Investigating the Fate of Ammonia in Breast Cancer

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Investigating the Fate of Ammonia in Breast Cancer

A dissertation presented

by

Jessica Brooke Spinelli

to

The Graduate School of Arts and Sciences

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Chemical Biology

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Investigating the Fate of Ammonia in Breast Cancer

Abstract

Ammonia (NH₃) is a ubiquitous by-product of cellular metabolism that is copiously generated by proliferating cells, especially cancer cells. Although NH₃ is classically conveyed as toxic metabolic waste, it accumulates in the tumor microenvironment (TME) 10-fold higher than what is considered toxic to healthy tissue. Thus, we hypothesized that NH₃ functions beyond a mere waste product in tumor biology. However, the fate of NH₃ generated by tumor metabolism had never been explored. To address this, we developed a novel LC-MS assay to detect ¹⁵N and ¹⁴N-isotopologues of NH₃ (Spinelli et al., Sci. Rep. 2017), and performed stable isotope tracing experiments in breast cancer cells to track the fate of NH₃. We discovered that breast cancer cells recycled NH₃ generated by tumor metabolism with 60% efficiency through a “reverse” catalytic activity of glutamate dehydrogenase (GDH) to generate glutamate (Spinelli et al., Science. 2017). This nitrogen-scavenging pathway accelerated proliferation of breast cancer cells grown in 3D culture models and in vivo xenograft tumor models. Furthermore, using a novel method for rapid mitochondrial isolation for metabolomics, we tracked the compartmentalized nature of NH₃ metabolism in breast cancer cells (Spinelli et al., submitted). Mitochondrial metabolomics revealed that NH₃ is assimilated inside this compartment as a local nitrogen source for glutamate. Importantly, mitochondrial localization of nitrogen recycling is required for NH₃ to stimulate proliferation, unveiling a limitation in the mitochondrial glutamate pool for breast cancer proliferation. We determined that the levels of mitochondrial glutamate dictate the rate of local translation. Thus, NH₃ assimilation to produce mitochondrial glutamate increases cellular respiratory capacity to enable accelerated proliferation. Taken together, this thesis work reveals that breast cancer cells recycle the waste product NH₃ to generate mitochondrial glutamate, which regulates local protein translation to accelerate proliferation.
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Chapter One: Introduction to Cancer Metabolism
I. A Historical Overview of Cancer Metabolism

Otto Meyerhof, and Archibald V. Hill (in extension of work done by Louis Pasteur) were celebrated with the 1922 Nobel Prize in Physiology or Medicine for their fundamental discoveries that the metabolic utilization of glucose is dictated by oxygen availability in yeast and muscle cells, respectively.¹ They demonstrated that when oxygen is abundant, cells fully oxidized glucose by shuttling this carbon through glycolysis, into the mitochondria, and through the tricarboxylic acid (TCA) cycle. In hypoxia, glucose is diverted away from the TCA cycle and shunted into lactate synthesis. This oxygen-dependent plasticity of cellular metabolism is fundamental to mammalian metabolism.

However, in stark contrast to these findings, Otto Warburg and Seigo Minami observed in 1923 that tumors acidified the “Ringer” solution (an isotonic salt solution with NaHCO₃) in normal levels of oxygen.² Thus, they identified that glycolytic flux in tumors is not merely dictated by oxygen abundance. Warburg and colleagues further measured this glycolytic flux in vivo in rats with hepatomas and sarcomas, showing higher lactate concentrations in blood from vessels leaving the tumor than those detected in vessels entering the tumor.³ In addition to their in vivo validation, Carl and Gerty Cori measured lactate levels in chicken wings, one with a tumor and the other without, and found higher lactate levels in the tumor-bearing wing.⁴ Thus, the observation that oxygen does not suppress glycolysis in tumors spanned both in vitro and in vivo models. Warburg and Minami’s study was the first to demonstrate for the first time that cancer cells engage a unique program of metabolic flux compared to healthy tissue, and paved the way for the next 100 years of research on cancer metabolism.

Warburg speculated that damaged mitochondria inhibited cellular respiration, creating a selective pressure for cells that were capable of aerobic glycolysis. He believed this was a driving factor in tumorigenesis.\(^5\) Warburg published his theory on prohibited respiration in his seminal manuscript "On the Origin of Cancer Cells" in 1956.\(^6\) However, Warburg’s theory that damaged respiration machinery caused glycolysis in tumors was highly disputed by Sidney Weinhouse and colleagues, who demonstrated that neoplastic tissue could respire if supplemented with NAD\(^+\).\(^7\) Thus, the respiratory chain was not dysfunctional, but rather respiration was altered based on re-wired tumor metabolism.\(^8\)

As Warburg and Weinhouse debated respiratory capacity in tumors throughout the early 1900’s, a new era of cancer biology emerged that would forego this debate and drastically affect how we interpret tumor metabolism. In the mid 1900’s the discovery of oncogenes and tumor suppressors as drivers and suppressors of tumorigenesis sparked widespread interest in cancer genetics. Importantly, research began to link the metabolic alterations of tumors that were identified by Warburg and Weinhouse with genetic changes caused by oncogenes and tumor suppressors.\(^9\) For example, it was found that enzymes in glycolysis are transcriptionally activated as part of the program of gene expression changes induced by the oncogenes Ras, Src, and HER-2/Neu, and the tumor suppressor p53 through hypoxia inducible factor-1 alpha (HIF1a) stabilization.\(^10\) Furthermore, c-MYC, which is the most ubiquitously induced transcription factor in cancer, activates lactate dehydrogenase A (LDHA) to facilitate the

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diversion of pyruvate into lactate for aerobic glycolysis.\textsuperscript{11} Thus, genetics was a major focus of the cancer field in the mid 1900’s, leading many to study how these genetic changes affected tumor metabolism. Publications in this era often discussed how genetic aberrations that induced cellular transformation were also leading to critical changes in metabolic flux. Studies showed that the combination of genetic changes and the downstream metabolic alterations enable rapid proliferation in tumors.

To expand upon how genetic alterations affect tumor metabolism, many studied how tumor metabolism affects cellular function. Research in the mid to late 1900’s centered on biochemistry, leading many to investigate how alterations in cancer metabolism affected enzyme kinetics and signal transduction pathways. Re-wiring of tumor metabolism directly affects the rate of cancer cell proliferation,\textsuperscript{12} capability for differentiation,\textsuperscript{13} response to DNA damage,\textsuperscript{14} ability to migrate and metastasize,\textsuperscript{15} and the overall function of the cell as part of the tissue of origin (i.e. pancreatic cancer and insulin secretion).\textsuperscript{16} Metabolism affects cellular function through its capacity to regulate signal transduction pathways. For example, ROS are a major product of mitochondrial respiration and are abundantly produced in cancer cells upon nutrient oxidation.\textsuperscript{17} ROS can directly oxidize solvent-exposed cysteine residues on proteins, and therefore at high concentrations can directly affect protein function.\textsuperscript{18} Such proteins include


HIF1-α, the p53 tumor suppressor, the protein-tyrosine phosphatase 1B (PTP1B), the tumor suppressor PTEN, and the PDGF-receptor associated phosphatase SHP-2. In addition to ROS-sensing mechanisms, signaling pathways sense cellular energy status and biosynthetic capacity. AMP-kinase is activated by elevations in AMP and phosphorylates components of the mTOR signaling pathway, ULK-mediated autophagy, lipid catabolism, and gluconeogenesis. Moreover, amino acids are limiting for biomass production in cancer cells, and their bioavailability is sensed by mTOR and GCN2 to regulate macromolecule synthesis or activate a “starvation” response. Finally, lipid and steroid abundance are sensed via SREBP for transcriptional regulation of their respective biosynthetic pathways. Thus, biochemistry in this era facilitated our understanding of the connections between rewired tumor metabolism and signaling cascades, which is requisite for cancer metabolism to affect cell fate and capacity to survive in the tumor microenvironment.

Modern day research on cancer metabolism has found that cancer metabolism is dynamic and extends far beyond glucose consumption and the Warburg Effect. In modern day research on cancer metabolism, technological advances have enabled us to more accurately investigate metabolic flux in cancer cells. Researchers are empowered by the cutting-edge technology for mass spectrometry and nuclear magnetic resonance (NMR) that enable studies

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on the fate of metabolites in tumor biology. Specifically, a deeper analysis of glycolytic flux in tumors revealed that the fate of glucose and subsequently pyruvate was not only dictated by tumor genetics and oxygen levels, but also dependent on the bioavailability of other key nutrients. Importantly, when cancer cells were incubated with glucose and amino acids, there was a 70% increase in the utilization of glucose carbon for the pentose phosphate pathway, instead of fully oxidizing it through the TCA cycle for bioenergetics.\textsuperscript{13} It was also demonstrated that amino acids such as leucine and glutamine could be substrates for TCA cycle and are highly oxidized by cancer cells.\textsuperscript{28} Importantly, 20% of ATP in cancer cells are derived from glutamine oxidation.\textsuperscript{29} Thus, the finding that amino acid bioavailability dictates the utilization of glucose in cancer cells provided the first example of metabolic flexibility in cancer. Importantly, since these metabolites are catabolized inside of the mitochondria, these studies emphasize a key role for mitochondria in cancer metabolism.

Beyond the fate of a given metabolite, metabolic flexibility in cancer cells was also investigated in the context of fuel choice. For example, tumors take up lipids from their environment, which can be used in fatty acid oxidation to support bioenergetics.\textsuperscript{30} Moreover, it was demonstrated that tumors rewire metabolism, shuttling carbon from glucose oxidation, glutamine oxidation, and citrate into fatty acid synthesis to support rapid proliferation.\textsuperscript{31} The balance of fatty acid oxidation and fatty acid synthesis in cancer is a very active area of research. In addition to lipids, consumption of ketone bodies can support bioenergetics,\textsuperscript{32} glycine uptake is required to support the anabolic 1C metabolism pathway for de novo

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nucleotide synthesis, cysteine uptake supports reactive oxygen species (ROS) homeostasis through glutathione synthesis, and the process of macropinocytosis is utilized by cancer cells to consume large amounts of protein as a source of amino acids. Taken together, this emerging body of research demonstrates that cancer metabolism is dynamic, and importantly, the field appreciates that fuel utilization extends far beyond glucose consumption and the Warburg Effect.

Over the last century the field of tumor metabolism has drastically evolved in ways that coincide with the timely advances in technology and enhanced understanding of fundamental aspects of cell biology (signaling and genetics). The initial studies performed by Otto Warburg and colleagues still largely shape the field and have propagated an assumption that mitochondria are dysfunctional in cancer. In spite of technological advances and critical discoveries in genetics, signal transduction pathways, and fuel choice, many still consider mitochondrial dysfunction to be a major hallmark of tumorigenesis. It will be very important for researchers to revisit these foundational studies, which were performed prior to major advances in technology and knowledge of genetic contributors to tumor metabolism. Moreover, there is a major need for researchers to understand the composition of the tumor microenvironment, and assess metabolic flux and fuel choice in the context of TME conditions. Given the history of cancer metabolism research, it is very timely that factors such as low pH, hypoxia, presence of other cell types (including cancer associated fibroblasts and immune cells), and accumulating metabolic waste products be factored in to assessing fuel choice and metabolic flux in tumor biology. These findings will be critical for improving the efficacy of therapeutics targeted to metabolic dependencies in cancer cells.

II. Cancer Metabolism and Therapeutic Opportunities

Cancer cells rely on reprogrammed metabolic pathways to support their rapid growth and proliferation and to survive stressors of the tumor microenvironment. Since cancer cells often utilize distinct pathways for nutrient catabolism and biosynthesis of building blocks when compared to healthy cells of the same tissue, cancer metabolism may be a vulnerability that can be manipulated for therapy. Many efforts have been made to exploit tumor metabolism in cancer detection and treatment.

Metabolism inspired the development of the first chemotherapy, which was discovered by Sidney Farber in 1947. Farber and colleagues found that treating children that had acute lymphoblastic leukemia (ALL) with the folate analog Aminopterin caused remission, leading to the development of an entire class of antimetabolites that would be used as chemotherapies for decades to follow (Figure 1.1). Folate is a requisite cofactor for one-carbon transfer reactions such as those in de novo nucleotide synthesis. Since ALL cells proliferate rapidly, they require an accelerated rate of nucleotide synthesis to replicate the genome for generation of daughter cells. Therefore, the efficacy of antifolates observed by Farber and colleagues could be attributed to inhibition of nucleotide synthesis, leading to the impartial inhibition of proliferating cells (cancerous and healthy) in the human body. Due to the success of antifolates in treatment of ALL, additional therapies targeting nucleotide synthesis were developed for use as chemotherapies in a wide range of cancers. These include the purine analogs 6-mercaptopurine and 6-thioguanine, which are inhibitors of the first step in purine synthesis 5-mercaptopurine and 6-thioguanine, which are inhibitors of the first step in purine synthesis.

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phosphoribosyl-1-pyrophosphatase amidotransferase, the pyrimidine analog 5-fluorouracil which inhibits thymidylate synthase to block thymidine synthesis, and Brequinar and Leflunomide which are inhibitors of critical oxidation step in de novo pyrimidine synthesis dihydroorotate dehydrogenase. Moreover, the nucleotide analogs Cytarabine and Gemcystabine were developed to repress DNA elongation through direct incorporation into the genome, causing steric hindrance of DNA elongation by DNA polymerase.

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Figure 1.1. Therapeutic targets in cancer metabolism related to nucleotide and one carbon metabolism. Cancer cells rely on elevated consumption of glucose and glutamine (yellow stars) to support rapid production of energy and biomass. Glucose carbon can be diverted away from glycolysis into the pentose phosphate pathway for nucleotide synthesis and into serine synthesis. A large amount of nitrogen needed for nucleotide synthesis derives from glutamine. Many pathways are targeted to the de novo nucleotide synthesis pathway, especially one carbon metabolism, whereas less effort has been focused on salvage nucleotide metabolism. A wide range of therapies have been developed that target nucleotide metabolism in cancer (shown in red).
In addition to targeting *de novo* nucleotide synthesis, inhibitors have been developed for clinical use that target salvage nucleotide metabolism, since this pathway provides an additional source of nucleotides for DNA and RNA synthesis. For example, cells are capable of scavenging nucleotides from their environment which can be exploited in cancer. Interestingly, cells consume an abundance epigenetically modified cytosine (5-hydroxymethyl-2'-deoxycytosine and 5-formyl-2'-deoxycytosine) and incorporate these bases into the genome without repercussions.\(^{48}\) However, cancer cells that overexpress cytidine deaminase convert these bases into analogs of uracil, which are genotoxic.\(^{49}\) Thus, treatment with epigenetically modified cytosine analogs has been tested for use as chemotherapy. Additionally, allopurinol, which is an inhibitor of xanthine oxidase in the salvage purine pathway, has been demonstrated to decrease risk of prostate cancer.\(^{50}\) Finally, tumors (often glioblastomas and pancreatic cancers) that have deletion of the tumor suppressor CDKN2A result in loss of the salvage purine synthesis enzyme methylthioadenosine phosphorylase (MTAP), causing enhanced reliance on *de novo* purine biosynthesis and subsequent sensitization of these tumors to inhibitors of this pathway.\(^{51}\) Thus, although cancer cells mainly generate nucleotides through *de novo* synthesis, salvage nucleotide metabolism is another critical way to target nucleotide metabolism for cancer therapy.

In addition to its role in adenine recycling for purine metabolism, MTAP is a key enzyme in the methionine cycle that enables regeneration of methionine for S-adenosylmethionine (SAM) synthesis. Since SAM is an important cofactor for methyl-transfer reactions, MTAP loss also makes cancer cells particularly sensitive to methionine deprivation, since exogenous


methionine is required in these cells for SAM synthesis and subsequent one-carbon transfer reactions.\textsuperscript{52} Furthermore, the MTAP reaction, which cleaves adenine off methylthioadenoside (MTA) to generate methylthioribose (MTR), plays a critical role in regulating the levels of cellular MTA. Loss of MTAP in cancers causes MTA accumulation, leading to potent inhibition of the enzyme protein arginine methyltransferase (PRMT5).\textsuperscript{53} PRMT5 is responsible for producing symmetrical dimethylation on arginine residues on proteins including SNRPD1 and SNRPD3 of the splicosome.\textsuperscript{54} Therefore, cancer cells with loss of MTAP are more sensitive to slight inhibition of PRMT5 than healthy cells, and inhibitors of PRMT5 have been tested in clinical trials.\textsuperscript{55}

In addition to broadly targeting one carbon transfer reactions and nucleotide synthesis, therapies have also been developed targeting rewired central carbon metabolism. As observed by Warburg and colleagues, cancer cells rely on elevated glycolysis and lactate synthesis compared to normal tissue. Glycolysis has been targeted for treatment of cancer in a number of ways. 2-deoxyglucose (2-DG) is an analog of glucose that competes with glucose as a substrate of hexokinase (Figure 1.1).\textsuperscript{56} Since glucose phosphorylation into glucose-6-phosphate is required for its ability to be retained inside of the cell and utilized for glycolysis, treatment with 2-DG represses glucose uptake and has been shown to inhibit cancer cell proliferation.\textsuperscript{57} Downstream of glycolysis, Warburg and colleagues demonstrated that pyruvate was diverted away from the further oxidation inside of the mitochondria to be utilized in cytosolic

\textsuperscript{54} Meister, G. (2002). The EMBO journal 21, 5853-5863.
\textsuperscript{55} Wang, Y., Hu, W., and Yuan, Y. (2018). Journal of medicinal chemistry. DOI: 10.1021/acs.jmedchem.8b00598
lactate synthesis. It was recently demonstrated that the isoform of pyruvate kinase PKM2, which performs a kinetically slower reaction than PKM1, fosters this diversion of glucose carbon into lactate synthesis. \(^{58}\) Consequently, activators of pyruvate kinase activity (AG-348, TEPP-46) shunt pyruvate into mitochondrial oxidation and can repress cancer proliferation in a specific subset of tumors (Figure 1.2). \(^{59,60}\) Similar to this concept, inhibition of mitochondrial pyruvate carrier (MPC) and diversion of pyruvate from mitochondrial oxidation has been shown to propagate the Warburg Effect in colon cancer, and increase tumor proliferation. \(^{61}\) Thus, activators of MPC activity to enhance mitochondrial pyruvate oxidation may be tumor suppressive in certain cancer subtypes. Aerobic glycolysis has also been targeted in cancer through inhibition of lactate synthesis and secretion. Direct inhibition of lactate dehydrogenase A, which generates lactate from pyruvate, decreases proliferation in a number of cancer subtypes, including lung and colorectal cancer. \(^{62,63}\) Moreover, AZD3965 and SR13800, which are inhibitors that targets lactate secretion through monocarboxylate transporter isoform 1 (MCT1), have been developed and utilized for treatment of lymphoma. \(^{64,65}\)

Recent work identified that a major utilization of glucose carbon in cancer is for serine synthesis. \(^{66,67}\) Many cancers overexpress the enzyme phosphoglycerate dehydrogenase


(PHGDH), which converts the glycolytic intermediate 3-phosphoglycerate into 3-phosphohydroxypyruvate in the committed step for de novo serine synthesis. Enhanced reliance on de novo serine synthesis could support tumors through many mechanisms, including general protein synthesis, the one-carbon metabolic shuttle for nucleotide synthesis and balance of reducing equivalents, and the transsulfuration pathway to generate cysteine. Although the central role of serine synthesis in tumor biology is unclear, inhibitors of PHGDH NCT-503 and CBR-5884 have been developed for clinical use.68 Unfortunately, PHGDH inhibitors have limited therapeutic potential due to their toxicity to the central nervous system.69


Figure 1.2. Therapeutic targets in cancer metabolism related to central carbon metabolism. A wide range of therapies have been developed that target central carbon metabolism in cancer, including lactate synthesis, oxidative phosphorylation, amino acid catabolism, and lipogenesis. Importantly, mitochondria are the hub for pathways involved in central carbon metabolism.

Although not classically considered a major function of pyruvate in cancer, literature has demonstrated that a pool of pyruvate can enter the mitochondria for oxidation, promoting rapid proliferation in certain cancer subtypes such as pancreatic cancer.\(^7^0\) Pyruvate dehydrogenase

complex (PDH) catalyzes the initial oxidative decarboxylation of pyruvate into acetyl CoA as a source of carbon for the TCA cycle. Inhibition of PDH with CPI-613 has demonstrated efficacy in mouse models of pancreatic cancer, especially in combination with Gemcitabine.\textsuperscript{71}

Although Warburg considered mitochondria inert in tumor biology, a large body of literature has demonstrated that mitochondrial metabolism is indispensable for cancer cells.\textsuperscript{72} Importantly, genetic mutations to the mitochondrial genome (which encode components of the respiratory chain) are negatively selected against in tumors, suggesting mitochondrial respiration is critical for tumorigenesis and proliferation.\textsuperscript{73} Inhibition of the electron transport chain (ETC) with diverse compounds can repress tumor growth.\textsuperscript{74,75} For example, the FDA-approved type two diabetes therapy Metformin, which inhibits complex 1 of the ETC, improved outcomes when used in patients as a monotherapy,\textsuperscript{76,77} and when used in combination with chemotherapy.\textsuperscript{78} Although metformin has numerous potential mechanisms of action for repression of tumor growth,\textsuperscript{79} its antitumor efficacy can partially be attributed to inhibition of complex 1 because other complex 1 inhibitors such as rotenone and piericidin are effective in repressing cancer cell proliferation.\textsuperscript{80} Additionally, treatment of leukemias with oligomycin, which

\textsuperscript{72} Vyas, S., Zaganjor, E., and Haigis, M.C. (2016). Cell 166, 555-566.
\textsuperscript{74} Kroll, W., Loffler, M., Schneider, F. (1983). Z Naturforsch C 7-8, 604-612.
\textsuperscript{75} Zhang, X., et al. (2014). Nature communications 5, 3295.
is an inhibitor of ATP synthase (complex 5), increases the efficacy of tyrosine kinase inhibition.\textsuperscript{81} Furthermore, inhibition of respiration in BRAF\textsuperscript{V600E} melanoma models decreases the prevalence of resistant JARID1B (high) cells after treatment with cisplatin or vemurafenib.\textsuperscript{82}

A major role of the ETC is regeneration of NAD\textsuperscript{+} from NADH to facilitate continued oxidative catabolism of nutrients. Since tumors are highly reliant on nutrient catabolism to support rapid biomass and energy production, efforts have been made to directly target NAD\textsuperscript{+} synthesis. Similar to nucleotide metabolism, there is \textit{de novo} and salvage biosynthetic routes for NAD\textsuperscript{+} production. Interestingly, efforts have only been made in targeting the salvage pathway. Nicotinamide phosphoribosyltransferase (NAMPT) catalyzes the fate-determining step of the salvage pathway, in which nicotinamide (NAM) is linked to PRPP to generate nicotinamide mononucleotide (NMN), which can subsequently be converted into NAD.\textsuperscript{83} Colorectal, ovarian, breast, PDAC, gastric, prostate, and glioma tumors have been shown to induce NAMPT expression to enhance NAD biosynthesis, leading to inhibitors being developed to target this pathway.\textsuperscript{84} Preclinical examination of NAMPT inhibitors demonstrated impressive antitumor effects,\textsuperscript{85,86} however clinical trials of NAMPT inhibitors as single agents did not lead to remission.\textsuperscript{87} Further studies are testing NAMPT inhibitors in combination with other NAD\textsuperscript{+}-dependent pathways such as PARP.\textsuperscript{88,89}


The mitochondrial TCA cycle can perform oxidative reactions on metabolites to generate reduced electron carriers that feed the respiratory chain. It can also perform reductive metabolism, consuming these electron carriers to support biosynthesis of amino acids, fatty acids, and heme. Since tumors must balance programs of catabolic and anabolic metabolism, the TCA cycle provides tumors with flexibility of carbon sources, and is therefore an excellent target for cancer therapy. For example, a broad study of TCA cycle enzymes in cancer demonstrated many cancer subtypes are dependent on oxoglutarate dehydrogenase (OGDH). CPI-613, which is the inhibitor previously described to target PDH, is a lipoate analog that also targets the oxoglutarate dehydrogenase complex. This compound has been tested in phase 1 clinical trials and demonstrates efficacy as a single agent in hematological malignancies.

Another example of a TCA cycle enzyme being targeted for cancer treatment is isocitrate dehydrogenase (IDH2). IDH is commonly mutated in glioma and acute myeloid leukemia (AML), leading to a neomorphic enzymatic activity that generates the oncometabolite D-2-hydroxyglutarate (D-2HG) instead of 2-oxoglutarate. D-2HG competes with 2-oxoglutarate for interaction with 2-OG-dependent dioxygenases, including the epigenetic modifying histone and DNA methylation enzymes. Thus, IDH mutation promotes tumor progression through D-2HG production, potentially through altered epigenetic modification. To

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repress D-2HG production, selective inhibitors targeting mutant IDH have been developed. The IDH2 inhibitor AG-221 increased survival in IDH2 mutant AML mouse models and was transitioned into clinical trials for AML patients in which clinical benefit was also observed.\textsuperscript{98}

Interestingly, the TCA cycle enzymes succinate dehydrogenase (SDH) and fumarate hydratase (FH) function as tumor suppressors.\textsuperscript{99,100} Loss of SDH and FH function leads to renal cell cancer through accumulation of succinate and fumarate, which compete with 2-oxoglutarate to inhibit the activity of numerous dioxygenases.\textsuperscript{101} These epigenetic modifications are thought to contribute to their role in tumorigenesis. Interestingly, in FH-null tumors, there is a compensatory flux of TCA cycle carbon into heme biosynthesis through succinyl CoA, enabling continued utilization of the TCA cycle in the absence of FH.\textsuperscript{102} The downstream degradation of heme into bilirubin is critical for disposal of excess carbon through this pathway, and therefore pharmacological inhibition of heme oxygenase 1 (critical for heme degradation) has been tested in FH null cancer cells. Additionally, FH-null tumors, which have elevated fumarate levels, cause the reversal of the urea cycle enzyme argininosuccinate lyase and consequently, increased consumption of arginine.\textsuperscript{103} Thus, FH-null tumors are sensitive to arginine deprivation. Similarly, metabolic rewiring in SDH-null tumors causes a severe depletion of aspartate due to lack of flux through the TCA cycle to generate oxaloacetate.\textsuperscript{104} Consequently, SDH-null tumors are dependent on pyruvate carboxylase (PC) as an alternative source of


\textsuperscript{100} Alam, N.A. (2003). Human Molecular Genetics 12, 1241-1252.


\textsuperscript{103} Zheng, L., et al. (2013). Cancer & metabolism 1, 12.

oxaloacetate for aspartate synthesis.\textsuperscript{105} SDH-null cells are sensitive to genetic depletion of PC; however, a small molecule specifically targeting this enzyme has not been developed yet.

Fatty acid metabolism is another key pathway that feeds into and utilizes TCA cycle carbon, depending on cancer subtype. Activation of fatty acid oxidation (FAO) enhances a cell’s capacity to survive detachment from the extracellular matrix (ECM) for migration.\textsuperscript{106} Furthermore, inhibition of FAO through etomoxir, which targets the rate-limiting step of fatty acid oxidation carnitine palmitoyl transferase (CPT1), repressed tumor growth in xenograft models of prostate cancer.\textsuperscript{107,108} Interestingly, etomoxir has been recently shown to target and inhibit complex I and the adenine nucleotide translocator.\textsuperscript{109} Whether the effects of etomoxir on tumor growth stemmed from inhibition of FAO or broadly inhibiting ATP production via the ETC is unclear. Moreover, the therapeutic potential of targeting FAO in cancer is an area of active research, since cancer cells must balance FAO with requirements for fatty acid synthesis.\textsuperscript{110}

Unlike normal tissue (aside from adipose, liver, and lactating breast), cancer cells generate a majority of lipids through \textit{de novo} synthesis.\textsuperscript{111} Citrate export from the mitochondria and subsequent cleavage into acetyl CoA through ATP citrate lyase (ACLY) provides carbon for lipogenesis and histone acetylation.\textsuperscript{112,113,114} Acetyl CoA is converted into malonyl CoA through

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acetyl coA carboxylase (ACC), which is then assimilated into the fatty acyl chain through fatty acid synthase (FASN). Inhibitors targeting ACLY, ACC and FASN have been tested with success in breast and lung cancer models,\textsuperscript{115,116} and the FASN inhibitor TVB-2640 has caused partial response and in some cases stable disease in clinical trials when combined with paclitaxel.\textsuperscript{117} Although fatty acid synthesis inhibitors have demonstrated efficacy in anti-tumor responses in mouse models and in patients, the mechanism for this process remains unclear. Future studies investigating the specific lipid species required for tumor growth will better inform therapeutic options.

