the committed serine biosynthetic enzyme[18, 20]. The present data suggest that the importance of serine arises from its dual roles as a source of both one carbon units and NADPH. In this respect, it is intriguing that PKM2, in addition to sensing serine[107, 108], is inactivated by oxidative stress[109]. Such inactivation should increase 3-phosphoglycerate and thus potentially serine-driven NADPH production.

In addition to synthesizing serine, rapidly growing cells avidly consume glycine[58]. Intriguingly, while only intact glycine (and not glycine-derived one carbon units) are incorporated into purines, knockdown of the glycine cleavage system impairs cancer growth[103]. We find that most glycine-derived one-carbon units are oxidized fully to CO₂ (Figure 4.3H), arguing against the glycine cleavage system’s primary role being to support mitochondrial methylation. Instead, its function may be simultaneous elimination of unwanted glycine and production of mitochondrial NADPH.
Understanding NADPH’s production and consumption routes is essential to global understanding of metabolism. The approaches provided here will enable evaluation of these routes in different cell types and environmental conditions. Analogous measurements for ATP, achieved first more than a half century ago[5], have formed the foundation for much of subsequent metabolism research. Given NADPH’s comparable role in medically important processes including lipogenesis, oxidative stress, and tumor growth[38], quantitative analysis of its metabolism may prove of similar importance.

4.3. Methods

4.3.1. Cell lines and culture conditions

HEK293T and MDA-MB-468 were purchased from ATCC. Immortalized baby mouse kidney epithelial cells (iBMK) with and without myr-AKT were a gift of Eileen White [47, 62]. All cell lines were grown in Dulbecco’s modified eagle media (DMEM) without pyruvate (CELLGRO), supplemented with 10% dialyzed fetal bovine serum (Invitrogen) in a 5% CO2 incubator at 37°C. Knockdown of enzymes were by infection with lentivirus expressing the corresponding shRNA (shMTHFD1,#1:CCGGGCTGAAGAGATTGGGATCAAACCTCGAGTTTGATCCCCAATCTCTTCAGCTTTTTTG,#2:CCGGGCTGAAGAGATTGGGATCAAACCTCGAGTTTGATCCCCAATCTCTTCAGCTTTTTTG;shMTHFD2,#1:CCGGGCCATTGATGCTCGGATATTTCTCGAGAAATATCCGAGCATCAATGGCTTTTTG,#2:CCGGGCCATTGATGCTCGGATATTTCTCGAGAAATATCCGAGCATCAATGGCTTTTTG;shG6PD,#1:CCGGCAACAGATACAAGAACGTGGAGCTCGAGTTCTTGTATCTGTTGTTTTTG,#3:CCGGGGCTGATGAAGAGATCGAGTTCTTGTATCTGTTGTTTTTG).
GGGTTTCTCGAGAAACCCACTCTTCTTCATCAGCTTTTTT;shNNT:CCGGCCCTATGGTT
AATCCAACATTCTCGAGAATGTTGGATTAACCATAGGGTTTTTG;shME1,#1:CCGGGC
CTTCAATGAACGCGCTATTTCTCGAGAATAGGCCGTTCATTGAAGGCTTTTT;#2:CCG
GCCAACAATATAGTTGTGGTTCTCGAGAACCACAAAAACTATATTTGTGGTTTTTG)
and puromycin selection. To obtain the shRNA-expressing virus, pLKO-shRNA vectors (Sigma-Aldrich) were cotransfected with the third generation lentivirus packaging plasmids (pMDLg, pCMV-VSV-G and pRsv-Rev) into HEK293T cells using FuGENE 6 Transfection Reagent (Promega), fresh media added after 24 h, and viral supernatants collected at 48 h. Target cells were infected by viral supernatant (diluted 1:1 with DMEM; 6 µg/ml polybrene), fresh DMEM added after 24 h, and selection with 3 µg/ml puromycin initiated at 48 h and allowed to proceed for 2 – 3 days. Thereafter, cells were maintained in DMEM with 1 µg/ml puromycin. For IDH1 and IDH2 knockdown, siRNA targeting IDH1 and IDH2 (Thermo Scientific, 40nM) were transfected into H293T cells using Lipofectamine™ RNAiMAX (Invitrogen). Knockdown of the enzymes was confirmed by immunobloting with commercial antibodies: G6PD (Bethyl Laboratories), MTHFD1 and MTHFD2 (Abgent), IDH1 (Proteintech Group) and IDH2 (Abcam) or Quantitive RT-PCR probes (ME1 and NNT, Applied Biosystems) (Appendix Figure B24). For enzymes with more than one successful knockdown sequences, knock down data presented here are mean ± SD of two independent experiments using two different shRNA sequence for each target gene.
4.3.2. Measurement of metabolite concentrations and labeling patterns

Cells were harvested at ~80% confluency. For metabolomic experiments, medium was replaced every 2 days and additionally 2 h before metabolome harvesting and/or isotope tracer addition. Metabolism was quenched and metabolites extracted by aspirating media and immediately adding -80°C 80:20 methanol:water. Supernatants from two rounds of methanol:water extraction were combined, dried under N₂, resuspended in HPLC water, placed in 4°C autosampler, and analyzed within 6 h to avoid NADPH degradation.

The LC-MS method involved reversed-phase ion-pairing chromatography coupled by negative mode electrospray ionization to a stand-alone orbitrap mass spectrometer (Thermo Scientific) scanning from m/z 85-1000 at 1 Hz at 100,000 resolution [21, 65, 66] with LC separation on a Synergy Hydro-RP column (100 mm × 2 mm, 2.5 μm particle size, Phenomenex, Torrance, CA) using a gradient of solvent A (97:3 H₂O/MeOH with 10 mM tributylamine and 15 mM acetic acid), and solvent B (100% MeOH). The gradient was 0 min, 0% B; 2.5 min, 0% B; 5 min, 20% B; 7.5 min, 20% B; 13 min, 55% B; 15.5 min, 95% B; 18.5 min, 95% B; 19 min, 0% B; 25 min, 0% B. Injection volume was 10 μL, flow rate 200μL/min, and column temperature 25 °C. Data were analyzed using the MAVEN software suite[67]. Data from ¹³C-labeling experiments were adjusted for natural ¹³C abundance and impurity of labeled substrate; those from ²H-labeling were not adjusted (natural ²H abundance is negligible)[110]. Absolute concentration of 6-phospho-gluconate was quantified by comparing the signal of intracellular compound to signal of isotopic labeled internal standard.
4.3.3. Fractional labeling of NADPH redox active site

The fractional NADPH redox active site labeling ($x$) was measured from the observed NADPH and NADP$^+$ labeling patterns from the same sample. We calculated $x$ to best fit the steady-state mass distribution vectors of NADPH and NADP$^+$ ($M_{NADPH}$ and $M_{NADP^+}$) by least square fitting in MATLAB (function: lsqcurvefit).

\[
M_{NADP^+} = \begin{bmatrix}
m_0 & m_1 & m_2 & \cdots & m_N \\
M+0 & M+1 & M+2 & \cdots & M+N
\end{bmatrix}
\]

\[
M_{NADPH} = \begin{bmatrix}
m_0 \times (1 - x) \\
m_1 \times (1 - x) + m_0 \times x \\
m_2 \times (1 - x) + m_1 \times x \\
\vdots \\
m_N \times (1 - x) + m_{N-1} \times x \\
m_N \times x
\end{bmatrix}
\]

4.3.4. Network analysis of potential NADPH producing pathways

To assess the potential contribution of various metabolic pathways to NADPH production, we analyzed feasible steady-state fluxes of a genome-scale human metabolic network model[60]. The glucose (98 nmol/(uL*h)), glutamine (40 nmol/(uL*h)), and oxygen uptake rates (21 nmol/(uL*h)), and lactate (143 nmol/(uL*h)), alanine (2 nmol/(uL*h)), pyruvate (15 nmol/(uL*h)), and formate (< 0.25 nmole/(µL*h)) excretion rates were set to experimental
measured fluxes in the iBMK cell line, as measured by a combination of electrochemistry (glucose, glutamine, lactate on YSI7200 instrument, YSI, Yellow Springs, OH), LC-MS (alanine, pyruvate with isotopic internal standards), fluorometry (oxygen on XF24 flux analyzer, Seahorse Bioscience, North Billerica, MA), and NMR (formic acid by $^1$H 500 MHz, Bruker, 10 µM limit of detection). The uptake of amino acids from DMEM media were bounded to not more than a third of that of glutamine, which is a loose constraint relative to experimental observations in iBMK cells and in NCI-60 cells[58]. Biomass requirements were based on the experimentally determined growth rate of the iBMK cell-line (Appendix Table B 3) with protein, fatty acids and nucleotides accounting for 60%, 10% and 10% of the total cellular dry mass, respectively, based on experimental measurements. Steady-state intracellular fluxes that best fit these experimental constraints were then selected by solving the flux balance equations in MATLAB with the objective function formulated to minimize the sum of total fluxes[61].

### 4.3.5. Correction for deuterium’s kinetic isotope effect

Correction for the deuterium kinetic isotope effect was based on the assumption that total metabolic fluxes are not impacted. This assumption minimizes the correction factor for the kinetic isotope effect. Let $x$ be the fractional labeling of the relevant substrate hydrogen, $F_U$ be the NADPH production flux from unlabeled substrate and $F_L$ be the NADPH production flux from the labeled substrate.

\[
\frac{F_L}{F_U} = \frac{x / (V_H / V_D)}{1-x} \quad \text{(Eqn.1)}
\]

\[
F_{\text{reaction}} = F_L + F_U = F_L \frac{V_H / V_D}{x} + x(1-V_H / V_D) \quad \text{(Eqn.2)}
\]
\( F_l / x \) is the flux in cases without a discernible kinetic isotope effect (e.g., for \(^{13}\)C). The remaining term is the correction factor for the kinetic isotope effect:

\[
C_{KIE} = V_H / V_D + x (1 - V_H / V_D) \quad \text{(Eqn.3)}
\]

For results assuming that total metabolic flux is decreased by the introduction of \(^2\)H-labeled tracer (i.e., that \( C_{KIE} = V_H / V_D \)), see Appendix Figure B4.

4.3.6. **ROS measurement, cell proliferation and cell death assay**

ROS measurement followed published protocols[111]. Briefly, HEK293T cells were incubated with 5 \( \mu \)M CM-H2DCFDA (Invitrogen) for 30 min. Cells were trypsinized, and mean FL1 fluorescence was measured by flow cytometry. Cell proliferation was measured by trypsinizing cells and counting using a Beckman's Multisizer 4 COULTER COUNTER. To measure cell death, cells were stained with Trypan Blue. Stained/unstained cells were counted and cell death percentages tabulated.