Amino acids account for the majority of biomass in proliferating cells and provide a critical source of nitrogen necessary for rapid growth in cancer cells.\textsuperscript{118} Therefore, it is not surprising that many therapies have been developed that target rewired amino acid metabolism in cancer cells. Cancer cells have a particular reliance on elevated consumption of asparagine, arginine, methionine, serine, cysteine and glutamine, leading to the development of therapies targeting their metabolic pathways.

Asparagine is abundant in human plasma and can also be generated in cells through asparagine synthetase (ASNS). Interestingly, ALL cells are asparagine auxotrophs, whereas healthy cells can rely on \textit{de novo} synthesis entirely for their pool of asparagine when this amino acid is depleted from plasma.\textsuperscript{119} Although the mechanism for ALL dependency on asparagine is unclear (and unconnected to ASNS function in ALL cells),\textsuperscript{120} L-asparaginase therapy, which

\begin{thebibliography}{120}
\bibitem{118} Hosios, A.M., et al. (2016). Developmental cell 36, 540-549.
\end{thebibliography}
degrades plasma asparagine into aspartate and ammonia, is utilized in combination with chemotherapy to treat ALL patients.\(^{121}\) Additionally, many cancers are reliant on consumption of the amino acid cysteine, which is a critical component of the tripeptide glutathione for maintenance of reactive oxygen species (ROS), especially during tumor initiation.\(^{122}\) In a similar approach to asparagine depletion, clinical trials on treatment with cysteininases are being performed to deplete plasma of cysteine, rendering cancer cells susceptible to the toxicity of accumulating ROS.\(^{123}\) Arginine auxotrophy is a third example of an amino acid dependency in cancer cells. Argininosuccinate Synthetase (ASS1) deficient tumors are incapable of sufficient arginine synthesis and therefore rely on consumption of exogenous arginine to support growth.\(^{124}\) Clinical trials in melanoma and hepatocellular carcinoma have tested the use of arginine deiminase, which degrades plasma arginine into citrulline and ammonia, as a therapy.\(^{125}\) Although some therapeutic benefit was observed with this treatment, some tumors are capable of re-expressing ASS1 in adaptation to exogenous arginine depletion.\(^{126}\)

Glutamine is the most abundant amino acid in plasma and is highly consumed by cancer cells as both a nitrogen source and an anaplerotic substrate of the TCA cycle for lipid synthesis and bioenergetics.\(^{127}\) As previously described, glutamine contributes nitrogen to nucleotide synthesis, which is critical for cancer proliferation. Therefore, treatment with the glutamine analogs 6-Diazo-5-oxo-L-norleucine (L-DON) or azaserine represses cancer cell proliferation in


vitro and in vivo mouse models through inhibition of nucleotide synthesis. However, toxicity of glutamine analogs precludes clinical studies on these therapies.

Although glutamine is a nitrogen reservoir for cancer cells, it is consumed in quantities that exceed cellular nitrogen demands, especially in vitro. In addition to providing nitrogen for cells, glutamine contributes to TCA cycle intermediates, where it is oxidized to support bioenergetics. In particular, cancer cells with c-MYC overexpression enhance flux of glutamine carbon into the TCA cycle through overexpression of glutaminase (GLS). To target glutamine anaplerosis of the TCA cycle in cancer, inhibitors of GLS were developed and tested in a wide range of cancer subtypes. Although these inhibitors are highly effective in vitro, their efficacy is limited in vivo due to a lack of glutamine anaplerosis of the TCA cycle in human tumors. Furthermore, it was identified that extracellular cystine levels dictate dependency on glutamine catabolism. Thus, further investigation into the mechanisms driving glutamine addiction in cancer will elucidate key targets for therapeutic development.

Finally, beyond targeting cancer metabolism for therapy, enhanced nutrient uptake is exploited for detection of tumors in the human body. The most commonly used tracer is the glucose analog 2-deoxy-2-\((^{18}\text{F})\)fluoro-D-glucose (2-\(^{18}\text{FDG}\)), which can be detected with Positron Emission Tomography (PET) imaging.\(^{139}\) Relative to healthy tissue, tumors consume more glucose and therefore can be detected by PET imaging after treatment with 2-\(^{18}\text{FDG}\). This applies to all cancers except those of the brain and kidney because of the high rate of consumption of glucose in healthy cells from these organs.\(^{140,141}\) To address detection of tumors in kidney and brain, a PET-active analog of glutamine was developed and is currently used for glioma and glioblastoma detection.\(^{142}\) Therefore, elevated consumption of glucose and glutamine can be utilized clinically for tumor detection.

While it is clear that cancer cells rely on elevated metabolic fitness to support rapid growth, proliferation, and survival, it is challenging to target metabolic pathways in isolation because of adaptability through alternative sources of metabolites. It is important for future research to identify pathways that are less adaptable in cancer cells so that metabolic therapies will be more effective. Evidence for this hypothesis is that the most effective therapies are those that inhibit cellular uptake of metabolites, and therefore suppress multiple pathways simultaneously.\(^{143,144,145,146}\) Repression of metabolite uptake is not adaptable, and is therefore detrimental to tumors. However, these inhibitors (L-DON, 2-DG) are broadly cytotoxic to cancer


and healthy tissue, and are therefore not feasible therapies for use clinically. Another ubiquitous feature of cancer metabolism, which comes as an effect of elevated metabolic fitness, is the need to handle accumulating metabolic waste products. This particular feature of cancer metabolism is especially important in solid tumors, which accumulate by-products as a consequence of poor perfusion and high metabolic flux. Healthy cells do not typically accumulate their metabolic waste products, and therefore approaches that target waste management will likely have efficacy similar to inhibitors of nutrient uptake without the cytotoxic side effects in normal cells. Identification of the pathways relevant to metabolic waste clearance will be key for therapeutic development.

III. Metabolic Waste in Tumor Metabolism

Cancer is initiated when healthy cells acquire mutations to oncogenes and tumor suppressors, causing cells to transform into a state of unchecked growth and proliferation. Metabolism is fundamental to maintaining this transformed state, and as previously described, is often targeted and/or exploited for cancer treatment. The combination of high nutrient consumption, increased metabolic fitness, and poor organization of tumor vasculature creates a distinct microenvironment. As a consequence of increased demand for cancer cells to generate energy and biomass, a key feature of this microenvironment is the accumulation of metabolic waste products. Importantly, the tumor microenvironment (TME) plays a critical role in disease progression; however very few components of the TME have been studied in depth for their role in tumor biology.

Lactate, which is the by-product of aerobic glycolysis (The Warburg Effect), is abundantly secreted by cancer cells and consequently accumulates in the microenvironment of

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solid tumors. Lactate accumulation in the tumor microenvironment not only has profound effects on tumor biology, but also the other cells in the TME. Excess lactate causes the pH in the TME to be lowered to 6.0 - 6.5 compared to plasma levels that are approximately 7.4. Acidification caused by lactate in the TME suppresses tumor-infiltrating lymphocytes, increases angiogenesis through VEGF/VEGFR2 signaling, enables metastasis, and alters metabolic flux in associated fibroblasts and mesenchymal stem cells. Importantly, recent studies have revealed that in addition to being a secreted metabolic waste product, lactate can be taken up by tumor cells and recycled as a primary carbon source for pyruvate and TCA cycle intermediates. Thus, the implications of lactate in the TME are well established.

Beyond lactate, the effects of metabolic by-products on the TME have not been studied in depth. Glucose provides tumor cells with carbon and oxygen, which is not sufficient for biosynthesis of macromolecules such as protein, RNA, and DNA that require nitrogen. To support the nitrogen demand of rapid proliferation, tumor cells take up amino acids such as glutamine, asparagine, serine, and alanine. Amino acids that are taken up by

tumor cells can be directly utilized for synthesis of protein and nucleic acids or can be catabolized for energy. Paradoxically, in amino acid and nucleotide catabolism, nitrogen atoms are cleaved and the metabolic waste product ammonia (NH$_3$) is released.$^{161}$ NH$_3$ is also generated in the TME during chemotherapy treatment by Tumor Lysis Syndrome, which involves rapid discharge of cell lysates, causing total ionic load (including NH$_4^+$) to rise steeply.$^{162}$

NH$_3$ has been classically conveyed as a toxic metabolic waste product in tumor metabolism.$^{163}$ Notably, all studies on NH$_3$ toxicity have been demonstrated in astrocytes, such that NH$_3$ treatment causes astrocyte swelling.$^{164,165}$ Systemic hyperammonemia, which is the elevation of plasma NH$_3$ levels above 50 µM, leads to seizures and encephalopathy.$^{166,167}$ In contrast to systemic ammonia levels, solid tumors, which are poorly vascularized, accumulate NH$_3$ to concentrations that range between 1 mM and 5 mM.$^{168}$ The ability for NH$_3$ to accumulate in the TME suggests that NH$_3$ is not toxic to tumor cells. In U2OS cells, it was demonstrated that supraphysiological concentrations of NH$_4$Cl stimulate autophagy through LC3-II lipidation.$^{169}$ However, a thorough investigation of the role of physiological ammonia accumulation in the TME on tumor biology has never been performed.

Although mammalian cells possess many reactions capable of generating NH$_3$, only three reactions are capable of assimilating NH$_3$. These three enzymes are carbamoyl phosphate synthetase 1 (CPS1), glutamate dehydrogenase (GDH), and glutamine synthetase (GS).$^{170}$ CPS1 is a mitochondrial enzyme mainly expressed in liver cells that catalyzes the committed step of the urea cycle, producing carbamoyl phosphate from free NH$_4^+$ and carbonate. GDH and GS are ubiquitous enzymes that utilize cataplerosis from the TCA cycle in iterative amination reactions on $\alpha$-ketoglutarate to sequester NH$_3$ for glutamate and glutamine, respectively. Whether these reactions can be utilized by cancer cells to assimilate ammonia as a nitrogen source had never been explored. Furthermore, strategies for targeting ammonia production and or assimilation in cancer cells have never been explored. Finally, the effect of ammonia in other cell types (beyond cancer cells) within the TME has never been investigated. A major reason for the dearth of research on ammonia metabolism in cancer is the lack of tools available for (1) specifically and quantitatively measuring ammonia levels in metabolic lysates and (2) distinguishing $^{15/14}$N-isotopologues of ammonia for metabolic tracing and mass spectrometry analysis.

Chapter Two: An LC-MS Approach to Quantitative Measurement of Ammonia Isotopologues

I. Abstract. Ammonia is a fundamental aspect of metabolism spanning all of phylogeny. Metabolomics, including metabolic tracing studies, are an integral part of elucidating the role of ammonia in these systems. However, current methods for measurement of ammonia are spectrophotometric, and cannot distinguish isotopologues of ammonia, significantly limiting metabolic tracing studies. Here, we describe a novel LC-MS-based method that quantitatively assesses both $^{14}$N- and $^{15}$N-isotopologues of ammonia in polar metabolite extracts. This assay (1) quantitatively measures the concentration of ammonia in polar metabolite isolates used for metabolomic studies, and (2) accurately determines the percent isotope abundance of $^{15}$N-ammonia in a cell lysate for $^{15}$N-isotope tracing studies. We apply this assay to quantitatively measure glutamine-derived ammonia in lung cancer cell lines with differential expression of glutaminase.

II. Introduction. Quantitative measurement of ammonia is paramount in a broad spectrum of research topics. As the most reduced form of inorganic nitrogen in nature, ammonia plays a pivotal role in the nitrogen cycle among aquatic life, plants, animals and microorganisms, as well as in the geochemical formation of the early Earth.$^{171}$ In addition, ammonia is an essential nitrogen source for yeast and E. coli, and deprivation of ammonia impairs their growth and survival.$^{172,173}$ Ammonia is also a normal by-product of nucleotide and amino acid metabolism, and systemic organismal levels are tightly regulated.$^{174,175}$ Furthermore, the microbiome generates an abundance of ammonia, and contributes to >50% (~3g/day) of the total ammonia.


in mammals. Dysfunction of ammonia metabolism underlies many diseases including cancer, urea cycle disorders, hepatic encephalopathy, and cerebral dysfunction such as, intracranial hypertension, seizures, and ataxia.\textsuperscript{176,177} Thus, assays to accurately measure ammonia would deepen our understanding of ammonia in biology.

Current techniques for ammonia quantification include ion-selective electrodes (ISEs), enzyme-based assays, and colorimetric assays. However, these methods for ammonia measurement have limitations, and there is no technique available that specifically and sensitively detects ammonia. ISEs are complicated by cross-ion interferences, while enzyme-based assays may be sensitive to factors such as salt concentration and metabolites that compete with or allosterically modulate enzyme activity.\textsuperscript{179,180} In addition, metabolites commonplace to biology interfere with readouts in colorimetric assays through overlapping absorption spectra.\textsuperscript{181} Therefore, with current technology, ammonia cannot be specifically measured in biological samples. Moreover, while many assays measure ammonia, there is no assay capable of distinguishing between isotopologues of ammonia (\textsuperscript{14}NH\textsubscript{3} and \textsuperscript{15}NH\textsubscript{3}) for metabolic tracing studies.

Metabolic tracing experiments are commonly used to determine the biosynthetic pathways utilized by a cell to generate a metabolite.\textsuperscript{182} In a metabolic tracing assay, cells are incubated with a stable isotopologues (usually containing \textsuperscript{13}C or \textsuperscript{15}N) of the metabolite of interest. Polar metabolites are then extracted and analyzed with liquid chromatography coupled

\textsuperscript{180} Ye, H., et al. (2017). ACS nano 11, 2052-2059.
\textsuperscript{182} Chokkathukalam, A., et al. (2014). Bioanalysis 6, 511-524.
to mass spectrometry (LC-MS). The derivatives of the labeled metabolite acquire $^{13}$C and/or $^{15}$N, and therefore are identified by a shift in mass-to-charge ratio (m/z). Such experiments have been utilized to determine TCA cycle anaplerosis, and nitrogen sources for nucleotide synthesis. However, unlike most polar metabolites, ammonia is not detected by LC-MS and is therefore neglected in metabolic tracing studies.

The Berthelot reaction is a colorimetric assay utilized to measure ammonia, which was originally developed by chemist Pierre Eugene Marcellin Berthelot (1827-1907). In this reaction, an oxidative phenolic coupling generates the compound indophenol from ammonia (Figure 2.1a). Indophenol is highly conjugated, and absorbs strongly between 630 and 720 nm, enabling spectrophotometric detection. This reaction has been applied to measure ammonia in complex matrices, such as blood, soil, and seawater. This assay is also the standard analysis used in hospital Blood Urea Nitrogen (BUN) exams, in which urea is converted to ammonia via Urease and subsequently converted to indophenol for detection. However, similar to the limitations of other colorimetric assays, the Berthelot reaction for ammonia detection is complicated by competing molecules that interfere with the reaction or absorb on the same spectra as indophenol, such as chlorophyll.

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We hypothesized that indophenol generated in the Berthelot reaction could be amenable to LC-MS, allowing for the direct and specific quantification of ammonia in a manner that filters out background reactions. We developed an LC-MS coupled Berthelot assay, which can be performed on polar metabolite extracts and other complex biological samples. Furthermore, we showed that the MS Berthelot assay allowed detection of isotopologues of ammonia ($^{14}$NH$_3$ and $^{15}$NH$_3$). In this study we applied this assay to quantify the contribution of $^{15}$N-(amide)-glutamine to total cellular ammonia.

III. Results

IIla. The Berthelot Reaction Generates Indophenol and is Sensitive Detected by LC-MS

We developed a mass spectrometry-based (MS) Berthelot assay to quantitatively measure ammonia. First, we assayed a pure indophenol standard using LC-MS (Figure 2.1b,c). Next, we assayed products from the Berthelot reaction, which we adapted to be performed in 80:20 methanol:water, allowing for compatibility with polar metabolite extracts. We observed the same peak and MRM transition (198.1-167.1) as the pure indophenol standard (Figure 2.1d,e). Next, we optimized the molar ratio of the reactant phenol to ammonia being measured (Figure 2.2a). In this assay, we tested 50 mM NH$_4$Cl, which is far greater than any physiological concentration observed in biology.\textsuperscript{193} To ensure reaction completion, a minimum volumetric ratio of 4:1 for each reactant solution to ammonia was required. Therefore, to ensure all measurements are quantitative, we used a volumetric ratio of 5:1 in our assays. Next, we assessed the sensitivity of this assay to low ammonia concentrations (Figure 2.2b) and determined the lower limit of ammonia detection to be 500 nM, with a limit of linearity of 0.4 mM. Therefore, this assay is optimal for quantitatively measuring ammonia concentrations in physiological ranges for human plasma (~35 µM) and in fresh water, in which ammonia concentration is very low.

\textsuperscript{193} Dasarathy, S. \textit{et al.} (2016) \textit{Metabolic brain disease}, 3
Figure 2.1. Indophenol Generated from The Berthelot Reaction is detected with LC-MS.

A. Schematic of the Berthelot Reaction, which generates indophenol from ammonia. B. Total ion chromatogram of an indophenol standard (1μg/mL). C. Mass spectrum for the retention time window 15.7-16.9 minutes of the total ion chromatogram of the indophenol standard. D. Total ion chromatogram of 500 μM ammonia after the Berthelot reaction. E. Mass spectrum for the retention time window 15.7-16.9 minutes of the total ion chromatogram of the Berthelot reaction.
IIIb. The MS Berthelot Assay Accurately Detects Ammonia Isotopologues ($^{14}$NH$_3$ and $^{15}$NH$_3$)

We tested whether ammonia isotopologues ($^{14}$NH$_3$ and $^{15}$NH$_3$) can be distinguished by LC-MS (Figure 2.2c). We performed the Berthelot reaction on both isotopologues of ammonia and found distinct mass spectra: $^{14}$NH$_3$ generates $^{14}$N-indophenol with an MRM transition of 198.2-167.9, and $^{15}$NH$_3$ generates $^{15}$N-indophenol with an MRM transition of 199.2-168.9 (Figure 2.2d). Next, we tested whether the assay can accurately calculate the percent abundance of $^{14}$N versus $^{15}$N-labeled ammonia (Figure 2.2e). The Berthelot reaction was performed with ratios of 500 mM $^{14}$N- and $^{15}$N- ammonia mixtures. The resulting standard curve was linear, $R^2=0.9989$, and therefore accurately measures ratios of $^{14}$N: $^{15}$N-ammonia.
Figure 2.2. The Berthelot Reaction Paired with LC-MS Analysis Sensitively Measures Ammonia

**A.** Optimization of the molar ratio of phenol to ammonia for maximal readout of indophenol after the Berthelot reaction. **B.** Concentration curve of ammonia after the Berthelot reaction measured as indophenol with LC-MS. Ammonia standards were made in 80% methanol, n=3 samples per concentration. LOD: Limit of detection, LOL: limit of linearity. **C.** Schematic of the Berthelot reaction on NH₃ and ¹⁵NH₃, which generate indophenol and ¹⁵N-indophenol respectively. **D.** Mass spectra of 500µM NH₃ (top) and 500 µM ¹⁵NH₃ after the Berthelot reaction in 80% methanol. **E.** Standard curve of percent ¹⁵NH₃ : NH₃ in a 500 µM ammonia solution generated in 80% methanol treated with the berthelot reaction. The resulting ratio of ¹⁵N-indophenol:¹⁴N-indophenol are plotted as a function of the expected percent ¹⁵N-ammonia.
Illic. *The MS Berthelot Assay Accurately Measures Ammonia in Cell Lysates*

We tested whether the MS Berthelot assay could quantify ammonia from cell lysates, a more complex matrix comprised of many metabolites that may potentially interfere with the Berthelot reaction. Importantly, we performed the MS Berthelot assay using lysates extracted in 80% methanol, amenable for metabolite profiling (Figure 2.3a). To ensure completion of the reaction in a cell lysate, we used a volumetric ratio of 10:1 of each solvent to lysate. First, we assayed the specificity of indophenol for the MS Berthelot assay. Indophenol was undetectable in mammalian cell lysates, and was only detected after the Berthelot reaction (Figure 2.3b). Thus, as indophenol is not a mammalian metabolite, it can be distinctly measured after reaction with ammonia in the complex matrix of a polar metabolite extraction.

We also evaluated the sensitivity of ammonia detection from mammalian cells. We spiked a concentration curve of ammonia into 293T lysates, generating a linear standard curve with an $R^2$ value of 0.998 (Figure 2.3c). Furthermore, ammonia increased linearly with a dynamic cell range of 500,000 to 2.5 million cells ($R^2 = 0.9991$) (Figure 2.3d). To directly assay ammonia concentrations within mammalian cells, we performed the MS Berthelot assay from a cell lysate not spiked with ammonia. We used the standard addition technique to generate a standard curve by spiking ammonia into 293T cell lysates (0.0-0.8 mM ammonia) (Figure 2.3e). In 293T cells, we measured 100 femtomoles of ammonia per cell (Figure 2.3f), consistent with levels detected using a commercial ammonia assay.
A

Workflow

1- Seed Cells

2- Isolate Metabolites (80% MeOH)

3A- The Berthelot Reaction

Lysate

NaOH

HCl

3B- OR

Steady-State Metabolomics

B

Lysate Before Berthelot Reaction

Lysate After Berthelot Reaction

C

Ammonia Concentration Curve

D

Standard Curve Cell Number

E

Standard Curve for Percent $^{15}$NH$_4$Cl in Lysate

$Y = 223840X + 57728$

$R^2 = 0.998$

$Y = 112369X + 240243$

$R^2 = 0.9991$

$Y = 0.008376X + 0.07427$

$R^2 = 0.995$

$Y = 0.008376X + 0.07427$
Figure 2.3. Assay development in cellular lysates. A. Schematic of the work flow for measuring ammonia from a cellular lysate. B. Total ion chromatogram for the MRM transition of 198.2 : 167.9 in a cellular lysate extracted with 80:20 methanol:water (top) and a cellular lysate after the Berthelot reaction (bottom). C. Concentration curve of the Berthelot reaction on samples with increasing ammonia spiked into cellular lysate from 3x10^6 293T cells. D. Standard curve of the Berthelot reaction performed on cellular lysates from increasing cell number. E. Standard curve of the Berthelot reaction performed on cellular lysates with spiked in concentrations of ammonia. F. Measurement of moles of ammonia per cell using the standard addition technique with the Indophenol assay, and in parallel from the same lysate, measurement of ammonia using a standard assay (n=3 replicates).
Illd.  The MS Berthelot Assay Accurately Distinctly Measures Ammonia Isotopologues in Cell Lysates

Next, we tested whether the MS Berthelot assay accurately measured ratios of $^{14}$N/$^{15}$N-ammonia spiked into a cell lysate. Indeed, with $R^2=0.995$, this assay accurately measures the ratio of ammonia isotopologues (Figure 2.4a). We next applied isotope-tracing studies to test whether ammonia as a by-product of metabolic reactions can be detected using a biologically relevant cellular system. Glutamine is commonly catabolized by the ubiquitous enzyme glutaminase (GLS) to generate glutamate and ammonia (Figure 2.4b). Lung cancer cell lines vary in GLS expression, and therefore represent an excellent model to study glutamine-derived ammonia using this assay (Figure 2.4c). We treated GLS high (Calu6, H2030) and GLS low (H23, H1299) lung cancer cell lines with the stable isotope $^{15}$N-amide-glutamine, which liberates $^{15}$N-ammonia in the process of glutaminolysis. We performed the MS Berthelot assay on media from cultured cells, as well as cell lysates. As anticipated, cell lines with high GLS expression contributed more glutamine-derived ammonia to the total ammonia pool in media (~55%) and in cells (~45%), compared to GLS low cell lines, which contributed 45% and 30% to media and cellular ammonia, respectively (Figure 2.4d).
Figure 2.4. Application of Indophenol Assay to Quantitatively Measure Glutamine-derived Ammonia in Lung Cancer Cell Lines. 

A. Standard curve of percent $^{15}$NH$_3$ : NH$_3$ in a 500 $\mu$M ammonia solution generated in cellular lysate from 293T cells treated with the Berthelot reaction. The resulting ratio of $^{15}$N-indophenol : $^{14}$N-indophenol are plotted as a function of the expected percent $^{15}$N-ammonia. 

B. Schematic of the metabolic tracing experiment to elucidate glutamine contribution to cellular ammonia pools. Blue represents the $^{15}$N-isotope. 

C. mRNA levels of glutaminase in lung cancer cell line panel. $n=3$ replicates 

D. Percent $^{15}$N-ammonia after treatment with 2.0 mM $^{15}$N-(amide)-glutamine. Measurements were taken in the media and in cellular lysates of the lung cancer panel, $n=3$ replicates per cell line.
IV. Discussion: Here we describe the development of a novel mass spectrometry assay, MS Berthelot assay, which sensitively measures ammonia in both pure solutions and cell lysates. This method utilizes the Berthelot reaction to convert ammonia into indophenol, which is directly detected by LC-MS. In samples that have complex matrices, such as cell lysates (as shown in Figure 2.3 and 2.4), the MS Berthelot assay generates a distinct molecule, indophenol, which can only be derived from ammonia. Thus, indophenol provides a direct readout of ammonia levels. Therefore, the MS Berthelot assay significantly advances current methods for ammonia detection by filtering out the interfering molecules that often complicate analysis of ammonia in complex matrices such as soil, blood, seawater, and cell lysates. We speculate that the MS-Berthelot assay can be applied to these systems for a more sensitive and direct method to measure ammonia compared to techniques that are currently available.

The MS Berthelot assay has several advantages over current ammonia detection strategies, including specificity, sensitivity, and the ability to detect isotopologues. The colorimetric Berthelot reaction is complicated by interfering background reactions that absorb in the same spectrum as indophenol. In the MS Berthelot assay, the triple quadrupole mass spectrometer filters out background reactions by selectively measuring ion counts of the mass:charge ratio of indophenol. Therefore, the MS-Berthelot assay resolves one of the major hurdles of ammonia measurement by eliminating the interference of background reactants in the readout. Furthermore, the colorimetric Berthelot assay for ammonia measurement has a lower limit of detection of ~7 µM, whereas the MS-Berthelot assay measures ammonia as low as 500 nM, increasing the sensitivity for ammonia detection 14-fold.\(^\text{194}\) This sensitivity is not only an improvement on the colorimetric Berthelot assay, but also an improvement on commercial assays, which often detect a lower limit of ~10 µM ammonia.

In this study, the MS-Berthelot assay was performed on cell lysates that were isolated with a polar metabolite extraction, showing the potential for this assay to be multiplexed with metabolite profiling studies. For this type of analysis, the Berthelot Reaction must be performed on an aliquot of the initial metabolite isolation, and metabolite profiling be performed on the remaining unreacted portion, since the highly oxidizing conditions of the Berthelot reaction will alter the contents of the metabolite isolate (Figure 2.3A). The MS-Berthelot assay requires only 2% of the initial metabolite isolation, and is therefore feasible to do in parallel with steady-state metabolomics experiments without negatively impacting metabolite detection.

Finally, the MS Berthelot assay was used to distinctly measure ammonia isotopologues (\(^{14}\text{NH}_3\) and \(^{15}\text{NH}_3\)). The MS Berthelot assay accurately measures ratios of ammonia isotopologues by analyzing \(^{14}\text{N}\)-indophenol (m/z:198) to represent \(^{14}\text{NH}_3\) and \(^{15}\text{N}\)-indophenol (m/z: 199) to represent \(^{15}\text{NH}_3\). Importantly, this analysis was performed on cellular lysates isolated by methanol extraction, and therefore, measurement of ammonia isotopologues can be integrated with nitrogen metabolic tracing studies. In this study, we provide one example of this in which the stable isotopologue of glutamine (\(^{15}\text{N}\)-(amide)-glutamine) was used. We showed that using the MS Berthelot assay, cells with high glutaminase expression generate more ammonia from glutaminolysis than cells with low glutaminase expression. We detected this in both cell lysates and the media. In addition to glutamine, this technique can be applied to wide-range of metabolic tracing experiments involving other nitrogen-containing metabolites such as branched chain amino acids and nucleotides. Beyond mammals, the MS Berthelot assay is applicable to all organisms, as ammonia is a ubiquitous by-product of metabolism, and will significantly advance mechanistic studies of ammonia metabolism.

V. Supplemental Material

Va. Materials and Methods

*Mass Spectrometry.* Samples were analyzed on a reverse phase ion-pairing chromatography coupled to tandem mass spectrometry (Agilent LC-MS). Analytes were eluted in buffer A (97 %
H₂O, 3% MeOH, 10 mM Tributylamine, 15 mM Glacial Acetic Acid, pH 5.5) and buffer B (10 mM Tributylamine, 15 mM Glacial Acetic Acid in 100% MeOH). Samples were run on a ZORBAX Extend-C18, 2.1 x 150 mm, 1.8 µm (Agilent) starting with a flow rate of 0.25 mL/min for 2.5 minutes of buffer A, followed by a linear gradient (100% buffer A to 80% buffer A) for 5 minutes, followed by a linear gradient (80% buffer A to 55% buffer A) for 5.5 minutes, followed by a linear gradient (55% buffer A to 1% buffer A) for 7 minutes, followed by 4 minutes with (1% buffer A). Following each run, an acetonitrile backwash was utilized to clean the column followed by a 8 minute re-equilibration period of 100% buffer A. Samples were ionized (with negative polarity) using Agilent Jet Spray ionization; nebulizer 45 psi, capillary –2000 V, nozzle voltage: 500 V, sheath gas temperature 325°C, and sheath gas flow 12 L/min. An Agilent 6470 Triple Quadrupole mass spectrometer was used for mass detection with a targeted method for indophenol. Using a 1 ug/mL indophenol standard (Sigma), the method was developed with a fragmentor voltage of 80 V, collision energy of 30 V, parent ion 198.1 and fragment 168.9. For 15N-indophenol, fragmentor voltage of 80 V, collision energy of 30 V, parent ion 199.1 and fragment 169.9 were utilized. Peaks were integrated in Mass Hunter (Agilent).

**Cell Culture.** All cell lines were cultured in DMEM (Life Technologies) supplemented with 10% FBS (Life Technologies) and 1% penicillin and streptomycin (Invitrogen). For tracing studies using isotopic glutamine, glutamine-free RPMI (Life Technologies) supplemented with 2.0 mM 15N-(amide)-glutamine (Sigma), 10% FBS and 1% penicillin and streptomycin was used.

**RNA Isolation and RT-PCR.** RNA was extracted using Direct-zol RNA Miniprep Kit (Genesee Scientific), and cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad). Real-time qPCR was performed on a Light Cycler 480 (Roche) using PerfeCTa SYBR Green Fast Mix (Quanta BioSciences). Primers: RPLP0 (F): acgggtacaacaggtctcg, RPLP0 (R): cgactttccttgcttcaa, GLS (F): tgtcagctctgcttgcttg, GLS (R): tcatagtctatgcttcaaag.

**Polar metabolite isolation.** Metabolites were isolated directly from adherent cells. Media was aspirated and wells were quickly washed with 1 mL ice-cold PBS. Then, 1 mL of 80% HPLC-
grade Methanol (BT Baker) for a 10 cm dish or 500 uL of ice-cold 80% HPLC-grade Methanol (BT Baker) for a 6-well dish were used to extract polar metabolites. Tissue culture dishes were incubated at -80 for 10 minutes, and scraped on dry ice. Lysates were centrifuged at 10,000 x g for 10 minutes and supernatants were either used for assays or stored at -80.

Metabolic tracing. Lung cancer cell lines were incubated with 2.0 mM ^15N-(amide)-glutamine for 24 hours. Metabolites were isolated from the media in a 10:1 80% MeOH: lysate ratio and from cells as previously described. The Berthelot reaction was performed on isolates and analyzed by LC-MS.

The MS Berthelot assay. The Berthelot reaction was performed on pure ammonia standards made in 80:20 methanol:water or cellular lysates generated using the previously described method for polar metabolite extraction. Reactions in 80% MeOH were performed with a volumetric ratio of 10:1 (reactant mixture: sample). For example, 20uL of sample was reacted with 100uL Solution #1 and 100 uL Solution #2. In a cell lysate, the volumetric ratio used was 20:1 (reactant mixture: sample), in which 20 uL of sample would be reacted with 200 uL Solution #1 and 200 uL Solution #2. Solution #1 is composed of 100 mM Phenol (Sigma) and 50 mg/L sodium nitroprusside (Sigma). Solution #2 is composed of 0.38 M Dibasic Sodium Phosphate (Sigma), 125 mM NaOH, 1% sodium hypochlorite, available chlorine 10-15% (Sigma). Upon addition of the two reactant solutions, the samples are mixed and incubated at 37° C for 40 minutes. All solutions and reactions should be stored at 4° C until use for LC-MS analysis.

Ammonia Measurement. Ammonia was measured using the Berthelot method as described above or using a commercial colorimetric assay (Abcam #ab83360).

Statistical Analysis. Two-tailed student’s t-test was used to compare the means among experimental subgroups. All statistical tests had an alpha of 0.05 as the significance threshold. * = P<0.05, ** = P<0.01, *** = P<0.005, **** = P<0.001, ***** = P<0.0001.
Vb. Acknowledgements. We thank all members of the Haigis lab for thoughtful discussion. M.C.H. is funded by the Ludwig Center at Harvard. J.B.S. is funded by the National Science Foundation Graduate Research Fellowship Program (Grant # DGE1144152).
Chapter Three: Metabolic Recycling of Ammonia Via Glutamate Dehydrogenase Supports Breast Cancer Biomass

I. **Abstract:** Ammonia is a ubiquitous by-product of cellular metabolism, however the biological consequences of ammonia production are not fully understood, especially in cancer. We find that ammonia is not merely a toxic waste product, but is recycled into central amino acid metabolism to maximize nitrogen utilization. Cancer cells primarily assimilated ammonia through reductive amination catalyzed by glutamate dehydrogenase (GDH), and secondary reactions enabled other amino acids, such as proline and aspartate, to directly acquire this nitrogen. Metabolic recycling of ammonia accelerated proliferation of breast cancer. In mice, ammonia accumulated in the tumor microenvironment, and was used directly to generate amino acids through GDH activity. These data show that ammonia not only is a secreted waste product, but a fundamental nitrogen source that can support tumor biomass.

II. **Main Text:** Increased nutrient consumption can supply carbon, nitrogen, oxygen, and sulfur to accommodate the extensive bioenergetic, biosynthetic, and pro-survival requirements of rapidly proliferating cells.\(^{195,196,197}\) As a consequence, such cells generate an excess of metabolic waste which are cleared in mammals through the excretory system. However, in the tumor microenvironment, metabolic waste such as lactate and ammonia accumulate.\(^{198,199}\) Although lactate is well studied in cancer, little is known about the mechanisms by which cancer cells manage increased amounts of ammonia (NH\(_3\)) generated by glutamine and asparagine catabolism, de novo cysteine synthesis through the transsulfuration pathway, and salvage


\(^{196}\) Vyas, S., Zaganjor, E., Haigis, M.C. (2016). Cell 166, 555-566

\(^{197}\) Coloff, J.L., et al. (2016). Cell metabolism 23, 867-880


nucleotide metabolism. Ammonia has been considered a toxic by-product that must be exported from cells and is subsequently cleared through urea cycle activity in the liver.

Glutamine has been called a “nitrogen reservoir” for cancer cells because of its anabolic role in nucleotide synthesis. However, the role of glutamine as a nitrogen reservoir is contradicted in catabolic glutamine metabolism, because nitrogen is liberated as the by-product ammonia. The fate of ammonia in metabolism of proliferating cells and tumors remains unclear. We hypothesized that ammonia might be re-assimilated into central metabolism to maximize the efficiency of nitrogen utilization. In this study we sought to clarify roles of ammonia as 1) a toxic waste product or 2) a biosynthetic metabolite (Figure 3.1a).

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**Figure 3.1.** Glutamine-Derived ammonia is recycled.  

**A.** Schematic of fates of ammonia in cancer.  

**B.** mRNA expression of ammonia-assimilating enzymes from The Cancer Genome Atlas in cancer compared to its normal tissue. Fold-change (cancer/normal) for GS (Glutamine Synthetase), GDH1 (Glutamate Dehydrogenase), and CPS1 (Carbamoyl Phosphate Synthetase 1) RNA levels were assessed using Oncomine.org. Values are the mean of fold change (cancer/normal) measured across the number of patients listed. (A) Ovarian Serous Cystadenocarcinoma, (B) Colon Adenocarcinoma, (C) Rectal Adenocarcinoma, (D) Lobular & Ductal Breast Carcinoma, (E) Lung Adenocarcinoma, (F) Squamous Lung Cell Carcinoma, (G) Endometrial Adenocarcinoma, (H) Bladder Urothelial Carcinoma, (I) Gastric Adenocarcinoma, (J) Glioblastoma, (K) Pancreatic Adenocarcinoma, (L) Hepatocellular Carcinoma, (M) Cutaneous Melanoma.  

**C.** Schematic of $^{15}$N-isotopologues after treatment with $^{15}$N-(amide) glutamine.  

**D.** Isotopologue abundance of unexpected $^{15}$N-(amide) glutamine derivatives +/- 1 uM BPTES in T47D and MCF7 cell lines. Values represent mean +/- SEM, n=4 per condition.  

**E.** Isotope abundance of $^{15}$N-(amide) glutamine-derived metabolites in control cells and cells depleted of GDH (shGDH#1 & shGDH#2). ND= $^{15}$N-Isotopologue not detected. Glu = Glutamate M+1, Pro = Proline M+1, Asp = Aspartate M+1, Cit = Citrulline M+1, Asa = Argininosuccinate M+1. Values represent mean +/- SEM, n=4 per condition.  

**F.** Schematic of ammonia recycling. For all comparisons two-tailed t test was used. *P < 0.05, **P < 0.01, ***P<0.001, ****P<0.0001.
Mammals have three enzymes that can overcome the thermodynamic hurdles of ammonia assimilation: carbamoyl phosphate synthetase I (CPS1), the ATP-dependent, rate-limiting step of the urea cycle; glutamate dehydrogenase (GDH), an NAD(P)H-dependent enzyme that catalyzes reductive amination of α-ketoglutarate; and glutamine synthetase (GS), which catalyzes the ATP-dependent amination of glutamate to generate glutamine (Figure 3.S1A). Analysis of transcriptomic data from The Cancer Genome Atlas for the ammonia-assimilating enzymes in healthy and cancerous tissues revealed that expression of GS and GDH mRNA was significantly increased across many cancer subtypes whereas CPS1 was only increased in colon (Figure 3.1b). Among healthy tissues, GS and GDH are ubiquitously expressed and CPS1 is only expressed in the liver (Figure 3.S1B). Breast cancers displayed increased expression of both GS and GDH. Specifically, Estrogen Receptor (ER) positive breast cancers have increased expression of GS and GDH compared to that in other subtypes. Therefore, we used ER positive breast cancer as a representative model to probe for ammonia assimilation.

To investigate the fate of glutamine-derived ammonia, we performed a metabolic tracing analysis with hydrophilic interaction liquid chromatography tandem mass spectrometry (HILIC-MS) and assessed the fate of $^{15}$N(amide)-glutamine, which liberates $^{15}$NH$_3$ through glutaminase activity. To identify the metabolic derivatives of $^{15}$N(amide)-glutamine in an unbiased manner, we developed a method to screen the nitrogen metabolome, which contained 211 $^{15}$N-isotopologues (table 3.S1). The majority of the nitrogen metabolome did not acquire $^{15}$N-

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labeling; of 211 $^{15}$N-isotopologues, only 33 metabolites were labeled (Figure 3.S2). Consistent with previous studies, $^{15}$N-(amide)-glutamine was incorporated into asparagine and nucleotides (Figure 3.1c & Figure 3.S3a).\textsuperscript{211} We also identified $^{15}$N-isotopologues of proline, aspartate, branched chain amino acids (BCAA), and glutamate, which have no previous biosynthetic connection to the amide-nitrogen on glutamine (Figure 3.1c & Figure 3.S3b). The labeled nitrogen was liberated as ammonia before production of these metabolites, suggesting that an ammonia recycling pathway may synthesize the other glutamine derivatives detected.

To test whether ammonia released during glutaminolysis was necessary for production of these unanticipated amide-nitrogen glutamine derivatives, cells were treated with the glutaminase inhibitor Bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide (BPTES), which at 1 mM is not cytotoxic or cytostatic in T47D and MCF7 cell lines (Figure 3.S4a-c).\textsuperscript{212} BPTES treatment significantly decreased $^{15}$N-isotopologues of glutamate, proline, and aspartate, whereas metabolites involved in direct glutamine metabolism such as nucleotides and asparagine remained labeled (Figure 3.1d & Figure 3.S4d). Addition of ammonia to BPTES-treated cells restored metabolites depleted by glutaminase inhibition, demonstrating the specific contribution of ammonia (Figure 3.S4e). This is consistent with findings that ammonia partially rescues proliferative defects in glutamine-deprived breast cancer cells.\textsuperscript{213}

We examined the potential mechanisms underlying assimilation of ammonia liberated during glutaminolysis. Because $^{15}$N-(amide)-glutamine did not elicit any isotopes of the urea cycle intermediates ornithine, citrulline, argininosuccinate and arginine, activity of CPS1 was ruled out as a mechanism for ammonia assimilation (Figure 3.S2). Instead, our data indicated that GDH was the primary point of ammonia assimilation because glutamate is upstream of


proline, aspartate, glutamine, and BCAA synthesis. However, the $K_m$ of GDH for ammonia is high (sub-mM) and GDH reportedly favors oxidative deamination over reductive amination in cancer cells.\textsuperscript{214,215,216,217} By contrast, GDH-catalyzed reductive amination is prevalent in the liver, where there is a sufficient concentration of ammonia to enable catalysis in this direction.\textsuperscript{218}

We wondered if increased concentrations ammonia in the tumor microenvironment might also permit GDH-catalyzed reductive amination.

To determine whether GDH assimilates ammonia generated by glutamine catabolism, we used shRNA to deplete cells of GDH, cultured them with $^{15}$N-(amide)-glutamine and then subjected them to nitrogen metabolome scanning (Figure 3.S4f). MCF7 and T47D cell lines express both GDH1 and GDH2 isoforms, and shRNAs targeted both (Figure 3.S4g). Abundance of $^{15}$N-isotopologues of glutamate and downstream metabolites (proline, aspartate) was significantly decreased in cells depleted of GDH (Figure 3.1e). Urea cycle intermediates (citrulline, argininosuccinate) remained unlabeled in cells lacking GDH, underscoring the lack of CPS1-mediated ammonia assimilation in breast cancer cells (Figure 3.1e). Re-expression of shRNA-insensitive GDH1 rescued labeling onto glutamate and downstream metabolites (Figure 3.S5).

Next, we measured the amount of $^{15}$NH$_3$ generated after 8 hour treatment with $^{15}$N-(amide)-glutamine using an LC-MS method for detection of $^{15}$NH$_3$ from $^{15}$N-(amide)-glutamine.\textsuperscript{219}

In MCF7 and T47D cells, only 3.5% of the total ammonia pool derived from glutaminolysis


(Figure 3.S6a-c). Since ~2% of the glutamate pool acquired this label in a GLS-dependent manner (Figure 3.S3b), we hypothesized that ammonia recycling from glutaminolysis is highly efficient. We quantified the efficiency of ammonia recycling from glutamine catabolism by incubating MCF7 cells with $^{15}\text{N}_{2}-^{13}\text{C}_5$ glutamine. Glutaminolysis generated $^{15}\text{N}-^{13}\text{C}_5$-glutamate (glutamate M+6), and ammonia recycling was measured by detection of $^{15}\text{N}$-glutamate (glutamate M+1) (Figure 3.S6d). We calculated the ratio of total amount of glutamate (glutamate M+6 & glutamate M+1) to glutamate directly generated in glutaminolysis (glutamate M+6) (Figure 3.S6e). In total, 1.57 molecules of glutamate (by nitrogen) were generated from a single reaction of glutaminolysis, indicating a 57% efficiency of ammonia recycling (Figure 3.S6f). Because both processes are mitochondrial, localization may support this high efficiency.$^{220}$ Since GDH is a bi-directional enzyme, we also tested whether the catalytic activity of oxidative deamination or reductive amination was prevalent. In GDH-depleted cells, ammonia recycling (glutamate M+1) was decreased, but a-ketoglutarate (M+5), was unchanged, suggesting a net activity of reductive amination in this system (Figure 3.S6g-h). In sum, these data indicate that ammonia derived from glutaminolysis is recycled by reductive amination catalyzed by GDH to support the synthesis of glutamate and downstream metabolites (Figure 3.1f).

Because numerous reactions generate ammonia in addition to glutaminolysis, we investigated whether free ammonia could be assimilated into metabolic pathways. To optimize NH$_4$Cl for tracing studies, we investigated whether exposure to increased concentrations of NH$_4$Cl was toxic to tumor cells. Physiological concentrations of ammonia in plasma range between 0-50 µM in healthy human adults, 50-150 m µM in newborns, and up to 1.0 mM in patients with hyperammonemia.$^{221}$ Supraphysiological concentrations of ammonia are toxic to


neurons, and sometimes assumed to also be toxic to tumor cells.\textsuperscript{222223224} However, NH$_4$Cl was not toxic to tumor cells, even at concentrations that were toxic to primary human astrocytes (Figure 3.2a, Figure 3.S7a). Previous reports have shown that high concentrations of ammonia induce autophagy in tumor cells.\textsuperscript{225} In MCF7 and T47D cell lines, LC3II lipidation is not induced until 10 mM is added to media, which is greater than levels of ammonia reported in the tumor microenvironment. (Figure 3.S7b) Moreover, ammonia concentrations of 0 to 10 mM did not alter uptake of glucose or glutamine, or basal respiration (Figure 3.S7c-e). The expression of ammonia assimilating enzymes GS, GDH and CPS1 was not affected by increasing ammonia concentration (Figure 3.S7f-i), nor did 10 mM ammonia alter the pH of the culture media (Figure 3.S7j). These data indicate that supra-physiological concentrations of ammonia did not induce toxicity or metabolic stress in breast cancer cells.

We also examined ammonia uptake by cells. When breast cancer cells were cultured in low concentrations of ammonia (0 to 1.0 mM) we observed a net output of ammonia, which reverted to net uptake as the extracellular concentration of NH$_4$Cl increased above 1 mM (Figure 3.S7k). At approximately 1.0 mM NH$_4$Cl, ammonia was taken up from the medium, such that ammonia entry may be regulated by diffusion. In agreement, the characterized mechanism of ammonium (NH$_4^+$) import and export is through facilitated diffusion with Rhesus glycoproteins (RhC and RhG).\textsuperscript{226} Also, ammonia (NH$_3$) can diffuse across the plasma membrane.

We performed steady-state and tracing experiments in the presence of 0.75 mM NH$_4$Cl because it is the inflection point of ammonia uptake and secretion and represents a low


\textsuperscript{224} Hillmann, P., Kose, M., Sohl, K., and Muller, C.E. (2008). Toxicology and applied pharmacology 227, 36-47.

\textsuperscript{225} Eng, C.H., et al. (2010). Science signaling 3, ra31

concentration of ammonia that is relevant to the tumor microenvironment. We used MetaboAnalyst 3.0 to perform an unbiased pathway analysis on the steady-state metabolites from cells cultured with or without ammonia (Figure 3.S8a). The most significantly altered pathway was glutamate, aspartate, and alanine metabolism. Exposure to NH₄Cl elicited a signature of increased transaminase activity, whereby abundance of ketoacids decreased and that of amino acids derived from them increased (Figure 3.2b). Although amounts of non-essential amino acids increased, the abundance of other amino acids remain unchanged by ammonia, indicating ammonia did not affect universal amino acid metabolism (Figure 3.S8b). Nor did ammonia alter the abundance of metabolites from the urea cycle and nucleotides (Figure 3.S8c-d).
Figure 3.2. Ammonia is assimilated by GDH to generate amino acids. A. Propidium Iodide (PI) staining of cells treated with a dose of NH₄Cl for 48 hours. Values represent mean +/- SEM, n=3. Representative experiment of three replicates. B. Heat map of fold-change in steady-state abundance of keto- and amino acids involved in transaminase reactions in T47D cells treated with 0.75 mM NH₄Cl. Values represent mean +/- SEM, n=4. Representative experiment of two replicates. C. Abundance of ¹⁵N-isotopologues in MCF7 and T47D cells after 8 hours of treatment with 0.75 mM ¹⁵NH₄Cl. (M+1) indicates a single nitrogen labeled and (M+2) indicates two nitrogen labeled. Values are scaled to account for total intracellular ammonia and represent mean +/- SEM, n=4. D. Isotopologue abundance of glutamate (M+1) in MCF7 and T47D cells treated for 8 hours with 0.75 mM ¹⁵NH₄Cl in control and GDH-depleted cells. Values are scaled to account for total intracellular ammonia and represent mean +/- SEM, n=4. E. Abundance of ¹⁵N-isotopologues for metabolites downstream of glutamate treated for 8 hours with 0.75 mM ¹⁵NH₄Cl in control and GDH depleted cells. Values are scaled to account for total intracellular ammonia and represent mean +/- SEM, n=4. For all comparisons two-tailed t test was used. *P < 0.05, **P < 0.01, ***P<0.001, ****P<0.0001.
MCF7 and T47D cells were treated with 0.75 mM $^{15}$NH$_4$Cl and scanned for $^{15}$N-isotopologues (Figure 3.S9). Isotopologue abundances were scaled to represent total ammonia pools, since treatment with 0.75 mM enriched for ~35% of the intracellular ammonia pool (table 3.S2). Consistent with tracing performed with glutamine-derived ammonia, we detected $^{15}$N-labeling of glutamate and downstream metabolites, such as proline and aspartate (Figure 3.2c-d). Upon tracing with low levels of ammonia, a striking 20% of the glutamate pool was labeled, implying an important role for ammonia assimilation in glutamate metabolism in cancer, as glutamate exists near sub-millimolar levels.$^{227}$ Tracing with high levels of ammonia that have been reported in the tumor microenvironment (3 mM), also elicited the same signature of ammonia assimilation (Figure 3.S10a).

Consistent with steady-state data, all of the amino acids labeled were generated through glutamate-dependent transaminase reactions, except proline and glutathione, which are made in direct synthetic pathways from glutamate (Figure 3.S10b). Other nitrogen-containing metabolites, particularly urea cycle intermediates, and essential amino acids were not labeled by ammonia (Figure 3.S9). Furthermore, in spite of ammonia generating $^{15}$N-isotopologues of glutamine, we detected no $^{15}$N-isotopologues of any nucleotides, which is distinct from ammonia metabolism in LKB low tumors.$^{228}$ We speculate that because labeled glutamine is generated in the mitochondria, this pool may not access the cytoplasm where de novo nucleotide synthesis occurs, rendering nucleotides unlabeled.

A time course of $^{15}$NH$_4$Cl tracing revealed that ammonia was rapidly converted into glutamate, the first metabolite to reach steady-state (Figure 3.S10c-f). Thus, ammonia appears to be primarily assimilated to generate glutamate and other labeled metabolites are produced in secondary reactions. Therefore, we investigated which metabolic derivatives of ammonia


required activity of GDH. In cells depleted of GDH, $^{15}$NH$_4$Cl labeling of glutamate was diminished, as was labeling of metabolites downstream of glutamate (Figure 3.2e and 3.2f). Indeed this labeling was rescued when shRNA-insensitive GDH1 was overexpressed (Figure 3.S10g-h). We did not observe adaptation through ammonia assimilating enzymes GS or CPS1 in cells lacking GDH. In both T47D and MCF7 cells, glutamine and asparagine labeling did not change in cells depleted of GDH (Figure 3.S11). Metabolites of the urea cycle were unlabeled in cells depleted of GDH, indicating that adaptive reprogramming of ammonia assimilation into the urea cycle is not important in cultured breast cancer cells (Figure 3.S9). Our data reveal a general mechanism by which free ammonia in the tumor microenvironment can be harnessed for biosynthetic pathways.

Ammonia assimilation in yeast has a fundamental role in supporting growth and proliferation.\textsuperscript{229,230} Because ammonia was not toxic to tumor cells (Figure 3.2a), we tested whether ammonia might facilitate growth and proliferation of breast cancer cells. As in yeast, addition of NH$_4$Cl to cell culture media increased proliferation in breast cancer cell lines (Figure 3.S12a-b). Media was changed daily to minimize ammonia accumulation in culture media, which we measure to be approximately 0.3 mM per day from glutamine degradation and cellular metabolism (Figure 3.S12c-f). Moreover, in 3D culture, addition of ammonia to media stimulated sphere growth and cell proliferation (Figure 3.3a-b & Figure 3.S12g). However, proliferation of primary human fibroblasts was not changed when ammonia was added to culture media (Figure 3.S13a). Using $^{15}$NH$_4$Cl tracing, we found that fibroblasts centrally assimilated ammonia to generate glutamine (Figure 3.S13b), in line with their high expression of glutamine synthetase.\textsuperscript{231} Moreover, $^{15}$N-amide-glutamine tracing revealed that fibroblasts did not recycle


\textsuperscript{230} Sunil Laxman, B.M.S., Lei Shi, Benjamin P. Tu (2014). Science signaling 7, ra120.

glutamine-derived ammonia to generate glutamate, aspartate or proline (Figure 3.S13c). Thus we hypothesized that, ammonia assimilation to generate glutamate through GDH may be important for its role in increased proliferation observed in breast cancer cells. Indeed, depletion of GDH prevented the accelerated growth of breast cancer cells treated with ammonia (Figure 3.3c). Interestingly, the glutamate derivatives proline, aspartate and glutathione are associated with proliferation and tumorigenesis.232,233,234,235,236

To assess the effect of tumor-generated ammonia on growth and proliferation, we compared the ability of cancer cells to grow in 3D cultures in which the medium was changed either daily or every 3 days, allowing ammonia to accumulate. The latter procedure provided a growth advantage for breast cancer cells, which correlated with ammonia accumulation in the media (Figure 3.3d-e). Therefore, we tested whether ammonia recycling through GDH was a critical aspect that influenced proliferation. Cells depleted of GDH had no growth defect when the culture media was changed daily, but the growth advantage when media was changed after 3 days was abrogated (Figure 3.S14a-c & Figure 3.3f). Furthermore, cells depleted of GDH secreted more ammonia into the medium, consistent with impairment of ammonia recycling (Figure 3.3g). In addition, treatment of MCF7 cells in 3D culture with high ammonia concentrations (3 mM NH₄Cl) also stimulated proliferation (Figure 3.S14d).

To examine the physiological relevance of ammonia in the tumor microenvironment in vivo, we measured concentrations of ammonia that accumulated in the interstitial fluids of ER(+) xenograft tumors. ER(+) xenografts accumulated 0.8 to 3 mM ammonia in the interstitial fluids of the tumor microenvironment, compared with plasma ammonia concentrations approximately

300 μM (Figure 3.4a). Importantly, this range of concentrations did not induce autophagy and was shown to accelerate growth and proliferation in vitro. Plasma ammonia concentrations in mice harboring tumors were not different than those in control mice (Figure 3.S15a).
A. MCF7 and T47D cells under control and (+) NH₄Cl conditions.