4.3.7. **Quantitation of NADPH consumption by reductive biosynthesis**

The general strategy for measuring consumption fluxes was as follows: (i) identifying the biomass components produced in cells grown in DMEM by NADPH-driven reductive biosynthesis (these are DNA, proline, and fatty acids); (ii) determining the biomass fraction of each component in each cell line; (iii) quantifying the cellular growth rate \( R_{growth} = \ln (2) / t_{1/2} \); (iv) measuring the fractional contribution of different biosynthetic routes to each biomass...
component via experiments with $^{13}$C-labeled glucose and/or glutamine and LC-MS analysis; (v) computing the average number of NADPH per unit of biomass component, which equals the sum of the fractional contribution of each route multiplied by the number of NADPH consumed by that route; and (vi) determining NADPH consumption as follows:

$$\text{Consumption flux} = \frac{\text{product abundance}}{\text{cell volume}} \times R_{\text{growth}} \times \frac{\text{average NADPH}}{\text{product}}$$

(Eqn. 4)

For analytical details related to DNA, see Appendix Figure B 18,22; proline, Appendix Figure B20; and fatty acids, Appendix Figure B19,21.

### 4.3.8. Measurement of $^{14}$C-CO$_2$ release

Radioactive CO$_2$ released by cells from positionally-labeled substrates was measured by trapping the CO$_2$ in filter paper saturated with 10 M KOH as previously described$^{14}$. Cells were grown in tissue culture flasks with DMEM medium with less than normal bicarbonate (0.74 g/L) and addition of HEPES buffer (6 g/L, pH 7.4). Amounts of tracers are listed in Appendix Table B 4. Total CO$_2$ release rate was calculated by normalizing the radioactive CO$_2$ production rate to packed cell volume and intracellular substrate labeling fraction, which was experimentally measured by culturing cells in 100% $^{13}$C-labeled substrates and LC-MS analysis.
4.3.9. **Contribution of 10-formyl-THF pathway to cytosolic NADPH production**

Quantitation of the fractional NADPH contribution of the 10-formyl-THF pathway was performed analogously to the oxPPP, feeding labeled serine instead of $1^{-2}\text{H}-\text{glucose}$:

\[
\text{Fraction}_{\text{NADPH from THF pathway}} = \frac{\text{NADP}^2\text{H}}{\text{Total NADPH}} \times \frac{\text{Total substrate}}{\text{H} - \text{substrate}} \times \text{Correction for kinetic isotope effect} \quad (\text{Eqn 5})
\]

The kinetic isotope effect of isolate methylene tetrahydrofolate dehydrogenase is large ($2 > V_H/V_D > 4$) [112]. Taking serine labeling as a surrogate for methylene-THF and 10-formyl-THF labeling, and assuming $V_H/V_D$ of 3, we obtain a fractional pathway contribution in the range of 10 – 40% of total NADPH.

4.3.10. **Fractional labeling of cytosolic formyl groups from U-$^{13}\text{C}$-serine**

Cells were cultured with media containing U-$^{13}\text{C}$-serine for 48 h, washed three times with cold PBS to remove extracellular serine, extracted, and the intracellular labeling pattern analyzed by LC-MS for ATP (representing purines; there is no labeling of ribose-phosphate based on LC-MS measurements), glycine, and serine. The purine ring has 5 carbons: 1 from $\text{CO}_2$, 2 from glycine, and 2 from formyl groups (10-formyl-THF). Assume that $\text{CO}_2$ labeling is negligible. Let $X_{\text{ATP},i}$ and $X_{\text{Gly},j}$ represent the experimentally observed fraction ATP and glycine with i and j labeled carbons. The cytosolic 10-formyl-THF labeling fraction, $x$, was fit by least squares:
\[ X_{\text{ATP-0}} = X_{\text{Gly-0}} \times (1-x)^2 \]
\[ X_{\text{ATP-1}} = 2 \times X_{\text{Gly-0}} \times x \times (1-x) \]
\[ X_{\text{ATP-2}} = X_{\text{Gly-2}} \times (1-x)^2 + X_{\text{Gly-0}} \times x^2 \]
\[ X_{\text{ATP-3}} = 2 \times X_{\text{Gly-2}} \times x \times (1-x) \]
\[ X_{\text{ATP-4}} = X_{\text{Gly-2}} \times x^2 \quad \text{(Eqn 6)} \]

**4.3.11. NADPH production from 10-formyl-THF pathway**

NADPH production from 10-formyl-THF pathway was quantified by tracking its end products: 10-formyl-THF consumed by purine synthesis and CO$_2$. (Formate excretion into media is below detection limit of NMR.) All 10-formyl-THF consumed by purine synthesis is generated in cytosol and associated with production of 1 NADPH. For each CO$_2$ released from glycine C2 or serine C3, 1 NADPH is produced from 10-formyl-THF oxidation, and either zero or one NADPH is produced via methylene tetrahydrofolate dehydrogenase (MTHFD1 uses NADP$^+$, but we do not know whether the CO$_2$-releasing pathway is active in the cytosol; MTHFD2 may use either NAD$^+$ or NADP$^+$).

Assuming equal production of NADPH and NADH by MTHFD2, and equal oxidation of serine to CO$_2$ in the cytosol and mitochondrion, NADPH production via 10-formyl-THF pathway is:

\[ Flux_{\text{NADPH from THF pathway}} = 2 \times Flux_{\text{purine synthesis}} + 1.75 \times Flux_{\text{CO}_2 \text{ from serine C3}} + 1.5 \times Flux_{\text{CO}_2 \text{ from glycine C2}} \quad \text{(Eqn. 7)} \]
4.4. Acknowledgement

The iBMK parental and Akt cell lines were generously provided by Dr. Eileen White. The $^{14}$C labeled CO$_2$ release experiments were conducted with the great help of Dr. Eric Suh and Dr. Hilary Coller. NMR measurement of formate was with help of Dr. Ian Lewis. We thank Dr. Hakim Djaballah and the High-Throughput Drug Screening Facility at MSKCC for supplying the hairpins, and Dr. Matthew Vander Heiden and his lab members for helpful discussions. This work was supported by Stand Up To Cancer and NIH R01 grants CA163591, AI097382, and CA105463, P01 grant CA104838, P50 grant GM071508. Jing Fan is a HHMI fellow supported by a Howard Hughes Medical Institute (HHMI) international student research fellowship. Jurre J. Kamphorst is a Hope Funds for Cancer Research Fellow supported by the Hope Funds for Cancer Research (HFCR-11-03-01).
Chapter 5
Human phosphoglycerate dehydrogenase (PHGDH) produces “oncometabolite” D-2-hydroxy-glutarate and influences histone methylation

5.1. Abstract

Human phosphoglycerate dehydrogenase (PHGDH), the first enzyme in the serine/glycine biosynthetic pathway, is genomically amplified in a wide range of tumors. Cells with such amplification are shown to be dependent on PHGDH. However, PHGDH knockdown does not significantly reduce the intracellular serine level, nor can it be rescued by addition of exogenous serine, suggesting that PHGDH has an additional important function that is independent of serine biosynthesis and downstream metabolic processes. Here we show that PHGDH has a moonlighting activity catalyzing the NADH dependent reduction of α-ketogutarate to D-2-hydroxy-glutarate, which has been recently identified as an oncometabolite and acts as a histone demethylation inhibitor. Knockdown of PHGDH significantly decreases cellular 2-hydroxy-glutarate (2-HG) level as well as histone methylation without significantly changing metabolites in serine/glycine metabolism or the TCA cycle. Reduced histone methylation in PHGDH knockdown cells can be reversed by addition of D-2HG ester. These results suggest that PHGDH plays an important role in epigenetic regulation and supports cancer growth by producing 2HG.
5.2. Introduction

Recent discoveries suggest that metabolic enzymes can behave like oncogenes[12]. Among them, isocitrate dehydrogenase (IDH) is one of the most well studied cases. Mutations of IDH1 and IDH2 are associated with many brain cancers and leukemia [8, 13]. The common feature of these mutations is the production of D-2-HG [9, 14], which influences epigenetic regulation, including histone demethylation and 5’-methylcytosine hydroxylation[15, 16], and alone is sufficient to promote leukemogenesis [17].

Another example is phosphoglycerate dehydrogenase (PHGDH). Human phosphoglycerate dehydrogenase (PHGDH) catalyzes the first reaction of de novo serine biosynthetic pathway, producing 3-phosphohydroxypyruvate (3-PHP) by NAD\(^+\) dependent oxidation of 3-phophoglycerate (3-PG). This conversion is reversible, and thermodynamically favors the direction from 3-PHP to 3-PG, while the reaction is driven towards serine synthesis by the irreversible production of serine. Recently PHGDH has been found genomically amplified in a wide range of tumors[37, 113], and the amplification enables the cell growth in serine free media. Especially, the amplification has been linked to the formation of melanoma and breast cancer. Suppression of PHGDH inhibits growth of PHGDH amplified cells both \textit{in vitro} and \textit{in vivo} [18, 20]. However, the mechanism by which PHGDH amplification supports cancer growth is not yet clear. The most direct hypothesis is that PHGDH amplification shunts metabolic flux away from glycolysis to \textit{de novo} serine synthesis, producing serine and glycine, which are not only important precursors for protein and glutathione, but can also be further metabolized to donate one carbon units required for nucleotide biosynthesis, methylation, and NADPH production [11, 114]. However, this metabolic role cannot completely explain the PHGDH dependence,
particularly because cells are able to take up substantial amount of serine and glycine from the extracellular environment. Even in PHGDH amplified cell lines, e.g. breast cancer cell line MDA-MB-468, BT-20 and melanoma cell line SK-MEL-28, only less than 20% of serine is generated from \textit{de novo} synthesis in standard culture condition (Appendix Figure C 1).

Interestingly, though PHGDH knockdown inhibits cell growth, it does not significantly reduce intracellular serine level, and the growth inhibition cannot be rescued by addition of exogenous serine or serine ester[18].

This led us to hypothesize that PHGDH carries another metabolic or regulatory function in addition to the production of serine and downstream metabolites. In this work, we show that PHGDH catalyzes the production of D-2-hydroxy-glutarate from $\alpha$-ketoglutarate, a structural analog of the thermodynamically favored natural substrate, 3-PHP. In investigated cancer cells, the PHGDH amplification further causes histone hyper-methylation likely due to the over-production of D-2-hydroxy-glutarate inhibiting histone demethylase.

In addition to providing a new mechanism by which PHGDH amplification contributes to cancer growth, our discovery identifies a new natural source of D-2HG. As D-2HG has been shown to have important epigenetic regulation functions, expression of PHGDH may play a significant regulatory role in cells without isocitrate dehydrogenase mutations.
5.3. Result

5.3.1. Moonlighting activities of Human PHGDH

PHGDH belongs to the D-isomer specific 2-hydroxyacid dehydrogenase family. The thermodynamically favored natural substrate of human PHGDH, 3-PHP, is structurally highly similar to various compounds in central metabolism, including pyruvate (one phosphate shorter), oxaloacetate (substitute phosphate group with carboxylic group), and α-ketoglutarate (one carbon longer) (Appendix Figure C 2). Previous studies have shown that the E.coli homolog of PHGDH, serA, is capable of producing 2-HG from AKG at appreciable rates[115], while such activity has not been found in the rat enzyme [116], or test for human enzyme. To test the activity of PHGDH using these compounds as alternative substrates, we measured enzyme activity by monitoring NADH decrease using recombinant human PHGDH in 200mM Tris buffer (pH=7.6) at 37C. Enzyme activity was observed using oxaloacetate or alpha-ketoglutarate (AKG) as substrates, but not pyruvate at up to 10mM (Figure 5.1A, Appendix Figure C 2).