B. Relative sphere area in MCF7 and T47D treated with control or NH₄Cl.

C. Relative sphere area in shControl, shGDH #1, and shGDH #2 treated with control or NH₄Cl.

D. MCF7 and T47D cells under control and conditioned conditions.

E. Ammonia levels in control and conditioned media.

F. Relative sphere area in shControl, shGDH #1, and shGDH #2 treated with control or conditioned media.

G. Ammonia secretion in conditioned media for shControl, shGDH #1, and shGDH #2.
Figure 3.3. Ammonia stimulates breast cancer growth and proliferation. A. Representative images of 3D culture models of MCF7 and T47D cells treated with 0.5 mM NH₄Cl compared to control conditions. B. Quantification of average sphere area of 100-200 spheres per well in 3D culture models of MCF7 and T47D cells treated with ammonia and control conditions for 7 days. Values represent mean area +/- SEM, n=4. Representative experiment of five replicates. C. Quantification of average sphere area of 200-250 spheres per well in 3D culture models of MCF7 cells harboring stable shRNA-mediated knockdown of GDH or control hairpin. Cells were treated for 8 days. Values represent mean area +/- SEM, n=4. Representative experiment of three replicates. D. Representative images of MCF7 and T47D cells in control conditions (daily media change) and conditioned media (media changed every 72 hours). Cells were treated for 8 days. E. Ammonia measurement in conditioned media compared to control after 8 days. F. Quantification of average sphere area of 200-250 spheres per well in 3D culture models of MCF7 control cells or cells depleted of GDH. Cells were treated in control or conditioned media for 8 days. Values represent mean area +/- SEM, n=4. Representative experiment of three replicates. G. Nmoles ammonia secreted per cell after 72 hours in control cells or cells depleted of GDH. Values represent mean +/- SEM, n=3. For all comparisons two-tailed t test was used. *P < 0.05, **P < 0.01, ***P<0.001, ****P<0.0001.
We tested whether accumulated ammonia in the tumor microenvironment was assimilated into metabolic pathways \textit{in vivo}. Mice harboring subcutaneous T47D breast tumors were intraperitoneally injected with $^{15}$NH$_4$Cl and the tumor, liver, and plasma were assessed for $^{15}$N-isotopologues over the next 1 to 4 hours (Figure 3.S15b). The liver and tumor used distinct metabolic pathways for ammonia assimilation (Figure 3.4b and Figure 3.S16, 3.S17a). In the liver, ammonia was sequestered into the urea cycle, leading to labeling of ornithine, citrulline, argininosuccinate and arginine (Figure 3.S16). Although these labeled intermediates of the urea cycle were also detected in the plasma, they were undetectable in the tumor, indicating that these breast tumors did not engage the urea cycle for ammonia assimilation \textit{in vivo} (Figure 3.4b & Figure 3.S16). Proline and aspartate, which were identified \textit{in vitro} as metabolic derivatives of ammonia, were also labeled in the tumor \textit{in vivo}. The metabolic pathways enabling proline and aspartate labeling were likely from tumor autonomous metabolism, as labeled proline and aspartate were not detected in the blood (Figure 3.4b).

We also observed labeling of glutamine and glutamate in the tumor. Because labeled glutamine and glutamate were also found in the liver and plasma, it is not clear whether these $^{15}$N-isotopologues were generated tumor autonomously. Furthermore, the kinetics of glutamine labeling in the tumor implied that a subset of the labeled glutamine pool in the tumor may be taken up from the plasma (Figure 3.4b).

To distinguish systemic contributions of ammonia metabolism from tumor autonomous metabolic pathways we traced $^{15}$NH$_4$Cl and $^{15}$N-(amide)-glutamine in tumors \textit{ex vivo} (Figure 3.S17b-d). With $^{15}$NH$_4$Cl, labeling of glutamate, aspartate, proline and glutamine was recapitulated. Consistent with \textit{in vivo} studies, the urea cycle intermediates, nucleotides, and other nitrogen-abundant metabolites were not labeled. These data underscore a fundamental role of ammonia for amino acid synthesis, particularly glutamate, aspartate and proline \textit{in vivo}. 

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Figure 3.4. Contributions of systemic and tumor autonomous ammonia metabolism to amino acid synthesis. A. Measurement of ammonia in the interstitial fluids of the tumor microenvironment (TME) compared to plasma isolated from ER(+) breast cancer xenograft models. Lines connect values of ammonia in the plasma to that in the interstitial fluid of the TME. B. Isotope abundance of $^{15}$N-isotopologues isolated from the liver, plasma and tumor of mice IP injected with a bolus (9.0 mmol/kg) of $^{15}$NH$_4$Cl. Tissues were harvested 1, 2, or 4 hours after injection. Values represent mean +/- SEM, n=4. $^{15}$N-isotopologues were corrected for natural abundance of tissues harvested from a control mouse treated with 9.0 mmol/kg NH$_4$Cl for 4 hours. C. Western blot of GDH knockdown in T47D xenograft tumors. D. In vivo tumor growth of T47D control and GDH-depleted xenograft models (n=15 mice per group). Values represent mean tumor volume +/- SEM. E. In vivo tracing of $^{15}$NH$_4$Cl in T47D control and GDH-depleted xenograft models. Values represent mean isotopologue abundance +/- SEM, n=4. F. Schematic of systemic and tumor autonomous ammonia metabolism.

For all comparisons two-tailed t test was used. *P < 0.05, **P < 0.01, ***P<0.001, ****P<0.0001.
Consistent with in vitro experiments, tumors treated with $^{15}$N-(amide)-glutamine generated labeled glutamate and the downstream metabolites proline and aspartate, suggesting that glutamine-derived ammonia may be recycled in solid tumors (Figure 3.S18a). In contrast to free $^{15}$NH$_4$Cl, which did not label metabolites of the urea cycle in cells, in vivo or in solid tumors ex vivo, $^{15}$N-(amide)-glutamine, when added to the tumors ex vivo, elicited labeling of the urea cycle intermediate citrulline (Figure 3.S17d). Thus, an alternative pathway that does not require ammonia may exist that connects the amide nitrogen on glutamine to citrulline production ex vivo.

We next generated a GDH—depleted xenograft model to further investigate the mechanism of ammonia assimilation in vivo (Figure 3.4c). Tumor-specific depletion of GDH significantly decreases tumor growth in vivo, consistent with our findings that GDH-depleted cells grow slower in conditioned media and are insensitive to ammonia-induced growth in vitro (Figure 3.4d, Figure 3.S18a). Since GDH- catalyzed ammonia assimilation mediates growth and proliferation in vitro, we tested whether GDH also assimilates ammonia in vivo using IP injection of $^{15}$NH$_4$Cl in control and GDH-depleted xenograft models. Glutamate, aspartate and proline labeling are significantly decreased in GDH depleted tumors compared to control tumors (Figure 3.4e). Importantly, GDH-depletion did not abrogate glutamine labeling, underscoring the specificity of GDH for ammonia assimilation to generate glutamate and the downstream metabolites proline and aspartate. To further validate that GDH-mediated ammonia assimilation is tumor autonomous, ammonia was traced ex vivo in control and GDH-depleted tumors. GDH-depletion significantly decreases glutamate, aspartate and proline labeling (Figure 3.S18b). Taken together, these data show that both tumor autonomous metabolism of ammonia, as well as systemic assimilation support tumor biomass, especially for glutamate-derived amino acids (Figure 3.4f).

Ammonia accumulates in the tumor microenvironment because tumors are poorly vascularized, making this a unique niche for ammonia metabolism in the human body. Since
ammonia transport is mediated by diffusion, elevated ammonia in the microenvironment leads to its accumulation inside of tumor cells (Figure 3.S18c). Therefore, the ability to re-assimilate this ammonia into metabolic pathways is critical in this context. In contrast, the liver re-assimilates ammonia to generate urea, which is a sink for excess nitrogen and is excreted as metabolic waste to protect against toxicity associated with systemic ammonia accumulation. Tumor cells strictly recycle this nitrogen to generate amino acids downstream of glutamate and do not engage the urea cycle.

In sum, we identified that ammonia is an important nitrogen source for breast cancer metabolism. Ammonia is not simply a metabolic waste product, and it can be recycled to support the high demand for amino acid synthesis in rapidly proliferating cells. Although ammonia is sometimes considered a toxin, it stimulated growth and proliferation in breast cancer cells. This stimulatory effect appears to be mediated by GDH-catalyzed ammonia assimilation. Furthermore, ammonia accumulated in the tumor microenvironment, and was used by cancer cells for amino acid synthesis in vivo. These biosynthetic pathways are supported in both systemic and tumor autonomous metabolism. Thus, we showed that metabolic recycling of ammonia provides an important source of nitrogen for breast cancer biomass.

III. Supplemental Material

A- Supplemental Figures (Figures 3.S1 - 3.S18)
Figure 3.S1. Ammonia-assimilating enzymes in cancer. A. Enzymatic reactions of the ammonia assimilating enzymes: carbamoyl phosphate synthetase 1 (CPS1), glutamate dehydrogenase (GDH), and glutamine synthetase (GS). B. Protein Atlas expression score for ammonia assimilating enzymes in healthy tissue.
Figure 3.S1. Heatmap of Metabolic Derivatives of $^{15}$N-(amide)-glutamine. Nitrogen metabolome-heat map of % isotopologue abundance of metabolites with $^{15}$N-mass shifts in MCF7 and T47D cells after 8 hour treatment with $^{15}$N-(amide)-glutamine. 211 metabolites were screened for $^{15}$N- isotopic mass shift using mass spectrometry. Each column is an individual replicate (n=4) and values are were adjusted to subtract out for natural abundance of $^{15}$N- detected in samples treated with $^{14}$N-(amide)-glutamine.
Figure 3.S2. Metabolic Derivatives of $^{15}$N-(amide)-glutamine in MCF7 and T47D. A. Isotope abundance of expected $^{15}$N -isotopologues after treatment with $^{15}$N -(amide)-glutamine. These metabolites are made in direct, enzymatically catalyzed reactions of glutamine with other metabolites. Values are the mean +/- SEM of 4 replicates. B. Isotope abundance of novel $^{15}$N -isotopologues after treatment with $^{15}$N -(amide)-glutamine. These metabolites are unexpected because the labeled nitrogen on glutamine is liberated as ammonia prior to their synthesis. Values are the mean +/- SEM of 4 replicates.
Figure 3.S3. Characterization of the glutaminase inhibitor BPTES in ER(+) breast cancer cell lines. A. Cytotoxicity assay by Propidium iodide staining of cells treated with a dose of BPTES for 48 hours. Values represent the mean +/-SEM of 3 replicates per concentration. B. Cell Number after treatment with a dose of BPTES for 48 hours. Values represent the mean +/-SEM of 3 replicates per concentration. C. Steady-state abundance of glutamate, analyzed by mass spectrometry, in MCF7 and T47D cells treated with 1 μM BPTES. Values represent the mean +/-SEM of 4 replicates. D. Abundance of expected 15N-isotopologues after treatment with 2.0 mM 15N-g-lutamine and 1 μM BPTES. Values represent the mean +/-SEM of 4 replicates. E. Steady-state metabolite abundance of MCF7 cells treated with 1 μM BPTES in the presence or absence of 0.75 mM NH4Cl. Values represent the mean +/-SEM of 4 replicates. F. Western blot depicting shRNA-mediated GDH knockdown. G. RT-PCR of GDH isoforms (GLUD1 and GLUD2) in MCF7 and T47D cells. Values represent the mean +/-SEM of 3 replicates.
Figure 3.S4. shRNA-insensitive GDH1 rescue of ammonia recycling. A. Western blot depicting GDH1 levels in MCF7 cell lines with stable expression of control or sh#2 GDH hairpins that have been transfected with empty vector (shControl and shGDH) or shRNA-resistant GDH1 (GDH add back). B. Isotopologue abundance of representative metabolites for ammonia recycling in control, GDH knockdown and GDH1 rescue after 8 hours of treatment with 2.0 mM $^{15}$N-(amide)-glutamine. N=3 per condition, data represent the average +/- SEM. Representative experiment of two replicates.
A. MS Berthelot Assay Work Flow
(For Measurement of $^{15}$N enrichment)

1. Treat with 2.5mM $^{15}$N-(amide)-Gln for 8 hours and isolate metabolites

2. The Berthelot Reaction

   $\text{NH}_3 + \rightarrow \text{Indophenol}$

   $^{15}\text{NH}_3 + \rightarrow ^{15}\text{N}-\text{Indophenol}$

3. LC-MS to measure enrichment of $^{15}$N-Indophenol

4. Calculate:

   \[ \text{% Enrichment of } ^{15}\text{N} = \left( \frac{\text{ions } ^{15}\text{N-Indophenol}}{\text{ions } ^{15}\text{N-Indophenol} + \text{ions Indophenol}} \right) \times 100 \]

B. Standard Curve for MS Berthelot Assay

\[ Y = 0.006245X + 0.1178 \]

R² = 0.9932

C. % Enrichment of $^{15}$NH₃ after 15N-(amide)-Gln treatment

3.43% +/- 1.29

D. Glutamine → Glu (M+6)

E. Glutamates / Glutaminolysis = \[ \frac{\text{Glu (M+6) Ion Counts}}{\text{Glu (M+1) Ion Counts}} + \frac{\text{Glu (M+6) Ion Counts}}{\text{Glu (M+1) Ion Counts}} \]

F. MCF7 Glu/Gln:

1.572 +/- 0.011

G. \( \alpha\)-Ketoglutarate (M+6) and (M+1)

H. Glutamate (M+1)
Figure 3.S5. Metabolic recycling of glutamine-derived ammonia is efficient. A. Schematic of the MS Berthelot assay. T47D cells were treated with 2.0 mM $^{15}$N-(amide)-glutamine for 8 hours, polar metabolites were extracted, and ammonia was derivatized to indophenol, which is measured quantitatively by LC-MS. B. Standard curve used for calculating percent enrichment of $^{15}$NH$_3$. Different ratios (0% to 80%) of $^{15}$NH$_3$ were spiked into untreated lysate, derivitized to generate indophenol and run on analyzed by LC-MS. C. Percent $^{15}$NH$_3$ in T47D cell lysates after treatment with $^{15}$N-(amide)-glutamine. Values represent mean +/- SEM, n=4. Representative experiment of three replicates. D. Schematic of experiment to measure recycling efficiency. MCF7 cells were incubated with 2.0 mM $^{15}$N$_2$$^{13}$C$_5$-glutamine (glutamine (M+7)) for 8 hours. Glutaminolysis was measured by the ion counts of glutamate (M+6). Ammonia recycling was measured by glutamate (M+1). Purple circles indicate $^{13}$C isotopes and yellow circles indicate $^{15}$N isotopes. E. The equation for quantification of total glutamates (by nitrogen) generated in glutaminolysis. F. The ratio of glutamates generated by glutaminolysis in MCF7 cells. The error is SEM over four replicates. G. Control and GDH depleted MCF7 cells treated with $^{15}$N$_2$$^{13}$C$_5$-glutamine (glutamine (M+7)) for 8 hours. α-Ketoglutarate (M+5) is a measure of GDH oxidative deamination. Values are mean +/- SEM, n=4. H. Control and GDH depleted MCF7 cells treated with $^{15}$N$_2$$^{13}$C$_5$-glutamine (glutamine (M+7)) for 8 hours. Glutamate (M+1) is a measure of GDH reductive amination. Values are mean +/- SEM, n=4.
Figure 3.S6. The effect of NH$_4$Cl on basal metabolic phenotypes. A. Propidium Iodide staining of Normal Human Astrocytes treated with a dose of NH$_4$Cl for 48 hours. Values are mean +/- SEM, n=3 per concentration. B. Western blot of LC3II in MCF7 and T47D cells treated with NH$_4$Cl (0-10 mM). Chlor = Chloroquine (40 mM). C. Glucose consumed from media after 24 hours in T47D and MCF7 on a dose of NH$_4$Cl (0-50 mM). Normalized to cell number. Values are mean +/- SEM, n=3. D. Glutamine consumed from media after 24 hours in T47D and MCF7 on a dose of NH$_4$Cl (0-50 mM) and n. Normalized to cell number. Values are mean +/- SEM, n=3. E. Basal oxygen consumption rate (OCR) in T47D and MCF7 on a dose of NH$_4$Cl (0-50 mM) and normalized to cell number. Values are mean +/- SEM, n=6. F. Western blot of ammonia assimilating enzymes: Carbamoyl phosphate synthetase 1 (CPS1), Glutamate Dehydrogenase (GDH) and Glutamine Synthetase (GS) with NH$_4$Cl (0–5 mM). G. RT-PCR of CPS1 in MCF7 and T47D with NH$_4$Cl (0-5 mM). Values are mean +/- SEM, n=3. H. RT-PCR of GLUD1/2 in MCF7 and T47D with NH$_4$Cl (0-5 mM). Values are mean +/- SEM, n=3. I. RT-PCR of GS in MCF7 and T47D with NH$_4$Cl (0-5 mM). Values are mean +/- SEM, n=3. J. pH measurement of media treated with a dose of NH$_4$Cl (0-50 mM). K. Ammonia uptake/output from media after 24 hours in T47D and MCF7 cell treated with a dose of NH$_4$Cl (0-50 mM). Normalized to cell number, values are mean +/- SEM, n=3. Blue bars represent net ammonia output and red bars represent net ammonia uptake. Representative experiment of three replicates.
Figure 3.S7. Steady-state metabolite profiling of cells treated with ammonia. A. Metabolites that were significantly altered (p<0.05 by two-tailed t test) in cells treated with 0.75 mM NH₄Cl compared to control were analyzed using Metaboanalyst 3.0 pathway analysis. B. Relative abundance of essential amino acids in cells treated with 0.75 mM NH₄Cl compared to control. Values are mean +/- SEM, n=4. C. Relative abundance of nucleotides in cells treated with 0.75 mM NH₄Cl compared to control. Values are mean +/- SEM, n=4. D. Relative abundance of urea cycle intermediates in cells treated with 0.75 mM NH₄Cl compared to control. Values are mean +/- SEM, n=4. For all comparisons two-tailed t test was used.
Figure 3.S8. Heatmap of Metabolic Derivatives of $^{15}$NH$_4$Cl. Percent isotope abundance of $^{15}$N- isotopologues in T47D and MCF7 cells treated with 0.75 mM $^{15}$NH$_4$Cl. This data is unscaled and represents 38% and 43% enrichment of $^{15}$NH$_3$ in MCF7 and T47D cell lysates respectively. (Table 3.S2)
Figure 3.S9. $^{15}$NH$_4$Cl Tracing in MCF7 and T47D cell lines. A. Abundance of $^{15}$N-isotopologues in MCF7 and T47D cells after 8 hours of treatment with 3.0 mM $^{15}$NH$_4$Cl. ND= $^{15}$N-isotopologue not detected. Isotopologue abundances were not scaled. Values represent mean +/- SEM, n=4. B. Schematic of metabolic pathways by which $^{15}$N-isotopologues acquire labeling from $^{15}$NH$_4$Cl. C-F. Isotopologue abundance of metabolites labeled after treatment with 0.75 mM $^{15}$NH$_4$Cl in T47D on a time course at the indicated times. Isotopologue abundances were not scaled. Values represent mean +/- SEM, n=4. G. Western blot depicting GDH1 levels in MCF7 cell lines with stable expression of control or sh#2 GDH that have been transfected with empty vector (shControl and shGDH) or shRNA-resistant GDH1 (GDH Add Back). H. Isotopologue abundance of metabolites in control, GDH knockdown and GDH1 rescue after 8 hours of treatment with 0.75 mM $^{15}$NH$_4$Cl. n=3 per condition, values represent the mean +/- SEM and are scaled to represent the total ammonia pool. Representative experiment of two replicates.
Figure 3.S10. GDH knockdown does not affect $^{15}$N-isotopologues that derive from GS. 

A. Isotope abundance of $^{15}$N-isotopologues in T47D and MCF7 cells after 8 hours of treatment with 0.75 mM $^{15}$NH$_4$Cl +/- GDH. Percent labeling is scaled using the MS Berthelot assay to represent the full ammonia pool. 

B. Schematic of GS-derived $^{15}$N-isotopologues.
Figure 3.S11. Ammonia accelerates proliferation in 2D and 3D culture. A. Representative growth curves of T47D cells treated with control or NH₄Cl. Values represent mean +/- SEM, n=3 per time point. B. Calculated doubling times for growth curves in ER(+) breast cancer cell lines (MCF7 and T47D) treated with a dose (0.0 mM, 0.1 mM, 0.5 mM) NH₄Cl in 2D cell culture. Each growth curve was replicated three or more times. C. Ammonia quantification in media after incubation for 24 hours with MCF7 and T47D cells. Values represent mean +/- SEM, n=4. D. Ammonia measurement in media (without cells) on a time course in a 37°C incubator. E. Glutamine measurement in media (without cells) on a time course in a 37°C incubator. F. Glucose measurement in media (without cells) on a time course in a 37°C incubator. G. Proliferation in 3D culture, measured by cell number of MCF7 cells in control (0.0 mM NH₄Cl) or ammonia treated (0.5 mM NH₄Cl) conditions for 8 days of treatment. Values represent mean +/- SEM, n=4. Representative experiment of three replicates. *P < 0.05, **P < 0.01, ***P<0.001, ****P<0.0001.
Figure 3.S12. Ammonia metabolism in fibroblasts. A. Representative growth curve of primary human fibroblasts treated with control or 0.5 mM NH$_4$Cl. Values represent mean +/- SEM, n=3 per time point. Representative experiment of two replicates. B. Abundance of $^{15}$N-isotopologues in primary human fibroblasts after 8 hours of with 0.75 mM $^{15}$NH$_4$Cl. ND = $^{15}$N-isotopologue not detected. Data is unscaled. Values represent mean +/- SEM, n=4. C. Abundance of $^{15}$N-isotopologues in primary human fibroblasts after 8 hours of treatment with 2.0 mM $^{15}$N-(amide)-glutamine. ND = $^{15}$N-isotopologue not detected above natural abundance. Values represent mean +/- SEM, n=4.
Figure 3.S13. GDH knockdown does not alter basal growth and proliferation in 3D culture with daily media change. A. Western blot depicting shRNA-mediated knockdown of glutamate dehydrogenase (GDH) compared to control hairpin in MCF7 cells. B. Average sphere area of MCF7 cells harboring control hairpin, or GDH knockdown (shGDH #1 or shGDH #2). Cells were treated in control conditions, with daily media change for 8 days. Values represent mean +/- SEM, n=3. C. Cell count of MCF7 cells harboring control hairpin, or GDH knockdown (GDH#1 or GDH#2). Cells were treated in control conditions, with daily media change for 8 days. Values represent mean +/- SEM, n=3. D. Quantification of average sphere area of 200-250 spheres per well in 3D culture models of MCF7 cells treated in control conditions or with 3.0 mM NH₄Cl. Values represent mean +/- SEM, n=3. For all comparisons two-tailed t test was used. *P < 0.05, **P < 0.01, ***P<0.001, ****P<0.0001.
Figure 3.S14. Plasma ammonia measurements in T47D xenograft model. A. Ammonia measurement in plasma isolated from control mice, without tumors and mice harboring a subcutaneous tumor >100mm³. Values represent mean +/- SEM, n=6 mice per condition. Two-tailed t test was used for comparison. B. Ammonia measurement in plasma isolated from mice harboring a subcutaneous tumor >100mm³ on a time course of bolus IP injection (9.0 mmoles/kg) of ¹⁵NH₄Cl.
Figure 3.S15. Heat Maps of $^{15}$N-isotopologues in the Tumor, Plasma and Liver after $^{15}$NH$_4$Cl Tracing in vivo. Mice harboring a subcutaneous tumor >100 mm3 were given a bolus IP injection (9.0 mmoles/kg) of $^{15}$NH$_4$Cl, and metabolites isolated from the tumor, plasma and liver were scanned for $^{15}$N-isotopologues. Percent isotope abundance of $^{15}$N-isotopologues was determined for tumor, plasma and liver tissue harvested 1, 2, and 4 hours after IP injection. Each column represents an individual mouse, n=4 mice per time point.
Figure 3.16. $^{15}$N-isotopologues in the Tumor, Plasma and Liver after $^{15}$NH$_4$Cl Tracing in vivo and ex vivo. A. Abundance of $^{15}$N-isotopologues isolated from the liver, plasma and tumor of mice IP injected with a bolus (9.0 mmol/kg) of $^{15}$NH$_4$Cl. Tissues were harvested 1, 2, or 4 hours after injection. $^{15}$N-isotopologues were corrected for natural abundance of tissues harvested from a control mouse treated with 9.0 mmol/kg NH$_4$Cl for 4 hours. Values represent mean +/- SEM, n=4 mice per condition. B. Heatmap of $^{15}$N-isotopologues after 4, 8, 16 hours of ex vivo tracing in T47D xenograft tumors. Each column represents an individual mouse, n=4 mice per time point. C. Abundance of $^{15}$N-isotopologues isolated from the tumors treated with
0.75 mM $^{15}$NH$_4$Cl *ex vivo*. Values represent mean +/- SEM, n=4 mice per condition. **D.** Abundance of $^{15}$N-isotopologues isolated from the tumors treated with 2.0mM $^{15}$N-(amide)-glutamine. Values represent mean +/- SEM, n=4 mice per condition.
Figure 3.S17. GDH Depletion in vivo Mediates Growth and Ammonia Metabolism. A. Image of control hairpin and GDH-depleted tumors after 28 days of growth in vivo. B. Abundance of $^{15}$N- isotopologues after 8 hours of ex vivo tracing with 0.75 mM $^{15}$NH$_4$Cl in T47D xenograft tumors. Values are scaled to represent the entire ammonia pool (Table S2). Values represent the mean +/- SEM, n=4. *P < 0.05, **P < 0.01, ***P<0.001, ****P<0.0001. C. Schematic of ammonia accumulation in the tumor microenvironment. Tumor metabolism generates an abundance of ammonia, some of which is recycled via GDH to generate glutamate and the rest, which is in equilibrium with the microenvironment. Poor vascularization of the tumor decreases ammonia clearance, allowing it to accumulate.
**Figure S. Table 1. Comprehensive List of $^{15}$N-isotopologues in “Nitrogen Scanning”**

**Method.** Nitrogen abundant metabolites screened for in nitrogen scanning. $M+1$ indicates a single $^{15}$N-mass shift, $M+2$ indicates two $^{15}$N-mass shifts, $M+3$ indicates three $^{15}$N-mass shifts, $M+4$ indicates four $^{15}$N-mass shifts, $M+5$ indicates five $^{15}$N-mass shifts, $M+6$ indicates six $^{15}$N-mass shifts, $M+7$ indicates seven $^{15}$N-mass shifts.
Table 2. Scaling factors for $^{15}$NH$_4$Cl tracing studies. Metabolites were isolated after incubation with the listed concentration of $^{15}$NH$_4$Cl and the MS Berthelot Assay (Figure 4.S6) was performed to determine the enrichment of $^{15}$NH$_3$ in the total intracellular ammonia pool.

<table>
<thead>
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<th>Sample</th>
<th>$[^{15}\text{NH}_4\text{Cl}]$ Treatment (mM)</th>
<th>$[^{15}\text{NH}_3$ Enrichment</th>
<th>Scaling Factor to reach 100%</th>
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</thead>
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<tr>
<td>T47D</td>
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<td>2.31</td>
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<td>8.78</td>
</tr>
<tr>
<td>MCF7 shGDH#1</td>
<td>0.75</td>
<td>9.9</td>
<td>10.10</td>
</tr>
<tr>
<td>MCF7 shGDH#2</td>
<td>0.75</td>
<td>9.3</td>
<td>10.75</td>
</tr>
<tr>
<td>MCF7 GDH Add Back</td>
<td>0.75</td>
<td>16.9</td>
<td>5.97</td>
</tr>
<tr>
<td>T47D shControl</td>
<td>0.75</td>
<td>16.4</td>
<td>6.08</td>
</tr>
<tr>
<td>T47D shGDH#1</td>
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<td>21.7</td>
<td>4.61</td>
</tr>
<tr>
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<td>0.75</td>
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<td>5.51</td>
</tr>
<tr>
<td>T47D shControl ex vivo</td>
<td>0.75</td>
<td>9.63</td>
<td>10.38</td>
</tr>
<tr>
<td>T47D shGDH ex vivo</td>
<td>0.75</td>
<td>9.56</td>
<td>10.45</td>
</tr>
</tbody>
</table>
C- Materials and Methods

Cell Culture

2D cell culture: All breast cancer cell lines and normal human astrocytes (NHA) were cultured in glutamine-free RPMI (Life Technologies) supplemented with 5% FBS (Life Technologies) and 1% penicillin and streptomycin (Invitrogen). 2 mM L-glutamine (Sigma) was added to the media on the day of the experiment to minimize glutamine degradation and subsequent ammonium accumulation in stored media. Media was changed every 24 hours to minimize ammonium accumulation. Inhibitors and other supplements: 0-50 mM ammonium chloride (Sigma), 1 mM BPTES (Sigma), 40 mM chloroquine (Sigma).

3D cell culture: Cells were maintained with modifications to previously described protocols (http://brugge.med.harvard.edu/protocols). Briefly, MCF7 and T47D cells that have been adapted to culture conditions for four days were seeded in 8-well glass chamber culture slides (BD Falcon) on a bed of LDEV -free MatriGel (Corning) in RPMI supplemented with 5% FBS, 1% penicillin/streptomycin and 2% MatriGel. Two days after seeding cells, media was replaced in all conditions. For control and ammonium-treated conditions media was changed daily for the duration of the experiment. For studies on conditioned media, media was changed every three days. Images were taken on a Nikon Eclipse TE2000-U Microscope after 8 days or 11 days for MCF7 and T47D cells, respectively. Sphere area was quantified using ImageJ on 200-300 colonies per replicate. Cells were harvested after incubation in Cell Recovery Solution (Corning: 354253) for one hour at 4 °C and counted with a Beckman Coulter Counter.

Proliferation Assays: Cell lines were adapted to medium conditions containing 0.0 mM, 0.1 mM, or 0.5 mM NH₄Cl for four days prior to experimentation. 25,000 cells were seeded in triplicate in 6-well dishes and counted daily for approximately one week on a Beckman Coulter Counter. Points were fitted to the exponential growth equation $Y=Y_0 e^{(kX)}$ to obtain the rate constant (k). Then, the equation $DT = \ln(2)/k$ was used to calculate doubling time (DT).

Bioinformatics
Oncomine Database Analysis: Patient data from The Cancer Genome Atlas (TCGA) was analyzed using the “Cancer Versus Normal” analytical tool on the public database Oncomine (www.oncomine.org). Datasets were filtered for a threshold P-value < 0.0001 and assessed for both Over-expression Fold-Change and Under-expression Fold-Change in mRNA levels relative to healthy tissue as measured with a Human Genome U133A Array.

Generation of Stable Cell Lines

Plasmids and Stable Cell Lines: shRNAs against GDH1/2 were subcloned into the pLKO.1 puro vector (Addgene Plasmid #8453) at EcoRI and AgeI sites:

shcontrol: 5’-
CCGGCCGTCATAGCGATAACGAGTTTCGAGAATCTCGCTATGACCGGTGTTTTT G-3’
sh #1 GDH: 5’-
CCGGGCCATTGAGAAAGTCTTAACTCGAGGTTTGAAGACTTTCTCAATGGCTTTTT G-3’
sh#2 GDH: 5’-
CCGGGCCTACACTCTATGAGATATTCTCGAGAATATCTCATAGGTAGGCTTTTT G-3’

Subcloned plasmids were transfected into HEK293T cells with lentiviral packaging vectors. MCF7 and T47D cells were subsequently infected with the lentivirus, generating stable GDH knockdown cell lines. GDH rescue experiments were performed with stable cell lines transfected with an overexpression vector of shRNA-resistant GDH1. pCMV-SPORT6-GLUD1 (HsCD00339304) was purchased from the Harvard PlasmID repository, PCR amplified with primers:

(F)- 5’-
GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGTACCGCTACCTGGGC-3’
(R)- 5’-
GGGGACCACTTTGTACAAGAAAGCTGGGTTTAAGTGATTAAAGGCAATTTTATCACA-3’

GDH1 was subcloned into pcDNAeGFP. Silent mutations were generated at two sites of the shRNA#2 target sequence using primers: (F)- 5’-CATGAGATATTACATCGTTTTGGTGC-3’
(R)- 5’- GAGTGCAGGCCCCACATTACAAATCC-3’. MCF7 cells harboring stable knockdown of GDH were transfected with shRNA-insensitive GDH1. Sequencing reactions were carried out with an ABI3730xl DNA analyzer at the DNA Resource Core of Dana-Farber/Harvard Cancer Center (funded in part by NCI Cancer Center support grant 2P30CA006516-48).

**Western Blots:** Adherent cells were lysed directly on the cell culture dish with lysis buffer (1% NP40, 1 mM DTT, 0.2% phosphatase inhibitor cocktail #2 & #3(Sigma), 1 cOmplete protease inhibitor tablet (Sigma). Protein content was quantified using a BCA Assay (Thermo Scientific) and equal amounts of protein were run on a 10 -20% Tris-HCl Gel (BioRad). Protein was transferred overnight (4 °C) to a nitrocellulose membrane (BioRad). Primary antibodies were used at the following dilutions: GLUD1 (Proteintech; 1:1000), α-Tubulin (Santa Cruz, 1:1000), LC3II (MBL Life Science, 1:1000), CPS1 (Santa Cruz, 1:500), GS (BD Biosciences, 1:1000). Secondary antibodies: Anti-rabbit IgG HRP-linked Antibody (1:5000, Cell Signaling), Anti-mouse IgG HRP-linked Antibody (1:5000, Cell Signaling), Anti-goat IgG HRP-linked Antibody (1:5000, Santa Cruz). Blots were developed using Pierce ECL Western Blotting Substrate (Thermo Scientific) and were exposed using autoradiography film.

**RNA Isolation and RT-PCR**

RNA was extracted with the Direct-zol RNA Miniprep Kit (Genesee Scientific) directly from adherent cells. cDNA was synthesized from 1 µg of RNA using the iScript cDNA synthesis kit (Bio-Rad). Real-time qPCR was performed on a Light Cycler 480 (Roche) using PerfeCta SYBR Green Fast Mix (Quanta BioSciences). Primers : RPLP0 (F): acgggtacaaacgagtctcg , RPLP0 (R): cgactttcttggtctcaa , GLUD1/2 (F): ggtcatgaaggtctacg , GLUD1/2 (R): tcagtgcgttaacgggatactc, GLUL (F): cttcactgtacggctagtc, GLUL (R): tgccttcccccaacacacca, CPS1 (F)- acttcagtggatcattatgcc, CPS1 (R)- ggaacggatcactggtgtag, GLUD1 (F): cctggcgaagcgtgttgc, GLUD1 (R): gggctgtccggggcggca, GLUD2 (F): tggccaaagcgctgtgcc, GLUD2 (R): gctgtccgggccccg.

**Metabolite Profiling and Tracing Studies**
**Steady-State Metabolite Extraction:** Prior to experiments, cells were adapted to 0.0 mM NH₄Cl or 0.75 mM NH₄Cl for four days. MCF7 and T47D were seeded in 6-cm plates at densities that would reach 70% confluency after 24 hours based on their calculated doubling times in each condition. Samples were plated in quadruplicate for metabolite extraction and in quadruplicate for cell count normalization. After 24 hours, cells were washed once with ice-cold PBS, and polar metabolites were extracted directly on the dish using 1 mL ice-cold 80% methanol.

**Glutamine and Ammonia Tracing:** Prior to experiments, cells were adapted to respective media conditions for four days. Following adaptation, cells were seeded in 6-cm plates as previously described. After 24 hours media was replaced with RPMI supplemented with 2 mM L-Glutamine glutamine (amide-¹⁵N) (Sigma, 98% isotopic purity), or 2 mM L-Glutamine glutamine (¹³C₅,¹⁵N₂) (Sigma, 98% isotopic purity), 0.75 mM or 3.0 mM ¹⁵NH₄Cl (Sigma, 98% isotopic purity). Cells were incubated with metabolic isotopes for on a time course (0-12 hours) or for 8 hours and polar metabolites were extracted as previously described.

**Mass Spectrometry:** Metabolites were extracted using 80% MeOH and analyzed using two hydrophilic interaction liquid chromatography tandem mass spectrometry (HILIC-MS) methods to profile metabolites in negative and positive ionization modes. Data were acquired using a Nexera UHPLC (Shimadzu) coupled to a Q Exactive Plus hybrid quadrupole orbitrap mass spectrometer (Thermo Fisher Scientific). For negative ion mode analyses, extracts (10 µL) were injected onto a Luna NH₂ column (150 x 2.0 mm; Phenomenex). The column was eluted at a flow rate of 400 µL/min with initial conditions of 10% mobile phase A (20 mM ammonium acetate and 20 mM ammonium hydroxide in water) and 90% mobile phase B (10 mM ammonium hydroxide in 75:25 v/v acetonitrile/methanol) followed by a 10 min linear gradient to 100% mobile phase A. For positive ion mode analyses, 100 µL of each cell extract was dried under nitrogen gas and resuspended in 10 µL of water and 90 µL of 74.9:24.9:0.2 v/v/v acetonitrile/methanol/formic acid containing stable isotope-labeled internal standards (valine-d8,
Sigma-Aldrich; and phenylalanine-d8, Cambridge Isotope Laboratories). Samples were injected onto an Atlantis HILIC column (150 x 2 mm, 3 µm; Waters) and the column was eluted isocratically at a flow rate of 250 µL/min with 5% mobile phase A (10 mM ammonium formate and 0.1% formic acid in water) for 0.5 minute followed by a linear gradient to 40% mobile phase B (acetonitrile with 0.1% formic acid) over 10 minutes. All MS analyses are carried out using electrospray ionization and a resolution setting of 70,000. Negative ion mode settings were: sheath gas 55, auxiliary gas 10, sweep gas 7, spray voltage -3.0 kV, capillary temperature 350°C, S-lens RF 50, and heater temperature 325°C. Positive ion mode settings were: sheath gas 40, auxiliary gas 15, sweep gas 2, spray voltage 3.5 kV, capillary temperature 350°C, S-lens RF 40, and heater temperature 300°C. Peak identities were confirmed matching masses and retention times to authentic reference standards. 176 compounds (118 positive ion and 58 negative ion) were integrated using TraceFinder 3.3 software (Thermo Fisher Scientific). For tracing studies, masses for $^{15}$N-isotopologues were assessed on every targeted metabolite containing nitrogen atom(s). To confirm isotopologue peaks, mass spectrum from control samples (not treated with metabolite isotopes) were scanned for the same $^{15}$N-isotopologues.

**Calculation of Percent Isotopologue Abundance:** All metabolic tracing experiments were performed as previously described. Four replicates were treated with the $^{15}$N-isotope of either glutamine or ammonia and four replicates were not treated with the $^{15}$N-isotope but kept in biologically equivalent conditions. This untreated control was used to subtract out the natural isotope abundance of $^{15}$N-metabolites. Control (untreated) samples were averaged and subtracted out of each treated sample to quantify the abundance of metabolic isotopes above natural abundance after treatment.

**Metaboanalyst Pathway Analysis:** Metabolites were sorted based on their statistical significance (students two-tailed T-Test) on fold-change of relative abundance (normalized peak area) in ammonium-treated cells compared to control. Metabolites altered with the statistical cutoff p<0.05 were submitted to MetaboAnalyst 3.0 Pathway Enrichment Analysis Software.
Quantification of $^{15}\text{NH}_3$ Isotope Enrichment by LC-MS

The Berthelot Reaction: The MS-Berthelot assay was performed as previously reported. Briefly, a volumetric ratio of 20:1 (reactants: analyte) was used to ensure completion of the Berthelot Reaction in metabolite isolates. 20 µL of metabolite isolates were incubated with 200 µL Solution #1 (100 mM Phenol, 50 mg/L Sodium Nitroprusside) and 200 µL Solution #2 (0.38 M Dibasic Sodium Phosphate, 125 mM NaOH, 1% sodium hypochlorite, available chlorine10-15%) and incubated at 37 °C for 40 minutes. Samples were immediately analyzed by LC-MS.

LC-MS Analysis of Indophenol: Samples were analyzed on a reverse phase ion-pairing chromatography coupled to tandem mass spectrometry (Agilent LC-MS). Analytes were eluted in buffer A (97 % H2O, 3% MeOH, 10 mM Tributylamine, 15 mM Glacial Acetic Acid, pH 5.5) and buffer B (10 mM Tributylamine, 15 mM Glacial Acetic Acid in 100% MeOH). Samples were run on a ZORBAX Extend-C18, 2.1 x 150 mm, 1.8 µm (Agilent) starting with a flow rate of 0.25 mL/min for 2.5 minutes of buffer A, followed by a linear gradient (100% buffer A to 80% buffer A) for 5 minutes, followed by a linear gradient (80% buffer A to 55% buffer A) for 5.5 minutes, followed by a linear gradient (55% bufferA to 1% buffer A) for 7 minutes, followed by 4 minutes with (1% buffer A). Following each run, an acetonitrile backwash was utilized to clean the column followed by a 8 minute re-equilibration period of 100% buffer A. Samples were ionized (with negative polarity) using Agilent Jet Spray ionization; nebulizer 45psi, capillary -2000 volts, nozzle voltage: 500volts, sheath gas temperature 325°C, and sheath gas flow 12 L/min. An Agilent 6460 Triple Quadrupole mass spectrometer was used for mass detection with a targeted method for indophenol. Ammonia was detected as indophenol (m/z 198.1) and $^{15}\text{NH}_3$ was detected as $^{15}\text{N}$-indophenol (m/z 199.1). Peaks were integrated in Mass Hunter (Agilent).

Metabolic Assays

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Respiration: Respiration was assessed using the Seahorse XFe-96 Analyzer (Seahorse Bioscience). MCF7 and T47D cells were pre-treated for 1 hour in normal media conditions with a dose of ammonium chloride (0 - 50 mM). Following this incubation, media was changed to a non-buffered, serum-free Seahorse Media (Seahorse Bioscience, Catalog #102353) supplemented with 5 mM glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, and the appropriate ammonium concentration. Oxygen consumption rate (OCR) was measured over a period of 30 minutes, and values were normalized to cell number.

Metabolite Uptake/Secretion Analysis: Glucose and glutamine uptake and lactate and ammonium secretion were assessed using the NOVA BioProfile Flex Analyzer (NOVA Biomedical). Control media (no cells) and cells treated with a dose of ammonium chloride (0 mM-50 mM) were incubated for 24 hours and run on the Bioanalyzer. Values for glucose, glutamine, lactate and ammonium in the experimental conditions were subtracted from the values in the respective control media and normalized to cell number.

In Vivo and Ex vivo Mouse Experiments

Xenograft Model: All mouse protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Harvard University. Foxn1nu athymic nude mice (Nu/J strain # 002019) were purchased from The Jackson Laboratory and housed in the New Research Building Animal Facility at Harvard Medical School. 10 week old mice were injected subcutaneously with six million T47D cells in 50% Hanks’ Balanced Salt solution (Sigma H6648) and 50% LDEV-free MatriGel (Corning). To sustain tumor growth, USP approved 17β-estradiol (Sigma E1024) was administered daily using a peroral method. Briefly, mice were fed 56 ug/kg/day 17β-estradiol mixed into 60 mg of the hazelnut cream Nutella. Tumor growth was measured every three to four days and tumor volume was quantified using the equation: Volume = (π/6)(S^2)(L) where S

represents the shorter length, and L represents longer length. All experiments were performed on mice with tumors >100mm³.

In vivo Ammonia Tracing: Mice harboring subcutaneous tumors >100 mm³ were injected intraperitoneal with a bolus of ammonium chloride (9.0 mmoles/kg) in two 50uL injections. For tracing studies, twelve mice were injected with ^15^NH₄Cl in Hanks’ Balanced Salt solution (Sigma H6648) and sacrificed 1 hour, 2 hours and 4 hours post-injection. For GDH knockdown experiments, 7.0 mmoles/kg ^15^NH₄ Cl was injected and tumors were harvested after 30 minutes. A control mouse was injected with an equivalent amount of NH₄Cl and sacrificed two hours after injection, a time determined to be the peak of ammonium levels in plasma after ammonium injection. Livers and tumors were excised, flash-frozen and powderized. Polar metabolites were extracted from 8mg tissue in 80% MeOH and profiled on LC/MS as previously described. Blood was collected from each mouse via heart puncture into heparin tubes and centrifuged at 1500 x g to separate plasma. Metabolites were extracted from plasma in 1:10 (v:v) plasma: 80% MeOH and spun at 10,000 x g for 10 minutes at 4°C. Supernatant was run on LC/MS to profile metabolites as previously described.

Ex vivo Metabolic Tracing: Tumors >100 mm³ were excised and washed with 1 mL PBS. Tumors were cut in half divided and incubated in RPMI supplemented with 5% FBS, 1% Pen/Strep and one of the following conditions: 2 mM glutamine only alone, 2 mM glutamine and 0.75 mM NH₄Cl, 2 mM ^15^N-(amide)-glutamine only alone, or 2 mM glutamine and 0.75 mM ^15^NH₄Cl. Incubations took place in a 37°C incubator with ambient 5% CO₂ and constant stirring. After incubation, tumors were powderized and metabolites were extracted as previously described.

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Isolation of Interstitial Fluids: Interstitial fluid from the tumor microenvironment was isolated using a validated based on a published protocol.\textsuperscript{241} Briefly, tumors >100mm\textsuperscript{3} were excised, washed with 1 mL PBS, and blotted dry with a kim wipe. Tumors were cut in half and centrifuged at 400 x g on a Nylon Mesh filter with 20 µm pores (EMB catalog #NY2004700). 2-5uL of fluid was isolated from each tumor. Ammonium was immediately measured using a colorimetric assay (Abcam #ab83360).

Ammonia Measurement in Plasma: Plasma was isolated as previously described. Ammonia levels were measured in two distinct assays run in parallel: a colorimetric assay (Abcam #ab83360) and a NADH-dependent spectrophotometric assay (Sigma #AA0100).

Cytotoxicity: Cell viability was assessed using a standard Propidium Iodide and Flow cytometry protocol.\textsuperscript{242} Briefly, cells were trypsinized, washed, re-suspended in PBS and treated with 1 ug/mL Propidium propidium Iodide iodide (Sigma). Samples were run on an LSR II Flow Cytometer (BD Biosciences) and cell populations were gated dependent on fluorescence with a 488 nm laser.

Statistics
Two-tailed student’s t-test was used to compare the means among experimental subgroups. All statistical tests had an alpha of 0.05 as the significance threshold. * = P<0.05, ** = P<0.01, *** = P<0.005, **** = P<0.001, ***** = P<0.0001.


Chapter Four: The Multifaceted Contributions of Mitochondria to Cellular Metabolism

I. **Abstract.** Although classically appreciated for their role as the powerhouse of the cell, the metabolic functions of mitochondria reach far beyond bioenergetics. Mitochondria catabolize nutrients for energy, generate biosynthetic precursors for macromolecules, compartmentalize metabolites for the maintenance of redox homeostasis, and function as hubs for metabolic waste management. We discuss the importance of these roles in both normal physiology and in disease.

II. **Introduction.** The transition to a highly oxidizing atmosphere in early earth development created a selective pressure that favored organisms with respiratory capacity,\(^{243,244}\) including heterotrophic anaerobes, which consumed aerobic prokaryotic microbes (protomitochondrion).\(^{245}\) Following endosymbiosis, mitochondrial signals have been synchronized with the eukaryotic cell\(^4\). This integral relationship is demonstrated by the compartmentalized nature of cellular metabolism, in which mitochondrial reactions are required components of metabolic pathways.

Mitochondria coordinate cellular adaptation to stressors such as nutrient deprivation, oxidative stress, DNA damage and ER stress.\(^{246}\) Although long known to be critical for bioenergetics, emerging research shows that mitochondrial metabolism is multifaceted, mirroring their diverse functions. In addition to ATP, mitochondria produce metabolic precursors for macromolecules such as lipids, proteins, DNA and RNA. Mitochondria also generate metabolic by-products, such as reactive oxygen species (ROS) and ammonia, and possess mechanisms to clear or utilize waste products.


In this review, we discuss the metabolic functions of mitochondria as bioenergetics powerhouses, biosynthetic centres, balancers of reducing equivalents, and waste management hubs. Metabolic compartmentalization is instrumental for mitochondria to perform these functions. We highlight how mitochondrial metabolism supports their diverse functions in cell biology and how metabolism is compartmentalized in normal physiology and disease. A deeper understanding of mitochondrial contributions to metabolism will further elucidate their roles in disease and may reveal co-dependent pathways to target in therapies.

III. Mitochondria are the Powerhouses of the Cell. Cells consume fuels such as sugars, amino acids and fatty acids to generate energy in the form of ATP and GTP.\textsuperscript{247} Nutrients are metabolized and shuttled into the tricarboxylic acid (TCA) cycle, and through iterative oxidations, electrons are stored in the reducing equivalents NADH and FADH\textsubscript{2}. These carriers deposit electrons into the electron transport chain (ETC) in the Inner Mitochondrial Membrane (IMM), and use electron flow to pump protons into the intermembrane space.\textsuperscript{248} Protons flow down their electrochemical gradient through F\textsubscript{1}F\textsubscript{0}-ATP synthase to generate ATP.\textsuperscript{249} Whereas oxidative phosphorylation is the largest source of cellular ATP, the potential energy generated by the ETC is also harnessed for biosynthetic purposes. Many diseases arise when the ETC is perturbed.\textsuperscript{250,251} We discuss how mitochondria integrate fuel metabolism to generate energy for the cell, encompassing both classical and unconventional fuel sources (Figure 4.1).

\textsuperscript{247} Walsh, C. T., Tu, B. P. & Tang, Y. (2017). Chemical reviews, doi:10.1021/acs.chemrev.7b00510


\textsuperscript{251} Dimauro, S. & Rustin, P. (2009). Biochimica et biophysica acta \textbf{1792}, 1159-1167
Figure 4.1. Mitochondria are the powerhouse of the cell. Mitochondria integrate fuel metabolism to generate energy in the form of ATP. Mitochondria oxidize pyruvate (derived from glucose or lactate), fatty acids and amino acids to harness electrons onto the carriers NADH and FADH2. NADH and FADH2 transport these electrons to the electron transport chain, in which an electrochemical gradient is formed to facilitate ATP production through oxidative phosphorylation. VDAC, voltage-dependent anion channel; IDH2, isocitrate dehydrogenase 2; OGDH, α-ketoglutarate dehydrogenase; SDH, succinate dehydrogenase; BCAT2, branched-chain amino transferase 2; ACS, acyl CoA synthetase. Electrons and reducing equivalents are shown in yellow.
A. **Pyruvate**

Pyruvate is generated by a number of sources, depending on nutrient availability and tissue, including glucose catabolism (thought to be a major source), and lactate.\(^{252,253,254}\) Pyruvate utilization in the cytosol versus mitochondria is one of the clearest examples of how compartmentalization is a major determinant of cellular bioenergetics. In healthy tissue, the fate of pyruvate is dependent on oxygen availability and mitochondrial respiratory capacity.\(^{255}\) In normoxia, pyruvate is generated via glycolysis and transported across the IMM through the Mitochondrial Pyruvate Carrier (MPC).\(^{256,257}\) Pyruvate is further catabolized inside mitochondria through the TCA cycle. During hypoxia, mitochondrial respiration is repressed, causing cells to adaptively sink electrons onto pyruvate through lactate dehydrogenase (LDH), generating lactate in the cytosol.\(^{258}\) This pathway is engaged in muscle during exercise, the intestines, and the renal medulla of the kidneys.\(^{259,260,261}\) Otto Warburg observed that cancer cells rewire glucose metabolism for lactate synthesis even in normoxia, known as the Warburg Effect.\(^{262}\) Additional studies must be performed to determine the net catalytic activity of LDH in tumors.

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given that metabolic tracing studies in lung cancer patients have demonstrated that lactate is a major source of TCA cycle intermediates.\textsuperscript{263} The extent of LDH-mediated pyruvate production may depend on \textit{in vitro} versus \textit{in vivo} models of tumor metabolism, emphasizing the need to test metabolic flux \textit{in vivo}.

The critical role of pyruvate compartmentalization in bioenergetics and metabolism is highlighted by recent elegant studies of the MPC.\textsuperscript{264,265} Pharmacological inhibition of MPC represses mitochondrial pyruvate uptake, shifting reliance to glycolysis for ATP production. This shift is evident in cancer cells, which repress MPC1 to promote the Warburg Effect, and in myocytes of diabetic mice, which elevate glucose consumption in response to MPC inhibition.\textsuperscript{266,267} Suppression of MPC accelerates proliferation in intestinal stem cells,\textsuperscript{268} suggesting that the role of MPC is context-dependent and sensitive to mitochondrial respiratory capacity and/or nutrient availability.

Within mitochondria, pyruvate may enter the TCA cycle via the activity of two distinct enzymes: pyruvate dehydrogenase complex (PDC), which generates acetyl CoA, and pyruvate carboxylase (PC), which generates oxaloacetate.\textsuperscript{269} Although PDC and PC both catalyze the flux of pyruvate into the TCA cycle, their enzymatic activities can be distinguished by stable isotope tracing,\textsuperscript{270,271} and their metabolic roles do not appear to be interchangeable. PDC

\begin{thebibliography}{99}
\bibitem{263} Faubert, B. \textit{et al.} (2017). \textit{Cell} 171, 358-371 e359
\bibitem{264} Herzig, S. \textit{et al.} (2012). \textit{Science} 337, 93-96
\bibitem{265} Bricker, D. K. \textit{et al.} (2012). \textit{Science} 337, 96-100
\bibitem{266} Schell, J.C., \textit{et al.} (2014). Molecular cell 56, 400-413.
\end{thebibliography}
deficiency is sufficient to rewire energy metabolism towards aerobic glycolysis despite the potential adaptive node for TCA cycle anaplerosis (a process to replenish TCA cycle intermediates), mediated by PC. Many cancers favor PC-mediated anaplerosis, although the factors that dictate the choice for pyruvate flux between PC and PDC are little studied. Therefore, these enzymes may have important functions beyond TCA cycle flux for bioenergetics.

B. Glutamine and Branched Chain Amino Acids (BCAAs)

The catabolism of glutamine, the most abundant amino acid, often starts in the mitochondria and its carbon and nitrogen atoms are distributed into macromolecules throughout the cell, including TCA cycle intermediates (important in bioenergetics), amino acids, nucleotides, glutathione, and lipids.

In mitochondria, glutaminase (GLS) converts glutamine into glutamate and ammonia. Either transaminase or glutamate dehydrogenase (GDH) converts glutamate into α-ketoglutarate. Glutamine anaplerosis sustains TCA cycle intermediates in conditions of limiting glucose and MPC inhibition, demonstrating the potential flexibility of these metabolic nodes. Glutamine anaplerosis is critical for meeting the energetic requirements of

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proliferative cells, such as T cells during the transition from quiescent naïve T cells to effector cells, and in cancers, particularly with MYC elevation.\textsuperscript{281,282,283} GLS inhibition suppresses proliferation, and GLS inhibitors are being evaluated in clinical studies for a number of cancers.\textsuperscript{284,285,286} However, sensitivity to GLS inhibition \textit{in vitro} is not always consistent \textit{in vivo}, and is dependent on extracellular cystine levels.\textsuperscript{287} This emphasizes the need for investigators to study the effect of the microenvironment on metabolic dependencies and to validate experiments \textit{in vivo}.

Although glutamine transporters at the plasma membrane have been identified,\textsuperscript{288} the mitochondrial glutamine transporter has not been fully characterized.\textsuperscript{289,290} This critical area of research is challenging to address because there are likely multiple mechanisms for glutamine import.

The BCAAs leucine, isoleucine, and valine are a major source of cellular energy via acetyl CoA and succinyl CoA generation.\textsuperscript{291} The tissue of origin dictates dependency on BCAA


catabolism in normal physiology and in cancer.\textsuperscript{292} In normal physiology, myocytes and adipocytes activate mitochondrial BCAA catabolic enzymes to support ATP production during exercise or fasting and differentiation, respectively.\textsuperscript{293,294} BCAA catabolism is repressed in maple syrup urine disease, which is caused by mutations to branched-chain keto acid dehydrogenase (BCKDH) and causes dysfunction of immune cells, skeletal muscle and the central nervous system.\textsuperscript{295} Although mitochondrial BCAA catabolism is critical in these pathologies, it is unknown how BCAAs are imported into the mitochondria. Identifying their transport mechanisms will be critical to our understanding of mitochondrial BCAA catabolism in cellular homeostasis.

C. Fatty Acid Oxidation

Palmitate, a 16-carbon fatty acid (FA), stores 39KJ/g of energy compared to 16KJ/g stored in glucose.\textsuperscript{296} Therefore, FAs are a major source of cellular energy, particularly under conditions of nutrient stress. Mitochondrial FA import is a rate-determining step for fatty acid oxidation (FAO) and demonstrates how metabolic compartmentalization adapts to cellular state. As long chain FAs are unable to cross mitochondrial membranes, mitochondria have evolved an intricate set of reactions and transporter activities to allow fat to access mitochondrial β-oxidation machinery. The outer mitochondrial membrane (OMM) enzyme carnitine palmitoyltransferase 1 (CPT1) forms acylcarnitines from fatty acyl CoAs.\textsuperscript{297} Acylcarnitines are shuttled into mitochondria through the carnitine–acylcarnitine translocase (SLC25A20) in the IMM. CPT2


\textsuperscript{295} Blackburn, P.R., et al. (2017). The application of clinical genetics 10, 57-66.


liberates FA from carnitine, initiating FAO. Acetyl CoA from FAO is used for the TCA cycle as well as for aspartate and nucleotide synthesis.