The products of these enzymatic reaction were further analyzed using LC-MS confirming that, similar to the reaction using 3-PHP as substrate, PHGDH reduces the α-keto group of oxaloacetate or α-ketoglutarate using NADH and converts them into malate and 2-hydroxyglutarate (2-HG) respectively (Figure 1B, Appendix Figure C 3). To confirm that this activity is indeed due to PHGDH, rather than contamination of other enzymes from the E.coli expression system, the same enzymatic assay was performed with human galactokinase, expressed and purified with the same protocol, as a control. The production of 2HG or malate was not detected with this control enzyme (Appendix Figure C 3). The 2HG production activity was further validated using PHGDH prepared from a different construct, and similar activity was
observed (Appendix Figure C 3). We also tested the same reactions using NADPH as cofactor. PHGDH shows very low activity for all three reactions using NADPH or NADP⁺ as cofactor. These results prove human PHGDH is capable of catalyzing the NADH dependent conversion between AKG and 2HG, or OAA to malate.

![Figure 5.1](image)

**Figure 5.1** Human PHGDH catalyzes NADH dependent alpha-ketoglutarate reduction to D-2-hydroxy-glutarate.

(A) Absorbance change with time after addition of 5mM AKG to reaction buffer with 1mM NADH, with or without PHGDH. The absorbance at 340nm is proportion to NADH concentration (B) D-2-hydroxy-glutarate level change in the same experiment was measured by mass spectrometer (C) chromatography of L-2HG standard, D-2HG standard, PHGDH reaction product and mixture of L- and D-2HG standard. Mean ± SD, N=3
5.3.2. Human PHGDH produces D-2-hydroxy-glutarate

Although the intracellular level of oxaloacetate (OAA) is very low in mammalian cells, α-ketoglutarate (AKG) is abundant, especially cancer cells. Therefore, it is likely that PHGDH carries substantial α-ketoglutarate reduction flux in vivo. This fact, together with the increasing number of recent studies that highlight the connection between 2HG and cancer, led us to hypothesize that production of 2HG by PHGDH is critical to the role of this enzyme in cancer cells.

There are two types of 2HG present in mammalian cells, L-2HG (i.e. S-2HG) and D-2HG (i.e. R-2HG). High levels of both D- and L-2HG inhibit histone and DNA methylation [15], while only D-2HG stimulates EglN prolyl hydroxylases[117]. Specifically, the level of D-2-hydroxyglutarate is greatly elevated in many cancer types harboring mutant isocitrate dehydrogenase 1 or 2 (IDH1 and IDH2), including gliomas and leukemias[9, 14]. Mutations in the active site of IDH cause the enzyme to gain the function of NADPH-dependent D-2HG production from α-ketoglutarate, an activity not present in wild type IDH[14]. A recent study shows that D-2HG but not L-2HG is sufficient to promote leukemogenesis, and its effects are reversible [17]. These discoveries established that D-2HG particularly acts as an “oncometabolite”[118], and might be more active for epigenetic regulation in general.

To determine the stereo-specificity of PHGDH, we performed GC-MS analysis of the enzyme product using a two step derivatization as described before [119]. Samples were derivatized with (R)-2-butanol followed by acetic anhydride before analysis with GC-MS. Reduction product of PHGDH elutes at the same time as D-2HG standard (Figure 5.1C), suggestion same as mutant
IDH, PHGDH superficially produces D-2HG. This is consistent with the fact that PHGDH contain a D-specific 2-hydroxy acid reductase domain. Furthermore, as a parallel approach, we tested the NAD⁺ dependent oxidative reaction using L- or D- malate and L- or D-2HG. We found PHGDH has activity to catalyze the NAD⁺ dependent D-2HG oxidation and D-malate oxidation, but does not act on the L- stereo form (Appendix Figure C 4). This further confirms that 2HG produced by PHGDH is specifically the D-form.

5.3.3. Kinetic parameters of various PHGDH activities

We measured the kinetic parameters of PHGDH catalyzing various reactions (37°C, pH 7.6), including (1) the main physiological function of 3PG oxidation, (2) OAA reduction, and (3) AKG reduction (Methods). The intracellular concentrations of related substrates 3-PG, NAD⁺, OAA, AKG, and NADH, were measured in MAD-MB-468 cell line by LC-MS using isotopic internal standards. (method, Table 1).

Table 5.1 Kinetic parameters of different reactions catalyzed by PHGDH, comparing with intracellular concentrations of the corresponding substrates in MDA-MB-468 cells

<table>
<thead>
<tr>
<th>Reaction</th>
<th>kinetic parameter</th>
<th>intracellular concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-PG -&gt; 3-PHP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kcat [1/min]</td>
<td>5.0 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>Km, NAD⁺ (mM)</td>
<td>0.02 ± 0.003</td>
<td>0.99 ± 0.01</td>
</tr>
<tr>
<td>Km, 3-phosphoglycerate (mM)</td>
<td>0.20 ± 0.25</td>
<td>0.18 ± 0.01</td>
</tr>
<tr>
<td>OAA -&gt; MAL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kcat [1/min]</td>
<td>13.4 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>Km, NADH (mM)</td>
<td>0.005 ± 0.01</td>
<td>0.37 ± 0.02</td>
</tr>
<tr>
<td>Km, OAA (mM)</td>
<td>6.2 ± 1.1</td>
<td>Not detectable</td>
</tr>
<tr>
<td>AKG -&gt; 2-HG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kcat [1/min]</td>
<td>5.2 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>Km, NADH (mM)</td>
<td>0.005 ± 0.001</td>
<td>0.37 ± 0.02</td>
</tr>
<tr>
<td>Km, alpha-ketoglutarate (mM)</td>
<td>0.7 ± 0.4</td>
<td>0.55 ± 0.02</td>
</tr>
</tbody>
</table>
The enzyme has the highest $K_{cat}$ for OAA reduction, the $K_{cat}$ values for 3-PG oxidation and AKG reduction are similar. As expected, affinities for different substrates correlate with similarities to the main reaction substrate. The enzyme is saturated for NAD and NADH in vivo, but the intracellular level of 3PG, OAA and AKG are all under the $K_m$. This is consistent with the observation that elevation of 3PG causing by inhibition pyruvate kinase M2, can lead to increased de novo serine synthesis flux[107]. The fact that the enzyme is unsaturated for AKG suggests the D-2HG production flux in the cells is sensitive to AKG level.

According to Michaelis–Menten kinetics, these kinetic parameter together with substrate concentrations suggest that in the cell, the 2HG production flux can be >10% of serine synthetic flux, which has been shown considerably high in many cancer cells[18, 20]. Considering that other than dilution, there is no known reaction consumes 2HG at a significant rate, this production flux is relatively high and can be crucial for determine steady state intracellular D-2HG level. Thus, 2HG production is an important function of PHGDH.

We also assayed kinetics for malate and 2HG oxidation. However, the activity is very low and thus the kinetic parameters cannot be determined accurately. This is consistent with the fact that PHGDH thermodynamically favors reduction directions. Furthermore, as the intracellular level of 2-HG and D-malate are low in cells without IDH mutation, their oxidation flux via PHGDH should be minimal.
5.3.4. **PHGDH is an important natural source for D-2HG *in vivo***

To test whether the production of 2-HG by PHGDH is significant in the cell, we generated two PHGDH knockdown cell lines from MDA-MB-468, a breast cancer cell line with genetically amplified PHGDH (Figure 5.2A). Consistent with previous observation, both knockdowns of PHGDH inhibited cell growth (Figure 5.2B). We performed metabolomic analysis of the control and knockdown cell lines. Significant drop of 3-phosphoserine, a direct downstream metabolite in *de novo* serine synthesis pathway, in the knockdown cells confirms the effective knock down of PHGDH enzymatic activity. Intracellular 2HG level decreases to ~50% upon knock down, suggesting PHGDH is an important source of 2HG *in vivo*. (note: though we can separate L- and D-2HG in enzymatic assay by GC-MS, cell extract has a lot less 2HG concentration and the sample is more complex, such analysis is difficult in cell extract. So what is showing here is combined D- and L-2HG level by LC-MS. Drop in D-2HG level may be more profound.) Level of other related compounds, including serine and TCA cycle intermediates AKG and malate, are not significantly affected by PHGDH knock down. This can be attributed to the fact that intracellular serine is mostly acquired by uptake from media. And there are normally high fluxes through AKG and malate via multiple enzymes, most importantly TCA cycle, thus PHGDH is relatively minor contributor in consuming or making these compounds. However, on the other hand, the basal flux through 2-HG is presumably low in cells without IDH mutation, PHGDH is a significant source and controls the level of 2HG. Similar results have been observed in another PHGDH amplified cell line, BT-20 (Appendix Figure C 5A). These results further support the hypothesis that it is most likely that the PHGDH amplification supports cancer growth by over producing D-2-HG. However, it is worth noting that PHGDH is not the only source that can produces 2HG, and to what extent PHGDH level controls intracellular 2HG level is cell line
dependent and related to availability of other potential 2HG producing enzyme. For example, in HCC70, another PHGDH amplified cell line which very high basal 2HG level, likely due to mutation in other enzymes, knock down of PHGDH does not significantly decrease 2HG level. (Appendix Figure C 5B).

**Figure 5.2** PHGDH knockdown in PHGDH amplified MDA-MB-468 breast cancer cell line causes decreased growth rate, 2-HG level, and histone methylation. (A) Confirmation of PHGDH knockdown by Western blot. (B) Relative cell number of control cell line and PHGDH cell line after 3 days. Cell number normalized to day zero. Mean ± SD, N=3 (C) Relative metabolite levels in PHGDH knock down cell lines, normalized to control cell line. Mean ± SD, N=3 (D) Histone H3 methylation level in control and PHGDH knock down cell.
5.3.5. **PHGDH level influences histone methylation**

Recent study revealed 2HG is an inhibitor of histone demethylation. As a result of high 2HG production, histone is hypermethylated in cells with mutant IDH[15]. To test if the 2HG level change caused by PHGDH amplification is sufficient to change histone methylation status, histone from control and PHGDH knockdown cells were tested for multiple types of methylation. A strong global decrease in histone methylation level was observed in both of the knockdown cell lines (Figure 5.3D), while there is no significant decrease in serine or SAM level in knockdown cells (Figure 5.3C). This observation suggests the decrease in histone methylation is not likely to be a result of lacking one carbon unit available for methylation, but rather a result of lower level of 2HG in PHGDH knockdown cells.