CPT1 activity is tightly controlled by a network of metabolites, linking it to cellular nutrient status. Malonyl CoA, generated by the enzyme acetyl CoA carboxylase (ACC), represses CPT1 to inhibit acylcarnitine import. Malonyl CoA is the initiating metabolite for FAS, and its levels dictate the balance of fat synthesis or oxidation within a cell. In low energy conditions, AMP-activated protein kinase (AMPK) phosphorylates and inhibits ACC, decreasing malonyl CoA and increasing CPT1 activity. ACC2 is also hydroxylated by the dioxygenase prolyl hydroxylase 3 (PHD3). Hydroxylation promotes ACC2 activity in nutrient abundance. These enzymes are altered in some cancers and human diseases as the mechanism that dictates fat utilization. PHD3 is suppressed in cancers that rely on FAO, such as AML and prostate cancer, and elevated in cancers that rely on FAS such as breast and non-small-lung-cell cancer. Reciprocally, AMPK is linked to fat utilization in diseases and cancers.

The dynamic regulation of FAO is key to cellular physiology. FAO is fundamental for the survival and function of memory CD8+ T cells, unlike effector cells that rely on glycolysis and

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glutaminolysis for energy. Likewise, FAO is activated in insulin resistance, in which free fatty acids provide a compensatory fuel for repressed glucose uptake.

**IV. Mitochondria are Biosynthetic Hubs.** Mitochondria participate in the biosynthesis of nucleotides, FAs, cholesterol, amino acids, glucose, and heme (Figure 4.2). These biosynthetic pathways are engaged in stress responses, and are often mis-regulated in disease. Rather than being dysfunctional, highly proliferative cells such as cancer cells and activated T cells rely on mitochondrial metabolites to form biomass. Below we review the mitochondrial compartmentalization of anabolic pathways and its role in cell stress responses and disease.

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Figure 4.2. Mitochondria are biosynthetic hubs. The mitochondria are a critical source of building blocks for biosynthetic pathways, including nucleotide synthesis, fatty acid and cholesterol synthesis, amino acid synthesis, and glucose and heme synthesis. Compartmentalization is a key feature of biosynthetic pathways. While many of the enzymes listed are bi-directional, arrows highlight the biosynthetic functions. Enzymes are circled in grey and brown. FTDH, formate dehydrogenase; TA, transaminase; GC, glutamate carrier; FLVCR, feline leukaemia virus subgroup C receptor 1.
A. *Nucleotides*

The 1C metabolic pathway involves a set of reactions that generate and transfer activated one carbon (1C) units for *de novo* nucleotide synthesis, compartmentalize amino acids, and contribute to redox homeostasis. The co-factor tetrahydrofolate (THF) is the carrier that mediates 1C transfer reactions for *de novo* nucleotide synthesis.\(^{316,317}\) Activated THF molecules are generated through an oxidative/reductive cycle that catabolizes serine (to generate glycine) in the mitochondria and synthesizes serine in the cytosol.

The carrier SLC25A32 imports THF into the mitochondria, where it is converted by serine hydroxymethyltransferase (SHMT2) into 5,10 methylene-THF and glycine. Like many enzymes in 1C metabolism, SHMT2 is bi-directional. SHMT2 favors production of glycine and 5,10 methylene-THF, and cells deficient in mitochondrial 1C metabolism are glycine auxotrophs.\(^{318}\) In the absence of SHMT2, cytosolic SHMT1 reverses flux to compensate demonstrating how metabolic flexibility among subcellular compartments is critical to stress adaptation.\(^{319}\)

Mitochondrial methylenetetrahydrofolate dehydrogenase (MTHFD2) converts 5,10 methylene-THF to 10-formyl-THF. MTHFD2 expression is regulated by mTORC1, and is critical for growth and proliferation.\(^{320}\) MTHFD2 is overexpressed in many human cancers,\(^{321}\) and mitochondrial biogenesis and SHMT2/MTHFD2 expression are promoted during T-cell

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activation to support proliferation.\(^{322}\) 10-formyl-THF has multiple fates: conversion into THF by 10-formyl-THF dehydrogenase, production of formyl-methionine for mitochondrial translation, or hydrolyzation to formate by MTHFD1L. Mitochondrial contributions to this pathway are critical, as mitochondrial formate is the main carbon source for cytosolic 1C metabolism.\(^{323}\)

The IMM enzyme dihydroorotate dehydrogenase (DHODH), which oxidizes dihydroorotate to orotate, is required for \textit{de novo} pyrimidine synthesis.\(^{324}\) Consistent with their reliance on 1C metabolism, T cells require DHODH for clonal expansion and differentiation into effector cells.\(^{325}\) DHODH is targeted in autoimmune disorders and inhibition suppresses myeloid differentiation of AML cells.\(^{326}\) DHODH activity is also elevated in response to DNA damage and upon genotoxic chemotherapy treatment to increase nucleotide synthesis for DNA repair.\(^{327,328}\)

B. \textit{Citrate}

In addition to generating electron carriers for the ETC, TCA cycle intermediates such as citrate regulate anabolic reactions. Mitochondrial citrate controls anabolic reactions by directly acting as the carbon source for FAs, cholesterol and ketone bodies through ATP citrate lyase (ACLY),\(^{329}\) and by allosteric modulation. Citrate is generated by citrate synthase (CS) or through the reduction of \(\alpha\)-ketoglutarate by isocitrate dehydrogenase (IDH).\(^{330,331,332}\) Mitochondrial citrate


is exported by the malate-citrate antiporter SLC25A1. In the cytosol, citrate is converted to acetyl CoA via ACLY, which can access many pathways, including conversion to malonyl CoA by the activity of ACC (as described above). Cytosolic citrate is a potent allosteric regulator of ACC by increasing its polymerization and activity.

Regulation of citrate export may provide a physiological node for the cell to communicate lipid homeostasis to the mitochondria. SLC25A1 is sensitive to membrane rigidity, and high levels of cholesterol or acidic phospholipids in the IMM repress mitochondrial citrate export. Moreover, fasting causes a 40% reduction in mitochondrial citrate export. Although these studies indicate that citrate export is affected by lipid abundance, it is unknown if repression of SLC25A1-mediated citrate export affects ACC2 polymerization and FAS initiation.

Acetyl CoA is required for epigenetic modifications such as histone acetylation. Thus, fat metabolism may be intimately linked with the epigenetic state, although it is unknown whether the connection is direct. The emerging role of mitochondrial metabolism in epigenetic reprogramming may extend beyond acetyl CoA to include other mitochondrial metabolites such

as succinate, fumarate, and ROS, which directly affect the activity of Fe (II)/α-KG-dependent dioxygenases, including hydroxylases, DNA demethylases and histone demethylases.\textsuperscript{340}

C. Amino Acids

The mitochondria is a hub for amino acid synthesis, including glutamine, glutamate, alanine, proline, and aspartate. Glutamine synthetase (GS) condenses glutamate and ammonia to make glutamine.\textsuperscript{341} GS has been reported to have activity in cytosol and mitochondria, and its biological role may differ depending on its subcellular localization. GS has a “weak” mitochondrial localization sequence and is imported into the mitochondria in the liver, whereas GS is cytoplasmic in astrocytes.\textsuperscript{342} In glioblastoma, GS generates a source of glutamine for \textit{de novo} purine synthesis.\textsuperscript{343} However, in breast cancer cells, GS-derived glutamine is not used for \textit{de novo} nucleotide synthesis.\textsuperscript{344} One possible explanation for this difference is the subcellular localization of GS in these systems.

Glutamate is generated by and utilized as a nitrogen source for numerous reactions.\textsuperscript{345} Glutamate metabolism stratifies in proliferating and quiescent cells; proliferating cells elevate the expression of glutamate-dependent transaminases, whereas quiescent cells suppress them.\textsuperscript{346} Many of the glutamate-dependent transaminases, such as glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) have two (cytosolic and


mitochondrial) isoforms. It will be key for future studies to elucidate the role of subcellular compartmentalization of glutamate metabolism in proliferation.

Proline and ornithine metabolism are centrally mitochondrial. The mitochondrial enzyme Pyrroline-5-carboxylate synthase (P5CS) generates pyrroline-5-carboxylate (P5C), which can be used for proline and ornithine production. Ornithine is made by ornithine amino transferase (OAT) and proline is produced through reduction of P5C by Pyrroline-5-carboxylate reductase (PYCR). The mechanisms underlying compartmentalization of proteinogenic amino acids, such as proline and glutamate are little studied.

D. Gluconeogenesis

Gluconeogenesis is predominantly a cytosolic process, although the initiating step by PC occurs inside the mitochondria. PC-derived oxaloacetate is converted to malate and exported from the mitochondria for the remaining steps of gluconeogenesis. This export can occur through SLC25A1 (citrate-malate antiporter), SLC25A11 (α-ketoglutarate-malate antiporter) or SLC25A10 (dicarboxylate-phosphate antiporter). The dominant mechanism for malate export in gluconeogenesis is unknown. Furthermore, it is unclear if metabolic stressors such as nutrient deprivation or hypoxia dictate this mechanism. In the cytosol, phosphoenolpyruvate carboxykinase (PCK) converts oxaloacetate into phosphoenol pyruvate (PEP) for

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gluconeogenesis. The mitochondrial isoform of this enzyme, PCK2, has no known connections to gluconeogenesis.

E. Heme

Heme metabolism illustrates an extraordinary example of metabolic compartmentalization. The committed step of the pathway is catalyzed by mitochondrial aminolevulinate synthase (ALAS), which generates ALA from glycine and succinyl CoA. ALA is exported via SLC25A38 and, through four cytosolic reactions, is converted into coproporphyrinogen III (CPGIII). Next, CPGIII enters the intermembrane mitochondrial space through the ATP-dependent transporter ABCB6 for further catalysis by coproporphyrinogen oxidase (CPOX). The intermembrane space is a region in which few metabolic reactions occur. The terminal step of heme synthesis is in the mitochondrial matrix, in which ferrochelatase (FECH) catalyzes the insertion of ferrous iron into the macrocycle. As heme biosynthesis generates $\text{H}_2\text{O}_2$ in the intermembrane region, we speculate that there may be direct links between heme metabolism and ROS-sensitive signaling pathways.

V. Mitochondria Balance Redox Equivalents. The mitochondria and cytosol have distinct requirements for $\text{NAD}^+$, and proper compartmentalization of redox equivalents is crucial for maintenance of cellular homeostasis and survival in response to environmental

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stressors. The cytosol is a more oxidizing environment in which the NAD⁺/NADH ratio ranges between 60-700. Conversely, mitochondria employ more reductive metabolic reactions, and the NAD⁺/NADH ratio is approximately 7-8. To sustain the imbalanced distribution of NAD, mammalian cells engage indirect pathways (Figure 4.3) because there is no known mammalian transporter for NAD⁺ or NADH, contrary to yeast which facilitate NAD transport through NDT1.

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Figure 4.3. Mitochondria Balance Redox Equivalents. In the absence of a direct mode for NAD transport, cells rely on compartmentalized flux of metabolites to support balance of reducing equivalents NAD$^+$/NADH and NADP/NADPH. Generally, redox shuttles favour cytosolic NAD$^+$ synthesis and mitochondrial NADH synthesis. ME1, malic enzyme 1; mGPDH/cGPDH, mitochondrial/cytosolic glycerol-3-phosphate dehydrogenase, respectively.
A. *Malate-Aspartate Shuttle*

The malate-aspartate shuttle is ubiquitously engaged to generate cytosolic NAD$^+$ and mitochondrial NADH. This cycle involves an oxidation or reduction catalyzed by malate dehydrogenase (MDH1: cytosolic, MDH2: mitochondrial), a transamination catalyzed by glutamate-oxaloacetate transaminase (GOT1: cytosolic, GOT2: mitochondrial), and two antiporters localized to the IMM (aspartate-glutamate antiporter AGC and malate α-ketoglutarate antiporter MaA). Compartmentalization of reducing equivalents through the malate-aspartate shuttle is key for survival in stress conditions such as exercise, in which cytosolic NAD$^+$ is required to promote glucose catabolism and mitochondrial NADH for ATP production. Moreover, in PDAC cancers with oncogenic KRAS, glutamine is fluxed through the malate-aspartate shuttle to raise the NADPH/NADP$^+$ ratio for glutathione synthesis. When oxidative phosphorylation is repressed, cells utilize the reverse flux of GOT1 to generate aspartate. In addition to its regulation of redox balance, the malate-aspartate shuttle may also contribute to cellular amino acid compartmentalization.

B. *Citrate-Malate Shuttle*

In contrast to the malate-aspartate shuttle, the citrate-malate shuttle functions equally (with respect to reducing equivalents), but is less studied in the context of disease. Similar to malate-aspartate shuttle, the citrate-malate shuttle utilizes both isoforms of MDH. However, MDH activity is paired with CS, ACLY, and the malate-citrate antiporter (CIC). Rather than

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elevating cytosolic aspartate, the citrate-malate shuttle increases cytosolic citrate levels. Therefore, flux through the citrate-malate shuttle promotes FAS through citrate compartmentalization. Thus, although both the malate-aspartate and citrate-malate shuttles balance reducing equivalents through MDH activity, these shuttles are not interchangeable. The implications of cytosolic citrate accumulation in the malate-citrate shuttle are yet to be defined beyond FAS. For example, flux through the citrate-malate shuttle may also affect epigenetics through ACLY activity and acetyl CoA production.

C. α-glycerophosphate Shuttle

The α-glycerophosphate shuttle is a unique redox balancing pathway, which intersects the mitochondria but does not directly affect mitochondrial NAD/NADH. The α-glycerophosphate shuttle is composed of cytosolic and mitochondrial α-glycerophosphate dehydrogenase (cGPDH and mGPDH). In this cycle, cGPDH utilizes NADH to reduce dihydroxyacetone phosphate (DHAP) to glycerophosphate (GAP) and generate cytosolic NAD+. GAP is subsequently oxidized to DHAP by the flavin-dependent mGPDH, which directly deposits electrons into the ETC. The α-glycerophosphate shuttle is tightly linked to glycolysis and is highly active in brown adipose tissue (BAT) to regenerate cytosolic NAD+ while simultaneously sinking electrons into the ETC for thermogenesis. As this pathway is engaged in highly glycolytic cells, it would be interesting for future studies to investigate the potential role of this redox shuttle in cancer.

D. One Carbon Metabolism

MTHFD is among the largest contributors to cellular NADPH, in addition to the pentose phosphate pathway and malic enzyme (ME).\textsuperscript{375} MTHFD isozymes are bi-directional, however, stable isotope tracing of NADPH revealed that the mitochondrial MTHFD favors NADPH production, and the cytosolic isoform favors NADP\textsuperscript+ production.\textsuperscript{376} The 1C metabolic pathway is an adaptive mechanism to survive oxidative stress. Upon ETC inhibition, flux through the mitochondrial arm of 1C metabolism is activated for NADPH/NADP\textsuperscript+ balance.\textsuperscript{377} NADPH is required for reduction of glutathione for clearance of ROS. In cancer cells, flux through the mitochondrial 1C pathway generates cytosolic NADPH for FAS.\textsuperscript{378}

VI. Mitochondria Orchestra\textsuperscript{t}e Waste Management. The by-products of metabolic reactions are often depicted as waste. However, emerging studies have revealed a functional role for metabolic by-products such as lactate, ammonia, ROS and hydrogen sulfide (H\textsubscript{2}S).\textsuperscript{379} The study of metabolic by-products is a growing area of research, especially in cancer, in which metabolic by-products accumulate in the tumor microenvironment (TME).\textsuperscript{380} (Figure 4.4A).

\begin{itemize}
\item \textsuperscript{376} Lewis, C.A., et al. (2014). Molecular cell 55, 253-263.
\item \textsuperscript{378} Tedeschi, P.M., et al. (2013). Cell death & disease 4, e877.
\item \textsuperscript{379} Hui, S. et al. (2017). Nature, 551, 115-118.
\item \textsuperscript{380} Faubert, B. et al. (2017). Cell 171, 358-371 e359
\end{itemize}
Mitochondria are indispensable in cellular waste management (Figure 4.4B-D). Below, we review the pathways that mitochondria utilize to re-purpose cellular waste.
Figure 4.4. Mitochondria orchestrate waste management. A. Tumour cells increase nutrient consumption and metabolic fitness relative to healthy tissue, leading to accumulation of waste products in the tumour microenvironment. To manage metabolic waste, cancer cells engage recycling pathways for these metabolic by-products. B. Production and metabolic clearance of ammonia (NH$_3$) in cell metabolism. NH$_3$ is generated by amino acid and nucleotide catabolism. NH$_3$ is assimilated in the mitochondria through GS, GDH, and CPS1. CPS1 initiates the urea cycle for production of the metabolic waste product urea. Urea can be re-catabolized by urease positive bacteria in the microbiome to regenerate NH$_3$. C. Production and metabolic clearance of hydrogen sulfide (H$_2$S) in cell metabolism. H$_2$S is generated by the mammalian enzymes CBS, CSE, 3MST and from the metabolic reactions in the microbiome. H$_2$S is cleared by iterative oxidation catalysed by SQR, TR, and SO. TR utilizes oxidized glutathione (GS$^-$) as a sink for electrons. Oxidations catalysed by SQR and SO are linked to mitochondrial ETC and
oxidative phosphorylation. **D.** Reactions that generate and sequester ROS. ROS are generated in the mitochondria through the ETC and NOX4. SOD2 converts superoxide into the less reactive molecule hydrogen peroxide (H$_2$O$_2$). In the mitochondria, H$_2$O$_2$ is turned over by combined functions of peridoxins (Prx) and thioredoxins (Trx). H$_2$O$_2$ also reacts with Fe$^{+2}$ (the Fenton reaction) to generate OH$^*$ in the mitochondria. ROS inflict oxidative damage to proteins in the mitochondria and cytosol, and also function as potent mitogen signalling agents.
A. Ammonia

Ammonia is generated in mammalian cells by amino acid lyases and nucleotide deaminases, however, the largest contributor to ammonia in mammals is the microbiome.\(^{385}\) Ammonia is a neurotoxin that is sustained below 50 µM in plasma of healthy adults, and can induce seizure when plasma levels become elevated.\(^{386}\) Moreover, high ammonia may induce autophagy in some cultured cells.\(^{387, 388}\) To evade toxicity, mammalian cells possess three ammonia-assimilating enzymes: carbamoyl phosphate synthetase 1 (CPS1), GS, and GDH.

The urea cycle is a sink for ammonia, ultimately generating urea, which cannot be metabolized by mammalian enzymes. CPS1 is the rate-limiting step of the urea cycle, generating carbamoyl phosphate (CP).\(^{389}\) N-acetyl glutamate (NAG) is an essential activator of CPS1, and congenital NAGS mutations cause hyperammonemia.\(^{390}\) CP is condensed with ornithine by ornithine carbamoyltransferase (OTC) to generate citrulline, which is exported through ORNT1, the citrulline-ornithine antiporter for the remaining steps of the cycle. Interestingly, in KRAS/LKB1 mutant cancer, CP from CPS1 is diverted into \textit{de novo} pyrimidine synthesis.\(^{391}\) The mechanism of CP export from the mitochondria is unknown and may be a potential therapeutic target.


Although urea is a metabolic waste product for mammalian cells, urease-positive bacteria in the microbiome re-catabolize 15-30% of urea to regenerate ammonia.\textsuperscript{392} Consequently, similar to congenital mutations in urea cycle enzymes, the microbiome can contribute to hyperammonemia.\textsuperscript{393,394} Beyond ammonia metabolism, many microbial metabolites intersect host biology and their roles remain an active area of research.\textsuperscript{395}

GDH and GS assimilate ammonia, generating glutamate and glutamine. Glutamate contributes to the urea cycle through conversion to aspartate by GOT2 and mitochondrial export via AGC1/2. GDH is a bidirectional enzyme, and high ammonia levels reverse the direction of GDH, favoring the reductive activity.\textsuperscript{396} This bi-directionality is particularly relevant in breast cancers, as ammonia accumulates in the TME, driving GDH towards glutamate synthesis.\textsuperscript{397} Beyond the TME, physiological niches with high ammonia levels (the microbiome, liver, and kidneys) may promote the reductive activity of GDH. Additionally, GDH-mediated ammonia assimilation requires NAD(P)H and therefore may contribute to redox balance.

B. ROS

Mitochondria generate, sequester and interconvert ROS in response to stressors such as hypoxia, nutrient availability, cytokine stimulation and changes in mitochondrial membrane potential.\textsuperscript{398} ROS are generated from the reduction of oxygen (O\textsubscript{2}) to superoxide (O\textsubscript{2}⁻), hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) and hydroxyl radical (OH⁻). Mitochondrial ROS are generated in reactions such

as NADPH oxidase (NOX4) and the Fenton Reaction, and through electron leak from ETC complexes,\textsuperscript{399} although NOX4 is not strictly localized to mitochondria.\textsuperscript{400} ROS are highly reactive and inflict oxidative damage to macromolecules.\textsuperscript{401}

Mitochondria rely on ROS clearance to protect the concentrated iron-sulfur clusters in the ETC and iron-dependent enzymes such as aconitase. Superoxide dismutase (SOD2) converts superoxide into a less reactive molecule, H$_2$O$_2$.\textsuperscript{402} Cellular H$_2$O$_2$ can be degraded to water by catalase, glutathione peroxidase (GPx), and peroxiredoxin (Prx), however mitochondria do not have catalase and only a single splice variant of GPx4 has been demonstrated to be localized to mitochondria.\textsuperscript{403}\textsuperscript{404} Mitochondria rely on the combined activities of peroxiredoxins (Prx3 and Prx5), thioredoxins (Trx2), and thioredoxin reductase 2 (TRXR2) to decompose the locally generated H$_2$O$_2$.\textsuperscript{405}

Beyond toxicity, ROS are potent mitogen signaling agents that foster proliferation, differentiation, and migration.\textsuperscript{406}\textsuperscript{407} Specifically, ROS oxidize cysteine residues, linking mitochondria to signaling cascades. ROS inactivates the catalytic cysteine of phosphatase 1B (PTP1B), enabling receptor tyrosine phosphorylation required for growth-factor signaling.\textsuperscript{408}


ROS inactivate PTEN, which represses the PI-3 Kinase/AKT signaling cascade and PHDs to repress HIF hydroxylation.\textsuperscript{409,410} In breast cancer, low levels of the mitochondrial sirtuin 3 promote HIF stabilization through ROS, stimulating the Warburg Effect.\textsuperscript{411} In macrophages, mROS promote the antibacterial innate immune response, and mice harboring mROS-deficient macrophages are susceptible to infection.\textsuperscript{412} Similarly, mitochondria provide ROS for B-cell and T-cell activation.\textsuperscript{413,414} ROS are thus critical to proliferating systems.

C. Hydrogen Sulfide

H\textsubscript{2}S is produced in the microbiome by sulfur-reducing bacteria and by mammalian cells through cystathionine β synthase (CBS), cystathionine γ lyase (CSE), and 3-mercaptopuruvate sulfurtransferase (3MST).\textsuperscript{415} H\textsubscript{2}S-producing enzymes are localized to the cytosol and mitochondria, depending on the tissue type.\textsuperscript{416}

High levels of H\textsubscript{2}S are toxic and repress respiration through complex IV inhibition.\textsuperscript{417} To dampen H\textsubscript{2}S toxicity, mitochondria sequentially oxidize H\textsubscript{2}S generating thiosulfate, sulfite, and ultimately sulfate.\textsuperscript{418} The first and last reactions catalyzed by flavin-dependent sulfide quinone reductase (SQR) and sulfite oxidase (SO) directly deposit electrons onto Coenzyme Q (CoQ) of


\textsuperscript{411} Finley, L.W., et al. (2011). Cancer cell 19, 416-428.


In CoQ deficiency, H$_2$S oxidation is significantly repressed.\textsuperscript{420} The intermediate oxidation step of H$_2$S is catalyzed by thiosulfate reductase (TR) and requires oxidized glutathione as an electron sink. Because the enzymes for glutathione synthesis are cytosolic, mitochondria must import glutathione for this process. Glutathione can utilize the dicarboxylate carrier SLC2510 and the α-kg carrier SLC25A11 for import, although a selective mechanism of transport remains unknown and may be pivotal for H$_2$S clearance.\textsuperscript{421}

H$_2$S metabolism is directly linked with oxidative phosphorylation.\textsuperscript{422} Hypoxia represses H$_2$S detoxification through respiratory chain inhibition.\textsuperscript{423} Interestingly, the microbiome, which has the highest H$_2$S levels, is hypoxic in some regions.\textsuperscript{424} The mechanism for H$_2$S tolerance in the microbiome remains unknown. H$_2$S production and clearance may be critical in diseases such as cancer and diabetes, which are associated with altered respiration.

VII. Future directions of Mitochondrial Metabolism in Cellular Homeostasis and Disease.

Here we discuss the multifaceted contributions of mitochondria to cell metabolism as bioenergetic powerhouses, biosynthetic centres, balancers of reducing equivalents and waste management hubs. Although mitochondrial pathways are well defined, the mechanisms by which metabolites are compartmentalized remain elusive. Identifying the transporters that coordinate metabolic flux for key pathways such as amino acid and glutathione import will be important directions for future research.\textsuperscript{425} Given that mitochondrial metabolism is critical to


\textsuperscript{420} Luna-Sanchez, M., \textit{et al.} (2017). EMBO molecular medicine 9, 78-95.


\textsuperscript{424} Rigottier-Gois, L. (2013). The ISME journal 7, 1256-1261.

many diseases, transporters that enable metabolic compartmentalization may be promising therapeutic targets.\textsuperscript{426,427,428,429,430} It will also be key to consider mitochondrial metabolite concentrations, which differ from whole cell concentrations,\textsuperscript{431} to better inform the kinetics of mitochondrial enzymes under different cellular stress conditions and in disease. Mitochondrial concentrations are critical when studying bi-directional enzymes such as transaminases and enzymes in 1C metabolism.

It will be important for future studies to probe the physiological contributions of mitochondria to cell biology. Metabolism is not always comparable when studying \textit{in vitro} and \textit{in vivo} models. These differences may dictate the efficacy of therapies, such as the glutaminase inhibitor in cancer.\textsuperscript{432} The extent to which a physiological niche alters mitochondrial contributions to metabolism and cell/tissue function has not been well explored. For example, metabolic by-products accumulate in the TME, increasing the necessity for cancer cells to engage waste management pathways.\textsuperscript{433,434,435} Disparities between model systems may be avoided by performing \textit{in vitro} studies in media with physiological metabolite concentrations, using model

\begin{thebibliography}{99}
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systems that represent the 3-dimensional architecture of the tissue being studied, and performing experiments \textit{in vivo}.\textsuperscript{436,437,438} Future studies in this exciting and growing field will continue to reveal the roles of mitochondrial metabolism in cellular homeostasis and disease.


Chapter Five: Mitochondrial Glutamate Levels Direct Local Translation to Promote Respiration and Proliferation in Breast Cancer

*Manuscript citation: Spinelli, J.B., et al. Submitted (in review).*
I. Abstract. Amino acids play a key role in tumor growth and proliferation, although the subcellular compartment in which amino acids are limiting has never been explored. Here we use in vitro, in vivo xenograft models, and primary breast cancer patient tissue to directly track metabolic flux of ammonia and the downstream amino acids through subcellular compartments. We show that glutamate generated in the mitochondria from ammonia assimilation is converted to aspartate by glutamate oxaloacetate transaminase 2 (GOT2), and can subsequently be secreted into the cytosol. Using genetic depletion of GOT2 and the mitochondrial glutamate transporter SLC25A22, we identified that breast cancer cells have a specific limitation in the mitochondrial glutamate pool for proliferation. Mechanistically, we found that mitochondrial glutamate directs local translation and subsequently cellular respiration. Thus, our data reveal that subcellular localization of amino acids is critical for their function in cellular biology.

II. Introduction. Cancer cells rewire metabolic pathways to support rapid growth and proliferation in the nutrient-limited tumor microenvironment. Numerous metabolic pathways that drive tumor growth span subcellular compartments. The mitochondria are a particular hub for metabolism and are a source of cellular energy, provide building blocks necessary for anabolic metabolism, facilitate balance of cellular reducing equivalents, and are a central location for recycling of metabolic waste products.

We recently discovered that breast cancer cells recycle the metabolic by-product ammonia via glutamate dehydrogenase (GDH) to generate glutamate and downstream amino acids.

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acids. Surprisingly generation of these non-essential amino acids via GDH was sufficient to increase proliferation in breast cancer cells, raising a question about their function in tumorigenesis and proliferation. As GDH is localized to the mitochondrial matrix, we reasoned that nitrogen recycling via GDH might serve a unique role to generate amino acids specifically within this cellular compartment. Moreover, while these non-essential amino acids are available in the media, the biological functions of these amino acids in specific subcellular compartments are not well studied. Since GDH is localized to the mitochondria, we hypothesized amino acid levels within the mitochondrial compartment may be limiting for cellular proliferation.

Here, we reveal the compartmentalized nature of nitrogen recycling. In hormone receptor positive (HR+) breast cancer cells, ammonia is initially assimilated within the mitochondria to increase local glutamate and many of the downstream amino acids. Using genetic knockdown of glutamate oxaloacetate transaminase (GOT2), which converts glutamate to aspartate and is requisite for export to the cytosol, we find that the mitochondrial pool of glutamate is essential for ammonia-stimulated cell proliferation. Elevations in glutamate stimulate the rate of mitochondrial translation and rapidly increase the abundance of mitochondrial-encoded respiratory chain subunits. We demonstrated mitochondrial glutamate compartmentalization via nitrogen recycling contributes to proliferation of breast cancer cells in culture, in vivo, and in primary tumors from patients. Taken together, this work illustrates the importance of gaining subcellular resolution of metabolism to understand how metabolites function in cell biology.

III. Results


A. Glutamate and Aspartate Stimulate Proliferation Downstream of Ammonia Assimilation

We previously demonstrated that ammonia is recycled in HR+ breast cancer cell lines in vitro and in vivo mouse models.\textsuperscript{445} To test the relevance in patient tumors, we examined ammonia assimilation in primary breast cancer patient tissue resected from patients with ER/PR (+), HER2 (-) tumors (Figure 5.1a). All patients were consented and tissue samples were de-identified as approved under the Dana Farber Cancer Institute IRB protocol #93-085. Quantification of ammonia from interstitial fluids in patient-matched healthy and tumor breast tissue revealed that ammonia levels were approximately 8-fold higher in the tumor microenvironment (TME) than in the interstitial fluids of healthy tissue from the same person, suggesting that ammonia is bioavailable for assimilation (Figure 5.1b). Ex vivo \textsuperscript{15}NH\textsubscript{4}Cl tracing on the patient tumors revealed that ammonia was assimilated to make amino acids, including glutamate, aspartate, proline, and alanine, but was not utilized to make nucleotides or urea cycle intermediates (Figure 5.1c). Thus, in breast cancer patient tumors, amino acids are the dominant product of ammonia assimilation, raising the need to better understand the functions of these amino acids in cancer cell biology.

As ammonia recycling in the mitochondria via GDH accelerates breast cancer cell proliferation, we sought to elucidate whether any of the amino acids generated downstream of GDH were limiting for proliferation and if there was a specific role for these amino acids in the mitochondrial versus cytosolic compartment (Figure 5.1d). To determine if glutamate, aspartate, proline, alanine, or leucine were sufficient to promote breast cancer proliferation, we supplemented cell culture media with these amino acids and measured proliferation rate in 3D culture. MCF7 cells treated with leucine, alanine, or proline displayed no growth advantage, whereas, cells treated with glutamate or aspartate increased spheroid area (Figure 5.1e, Figure

5.S1). Since these amino acids are generated downstream of ammonia assimilation, we tested whether they retained their ability to stimulate proliferation in GDH-depleted cells. Although GDH was required for ammonia to stimulate proliferation as previously described (Figure 5.1f-g), glutamate and aspartate significantly increased the rate of proliferation in the cells depleted of GDH (Figure 5.1h-i). Therefore, glutamate and aspartate stimulated proliferation downstream of GDH-mediated ammonia assimilation.
Figure 5.1. Glutamate and aspartate stimulate proliferation downstream of ammonia assimilation. A. Schematic of experiments performed on primary breast cancer patient tissue. B. Quantification of ammonia in the interstitial fluid of healthy and tumor tissue from three estrogen receptor (ER) positive breast cancer patients. C. Isotopologue abundance of $^{15}$N-metabolites in primary ER(+) breast cancer tumors after 24 hour ex vivo treatment with 3 mM $^{15}$NH$_4$Cl. Values represent mean +/- SEM, n = 3. D. Schematic depicting the underlying hypothesis that compartmentalization of amino acids downstream of ammonia assimilation plays a role in accelerated proliferation. E. 3D culture assay in MCF7 cells. Quantification of average sphere area of 100 spheres per well. Cells were treated for 8 days in control media
supplemented with 1 mM NH₄Cl, 20 mM aspartate, 20 mM glutamate, 20 mM alanine, 20 mM proline, or 10 mM leucine. Values represent mean +/- SEM, n = 3. F. Western blot depicting shRNA-mediated GDH knockdown in MCF7 cells. G. Cell count in 3D culture assay in control or GDH-depleted MCF7 cells treated for 8 days in media supplemented with 1 mM NH₄Cl, H. 20 mM glutamate, or I. 20 mM aspartate. Values represent mean +/- SEM, n = 3. *P < 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001. Differences in individual genes or between two groups (control group compared to experimental) were assessed using two-tailed t-tests (95% confidence interval). NS= not significant. All experiments were performed independently at least twice.
B. Glutamate and Aspartate Generated Downstream of Ammonia Assimilation are Compartimentalized

GDH assimilates ammonia in the mitochondria; mitochondrial glutamate-oxaloacetate transaminase (GOT2) converts this glutamate into aspartate, and cytosolic GOT1 can reconvert this aspartate into glutamate.\(^{446-447}\) However, it is unclear whether the amino acids generated in the mitochondria from ammonia assimilation are secreted to the cytosol, and subsequently, which compartment plays a role in ammonia-stimulated proliferation.

To test if amino acids generated downstream of ammonia assimilation localized to the mitochondria or were exported to the cytosol, we used a rapid immunoprecipitation (IP) protocol to purify epitope-tagged mitochondria from cells.\(^{448}\) We validated the purity of the mitochondrial isolation; mitochondrial proteins VDAC, GOT2, and GDH were enriched in purified mitochondria, whereas cytosolic GOT1 and AKT were not detected (Figure 5.2a). To test the model that ammonia recycling by GDH increases mitochondrial glutamate and aspartate, we profiled the abundance of mitochondrial metabolites from cells that were treated with NH\(_4\)Cl for 24 hours using liquid chromatography coupled to mass spectrometry (LC-MS). Ammonia treatment increased the abundance of mitochondrial glutamate and aspartate, which is consistent with ammonia assimilation in this organelle (Figure 5.2b). Importantly, ammonia decreased mitochondrial \(\alpha\)-ketoglutarate, which is consistent with its consumption by GDH, favoring reductive amination in this condition (Figure 5.2b).\(^{449}\) The effect of ammonia on mitochondrial amino acids was not ubiquitous, and the majority of amino acids did not change in response to


ammonia treatment, especially essential amino acids (Figure 5.2c). These data demonstrate that ammonia directly affects mitochondrial amino acid metabolism.

To consider whether ammonia stimulated proliferation via mitochondria or cytosolic functions of amino acids, we developed a strategy to test whether mitochondrial-generated glutamate and aspartate directly contributed to amino acids in the cytosol. We incubated cells with $^{15}\text{NH}_4\text{Cl}$, rapidly isolated mitochondria, and performed LC-MS on the mitochondrial to scan for $\sim$211 $^{15}$N-isotopologues, enabling the direct detection of ammonia assimilation into mitochondrial metabolites (Figure 5.2-d-e, Figure 5.S2b). $^{15}$N-isotopologues of glutamate and aspartate were detected in mitochondria, consistent with mitochondrial synthesis of these amino acids (Figure 5.2d). To determine if these amino acids were secreted from the mitochondria to generate metabolites in the cytosol, we screened for $^{15}$N-isotopologues identified in the whole cell lysate but were absent in the mitochondria (Figure 5.S2a). We reasoned that this labeling pattern would suggest that $^{15}$N-derivatives of ammonia were generated outside of the mitochondria. As expected, most isotopologues detected in the whole cell lysates were also detected in the mitochondrial lysates, demonstrating mitochondria are a hub for nitrogen recycling (Figure 5.S2b). Interestingly, $^{15}$N-alanine was only the metabolite uniquely detected in the whole cell lysate, suggesting that alanine was generated from labeled metabolites in the cytosol (Figure 5.2d-e, Figure 5.S2b). This lack of mitochondrial alanine labeling occurred in spite of sufficient mitochondrial pyruvate, which increased ~3-fold upon ammonia treatment (Figure 5.S2b-c), and adequate unlabeled ($^{14}$N-alanine) detection (Figure 5.S2d). Taken together, these data demonstrate that labeled alanine is generated outside of the mitochondria. Since glutamate is the nitrogen donor for alanine synthesis via glutamate-pyruvate transaminase (GPT), these data demonstrate that ammonia metabolism is compartmentalized.

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among the mitochondrial and cytosolic fractions. Cytosolic alanine synthesis may be dependent on the cancer subtype,\textsuperscript{451,452} however, in breast cancer cells, alanine labeling provides a readout for the export of amino acids, such as glutamate and aspartate, generated from mitochondrial ammonia assimilation.


Figure 5.2. Glutamate and aspartate generated downstream of ammonia assimilation are compartmentalized A. Western blot comparing whole cell lysates to lysates from rapidly IP’ed mitochondria (HA-tagged) or control IP (MYC-tagged). B. Steady-state abundance of metabolites in purified mitochondrial lysates in MCF7 cells treated with 1 mM NH$_4$Cl. Samples were normalized to protein levels of mitochondrial lysates, N=3 per condition. C. Steady-state abundance of metabolites in purified mitochondrial lysates in MCF7 cells treated with 1 mM NH$_4$Cl. Samples were normalized to protein levels of mitochondrial lysates, N=3 per condition. D. Venn diagram of labeled metabolites in the mitochondria and whole cell lysates in MCF7 cells treated with 3 mM $^{15}$NH$_4$Cl for 8 hours. E. Isotopologue abundance of $^{15}$N-amino acids in purified mitochondrial lysates compared to whole cell lysates in MCF7 cells treated with 3 mM $^{15}$NH$_4$Cl for 8 hours. F. Schematic depicting compartmentalization of ammonia metabolism. *P < 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001. Differences in individual genes or between...
two groups (control group compared to experimental) were assessed using two-tailed t-tests (95% confidence interval). NS= not significant. All experiments were performed independently at least twice.
C. GOT isozymes facilitate compartmentalization of ammonia metabolism in vitro and in vivo

To genetically probe this model of nitrogen distribution from the mitochondria, we investigated the mechanism of amino acid export from the mitochondria. Glutamate is the nitrogen donor for cytosolic alanine through the GPT, suggesting that alanine labeling from ammonia assimilation requires mitochondrial glutamate secretion to the cytosol. Although a mode for direct glutamate export is unknown, the combined activity of GOT2 and GOT1 may enable ammonia-derived nitrogen distribution from the mitochondria (Figure 5.2f). Therefore, we tested if the GOT isozymes facilitate the compartmentalization of ammonia metabolism.

GOT1 is a cytosolic enzyme that typically favors conversion of aspartate into glutamate, whereas GOT2 is the mitochondrial isozyme that typically favors conversion of glutamate to aspartate. Since these isozymes are bi-directional, we first examined the direction of their net-reactions in breast cancer cells stably depleted by shRNA-knockdown of either GOT1 or GOT2 (Figure 5.S3a). Depletion of GOT1 led to increased steady-state aspartate levels and no significant change in the contribution of labeled ammonia into aspartate, consistent with GOT1 favoring cytosolic glutamate synthesis in breast cancer cells (Figure 5.S3b-c). Conversely, depletion of GOT2 caused a decrease in steady-state aspartate abundance and a decrease in ammonia incorporation into aspartate, consistent with GOT2 favoring mitochondrial aspartate synthesis (Figure 5.S3b-c). Thus, these data demonstrate that GOT1 and GOT2 have opposing

net-reactions and may work in synchrony to facilitate glutamate export from the mitochondria, with aspartate as an intermediate.

To determine if the GOT isozymes work in concert to facilitate export of glutamate from the mitochondria, we measured $^{15}$NH$_4$Cl incorporation into alanine. Depletion of either GOT isozyme caused a significant decrease in the abundance of cellular alanine without affecting global amino acid pools. (Figure 5.3a, Figure 5.S3d). Moreover, depletion of GOT1 and GOT2 lead to a decrease in the incorporation of $^{15}$NH$_4$Cl into alanine (Figure 5.3b). Since alanine labeling occurs outside of the mitochondria, these data demonstrate that GOT1 and GOT2 facilitate the distribution of mitochondrial glutamate derived from ammonia assimilation.

To test the physiological relevance of mitochondrial nitrogen distribution, we utilized breast cancer xenograft mouse models that were depleted of either GOT1 or GOT2 (Figure 5.3c-d). Similar to in vitro experiments, depletion of GOT1 and GOT2 had opposing effects on the steady-state levels of aspartate in the tumor and in the tumor microenvironment, demonstrating that the net-catalytic activity of GOT1 and GOT2 are the same in vitro and in vivo (Figure 5.3e, Figure 5.S3f). Furthermore, depletion of either GOT1 or GOT2 caused accumulation of ammonia in the interstitial fluids, consistent with decreased ammonia assimilation (Figure 5.S3g). In addition to ammonia, numerous metabolites were statistically increased or decreased in the TME of GOT1 and GOT2-depleted tumors compared to control, demonstrating that metabolic changes reported are not caused by differences in tumor volume or input into the mass spectrometer (Figure 5.S3h). Importantly, the steady-state levels of alanine in either GOT1 or GOT2-depleted tumors were significantly lower than control tumors (Figure 5.3f). Additionally, ex vivo $^{15}$NH$_4$Cl tracing revealed decreased incorporation of $^{15}$N-labeling into alanine in both GOT1 and GOT2-depleted tumors relative to control (Figure 5.3g). Thus, these data demonstrate that GOT isozymes facilitate the distribution of mitochondrial glutamate derived from ammonia assimilation in vivo (Figure 5.3h).
Importantly, these findings may have relevance to breast cancer patients because ex vivo $^{15}$NH$_4$Cl labeling of primary patient breast tissues revealed substantial labeling on aspartate, glutamate, and alanine (Figure 5.1b), and western blot analysis of healthy breast versus tumor tissue from the same patient demonstrated elevated levels of GDH, GOT1, and GOT2 in tumor tissues (Figure 5.S3i). A pan-cytokeratin antibody was used to demonstrate that both healthy and tumor tissue were epithelial origin (Figure 5.S3i). The elevated GOT1 and GOT2 levels detected in the patient tumor are consistent with bioinformatic gene expression data from The Cancer Genome Atlas (TCGA), demonstrating that the expression of GOT1 and GOT2 is ~1.5-fold higher in breast cancer relative to healthy breast tissue (Figure 5.S3j).

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Figure 5.3. GOT Isozymes Facilitate Compartmentalization of Ammonia Metabolism in vitro and in vivo. A. Steady-state abundance of alanine in MCF7 cells depleted of GOT1 or GOT2. Samples were normalized to cell number, N=3 per condition. Values represent mean +/- SEM. B. Isotopologue abundance of $^{15}$N-alanine in MCF7 cells treated with 3 mM $^{15}$NH$_4$Cl for 8 hours. Values represent mean +/- SEM, n = 3. C. Schematic of xenograft mouse experiments. Mice harbor tumors with stable shRNA-mediated knockdown of GOT1 or GOT2. D. Western blot depicting shRNA-mediated GOT1 or GOT2 knockdown in MCF7 xenograft tumors. E. Steady-state abundance of aspartate in MCF7 xenograft tumors depleted of GOT1 or GOT2. Values represent mean +/- SEM, N=5 mice per condition. F. Steady-state abundance of alanine in MCF7 xenograft tumors depleted of GOT1 or GOT2. Values represent mean +/- SEM, N=5 mice per condition. G. Isotopologue abundance of $^{15}$N-alanine in MCF7 xenograft tumors treated ex vivo with 3 mM $^{15}$NH$_4$Cl for 8 hours. Values represent mean +/- SEM, n = 5 mice per condition. H. Schematic depicting the role of GOT isozymes in ammonia compartmentalization. I. Schematic of experiments performed on primary breast cancer patient tissue. *$P < 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$. Differences in individual genes or between two groups (control group compared to experimental) were assessed using two-tailed $t$-tests (95% confidence interval). NS= not significant. All experiments were performed independently at least twice.
D. Ammonia Stimulates Proliferation Through the Mitochondrial Glutamate Pool

Since ammonia assimilation provides nitrogen to mitochondrial glutamate and aspartate, which are subsequently exported to the cytosol, we used genetic perturbations of cytosolic GOT1 and mitochondrial GOT2 to determine how subcellular compartmentalization of glutamate and aspartate affect proliferation. Depletion of cytosolic GOT1 leads to an increase cellular aspartate, whereas depletion of mitochondrial GOT2 leads to a decrease in cellular aspartate (Figure 5.3e). In 2D and 3D cell culture, depletion of GOT1 caused a decrease in proliferation, consistent with previous reports (Figure 5.S4a Figure 5.4a).\textsuperscript{459} Re-expression of shRNA-resistant GOT1 in knockdown cells is sufficient to rescue proliferation, and overexpression of GOT1 caused increased proliferation (Figure 5.S4b). Surprisingly, depletion of GOT2 in 2D and 3D cell culture caused an increase in proliferation (Figure 5.4a). Re-expression of shRNA-resistant GOT2 in knockdown cells is sufficient to repress proliferation, and overexpression of GOT2 leads to decreased proliferation (Figure 5.S4c). These results demonstrate that the GOT isozymes have opposing effects on proliferation, and points to a potential role for the accumulation of glutamate within the mitochondria to support proliferation. To test if mitochondrial amino acids play a role in GOT-mediated proliferation, we supplemented GOT1 and GOT2-depleted cells with NH$_4$Cl, which directly increases mitochondrial amino acids (Figure 5.2a-c). Ammonia stimulated proliferation in GOT1-depleted cells, demonstrating that the effect of ammonia on proliferation is independent of GOT1 activity (cytosolic glutamate synthesis) (Figure 5.4b). Furthermore, glutamate and aspartate supplementation, which increased proliferation in control conditions, also increased the proliferation of GOT1-deficient cells, demonstrating their effect on proliferation is independent of GOT1 activity (Figure 5.S4f). By contrast, ammonia did not significantly change the rate of proliferation in GOT2-deficient cells (Figure 5.4c), suggesting either ammonia requires GOT2 activity to stimulate proliferation, or GOT2 and ammonia assimilation work via the same pathway, explaining lack of additivity.

Since GOT2 loss represses mitochondrial glutamate turnover, and ammonia assimilation directly generates mitochondrial glutamate, these data collectively support a model whereby mitochondrial glutamate supports cell proliferation.

It is worth noting that GOT isoforms have been reported to support proliferation through redox balance via the malate-aspartate shuttle in pancreatic cancer cells. In breast cancer cells, treatment with ammonia or depletion of either GOT1 or GOT2 had no effect on the NAD/NADH ratio (Figure 5.4d). Furthermore, supplementation with nicotinamide mononucleotide (NMN), which raises the NAD/NADH ratio, did not affect ammonia-stimulated proliferation or GOT1/2-depleted effects on proliferation (Figure 5.4e). Thus, the effects of GOT1 and GOT2-depletion in this system are independent of cellular NAD/NADH.

To determine if perturbation of the mitochondrial glutamate pool is sufficient to affect proliferation, we knocked down the mitochondrial glutamate carrier SLC25A22, which imports glutamate into the mitochondria. SLC25A22 overexpression has been previously shown to increase proliferation in colon cancer. Consistent with this finding, MCF7 cells depleted of SLC25A22 had decreased proliferation in 3D culture compared to control cells, suggesting that repressing mitochondrial glutamate import is sufficient to decrease proliferation (Figure 5.4d-e). Analysis of TCGA expression data of breast cancer patients revealed a 2-fold increase in SLC25A22 expression in breast cancer relative to healthy breast tissue, suggesting that mitochondrial glutamate import is critical for breast cancer growth (Figure 5.4f).

Since GOT2 loss and SLC25A22 loss had opposing effects on proliferation, we tested whether these perturbations had opposing effects on the mitochondrial glutamate levels by measuring metabolites in the mitochondrial compartment compared to whole cell lysates.

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Mitochondrial metabolite profiling revealed that GOT2 loss caused an increase in mitochondrial glutamate, whereas SLC25A22-loss caused decreased mitochondrial glutamate, demonstrating that mitochondrial glutamate levels correlate with proliferation rate (Figure 5.4g). However, mitochondrial aspartate levels were decreased in both GOT2- and SLC25A22-depleted cells, and most other metabolites detected were unchanged (Figure 5.S4g, Figure 5.4g).

We noted that mitochondrial glutamate changes were blunted in our measurements from whole cell lysates alone (Figure 5.4g-h). Strikingly, mitochondrial changes in glutamate levels were the opposite of those detected in whole cell lysates, such that GOT2-loss decreased glutamate and SLC25A22-loss increased glutamate (Figure 5.4h). In whole cell lysates, loss of GOT2 caused a decrease in cellular glutamate levels, underscoring the importance of this enzyme as the gatekeeper of nitrogen secretion from the mitochondria (Figure 5.4h). Opposingly, in whole cell lysates, loss of SLC25A22 lead to an increase in cellular glutamate, consistent with repressed mitochondrial glutamate import (Figure 5.4h). Interestingly, GOT2-depleted cells significantly decreased the protein levels of SLC52A22, and SLC25A22-depleted cells decreased GOT2 protein levels (Figure 5.4i). This suggests that mitochondrial glutamate levels are tightly controlled, such that high mitochondrial glutamate may suppress excess glutamate import and low mitochondrial glutamate may repress glutamate export (Figure 5.4j). Importantly, this demonstrates that the proliferation effects are dictated by the glutamate pools, and not by other activities or interactions of GOT2 and SLC25A22. These data demonstrate that modulating the mitochondrial glutamate pool is sufficient to affect proliferation in breast cancer cells. Thus, profiling the mitochondrial versus whole cell levels of glutamate provided the resolution to identify mitochondrial glutamate as a driver of proliferation.
Figure 5.4. Ammonia Stimulates Proliferation Through the Mitochondrial Glutamate Pool

A. Cell count in 3D culture assay in control, control treated with 1 mM NH₄Cl, GOT1, or GOT2-depleted MCF7 cells grown for 6 days. Values represent mean +/- SEM, n = 3.  
B. Cell count in 3D culture assay in control and GOT1-depleted MCF7 cells grown for 6 days in the presence or absence of 1 mM NH₄Cl. Values represent mean +/- SEM, n = 3.  
C. Cell count in 3D culture assay in GOT2-depleted MCF7 cells grown for 6 days in the presence or absence of 1 mM NH₄Cl. Values represent mean +/- SEM, n = 3.  
D. Western blot depicting shRNA-mediated knockdown of SLC25A22 in MCF7 cells  
E. Cell count in 3D culture assay in control and SLC25A22-depleted MCF7 cells grown for 6 days. Values represent mean +/- SEM, n = 3.  
F. mRNA expression data from The Cancer Genome Atlas of SLC25A22 in invasive breast
carcinoma compared to normal breast tissue. Fold-change (cancer/healthy) RNA levels were assessed using Oncomine.org G. Steady-state abundance of glutamate and aspartate in rapidly purified mitochondria from MCF7 cells with GOT2 or SLC25A22 depletion. Samples were normalized to protein levels of mitochondrial lysates, N=3 per condition H. Steady-state abundance of glutamate and aspartate in whole cell lysates of MCF7 cells harboring epitope-tagged mitochondria with GOT2 or SLC25A22 depletion. Samples were normalized to cell number, N=3 per condition. I. Western blot depicting SLC25A22 and GOT2 levels in GOT2 and SLC25A22-depleted MCF7 cells. J. Schematic of mitochondrial glutamate metabolism. *P < 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001. Differences in individual genes or between two groups (control group compared to experimental) were assessed using two-tailed t-tests (95% confidence interval). NS= not significant. All experiments were performed independently at least twice.
E. Ammonia assimilation stimulates mitochondrial translation and increases respiration

We next investigated the mechanism by which mitochondrial glutamate stimulates proliferation. Glutamate has multiple functions in a cell: it is a nitrogen donor to other amino acids, it is fundamental for glutathione synthesis as (1)- a direct component of the tri-peptide and (2)- enables cystine uptake, it can provide carbon for TCA cycle intermediates, contribute to cellular NAD/NADH compartmentalization, is an allosteric modulator of other enzymes, and is one of the 20 proteinogenic amino acids utilized for protein synthesis.\(^{464}\) As previously described, perturbations that raise mitochondrial glutamate such as ammonia treatment or GOT2-depletion had no effect on general amino acid levels (Figure 5.2c and Figure 5.S3d), nor did it affect the cellular NAD/NADH ratio (Figure 5.S4d-e). Furthermore, glutathione synthesis is a cytosolic process, and therefore can be ruled out as a mechanism driving this process. Thus, we tested the idea that nitrogen recycled from ammonia could support protein synthesis.

In addition to ammonia incorporation into free amino acids, we measured incorporation of \(^{15}\)NH\(_4\)Cl into the whole cell and mitochondrial proteome (Figure 5.5a-b). Ammonia-derived amino acids were incorporated into the mitochondrial proteome, corroborating assimilation in this compartment (Figure 5.5a). Many amino acids were detected in greater abundance in the whole cell proteome compared to the mitochondrial proteome consistent with high export of ammonia-derived nitrogen to the cytosol (Figure 5.5b). Furthermore, certain amino acids (glutamine and asparagine) were only detectable in the whole cell proteome (Figure 5.5b). Collectively, these data demonstrate that amino acids generated from nitrogen recycling in the mitochondria contribute to the mitochondrial and cytosolic proteome.

Since nitrogen from ammonia was highly incorporated into the mitochondrial proteome (Figure 5.5a), and mitochondrial glutamate stimulates cell proliferation, we tested whether ammonia treatment increased the rate of mitochondrial protein translation. There are 13 genes

in the mitochondrial genome that encode subunits which form complexes with nuclear-encoded subunits to generate the electron transport chain (ETC).\textsuperscript{465,466} To test if ammonia increased the level of mitochondrial-encoded proteins compared to nuclear-encoded proteins of the ETC, we performed a western blot on MCF7 cells treated on a time course with 1 mM NH\textsubscript{4}Cl, which is physiological to the tumor microenvironment.\textsuperscript{467,468} Treatment with ammonia increased the abundance of the mitochondrial-encoded proteins MT-ND6, MT-CO1, MT-ND1, and MT-ATP6 (Figure 5.5c, Figure 5.S5a). Surprisingly, NH\textsubscript{4}Cl treatment did not significantly alter the levels of the nuclear-encoded subunits of the ETC such as SDHA, UQRC2, COX4 and ATP5O (Figure 5.5c, Figure 5.S5a). NUDFS3, a complex I subunit, was the only increased nuclear-encoded protein measured (Figure 5.5c). Interestingly, the levels of this protein are responsive to changes in the rate of mitochondrial translation.\textsuperscript{469} Ammonia treatment had no effect on TOMM20 levels, MT-DNA copy number, or mitochondrial mass using MitoTracker (Figure 5.5d-e), demonstrating lack of global induction of mitochondrial number. Furthermore, NH\textsubscript{4}Cl treatment did not affect mRNA levels of MT-CO1, MT-ND6, and MT-ATP6, suggesting that this effect was not transcriptional (Figure 5.5f). Finally, we tested whether ammonia induced the mitochondrial unfolded protein response (UPR\textsuperscript{MT}), which is classically activated upon imbalance of mitochondrial and nuclear-encoded subunits of ETC complexes.\textsuperscript{470} A signature of this response is the induction of Heat shock factor 60 (HSP60), which is a mitochondrial chaperone

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that maintains proteostasis in the mitochondria.\textsuperscript{471,472} MCF7 cells treated with ammonia did not significantly alter HSP60 protein levels, suggesting that the UPR\textsuperscript{MT} is not activated by this condition (Figure 5.S5a).