![Western blot of histone H3 and different methylation forms](image)

**Figure 5.3** Reduced histone methylation in PHGDH knock down cells can by reversed by addition of D-2HG.

Western blot of histone H3 and different methylation forms in PHGDH knockdown of MDA-MB-468 cells treated with vehicle control (DMSO) or 250μM D-2-HG-TFMB for 48h, comparing with control MDA-MB-468 cells.
To further test if this decrease can be reversed by addition of 2HG, knockdown cells were treated with 250μM of cell permeable D-2HG TFMB ester. Generally, methylation level increases to the level similar to shNT cells upon treatment. These results reveal that 2HG level change in response to PHGDH activity is important for regulating histone methylation and downstream events. Thus, PHGDH amplification in cancer cells is likely to cause histone hyper-methylation.

5.4. Discussion

To our knowledge, this work provides the first direct evidence that human PHGDH has substantial activity of NAD dependent OAA reduction and αKG reduction. Particularly, PHGDH produces significant amount of D-2HG from AKG in vivo, which is reported to promote cell growth. In addition its connection with cancer, 2HG, especially D-2HG, may play may an important role in epigenetic regulation in a wider range of cell types. Though it is established that in cancer cells with IDH mutation, extremely high concentration of 2HG is a product of mutant IDH. The natural source of 2HG in cells without such mutation is not yet clear. One known enzyme to produce 2HG is malate dehydrogenase, whose main function, i.e. NADH-dependent OAA reduction to malate, has high similarity with PHGDH. MDH is shown to produce 2-hydroxyglutarate that causes 2-hydroxyglutaric aciduria, a toxic built up of 2HG that may lead to cancer, when the 2HG is not cleared effectively by 2-hydroxyglutarate dehydrogenase in brain [120]. However, malate dehydrogenase specifically produces L-malate and L-2HG. Our results identify PHGDH as an important source of D-2HG. Comparing to mutant IDH, the production of D-2HG via PHGDH does not require mutation. Thus the importance of this activity is not limited to only PHGDH amplified cancer cells, but may also play a significant role in epigenetic regulation of other cell type. In a more general term, this
observation provides an example that side reaction products generated naturally by metabolic reactions can be an important regulator for cellular function.

The enzyme kinetic study presented in this work suggests the D-2HG production flux via PHGDH to be >10% of de novo serine synthesis flux, and is sensitive to cellular AKG level. Thus both PHGDH level and AKG level are important for controlling intracellular D-2HG level. However, the measured $K_{\text{cat}}$ by in vitro enzymatic assay appear to be low for both main reaction using 3-PG as substrate or moonlight activity using AKG as substrate. It is shown that at least PHGDH carries high flux for its main activity supporting de novo synthesis of serine in vivo[20]. Achieving such flux would require extremely abundant enzyme. It is highly likely that the activity in vivo is actually much higher than measured in vitro. This could be a result of lost of activity during purification, or more likely, missing activating regulation. Though not tested for AKG reduction activity, previous study using PHGDH purified from HEK293T cells instead of E.coli expression system reported similar $K_{\text{cat}}$ for 3PG oxidation reaction [121], suggesting the low activity is not likely due to lack of covalent modification in human cells. It is more possible that there are critical small molecules act as allersteric activators. For example, recent study reported 2-phospho-glycerate is an activator of PHGDH, at least for the 3PG oxidation activity[122].

We have shown that in PHGDH amplified cells, 2HG level change corresponding to PHGDH level can influence histone methylation. However, the mechanism that 2-HG regulates histone methylation is not yet completely clear. It has been reported that 2-HG inhibits histone demethylase by competing with aKG [15]. It is surprising that the change in 2HG level upon
PHGDH knockdown result in such profound change in histone methylation. Given that 2HG is the only metabolite change significantly and consistently in both PHGDH knockdown cell lines that is known to influence histone methylation and growth (Appendix Figure C 6), our results points to the possibility that 2HG regulates histone methylation by other mechanism beyond competition, which is consistent with the fact we do not see significant changes in DNA methylation (Appendix Figure C 7), or that the 2HG produced by PHGDH is localized in specific compartment. It is also possible that availability of methyl-donor is another contributing factor given the close connection between PHGDH and serine. Though we did not see significant change in SAM level upon PHGDH knockdown, we further tested if cellular one carbon unit is preferably derived from serine made de novo versus uptake from media using U-13C-glucose labeling. The result does not support such metabolic channeling (Appendix Figure C 8).

In addition to previously proposed hypothesis that PHGDH may be important for purine and glutathion generation [19], facilitating glutamine utilization by TCA cycle[18], and act as a non-metabolic binding partner of FOXM1[123], our finding reveals production of 2HG is another mechanism to explain the PHGDH dependence of PHGDH amplified cancer cell. This is consistent with the observation that growth defect caused by PHGDH knockdown cannot be rescued by serine. In ordered to test if such growth inhibition can be rescue by addition of D-2HG, we treated MDA-MB-468 or BT-20 cells with or without PHGDH knock down with 250μM cell permeable D-2HG TMFB ester. Comparing with non-treated cells, the growth defect in PHGDH knockdown cells, normalized to control cells, reduced significantly (Appendix Figure C 9). However, 2HG ester also appears to have a toxic effect. The toxicity is likely a result of derirvertiztion reagent or contamination during ester synthesis (Appendix Figure C 9). To draw certain conclusion, more careful experiments with other form of D-2HG derivative are still
needed. Nevertheless, the demonstration of 2HG production activity and importance of such activity points to a potential therapeutic strategy targeting PHGDH amplified cells, i.e. targeting the side reaction using AKG as substrate instead of the main reaction. This strategy can be potentially of lower toxicity as it will not influence serine metabolism and downstream pathways, which is important to normal tissue as well, but superficially inhibit the production of the oncometabolite.

5.5. Methods

5.5.1. Enzyme activity assay

Two types of recombinant human PHGDH were tested for its side reaction activity: His-tagged PHGDH is purchased BPS Biosciences, strep-tag PHGDH is a gift from Dr. Olszewski. Similar AKG and OAA reduction activities are found with both PHGDH. The kinetic parameters were measured using enzyme from BPS Biosciences, in 200mM Tris buffer (pH=7.6) at 37°C. Reaction rate was measured by monitoring the NADH concentration change, which can be read out as absorption at 340nm (BioTek, Synergy HT). To test the 3PG oxidation reaction, 200mM of hydrazine was added to the buffer to drive this thermodynamically unflavored reaction continuously. K_m values were measured using 500 nmol enzyme, various concentration of one interested substrate, and saturated concentration of the other substrate. K_cat were measured by varying enzyme concentration from 0 to 1μmol and saturated substrates. Blank was measured and subtracted using same reaction set up but substitute PHGDH with same concentration of human galactokinase, which is expressed and purified with the same protocol as PHGDH. All
substrates were from Sigma. For mass spectrometer based analysis for reaction products, reactions were quenched with 4 volume of methanol, and centrifuged to remove protein. Supernatant was further diluted or derivertized for measurement by LC-MS or GC-MS.

### 5.5.2. Separation of L- and D-2-hydroxyglutarate

To determine the chirality of 2HG, samples were derivertized according to protocol modified from previous report [119]. Samples were dried down under N2 flow, then resuspended in (R)-2-butanol 1M HCl and heated for 2h at 100°C. Product was dried down under N2 flow, and resuspended in 1:1 pyridine : acetic anhydride, heated for 30min at 100°C.

After drying the product, samples were dissolved in chloroform, and analyzed by a GC-MS unit, a Aglient 7890 A GC system with Aux EPC column (30m*0.25mm, 0.25um film thickness) coupled with TOF mass spectrometer in negative mode. The temperature gradient used in this analysis is: 100°C for 3min, 4°C / min from 100°C to 230°C, 15 °C / min from 230 °C to 300 °C, 300 °C for 5min.

### 5.5.3. Cell lines and culture conditions

MDA-MB-468 and SK-MEL-28 cells were purchased from ATCC. All cell lines were grown in Dulbecco’s modified eagle media (DMEM) without pyruvate (CELLGRO), supplemented with 10% dialyzed fetal bovine serum (Invitrogen) in a 5% CO₂ incubator at 37°C.

Knockdown of enzymes were by infection with lentivirus expressing the PHGDH shRNA (#1: TRCN0000233031, #2: TRCN0000028532, sigma) and puromycin selection. To obtain the shRNA-expressing virus, shRNA vectors (Sigma-Aldrich) were cotransfected with lentivirus packaging plasmids (you can reference the following paper) into HEK293FT cells using X-
tremeGENE HP DNA Transfection Reagent (Roche, Catlog # 6366236001). Viral supernatants were collected 24h later and fresh medium as added to HEK293FT cells. Target cells were infected by viral supernatant (diluted 1:1 with fresh DMEM). Such treatment was repeated three times, followed by selection with 2 µg/ml puromycin initiated at day 4 and allowed to proceed for 2 – 3 days. Thereafter, cells were maintained in DMEM with 2 µg/ml puromycin.

5.5.4. **Measurement of metabolite concentrations**

Cells were harvested at ~80% confluency. For metabolomic experiments, medium was replaced every 2 days and additionally 2 h before metabolome harvesting. Metabolism was quenched and metabolites extracted by aspirating media and immediately adding -80°C 80:20 methanol:water. Supernatants from two rounds of methanol:water extraction were combined, dried under N₂, resuspended in HPLC water for analysis.

The LC-MS method involved reversed-phase ion-pairing chromatography coupled by negative mode electrospray ionization to a stand-alone orbitrap mass spectrometer (Thermo Scientific) scanning from m/z 85-1000 at 1 Hz at 100,000 resolution [21, 65, 66] with LC separation on a Synergy Hydro-RP column (100 mm × 2 mm, 2.5 µm particle size, Phenomenex, Torrance, CA) using a gradient of solvent A (97:3 H₂O/MeOH with 10 mM tributylamine and 15 mM acetic acid), and solvent B (100% MeOH). The gradient was 0 min, 0% B; 2.5 min, 0% B; 5 min, 20% B; 7.5 min, 20% B; 13 min, 55% B; 15.5 min, 95% B; 18.5 min, 95% B; 19 min, 0% B; 25 min, 0% B. Injection volume was 10 µL, flow rate 200µl/min, and column temperature 25 °C. Data were analyzed using the MAVEN software suite[67]. Absolute concentrations were quantified by comparing the signal of isotopic labeled intracellular compound to signal of internal standard as described before[68].
5.5.5. Immunoblot

The following antibodies were used: H3 (Cellsignal), H3K9Me2 (Active Motif), H3K27Me2 (Active Motif), H3K27Me3 (Active Motif), H3K4Me3 (Abcam), and PHGDH (Sigma).

5.5.6. Synthesis of D-2HG TFMB ester

To a solution of D-α-hydroxyglutaric acid disodium salt (38mg, 0.2mmol), N,N’-dicyclohexylcarbodiimide (2.5 equiv), 4-Dimethylaminopyridine (1 equiv) in dichloromethane (0.5ml) was added 3-(trifluoromethyl)benzyl bromide (0.15ml, 5 equiv). The mixture was allowed to stir at room temperature overnight. The organics were washed with water, saturated Na₂CO₃, saturated NaCl, and were dried over Na₂SO₄. Concentration was purified on silica gel, eluting with 7:3 hexane: ethyl acetate.

5.6. Acknowledgement

We thank Dr. Kellen Olszewski for generously sharing the enzyme and helpful discussion, and Sisi Zhang for help with GC-MS analysis. Jing Fan is supported by a Howard Hughes Medical Institute (HHMI) international student research fellowship.
Appendix
Appendix A  Additional Information for Chapter 2

Appendix Table A1. Doubling time of iBMK cells

<table>
<thead>
<tr>
<th></th>
<th>Parental</th>
<th>Ras</th>
<th>Akt</th>
<th>Parental-hypoxia</th>
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</thead>
<tbody>
<tr>
<td>doubling time / h</td>
<td>24</td>
<td>22</td>
<td>21</td>
<td>48</td>
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</table>

Appendix Table A2. Fatty acid contents in iBMK cells.
Fatty acid contents in units of nmol/μl cells.

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<tr>
<th></th>
<th>Parental</th>
<th>Ras</th>
<th>Akt</th>
<th>Parental-hypoxia</th>
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</thead>
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<tr>
<td>C16:0</td>
<td>5.5</td>
<td>7.8</td>
<td>8.1</td>
<td>10.2</td>
</tr>
<tr>
<td>C16:1</td>
<td>1.6</td>
<td>3.1</td>
<td>2.2</td>
<td>1.3</td>
</tr>
<tr>
<td>C18:0</td>
<td>2.8</td>
<td>3.0</td>
<td>4.4</td>
<td>7.8</td>
</tr>
<tr>
<td>C18:1</td>
<td>6.9</td>
<td>8.8</td>
<td>8.1</td>
<td>10.7</td>
</tr>
</tbody>
</table>
Appendix Table A 3 Metabolic flux distributions of parental, Ras, Akt cells in normoxia and parental iBMK cells in hypoxia.

Fluxes are obtained as described in the Methods. The table shows the best estimate and standard deviation. Fluxes are in units of nmole/ (h * μl cells).

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Reaction description</th>
<th>Atom mapping</th>
<th>Constrain</th>
<th>Parent</th>
<th>Ras</th>
<th>Akt</th>
<th>Parental</th>
<th>Parental</th>
<th>RasAct</th>
<th>Parental</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>1/2 glucose.ext -&gt; pyruvate</td>
<td>abc-&gt;abc</td>
<td>1</td>
<td>172.6</td>
<td>349.320</td>
<td>309.7</td>
<td>7.8</td>
<td>25.32.</td>
<td>0.1</td>
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<tr>
<td>F2</td>
<td>pyruvate + NAD^+ -&gt; acetyl-CoA (m) + CO2 + NADH</td>
<td>abc-&gt;bc + a</td>
<td>0</td>
<td>20.5</td>
<td>10.8245</td>
<td>4.7</td>
<td>&lt;0.1</td>
<td>0.5 0.1</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>F3</td>
<td>acetyl-CoA (m) + malate + NAD^+ -&gt; citrate + NADH</td>
<td>abcd+ef-&gt;dcbfea</td>
<td>0</td>
<td>26.4</td>
<td>11.3304</td>
<td>8.4</td>
<td>&lt;0.1</td>
<td>0.5 0.1</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>F4</td>
<td>citrate + NAD^+ -&gt; alpha-ketoglutarate + CO2 + NADH</td>
<td>abcd&gt;abc&gt;def</td>
<td>0</td>
<td>30.0</td>
<td>14.8326</td>
<td>35.2</td>
<td>0.1</td>
<td>0.9 0.1</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>F5</td>
<td>alpha-ketoglutarate + CO2 + NADH -&gt; citrate + NAD^+</td>
<td>abcd&gt;def&gt;abcdef</td>
<td>0</td>
<td>5.3</td>
<td>4.95</td>
<td>20.1</td>
<td>0.6</td>
<td>0.4 0.7</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>F6</td>
<td>alpha-ketoglutarate + NAD^+ + FAD -&gt; fumarate + CO2+</td>
<td>abcd&gt;1/2 bcde + 1/2</td>
<td>0</td>
<td>77.2</td>
<td>64.6846</td>
<td>57.1</td>
<td>&lt;0.1</td>
<td>1.7 &lt;0.</td>
<td>&lt;0.1</td>
<td></td>
</tr>
<tr>
<td>F7</td>
<td>malate/OAA + NAD^+ -&gt; pyruvate + CO2 + NADH</td>
<td>abcd -&gt; abc</td>
<td>0</td>
<td>7.2</td>
<td>12.0 &lt;0.1</td>
<td>&lt;0.1</td>
<td>3.8</td>
<td>8.0 &lt;0.</td>
<td>&lt;0.1</td>
<td></td>
</tr>
<tr>
<td>F8</td>
<td>pyruvate + CO2 + NADH -&gt; malate/OAA + NAD^+</td>
<td>abc + d -&gt; abcd</td>
<td>0</td>
<td>8.5</td>
<td>5.47</td>
<td>8.0</td>
<td>1.2</td>
<td>1.0 1.3</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>F9</td>
<td>citrate -&gt; malate + acetyl-CoA (c).ext</td>
<td>abcd-&gt;fcb+a + ed</td>
<td>0</td>
<td>2.1</td>
<td>1.5 4.3</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2 0.4</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>F10</td>
<td>other sources (e.g. fatty acid) -&gt; acetyl-CoA (m)</td>
<td>ab -&gt; ab</td>
<td>0</td>
<td>5.9</td>
<td>5.95</td>
<td>3.7</td>
<td>0.8</td>
<td>0.7 0.9</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>F11</td>
<td>glutamine -&gt; alpha-ketoglutarate</td>
<td>abcde -&gt; abcde</td>
<td>1</td>
<td>52.5</td>
<td>54.7570</td>
<td>42.0</td>
<td>&lt;0.1</td>
<td>1.6 &lt;0.</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>F12</td>
<td>pyruvate + NADH -&gt; lactate.ext + NAD^+</td>
<td>abc -&gt; abc</td>
<td>1</td>
<td>150.8</td>
<td>345.288</td>
<td>297.0</td>
<td>8.8</td>
<td>26.33</td>
<td>0.3</td>
<td></td>
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<tr>
<td>F13</td>
<td>malate+ NAD^+ -&gt; OAA + NADH</td>
<td>abcd -&gt; abcd</td>
<td>0</td>
<td>54.2</td>
<td>48.1658</td>
<td>56.9</td>
<td>1.0</td>
<td>3.7 1.6</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>F14</td>
<td>acetyl-CoA (c) -&gt; biomass</td>
<td>abcd&gt;abc&gt;def</td>
<td>0</td>
<td>0.4</td>
<td>0.1 1.6</td>
<td>6.9</td>
<td>0.8</td>
<td>0.2 1.0</td>
<td>0.5</td>
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<td>F15</td>
<td>citrate.ext -&gt; citrate</td>
<td>abcd&gt;abc&gt;def</td>
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<td>77.2</td>
<td>64.6846</td>
<td>57.1</td>
<td>&lt;0.1</td>
<td>1.7 &lt;0.</td>
<td>&lt;0.1</td>
<td></td>
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<tr>
<td>F16</td>
<td>malate/OAA -&gt; fumarate/OAA</td>
<td>abcd-&gt;1/2 abcd + 1/2</td>
<td>0</td>
<td>17.0</td>
<td>16.020</td>
<td>12.0</td>
<td>6.1</td>
<td>8.8 7.7</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>F17</td>
<td>1/2 glucose + NAD -&gt; serine + NADH</td>
<td>abc -&gt; abc</td>
<td>1</td>
<td>5.6</td>
<td>4.0 9.6</td>
<td>0.4</td>
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<tr>
<td>F18</td>
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<td>abc -&gt; abc</td>
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<td>306.2</td>
<td>224.351</td>
<td>204.5</td>
<td>11.0</td>
<td>6.3 0.0</td>
<td>0.0</td>
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<tr>
<td>F19</td>
<td>NADH + 1/2 O2 =&gt; NAD</td>
<td>abc -&gt; abc</td>
<td>1</td>
<td>306.2</td>
<td>224.351</td>
<td>204.5</td>
<td>0.0</td>
<td>6.3 0.0</td>
<td>0.0</td>
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</table>
### Appendix Table A 4  Metabolite concentrations in iBMK cells.

In units of nmole/ μl cells.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Parental mean</th>
<th>Parental stdev</th>
<th>Ras mean</th>
<th>Ras stdev</th>
<th>Akt mean</th>
<th>Akt stdev</th>
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</thead>
<tbody>
<tr>
<td>hexose-phosphate</td>
<td>0.8</td>
<td>0.2</td>
<td>1.2</td>
<td>0.2</td>
<td>0.8</td>
<td>0.03</td>
</tr>
<tr>
<td>ribose-phosphate</td>
<td>0.04</td>
<td>0.03</td>
<td>0.05</td>
<td>0.01</td>
<td>0.05</td>
<td>0.01</td>
</tr>
<tr>
<td>fructose-1,6-bisphosphate</td>
<td>1.1</td>
<td>0.07</td>
<td>1.5</td>
<td>0.14</td>
<td>1.5</td>
<td>0.33</td>
</tr>
<tr>
<td>3-phosphate-glycerate</td>
<td>0.2</td>
<td>0.05</td>
<td>0.5</td>
<td>0.1</td>
<td>0.3</td>
<td>0.06</td>
</tr>
<tr>
<td>pyruvate</td>
<td>4.6</td>
<td>0.3</td>
<td>4.4</td>
<td>0.2</td>
<td>5.9</td>
<td>0.2</td>
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<td>acetyl-CoA</td>
<td>0.02</td>
<td>0.004</td>
<td>0.02</td>
<td>0.006</td>
<td>0.03</td>
<td>0.01</td>
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<td>fumarate</td>
<td>0.3</td>
<td>0.06</td>
<td>0.4</td>
<td>0.06</td>
<td>0.3</td>
<td>0.01</td>
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<td>succinate</td>
<td>0.2</td>
<td>0.04</td>
<td>0.2</td>
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<td>0.03</td>
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<td>citrate</td>
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<td>aspartate</td>
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<td>4.9</td>
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<td>37.0</td>
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<td>alpha-ketoglutarate</td>
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## Appendix Table A 5 Constraints for MFA model

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<tr>
<th></th>
<th>Parental - normoxia</th>
<th></th>
<th>RAS</th>
<th></th>
<th>AKT</th>
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<th>Parental - hypoxia</th>
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<tbody>
<tr>
<td></td>
<td>Average</td>
<td>Std</td>
<td>Average</td>
<td>Std</td>
<td>Average</td>
<td>Std</td>
<td>Average</td>
<td>Std</td>
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<tr>
<td>Glucose uptake (μmole/μL cells/h)</td>
<td>0.098</td>
<td>0.008</td>
<td>0.183</td>
<td>0.020</td>
<td>0.170</td>
<td>0.020</td>
<td>0.157</td>
<td>0.006</td>
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<tr>
<td>Glutamine uptake (μmole/μL cells/h)</td>
<td>0.040</td>
<td>0.008</td>
<td>0.054</td>
<td>0.005</td>
<td>0.045</td>
<td>0.008</td>
<td>0.037</td>
<td>0.003</td>
</tr>
<tr>
<td>Lactate secretion (F12) (μmole/μL cells/h)</td>
<td>0.143</td>
<td>0.014</td>
<td>0.340</td>
<td>0.040</td>
<td>0.270</td>
<td>0.050</td>
<td>0.295</td>
<td>0.011</td>
</tr>
<tr>
<td>Pyruvate + alanine secretion (F18) (μmole/μL cells/h)</td>
<td>0.017</td>
<td>0.003</td>
<td>0.016</td>
<td>0.005</td>
<td>0.020</td>
<td>0.005</td>
<td>0.012</td>
<td>0.003</td>
</tr>
<tr>
<td>O2 consumption by OxPhos (F19) (nmole/μL cells/h)</td>
<td>170.100</td>
<td>16.200</td>
<td>124.500</td>
<td>16.600</td>
<td>195.300</td>
<td>27.900</td>
<td>113.581</td>
<td>19.567</td>
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<tr>
<td>Demand flux: glucose to ribose (nmole/μL cells/h)</td>
<td>2.900</td>
<td>0.580</td>
<td>3.163</td>
<td>0.633</td>
<td>3.103</td>
<td>0.621</td>
<td>1.450</td>
<td>0.290</td>
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<tr>
<td>Demand flux: proteomic gln/glu/pro (nmole/μL cells/h)</td>
<td>2.846</td>
<td>0.569</td>
<td>3.105</td>
<td>0.621</td>
<td>3.252</td>
<td>0.650</td>
<td>1.423</td>
<td>0.285</td>
</tr>
<tr>
<td>Demand flux: acetyl-CoA (F14) (nmole/μL cells/h)</td>
<td>2.077</td>
<td>0.415</td>
<td>1.958</td>
<td>0.392</td>
<td>4.806</td>
<td>0.961</td>
<td>0.200</td>
<td>0.040</td>
</tr>
<tr>
<td>Demand flux: serine biosynthesis (F17) (nmole/μL cells/h)</td>
<td>5.430</td>
<td>0.600</td>
<td>3.980</td>
<td>0.840</td>
<td>6.480</td>
<td>1.780</td>
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<td>0.030</td>
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Appendix Figure A 1 Metabolite labeling patterns in parental cell line in normoxia.

(A) Measured (blue bars) and simulated (red bars) steady-state labeling patterns for indicated metabolites after 72 h of cell growth in U-$^{13}$C-glucose (A1) or U-$^{13}$C-glutamine (A2). (B) Kinetic flux profiling after switch to U-$^{13}$C-glucose (B1) or U-$^{13}$C-glutamine (B2). Points are experimental data (mean ± 2 SD, N=2-4) and lines are simulation output.
Appendix Figure A 2 Metabolite labeling patterns in Ras cell line in normoxia.

(A) Measured (blue bars) and simulated (red bars) steady-state labeling patterns for indicated metabolites after 72 h of cell growth in U-$^{13}$C-glucose (A1) or U-$^{13}$C-glutamine (A2). (B) Kinetic flux profiling after switch to U-$^{13}$C-glucose (B1) or U-$^{13}$C-glutamine (B2). Points are experimental data (mean ± 2 SD, N=2-4) and lines are simulation output.
Appendix Figure A 3 Metabolite labeling patterns in Akt cell line in normoxia.

(A) Measured (blue bars) and simulated (red bars) steady-state labeling patterns for indicated metabolites after 72 h of cell growth in U-^{13}C-glucose (A1) or U-^{13}C-glutamine (A2). (B) Kinetic flux profiling after switch to U-^{13}C-glucose (B1) or U-^{13}C-glutamine (B2). Points are experimental data (mean ± 2 SD, N=2-4) and lines are simulation output.
Appendix Figure A 4 Metabolite labeling patterns in parental cell line in hypoxia. Measured (blue bars) and simulated (red bars) steady-state labeling patterns for indicated metabolites after 72 h of cell growth in U-13C-glucose (A) or U-13C-glutamine (B).

Appendix Figure A 5 ATP production rates from oxidative phosphorylation and glycolysis in parental, Ras, Akt iBMK cells in normoxia and parental cells in hypoxia. Oxidative ATP production rate is calculated based on the assumption that all cytosolic NADH are shuttled into mitochondrial through glycerol-phosphate shuttle, thus it is a lower bond of oxidative ATP production.
Appendix Figure A 6

(A) Effect of oxidative phosphorylation inhibitors on the NADH/NAD$^+$ ratio in Ras and Akt-driven cells in normoxia. NADH and NAD$^+$ levels were measured 5 min after addition of vehicle (DMSO), the complex III inhibitor antimycin A (4 μg/ml) or the ATP synthase inhibitor oligomycin (8 μg/ml) (mean ± SD of N = 3). (B) NADH/NAD$^+$ ratio in Ras and Akt in normoxia measured after switching cells to complete media or media lacking glucose or glutamine for 8 hours (mean ± SD of N = 3).

Appendix Figure A 7

Rates of ATP production by oxidative phosphorylation across the NCI-60 cell lines predicted based on flux balance analysis using the glycolysis and TCA cycle reaction network shown in
Appendix Table A4 and constrained by experimental data on metabolite uptake and excretion rates from Jain et. al.

Appendix Figure A 8
Rate of ATP production by oxidative phosphorylation across the NCI-60 cell lines predicted based on flux balance analysis using a genome-scale metabolic model and constrained by experimental data on metabolite uptake and excretion rates from Jain et. al. and biomass production rates. (A) With optimization of total ATP production. (B) With constraining oxygen consumption rates to a reasonable range without maximizing ATP production.

Appendix Figure A 9 Model to quantify \textit{de novo} serine synthesis flux.
Appendix Figure A 10 Metabolite pool sizes in Ras, Akt, and Raf cells relative to the parental cell line
Appendix Figure A 11 Contribution of glucose and glutamine to lipids in parental, Ras, Akt, and Raf cells.
Appendix Figure A 12 Changes in protein levels of metabolic enzymes upon oncogene activation
Appendix B  Additional Information for Chapter 4

Appendix Table B 1 Pool size of 6-phosphogluconate (pmole / µL cells). Mean ± SD, N=3.

<table>
<thead>
<tr>
<th>HEK293T</th>
<th>MDA-MB-468</th>
<th>iBMK-parental</th>
<th>iBMK-Akt</th>
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<tbody>
<tr>
<td>23 ± 6</td>
<td>16 ± 7</td>
<td>12 ± 3</td>
<td>15 ± 4</td>
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</table>

Appendix Table B 2  Cellular protein content (µg/µL cells). Mean ± SD, N=3.

<table>
<thead>
<tr>
<th>HEK293T</th>
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<th>iBMK-parental</th>
<th>iBMK-Akt</th>
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<tbody>
<tr>
<td>69 ± 5</td>
<td>69 ± 3</td>
<td>71 ± 3</td>
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Appendix Table B 3 Cell doubling times.
(A) Parental cell lines. (B) HEK293T with stable knockdown of indicated genes (results for different hairpins of the same gene were indistinguishable).

(A)

<table>
<thead>
<tr>
<th>HEK293T</th>
<th>MDA-MB-468</th>
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<th>iBMK-Akt</th>
</tr>
</thead>
<tbody>
<tr>
<td>22 h</td>
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(B)

<table>
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<th>shMTHFD2</th>
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</thead>
<tbody>
<tr>
<td>22 h</td>
<td>38 h</td>
<td>35 h</td>
<td>26 h</td>
<td>24h</td>
<td>22h</td>
</tr>
</tbody>
</table>
Appendix Table B 4  Radioactive tracers employed for monitoring $^{14}$C-CO$_2$ release.
The table states the amount of tracer in the medium. For each cell line, the amount was selected to be the minimum that gives a sufficient radioactive CO$_2$ signal to quantitate accurately. To perform the assay, cells in flasks were incubated in the $^{14}$C-labeled medium for 4 h to ensure pseudo-steady-state labeling of intracellular metabolites, after which the flask was sealed with a rubber stopper with a central well (Kimble Chase) containing a piece of filter paper saturated with 10 M KOH solution. The flasks were incubated at 37°C for 24 h. CO$_2$ released by cells was absorbed by the base (i.e., KOH) in the central well. Metabolism was stopped by injection of 1 mL 3 M acetic acid solution through the rubber stopper. The flasks were then incubated at room temperature for 1 h to ensure all the CO$_2$ dissolved in media was released and absorbed into the central well. The filter paper and all the liquid in central well was transfer to a scintillation vial containing 15 mL liquid scintillation cocktail (PerkinElmer Inc.). The central well was washed with 100 µL water twice, and the water was added to the same scintillation vial. Radioactivity was measured by liquid scintillation counting.

In parallel, the same experiments were performed using U-$^{13}$C-labeled nutrient (in amounts that fully replaced the unlabeled nutrient in DMEM) and the extent of labeling of the intracellular metabolite that is the substrate of the CO$_2$-releasing reaction was measured by LC-MS. To calculate absolute CO$_2$ release rates from the nutrients of interest, measured rates of $^{14}$C-CO$_2$ release were corrected by multiplying by the total nutrient concentration in DMEM divided by the concentration of added $^{14}$C-labeled nutrient, and divided by the fractional intracellular substrate labeling from U-$^{13}$C-nutrient.

<table>
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<td>1.0</td>
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<td>$^{14}$C-glycine</td>
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<td>1.0</td>
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<tr>
<td>$^{14}$C-glycine</td>
<td>0.5</td>
<td>1.0</td>
<td>0.5</td>
<td>0.2</td>
</tr>
</tbody>
</table>
Appendix Figure B 1. LC-MS chromatogram of M+0 and M+1 forms of NADPH and NADP⁺.
Note that the compounds are fully chromatographically resolved. Data were collected with negative electrospray ionization. Plotted values are 5 ppm mass window around each compound.
Appendix Figure B 2 Incomplete labeling of glucose-6-phosphate (G6P) from 1-\(^2\)H-glucose.

Unlabeled G6P arises due both to production of G6P from intracellular stores (e.g., glycogen) and to hydrogen exchange with water. (A) Incomplete labeling can occur due to influx from glycogen or H/D exchange. (B) Labeling fraction of hexose-phosphate and fructose-1,6-phosphate in iBMK cells with and without activated Akt. (C) Labeling fraction of fructose-1,6-phosphate and 6-phosphogluconate. Labeling fraction of fructose-1,6-phosphate reflects the labeling of hexose-phosphate, which gave too low a signal in HEK293T and MDA-MB-468 cells to allow precise quantitation of its labeling directly. The difference in the labeling fraction between hexose-phosphate and 6-phosphogluconate reflects the fraction of deuterium labeling specifically at position 1 of hexose-phosphate. Mean ± SD. Two independent experiments each with ≥ 2 biological replicates.
Appendix Figure B 3 Relative concentration of oxPPP intermediates with or without feeding of $^{1-2}H$-glucose.

No significant changes were observed upon feeding $^{1-2}H$-glucose, suggesting that feeding of the tracer does not create a bottleneck at any step of the pathway. Mean ± SD; two independent experiments each with ≥ 2 biological replicates.
Appendix Figure B 4  Impact of different mechanisms of correcting for the deuterium kinetic isotope effect on (A) fractional contribution of oxPPP to NADPH production and (B) total NADPH production rate.

The kinetic isotope effect ($V_H/V_D$) for isolated NADPH producing enzymes ranges from 1.8 – 4, with isolated G6PD and 6-phosphogluconate dehydrogenase having $V_H/V_D = 1.8$. However, cellular homeostatic mechanisms (including flux control being distributed across multiple pathway enzymes) may result in a lesser impact on labeling patterns in cells. The above figure shows results assuming (i) no kinetic isotope effect ($C_{KIE} = 1$), (ii) no impact on total pathway flux but preferential utilization of $^1$H over $^2$H-labeled substrate (Eqn. 4 of main text) (the smallest reasonable correction, and the one applied in the main text), or (iii) full kinetic isotope effect observed for the isolate enzyme with associated decrease in total pathway flux (the largest reasonable correction)., which is calculated by the equation

$$C_{KIE} = \frac{V_H/V_D}{1+2 \times (V_H/V_D - 1) \times X_{NADPH}}$$

Note that the impact of the kinetic isotope effect on NADP$^2$H production may be partially offset by an analogous (albeit smaller) kinetic isotope effect in NADP$^2$H consuming reactions. $V_H/V_D$ for fatty acid synthetase is ~1.1[124].
Appendix Figure B 5  Diagram of 1-$^{14}$C-glucose and 6-$^{14}$C-glucose metabolism through glycolysis and pentose phosphate pathway.

The oxPPP specifically releases glucose C1 as CO$_2$. When glucose is metabolized via glycolysis, C1 and C6 of glucose become equivalent after conversion of fructose-1,6-bisphosphate into two molecules of glyceraldehyde-3-phosphate (GAP): both C1 and C6 of glucose become C1 of GAP and C3 of 3-phosphoglycerate, pyruvate, and lactate. All CO$_2$-releasing reactions outside the oxPPP are downstream of GAP and will release equal amounts of $^{14}$C-CO$_2$ from 1-$^{14}$C-glucose and 6-$^{14}$C-glucose. Thus, the difference in CO$_2$ release rate from 1-$^{14}$C-glucose and 6-$^{14}$C-glucose is the oxPPP flux.
Appendix Figure B 6 Two independent measurement methods give consistent oxPPP fluxes. (A) CO₂ release rate from 1-¹⁴C-glucose and 6-¹⁴C-glucose across 4 cell lines. (B) Kinetics of 6-phosphogluconate labeling upon switching cells to U-¹³C-glucose (Mean ± SD, N=3). Simulated curve based on the flux that best fits the labeling data is shown in blue, and that for the flux from ¹⁴C-CO₂ release measurements in green. Labeling curves were simulated using differential equations as previously described[125], using the concentrations shown in Supplementary Table 1. (C) Calculated fluxes and 95% confidence intervals based on the U-¹³C-glucose labeling and positional ¹⁴C-glucose labeling. Note that both approaches gave consistent results, but the positional labeling with measurement of ¹⁴C-CO₂ release is more precise.
Appendix Figure B 7  Probing NADPH production via isocitrate dehydrogenase with 2,3,3-\(^{2}\text{H}\)-aspartate reveals no detectable labeling of NADPH’s redox active site.

(A) Pathway diagram showing potential for 2,3,3-\(^{2}\text{H}\)-aspartate to label NADPH via isocitrate dehydrogenase. (B) NADP\(^{+}\) and NADPH labeling patterns after 48 h incubation with 2,3,3-\(^{2}\text{H}\) -aspartate. The indistinguishable labeling of NADP\(^{+}\) and NADPH implies lack of redox active hydrogen labeling. Results are mean ± SD, N \(\geq\) 2 biological replicates from a single experiment; in addition, all results were confirmed in N \(\geq\) 2 independent experiments.

Appendix Figure B 8  Probing NADPH production via malic enzyme and glutamate dehydrogenase with 2,3,3,4,4-\(^{2}\text{H}\) -glutamine reveals no detectable labeling of NADPH’s redox active site.

(A) Pathway diagram showing potential for 2,3,3,4,4-\(^{2}\text{H}\) -glutamine to label NADPH via glutamate dehydrogenase and via malic enzyme. Labeled hydrogens are shown in red. (B) NADP\(^{+}\) and NADPH labeling patterns after 48 h incubation with 2,3,3,4,4-\(^{2}\text{H}\) -glutamine. The indistinguishable labeling of NADP\(^{+}\) and NADPH implies lack of NADPH redox active hydrogen labeling. Results are mean ± SD, N \(\geq\) 2 biological replicates from a single experiment; in addition, all results were confirmed in N \(\geq\) 2 independent experiments.
Appendix Figure B 9 Incomplete labeling at redox active hydrogen of malate after feeding 2,3,3,4,4-$^2$H-glutamine.

(A) Diagram of 2,3,3,4,4-$^2$H-glutamine metabolism through TCA cycle, tracing labeled hydrogen. Hydrogen atoms of lighter shade show the positions that can exchange with water and thereby lose labeling. (B) Malate and glutamate labeling fraction after cells were labeled with 2,3,3,4,4-$^2$H-glutamine for 48 h. Mean ± SD. Two independent experiments each with ≥ 2 biological replicates.
Appendix Figure B 10 Malic enzyme flux measurement by U-13C-glutamine labeling.

Cells were incubated with U-13C-glutamine for 48 h. M+3 pyruvate indicates malic enzyme flux, which may generate either NADH or NADPH. Similar results were obtained also for M+3 lactate, which was used as a surrogate for pyruvate in cases where lactate was better detected. Mean ± SD, N=3. These data provide an upper bound on NADPH production by malic enzyme:

\[
\text{Flux}_{\text{NADPH ME}} \leq \frac{\text{Pyruvate}(13C_3)}{\text{Total Pyruvate}} \times \frac{\text{Total Malate}}{\text{Malate}(13C_4) + 0.5 \text{Malate}(13C_3)} \times \text{Flux}_{\text{glycolysis}}
\]

The corresponding maximal possible malic enzyme-driven NADPH production rate ranges, depending on the cell line, from < 2 nmol uL⁻¹ h⁻¹ to 6 nmol uL⁻¹ h⁻¹.
Appendix Figure B 11

(A) Predicted contribution of folate metabolism to NADPH production based on flux balance analysis across different biomass compositions. The biomass fraction of cell dry weight consisting of protein, nucleic acid, and lipid was varied as follows: protein 50% - 90% with a step size of 10%; RNA/DNA 3%-20% with step size of 1%, and lipids 3% - 20% with step size of 1% (considering only those combinations that sum to no more than 100%). Note that with the constraint of experimentally measured biomass composition, yet without constraining uptake rate of amino acids other than glutamine to be \( \leq 1/3 \) of the glutamine uptake rate, the contribution of folate pathway to total NADPH production is predicted to be 23%.

(B) Range of feasible flux through NADPH producing reactions in Recon1 model computed via Flux Variability Analysis under the constraint of maximal growth rate. As shown, the model predicts that each NADPH producing reaction can theoretically have zero flux, with all NADPH production proceeding through alternative pathways. Only reactions whose flux upper bound is greater than zero are shown. Reactions producing NADPH via a thermodynamically infeasible futile cycles were manually removed. As shown, MTHFD has the highest flux consistent with maximal growth among all NADPH producing reactions.
Appendix Figure B 12  Feeding of 2,3,3-^{2}H-serine demonstrates NADPH production via the 10-formyl-THF pathway.

(A) Pathway diagram showing potential for 2,3,3-^{2}H-serine to label NADPH via methylene tetrahydrofolate dehydrogenase. (B) NADP\(^{+}\) and NADPH labeling pattern after 48 h incubation with 2,3,3-^{2}H-serine (no glycine present in the media). The greater abundance of more heavily labeled forms of NADPH relative to NADP\(^{+}\) indicates redox active hydrogen labeling. Results are mean ± SD, N \(\geq\) 2 biological replicates from a single experiment; in addition, all results were confirmed in N \(\geq\) 2 independent experiments.

Based on the data in panel (B), the contribution of MTHFD1 to cytosolic NADPH production can be estimated as follows:

\[
\text{Fraction}_{\text{NADPH}(c)} \text{ from MTHFD1} = \frac{\text{NADP}^{2}H(c)}{\text{total NADPH}(c)} \times \frac{\text{total methylene-THF}(c)}{2H-\text{methylene-THF}(c)} \times C_{\text{KIE}}(\text{MTHFD1})
\]

Existing methods do not allow direct measurement of methylene-THF labeling, but such labeling can be approximated based on intracellular serine labeling (formally, the \(^{2}\text{H}\)-serine labeling places an upper bound on \(^{2}\text{H}\)-methylene-THF labeling).

\[
\text{Fraction}_{\text{NADPH}(c)} \text{ from MTHFD1} \geq \frac{\text{NADP}^{2}H(c)}{\text{total NADPH}(c)} \times \frac{\text{total serine}}{2H-\text{serine}} \times C_{\text{KIE}}(\text{MTHFD1})
\]

MTHFD1 has deuterium kinetic isotope effect \(V_{H}/V_{D}\) of 3.

Note that the total contribution of the cytosolic 10-formyl-THF pathway to NADPH production exceeds that of MTHD1, as 10-formyl-THF dehydrogenase also produces NADPH.
Appendix Figure B 13 Most cytosolic formyl groups incorporated into purines via 10-formyl-THF are derived from serine C3.
Cells were cultured with U-^{13}C-serine for 48 h, and the intracellular labeling pattern of serine, glycine, and ATP was measured. Mean ± SD, N=3.
Appendix Figure B 14 High concentration of glycine further inhibits cytosolic one carbon unit production from serine, resulting in lower NADPH producing flux via THF-pathway. (A) Schematic of reversible reaction of serine hydroxymethyltransferase (SHMT). High cytosolic glycine to serine ratio inhibit the net production of 5,10-methylene-THF. Reversibility of SHMT1 is supported by labeling of intracellular serine and glycine from U-\textsuperscript{13}C-serine in the media (B), as well as labeling of intracellular glycine and serine from U-13C-glycine in the media (C). Mean ± SD, N=3.
Appendix Figure B 15 CO₂ release rate from serine and glycine measured by combination of ¹⁴C and ¹³C labeling.

(A) ¹⁴C-CO₂ release rate when cells are fed media with a trace amount of 3-¹⁴C-serine, 1-¹⁴C-glycine or 2-¹⁴C-glycine (as per Appendix Table B4, with the final concentration of serine and glycine the same as in standard DMEM, i.e. 0.4mM). (B) Fraction of intracellular serine labeled in cells grown in DMEM media containing 0.4 mM 3-¹³C-serine in place of unlabeled serine. The residual unlabeled serine is presumably from de novo synthesis. (C) Fraction of intracellular glycine labeled in cells grown in DMEM media containing 0.4 mM U-¹³C-glycine in place of unlabeled glycine. (D) CO₂ release rates from serine C3, glycine C1 or C2, which are calculated as:

\[
\text{Rate}_{\text{CO}_2 \text{ from source}} [\text{n mole/h/µl cell}] = \frac{\text{Rate}_{\text{CO}_2 \text{ from } ^{14}C\text{-labeled-tracer}} [\mu Ci/ h/ µl cell]}{\text{overall media activity} [^{14}C\text{-labeled-tracer}} [\mu Ci/ n mole] \times \text{fraction intracellular compound, from media}}
\]

. Mean ± SD, N=3.
Appendix Figure B 16 $^{14}$CO$_2$ release from labeled serine or glycine is through THF-pathway.

(A) Alternative pathway to metabolize glycine or serine into CO$_2$, via pyruvate. (B) Pyruvate labeling fraction after 48 h labeling with U-$^{13}$C-serine or U-$^{13}$C-glycine. The lack of labeling in pyruvate indicates that serine and glycine are not metabolized through this pathway. Mean ± SD. Two independent experiments each with ≥ 2 biological replicates.
Appendix Figure B 17 Knockdown of MTHFD2 blocks most CO₂ release from glycine C2. Mean ± SD.

Appendix Figure B 18 Cellular DNA and RNA contents.
Cellular DNA and RNA were extracted and separated with TRizol reagent (Invitrogen) and measured by Nanodrop spectrophotometer after purification. Mean ± SD, N=3.
Appendix Figure B 19 Fatty acid content in 4 cell lines.
Total cellular lipid was extracted and saponified after addition of isotope-labeled internal standards for the 4 indicated fatty acids. Samples were analyzed by negative ESI-LC-MS with LC separation on a C8 column. Concentrations of other fatty acids, for which isotope-labeled internal standard were not available, was by comparison to the palmitate internal standard. The calculated fatty acid concentrations were multiplied with a correction factor to account for incomplete lipid recovery in the first step of the sample preparation procedure. This correction factor was empirically determined to be 1.9 by experiments in which lipid standards were spiked into extraction solution. Data are mean ± SD, N=3.
Appendix Figure B 20  Quantitation of proline synthesis based on proline and glutamate labeling pattern after 24 h in U-^{13}C-glutamine media.

Mean ± SD, N=3. Proline can be made from either arginine or glutamate, with only proline produced from glutamate labeled by U-^{13}C-glutamine. Proline synthesis from either substrate requires two high-energy electrons at the step catalyzed by pyrroline-5-carboxylate reductase, which may use NADH or NADPH (for simplicity, we assume an equally contribution from each). Proline synthesis from glutamate consumes one additional NADPH. [126]:

\[
\text{Fraction of proline from glutamate} \quad X_{\text{Glu}} = \frac{\text{Fraction proline}^{^{13}\text{C}-\text{labeled}}}{\text{Fraction glutamine}^{^{13}\text{C}-\text{labeled}}}
\]

\[
\text{Flux}_{\text{NADPH for proline}} = \frac{\text{growth rate} \times \text{protein content}}{\text{average formula weight per residue}} \times \text{proline frequency} \times (1.5X_{\text{Glu}} + 0.5(1 - X_{\text{Glu}}))
\]
Appendix Figure B 21  Experimentally-observed fatty acid labeling and associated least square fitting of fraction fatty acid de novo synthesis, elongation, and import.

Cells were cultured in U-\(^{13}\)C-glucose and U-\(^{13}\)C-glutamine until pseudo-steady state, and fatty acids saponified from total cellular lipids and their labeling patterns measured (green bars). To quantitate the NADPH consumed by fatty acid synthesis and elongation, we simulated the different routes of production of each fatty acid. For example, for C18:0, the two routes are uptake (completely unlabeled) and elongation of C16:0 (labeling pattern based on observed labeling of acetyl-CoA and C16:0). The fractional contribution of each route is determined by least square fitting (pink bars). Data here are for the four fatty acids species (C16:0, C16:1, C18:0, C18:1), which together account for ~ 80% of total cellular fatty acids and > 90% of non-essential fatty acids (essential fatty acids are imported, not synthesized, and thus do not impact NADPH production). NADPH calculations also include similar data for all measurable fatty acids (data not shown). Mean ± SD, N=3. Continued on next page with data for iBMK cell lines.
Continued from prior page, which shows data for HEK293T and MDA-MB-468 cells. Cells were cultured in U-$^{13}$C-glucose and U-$^{13}$C-glutamine until pseudo-steady state, and fatty acids saponified from total cellular lipids and their labeling patterns measured (green bars). To quantitate the NADPH consumed by fatty acid synthesis and elongation, we simulated the different routes of production of each fatty acid. For example, for C18:0, the two routes are uptake (completely unlabeled) and elongation of C16:0 (labeling pattern based on observed labeling of acetyl-CoA and C16:0). The fractional contribution of each route is determined by least square fitting (pink bars). Data here are for the four fatty acids species (C16:0, C16:1, C18:0, C18:1), which together account for ~ 80% of total cellular fatty acids and > 90% of non-essential fatty acids (essential fatty acids are imported, not synthesized, and thus do not impact NADPH production). NADPH calculations also include similar data for all measurable fatty acids (data not shown). Mean ± SD, N=3.
Appendix Figure B 22 NADPH consumption rates by *de novo* DNA synthesis.
Mean ± SD, N=3.

Appendix Figure B 23 Total cytosolic NADPH production is decreased by oxidative stress. HEK293T cells were treated with 150 µM H₂O₂ for 5 h. Total cytosolic NADPH turnover was measured based on the absolute rate of oxPPP flux (A) and the fractional contribution of the oxPPP to total NADPH as measured using NADP²H formation from 1⁻²H-glucose, which is calculated as described before from measured NADP glucose-6-phosphate labeling at C1 position (B) and NADP²H labeling (C). Total cytosolic NADPH production is decreased by the treatment of H₂O₂ (D).
Appendix Figure B 24  Confirmation of knockdown efficiency by western blot or Q-PCR.

(A) Western blot of G6PD knockdown (B) Western blot of MTHFD1 and MTHFD2 knockdown.
(C) mRNA level of ME1 knockdown. (D) mRNA level of NNT knockdown. (E) Western blot of IDH1 and IDH2 knockdown. Note that ME3 expression was not detectable in HEK293T cells by either western plot or Q-PCR.
Appendix Figure B 25  Total cytosolic NADPH turnover rates in HEK293T cells after stable knockdown of the indicated genes.
NADPH turnover rates are measured as in Figure 4.1. Mean ± SD, N=3
Appendix C  Additional Information for Chapter 5

Appendix Figure C 1. Majority of intracellular serine comes from uptake from media. Intracellular serine labeling is measured after cells cultured in media with 100% U-\(^{13}\)C-serine for 24h and washed with PBS three times before extraction. Unlabeled serine mainly comes from *de novo* serine synthesis from glucose. Results are shown for three cell lines with PHGDH amplification: (A) MDA-MB-468 (B) BT-20, and (C) SK-MEL-28. Mean ± SD, N=3
Appendix Figure C 2. PHGDH has OAA and AKG reduction activity.

(A) pyruvate, OAA, AKG are structurally similar to the natural substrate of PHGDH, 3-PHP. Similar reaction can potentially be catalyzed by PHGDH. (B) pyruvate, OAA, AKG reduction activity were assayed by incubating 10mM these substrate with PHGDH and 1mM NADH. NADH oxidation was tested by monitoring absorbance changes at 340nm.
Appendix Figure C 3  PHGDH catalyzes the production of 2-HG from AKG and malate from OAA.

(A) 2HG level in enzymatic reaction products of PHGDH incubated with 5mM AKG and 1mM NADH for 30min. (B) malate level in enzymatic reaction products of PHGDH incubated with 5mM OAA and 1mM NADH for 30min. (37°C, pH 7.6) Similar amount of product is produced by two different preparations of PHGDH were used: his tagged PHGDH obtained from BPS Biosciences and strep tagged PHGDH (a gift from Dr. Olszewski). The enzymatic activity is compared with two controls: reaction with same amount of his tagged PHGDH without substrate (AKG or OAA), or reaction with same amount of substrates incubated with human galactokinase (expressed and purified with the same protocol as PHGDH) instead of PHGDH. Enzymatic reaction products are analyzed by LC-MS. Mean ± SD, N>=3
Appendix Figure C 4. PHGDH is D-specific.
PHGDH was incubated with 1mM NAD⁺ and 10mM L-2HG or D-2HG (37°C, pH 7.6). (A) Changes in absorbance at 340nm indicate the production of NADH. (B) AKG production after 1h was analyzed by LC-MS. Mean ± SD, N=3

Appendix Figure C 5. PHGDH knockdown reduces 2HG level in BT-20 (A) but not in HCC70 (B). Levels are normalized to shNT cells. Mean ± SD, N=3
Appendix Figure C 6 Metabolomic changes upon PHGDH knockdown in MDA-MB-468 cells.

Metabolite levels are averaged and normalized to cell number, then normalized to shNT cells. Results are log2 transformed and clustered, N=3.
Appendix Figure C 7. PHGDH overexpression does not cause significant changes in DNA methylation.
DNA samples are purified from MDA-MB-468 cells with PHGDH knockdown or control knockdown. DNA was quantified by nano-drop, 5-Methylcytosine (5-mC) level and 5-Hydroxymethylcytosine (5-hmC) level was tested by dot-blot. Antibodies were obtained from Activemotif (5-hmC) and Millipore (5-mc).
Appendix Figure C 8. PHGDH level does not significantly influence the availability of one carbon unit.

To track the source of 5-methyl-THF, dTTP labeling and UTP labeling patterns were measured when cells are labeled with (A) U-^{13}C-serine and (B) U-^{13}C-glucose for 48h. 5-Methyl-THF labeling was calculated accordingly (C). Labeling pattern of 5-methyl-THF is consistent with serine labeling pattern suggesting no metabolic channeling preferably use *do novo* synthesized serine as methyl-donor. Furthermore, methione, the direct precursor of SAM which donate methyl group in histone methylation, does not get labeled from serine, either *do novo* synthesized or taken-up. Mean + SD, N=3
Appendix Figure C 9. Growth phenotype when control and PHGDH knock down MDA-MB-468 treated with D-2HG ester.

(A) Relative cell number of PHGDH knockdown cells compared to shNT cells after 2 days. Cells are seeded at the same density, and both shNT cells and shPHGDH cells were treated with various doses of D-2HG-TFMB ester. Cell number of shPHGDH cells were normalized to shNT cells treated with same concentration of 2HG-TFMB. (B) shNT ans shPHGDH cells are seeded at the same density, and treated with vehicle control (DMSO), 500 µM 2HG-TFMB ester, or procedure blank of 2HG-ester synthesis. In 2 days, cells are counted and relative cell numbers are normalized to shNT cells treated with vehicle control. Mean ± SD, N=3
Appendix D  List of publications and conference presentations

D.1 Journal Articles


### D.2 Conference presentations


Bibliography


37. !!! INVALID CITATION !!!