Since ammonia treatment caused an increase in mitochondrial-encoded ETC subunits without changing their mRNA levels, we tested whether ammonia treatment directly affected the rate of translation. To do this, we measured the rate of incorporation of \(^{13}\)C-methionine into the mitochondrial proteome, which was isolated using epitope-tagged mitochondria for rapid immunoprecipitation. Cells treated with chloramphenicol, which inhibits the mitochondrial ribosome,\textsuperscript{473} were used as a control for incorporation of \(^{13}\)C-methionine into proteins specifically translated inside the mitochondria (Figure 5.5g). We found that treatment with 1 mM NH\(_4\)Cl caused a significant increase in the rate of mitochondrial translation relative to control conditions (Figure 5.5g). Importantly, ammonia did not stimulate cytosolic protein synthesis or mTOR function as a measure of phospho-S6, or phospho-CAD (Figure 5.S5b-c).\textsuperscript{474} Therefore, these data demonstrate that ammonia specifically activates a program of mitochondrial translation.

Since assimilation through GDH was required for ammonia to stimulate proliferation, we tested whether GDH was also required for ammonia to activate mitochondrial translation. Ammonia increased the levels of mitochondrial-encoded proteins within 5 minutes, which is a similar rate to that of ammonia assimilation, suggesting that GDH may mediate this effect (Figure 5.5h). GDH-depletion abrogated the effect of ammonia on mitochondrial translation (Figure 5.S5d). Furthermore, GDH-depletion alone was sufficient to decrease mitochondrial protein levels, without changing mitochondrial number (Figure 5.S5d-e). Unlike ammonia


treatment, GDH-depletion caused a decrease in the expression of both MT-CO1 and MT-ND6, which may be due to a feedback inhibition of transcription from stalled translation (Figure 5.S5f).

Likewise, MCF7 cells treated with glutamate or aspartate, which are generated downstream of ammonia assimilation, had increased levels of mitochondrial-encoded proteins relative to control media (Figure 5.S5g). Other amino acids such as alanine proline and leucine that did not stimulate breast cancer proliferation (Figure 5.S5h), did not alter MT-ND6 and MT-CO1 protein levels, demonstrating specificity in this response that coincides with their effects on proliferation (Figure 5.1e and 5.S5h). Taken together, these data demonstrate that ammonia assimilation through GDH stimulates mitochondrial translation.

To test the functional consequence of increased mitochondrial translation, we investigated the effect of ammonia on mitochondrial respiration. MCF7 cells treated with ammonia for 24 hours lead to an increase in respiration, which is dependent on GDH-mediated ammonia assimilation (Figure 5.5i). Importantly, repression of mitochondrial protein synthesis with the antibiotic chloramphenicol abrogates this increase in respiration (Figure 5.5j and 5.S5i). To understand the basis of increased mitochondrial respiration in the absence of mitochondrial biogenesis, we assessed mitochondrial morphology and cristae structure using transmission electron microscopy (TEM). Mitochondria were imaged in MCF7 cells treated with or without 1 mM NH₄Cl for 24 hours (Figure 5.5k). Ammonia treatment caused striking changes in mitochondrial morphology, leading to increased density of cristae and overall larger size (Figure 5.5k).

Next, since GDH-mediated ammonia assimilation stimulated respiration and proliferation in MCF7 cells, we tested whether elevated respiration caused the observed increase in proliferation. First, we tested if inhibition of mitochondrial protein synthesis affected ammonia-stimulated proliferation in breast cancer cells. Chloramphenicol treatment, which decreased the synthesis of mitochondrial proteins (Figure 5.S5g), repressed the ability for ammonia to

Lin, Y.F., and Haynes, C.M. (2016). Molecular cell 61, 677-682
stimulate proliferation (Figure 5.5l). Furthermore, inhibition of respiratory chain complex III using Antimycin was sufficient to abrogate ammonia-stimulated proliferation (Figure 5.5m). Thus, ammonia assimilation stimulated mitochondrial translation, which leads to increased proliferation in breast cancer cells.
Figure 5.5. Ammonia Assimilation Stimulates Mitochondrial Translation and Increases Respiration. A. Isotopologue abundance of $^{15}$N-amino acids in protein isolated from whole cell lysates of MCF7 cells treated with 3 mM $^{15}$NH$_4$Cl for 48 hours. Values represent mean +/- SEM. B. Isotopologue abundance of $^{15}$N-amino acids from protein isolated from purified mitochondria of MCF7 cells treated with 3 mM $^{15}$NH$_4$Cl for 48 hours. Values represent mean +/- SEM. C. Western blot depicting a time course of MCF7 cells treated with 1 mM NH$_4$Cl. SDHA, UQCRC2, and NDUFS3 are components of the respiratory chain encoded in the nucleus. MT-CO1 and MT-ND6 are components of the respiratory chain encoded by the mitochondrial genome. D. MT-DNA copy number in MCF7 cells treated with or without 1 mM NH$_4$Cl for 24 hours. Values are mean +/- SEM, N=3. E. Mitochondrial mass of MCF7 cells treated with or without 1 mM NH$_4$Cl for 24 hours measured using MitoTracker Green. Cells were sorted for single cells and RFU (Relative Fluorescence Units) was quantified. Values are mean +/- SEM, N=3. F. Real time PCR of mitochondrial encoded genes MT-ATP6, MT-ND6 and MT-CO1 in MCF7 cells treated with or without 1 mM NH$_4$Cl for 24 hours. Values are mean +/- SEM, N=3. G. Mitochondrial protein synthesis assay in 293T cells with HA-tagged mitochondria. Cells were treated with 1 mM NH$_4$Cl, or with 50 ug/mL chloramphenicol in the presence of $^{13}$C-methionine. Data represents rate of incorporation of $^{13}$C-methionine into mitochondrial proteins. N=3 for 1 minute and 10 minute time points, N=2 for all other conditions. H. Isotopologue abundance of $^{15}$N-glutamate in MCF7 cells treated with 3 mM NH$_4$Cl on a time course. Values are mean +/- SEM, N=3. I. Oxygen consumption rate of MCF7 cells depleted of GDH in the presence or absence of 1 mM NH$_4$Cl for 24 hours. Values are mean +/- SEM, N=6. J. Oxygen consumption rate of MCF7 cells in the presence or absence of 1 mM NH$_4$Cl or 50 mg/mL chloramphenicol for 24 hours. Values are mean +/- SEM, N=6. K. Transmission electron microscopy images of mitochondria in MCF7 cells treated in control conditions or with 1 mM NH$_4$Cl for 24 hours. L. Proliferation of MCF7 cells in 3D culture the presence or absence of 1 mM NH$_4$Cl or 50 mg/mL chloramphenicol for five days. Values are mean +/- SEM, N=3.
3D culture the presence or absence of 1 mM NH₄Cl or 0.25 mM Antimycin A for five days. Values are mean +/- SEM, N=3. *P < 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001. Differences in individual genes or between two groups (control group compared to experimental) were assessed using two-tailed t-tests (95% confidence interval). NS= not significant. All experiments were performed independently at least twice.
F. The Mitochondrial Glutamate Pool is a Toggle for Respiration

Our cumulative data suggest that mitochondrial pools of glutamate promote translation and respiration in this organelle leading to elevated proliferation. To further interrogate this model, we utilized genetic perturbations to directly affect mitochondrial glutamate (Figure 5.4). We utilized depletion of GOT2 (to increase glutamate) or SLC25A22 (to decrease glutamate) to test if glutamate toggles mitochondrial protein synthesis. GOT2 depletion, which increased mitochondrial glutamate (Figure 5.4g), caused an increase in respiratory chain subunits without affecting mitochondrial number or mitochondrial mass (Figure 5.6a, Figure 5.S6a-b). Importantly, depletion of GOT2 did not alter the mRNA levels of MT-CO1, MT-ATP6, and MT-ND6, suggesting that this effect is independent of mitochondrial transcription (Figure 5.S6c). Consistent with elevated MT-protein synthesis, loss of GOT2 caused an increase in respiration (Figure 5.6b). Furthermore, TEM analysis of MCF7 cells depleted of GOT2 demonstrate morphological changes with increased cristae density, similar to the effect of ammonia treatment (Figure 5.6c). To test if elevated mitochondrial protein synthesis and respiration drives GOT2-mediated proliferation, we perturbed these pathways. Inhibition of mitochondrial protein synthesis with chloramphenicol or inhibition of respiratory chain complex III using Antimycin was sufficient to abrogate the effect of GOT2-loss on proliferation (Figure 5.6d-e). Thus, increasing mitochondrial glutamate with either ammonia treatment or GOT2-depletion is sufficient to increase mitochondrial translation, causing elevated proliferation.

Conversely, depletion of SLC25A22, which decreased mitochondrial glutamate levels, caused a decrease in mitochondrial protein levels without changing MT-DNA copy number (Figure 5.6f, Figure 5.S6a). Interestingly, loss of SLC25A22 caused a decrease in expression of MT-CO1, MT-ATP6, and MT-ND6 (Figure 5.S6c). Furthermore, although loss of GOT2 did not affect mitochondrial mass, SLC25A22 caused a significant decrease in mitochondrial mass (Figure 5.S6b). Loss of SLC25A22, which decreased mitochondrial translation, caused a decrease in cellular respiration (Figure 5.6g). Thus, performing metabolite profiling with
resolution of subcellular compartments revealed an unexpected function of mitochondrial glutamate generated downstream of ammonia recycling in mitochondrial translation and respiration (Figure 5.6i).
Figure 5.6. Mitochondrial Glutamate is a Toggle for Mitochondrial Respiration

A. Western blot of MCF7 cells depleted of GOT2. B. Oxygen consumption rate of MCF7 cells depleted of GOT2. Values are mean +/- SEM, N=6. C. Transmission electron microscopy images of mitochondria in MCF7 cells with a control shRNA (left), depleted of GOT2 with shRNA#1 (top right), or depleted of GOT2 with shRNA#2 (bottom left). D. Proliferation of MCF7 cells depleted of GOT2 in 3D culture the presence or absence of 50 µg/mL chloramphenicol for five days. Values are mean +/- SEM, N=3. E. Proliferation of MCF7 cells depleted of GOT2 in 3D culture the presence or absence of 0.25 µM Antimycin A for five days. Values are mean +/- SEM, N=3. F. Western blot of MCF7 cells depleted of SLC25A22. G. Oxygen consumption rate of MCF7 cells depleted of SLC25A22. Values are mean +/- SEM, N=6. H. Schematic of mitochondrial glutamate as a toggle for respiration. *P < 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001. Differences in individual genes or between two groups (control group compared to experimental) were assessed using two-tailed t-tests (95% confidence interval). NS = not significant. All experiments were performed independently at least twice.
IV. Discussion. Here, we demonstrate that nitrogen recycling is compartmentalized to generate pools of amino acids in the mitochondria, which serve as a toggle for mitochondrial translation. Using ammonia tracing through subcellular compartments, and genetic knockdown of GOT2 and SLC25A22, we show that mitochondrial glutamate is sufficient to trigger mitochondrial translation, revealing an unexpected role for amino acid compartmentalization in cellular respiratory capacity. Taken together, this work reveals the importance of studying the localization of metabolites to better inform their function in cell biology.

A. An unexpected role for mitochondrial glutamate. Amino acids comprise the majority of biomass in proliferating cells, and increased amino acid synthesis is a key feature of the metabolic transition between quiescence and proliferation.\(^\text{476-477}\) Amino acids such as aspartate, asparagine, and branched chain amino acids have been shown to promote tumor growth in many cancer subtypes.\(^\text{478-479,480,481,482}\) However, the subcellular compartment in which amino acids are limiting has never been explored. Since ammonia is primarily assimilated into glutamate within mitochondria, and ammonia increases breast cancer cell proliferation, we utilized this nitrogen source as a tool to investigate the role of amino acid compartmentalization in cellular proliferation.

Using compartmentalized tracing of ammonia and genetic models to increase and decrease mitochondrial glutamate levels, our data demonstrate that mitochondrial glutamate is limiting for breast cancer proliferation. First, we developed mass spectrometry-based methods to trace ammonia assimilation into amino acids in the mitochondria and dissect their subsequent export into the cytosol. Furthermore, we used these tools along with genetic models to perturb mitochondrial glutamate levels to demonstrate that mitochondrial glutamate is limiting for proliferation. We postulate that mitochondria may be particularly sensitive to glutamate levels because it is a central metabolite in cellular anabolic metabolism, including lipid synthesis, production of other non-essential amino acids, nucleotide synthesis (through aspartate and glutamine), and glutathione. When glutamate and its derivatives are abundant in the cell, mitochondrial pools of glutamate are preserved and not exported to the cytosol. Thus, our data suggest that mitochondrial glutamate abundance is tuned to the anabolic state of the cell to enable rapid activation of respiration in nitrogen-replete environments. Emerging studies have identified downstream roles of the ETC in proliferation including ATP synthesis, production of reactive oxygen species (ROS), de novo pyrimidine synthesis, aspartate synthesis, and likely many other key pathways that support proliferation.483,484,485,486,487

B. Ammonia assimilation is a nitrogen-sensing pathway conserved throughout evolution. Ammonia assimilation is highly conserved throughout evolution and is the preferred nitrogen source for E. coli and many other microbial species.488489 Particularly in nutrient-limiting


environments, E. Coli rely on ammonia assimilation by GDH to generate glutamate. Furthermore, the bacterial response to ammonia-deprivation is conserved in yeast, demonstrating high penetrance of this pathway throughout evolution. Importantly, the higher organism yeast rely on cataplerotic reactions of mitochondrial TCA cycle intermediates to generate glutamate, aspartate, and glutamine to achieve a nitrogen-replete state. These results parallel our direct measurements of ammonia assimilation in the mitochondria, and the dependency of breast cancer cells on mitochondrial amino acid synthesis. Importantly, mitochondria have proteobacterial ancestry. Symbiotic association of proteobacteria with the eukaryotic cell provided a metabolic advantage that enabled survival in the severe atmospheric conditions of early earth development. It is possible that the mitochondria’s ability to sense and utilize ammonia as a nitrogen source, coupled with its ability to export amino acids, provided an additional selective advantage during endosymbiosis. We speculate that these pathways particularly enabled mammalian cell survival in niches with high ammonia concentrations such as the tumor microenvironment, the liver, and the microbiome.

C. Mitochondrial translation and cancer. Until recently, it was thought that The Warburg Effect in tumors, which diverts glycolytic flux to lactate synthesis, was accompanied by mitochondrial dysfunction. However, a growing body of work has revealed that mitochondria promote tumor growth through their signaling and metabolism. For example, metabolic

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pathways, such as ammonia assimilation, citrate export, and one-carbon metabolism, are important for tumor growth.\textsuperscript{496,497,498} Furthermore, new studies show these pathways are also upstream of respiratory chain function, either directly through promoting mitochondrial translation of respiratory chain subunits,\textsuperscript{499,500} or indirectly by generating reduced cofactors that deposit electrons into the respiratory chain, propagating the potential energy of the ETC.\textsuperscript{501}

Here, we show that ammonia assimilation leads to increased translation of mitochondrial-encoded subunits of the respiratory chain. These findings highlight a crucial role for mitochondrial translation in cancer cell proliferation, which may be a metabolic vulnerability in cells with high nitrogen recycling through GDH. Inhibition of mitochondrial translation ablates the respiratory chain complexes, which, aside from complex II, are composed of both nuclear and mitochondrial-encoded subunits.\textsuperscript{502} This imbalance of mitochondrial and nuclear components causes a decrease in respiration.\textsuperscript{503} Contrary to loss of the mitochondrial subunits, ammonia assimilation causes increased mitochondrial translation, and subsequently leads to an increase in respiration in the absence of mitochondrial biogenesis. These results are in support of the idea that the mitochondrial subunits are limiting for complex formation and overall respiratory capacity of mitochondria. Thus, the tumor microenvironment, which accumulates


ammonia, may cause cancer cells to be more sensitive to antibiotic treatment than healthy cells that reside in “low ammonia” microenvironments.

D. Mitochondrial nitrogen recycling is critical for survival in the TME. The tumor microenvironment is a distinctive niche in the human body characterized by limited bioavailability of nutrients such as glucose and glutamine, low pH, and accumulation of metabolic waste.\textsuperscript{504} Cancer cells adapt to this microenvironment in a multitude of ways, such as recycling metabolic waste products to salvage carbon and nitrogen for biomass.\textsuperscript{505,506} Here, we demonstrate that mitochondria are essential for nitrogen recycling to generate amino acids. This pathway is particularly important in the TME, in which amino acid availability is limited, and \textit{de novo} synthesis of amino acids is required to sustain protein translation. Thus, mitochondrial nitrogen recycling plays an important role in the adaptive reprogramming that cancer cells undergo to survive in the TME. Our data also reveals that ammonia, which directly raises mitochondrial glutamate levels, provides an additional advantage to cancer cells because it is a compartmentalized pathway. Ammonia in the TME contributes to a mitochondrial pool of glutamate that regulates cancer cell respiratory capacity.

V. Acknowledgements. We thank all members of the Haigis lab and Sejal Vyas for thoughtful discussion on this manuscript. J.B.S is funded by the National Science Foundation Graduate Research Fellowship (Grant # DGE1144152) and M.C.H is funded by NIH grant R01CA213062, the Ludwig Center at Harvard University, and the Breast Cancer Alliance Exceptional Project grant. J.B.S and M.C.H are co-inventors on the patent HMV-27560 submitted by Harvard Medical School that covers ammonia utilization in cancer.

V. Supplementary Materials


A. Supplemental Figures.
Figure 5.S1 (Related to Figure 5.1). Glutamate and Aspartate Stimulate Proliferation Downstream of Ammonia Assimilation. Representative images of 3D culture assay in MCF7 cells. Cells were treated for 8 days in control media supplemented with 1 mM NH$_4$Cl, 20 mM aspartate, 20 mM glutamate, 20 mM alanine, 20 mM proline, or 10 mM leucine.
Figure 5.S2 (Related to Figure 5.2). Glutamate and Aspartate Generated in the Mitochondria Downstream of Ammonia Assimilation are secreted to the Cytosol. A. Isotopologue abundance of $^{15}$N-amino acids in purified mitochondrial lysates compared to whole cell lysates in MCF7 cells treated with 3 mM $^{15}$NH$_4$Cl for 8 hours. B. Schematic depicting ammonia assimilation leading to alanine labeling. GDH: glutamate dehydrogenase, GPT: glutamate pyruvate transaminase. C. Steady-state abundance of pyruvate in purified
mitochondrial lysates in MCF7 cells treated with 1 mM NH₄Cl. Samples were normalized to protein levels of mitochondrial lysates, N=3 per condition. D. Raw ion counts for alanine (M+0) in mitochondrial lysates in MCF7 cells treated with 3 mM ^15^NH₄Cl for 8 hours. Values represent mean +/− SEM. *P < 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001. Differences in individual genes or between two groups (control group compared to experimental) were assessed using two-tailed t-tests (95% confidence interval). NS= not significant. All experiments were performed independently at least twice.
Figure 5.S3 (Related to Figure 5.3). GOT isozymes facilitate compartmentalization of ammonia metabolism in vitro and in vivo. A. Western blot depicting shRNA-mediated GOT1 or GOT2 knockdowns in MCF7 cells. B. Steady-state abundance of aspartate in MCF7 cells depleted of GOT1 or GOT2. Samples were normalized to cell number, N=3 per condition. Values represent mean +/- SEM. C. Isotopologue abundance of $^{15}$N-aspartate in MCF7 cells.
depleted of GOT1 or GOT2 treated with 3 mM $^{15}$NH$_4$Cl for 8 hours. Values represent mean +/- SEM, n = 3. D. Steady-state abundance of amino acids in MCF7 cells depleted of GOT1 or GOT2. Samples were normalized to cell number, N=3 per condition. Values represent mean +/- SEM. E. Volumes of tumors at the time of harvest. Values represent mean +/- SEM, n = 5 mice per condition. F. Steady-state abundance of aspartate in MCF7 xenograft tumor microenvironment (TME) depleted of GOT1 or GOT2. Values represent mean +/- SEM, N=5 mice per condition. G. Ammonia levels in MCF7 xenograft tumor microenvironment (TME) normalized to plasma levels in mice with tumors depleted of GOT1 or GOT2. Values represent mean +/- SEM, N=5 mice per condition. H. Volcano plot depicting metabolite changes in MCF7 xenograft tumor microenvironment (TME) samples of GOT1-depleted or GOT2-depleted tumors compared to control tumors. Values represent mean +/- SEM, n = 5 mice per condition. I. Western blot of healthy compared to tumor tissue from an individual patient with ER (+) breast cancer. J. mRNA expression data from The Cancer Genome Atlas of GOT1 and GOT2 in invasive breast carcinoma compared to normal breast tissue. Fold-change (cancer/healthy) RNA levels were assessed using Oncomine.org. *$P < 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$. Differences in individual genes or between two groups (control group compared to experimental) were assessed using two-tailed t-tests (95% confidence interval). NS= not significant. All experiments were performed independently at least twice.
Figure 5.S4 (Related to Figure 5.4). Ammonia Stimulates Proliferation Through the Mitochondrial Glutamate Pool

**A.** Cell count in 2D culture in control, GOT1-depleted cells, GOT1-depleted cells. Values represent mean +/- SEM and are normalized to control conditions, n = 3 per condition. Curve fitting performed with nonlinear regression.

**B.** Cell count in 3D culture in control, GOT1-depleted cells, GOT1-depleted cells with transient re-expression of shRNA-resistant GOT1, or overexpression (OE) of GOT1 only. Values represent mean +/- SEM and are normalized to control conditions, n = 3 per condition. The associated western blot is loaded in the same order as the experiment above.

**C.** Cell count in 3D culture in control, GOT2-depleted cells, GOT2-depleted cells with transient re-expression of shRNA-resistant GOT2, or overexpression (OE) of GOT2 only. Values represent mean +/- SEM and are normalized to control conditions, n = 3 per condition. The associated western blot is loaded in the same order as the experiment above.

**D.** NAD/NADH ratio in MCF7 cells treated with 1 mM nicotinamide mononucleotide (NMN) or 1 mM NH₄Cl for 24 hours and in cells depleted of GOT1 or GOT2.

**E.** Cell count in 3D culture assay in MCF7 cells depleted of GOT1 or GOT2 and treated with 1 mM NMN for 6 days. Values represent mean +/- SEM, n = 3.

**F.** Cell count in 3D culture assay in MCF7 cells depleted of GOT1 or GOT2 and treated with either 20 mM aspartate or 20 mM glutamate for 6 days. Values represent mean +/- SEM, n = 3.

**G.** Volcano plots depicting metabolite changes in isolated mitochondria from MCF7 cells with GOT2-depletion or SLC25A22-depletion. Values represent mean +/- SEM, n = 3. *P < 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001. Differences in individual genes or between two groups (control group compared to experimental) were assessed using two-tailed t-tests (95% confidence interval). NS= not significant. All experiments were performed independently at least twice.
A. Time Incubated with 1 mM NH₄Cl
   - ATP50
   - COX4
   - MT-ND1
   - MT-ATP6
   - HSP60

B. Time Incubated with 1 mM NH₄Cl
   - P-S6
   - S6

C. Time Incubated with 1 mM NH₄Cl
   - P-CAD
   - CAD

D. 1 mM NH₄Cl
   - Control
   - KD#1
   - KD#2
   - MT-ND6
   - NDUFS3
   - Actin
   - GDH

E. MT-DNA Content
   - Relative Mitochondrial DNA Copy Number

F. MT-gene expression
   - MT-ND6
   - MT-CO1

G. Control NH₃ Glu Asp
   - MT-ND6
   - MT-CO1
   - MT-ND1
   - SDHA
   - TOMM20
   - Actin

H. Control Glutamate Alanine Leucine
   - MT-ND6
   - MT-CO1
   - SDHA
   - TOMM20

I. Control Chlor
   - UQCRCl2
   - MT-CO1
   - TOMM20
Figure S5 (Related to Figure 5). Ammonia assimilation increases respiratory function through elevating mitochondrial protein levels. A. Western blot depicting a time course of MCF7 cells treated with 1 mM NH₄Cl. COX4 and ATP5O are components of the respiratory chain encoded in the nucleus. MT-ND1 and MT-ATP6 are components of the respiratory chain encoded my the mitochondrial genome. B. Western blot depicting a time course of MCF7 cells treated with 1 mM NH₄Cl. C. Western blot depicting a time course of MCF7 cells treated with 1 mM NH₄Cl. D. Western blot depicting MCF7 cells depleted of GDH in the presence or absence of 1 mM NH₄Cl for 6 hours. E. MT-DNA copy number in MCF7 cells depleted of GDH or treated with 1 mM NH₄Cl for 24 hours. Values are mean +/- SEM, N=3. F. Real time PCR of mitochondrial encoded genes MT-ND6 and MT-CO1 in MCF7 cells treated with 1 mM NH₄Cl for 24 hours or depleted of GDH. Values are mean +/- SEM, N=3. G. Western blot of MCF7 cells treated for 24 hours with 1 mM NH₄Cl, or 20 mM glutamate, or 20 mM aspartate. H. Western blot depicting MCF7 cells treated with 1 mM NH₄Cl, 20 mM aspartate, 20 mM glutamate, 20 mM alanine, 20 mM proline, or 10 mM leucine for 24 hours. I. Western blot depicting MCF7 cells treated with 50 µg/mL chloramphenicol for 24 hours. *P < 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001. Differences in individual genes or between two groups (control group compared to experimental) were assessed using two-tailed t-tests (95% confidence interval). NS= not significant.
Figure 5.S6 (Related to Figure 5.6). Ammonia assimilation increases respiratory function through elevating mitochondrial protein levels. A. MT-DNA copy number in MCF7 cells depleted of GOT2 or SLC25A22 or treated with 1 mM NH₄Cl for 24 hours. Values are mean +/- SEM, N=3. B. Mitochondrial mass of MCF7 cells depleted of either GOT2 or SLC25A22 measured using MitoTracker Green. Cells were sorted for single cells and RFU (Relative Fluorescence Units) was quantified. Values are mean +/- SEM, N=3. C. Real time PCR of mitochondrial encoded genes MT-ND6 and MT-CO1 in MCF7 cells treated with 1 mM NH₄Cl for 24 hours or depleted of GDH. Values are mean +/- SEM, N=3. *P < 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001. Differences in individual genes or between two groups (control group compared to experimental) were assessed using two-tailed t-tests (95% confidence interval). NS= not significant.
B. Materials and Methods.

**Cell Lines.** All cell lines were cultured in RPMI (Life Technologies) supplemented with 5% FBS (Life Technologies) and 1% penicillin and streptomycin (Invitrogen). Cell lines have not been authenticated. Media was changed every 24 hours to minimize ammonium accumulation. Inhibitors and other supplements: 0.0-1.0 mM ammonium chloride (Sigma), 20 mM aspartate, 20 mM glutamate, 20 mM proline, 20 mM alanine, 10 mM leucine, 50 ug/mL Chloramphenicol (Sigma), 0.25 mM Antimycin A (Sigma).

**In vivo Xenograft Mouse Models.** All mouse protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Harvard University. Foxn1nu athymic nude mice (Nu/J strain # 002019) were purchased from The Jackson Laboratory and housed in the New Research Building Animal Facility at Harvard Medical School. 7 week old female mice were used for all studies.

**Primary Breast Cancer Patient Samples.** All protocols are approved by the Dana-Farber Cancer Institute, IRB (DFCI Protocol No.: 93-085) in accordance with Federal regulations. Healthy and tumor tissue were resected from de-identified patients and used for these studies with their consent.\(^{507}\)

**METHOD DETAILS**

**3D cell culture:** Cells were maintained with modifications to previously described protocols (Debnath and Brugge, 2005) (http://brugge.med.harvard.edu/protocols).\(^{508}\) Briefly, cells were seeded in 8-well glass chamber culture slides (BD Falcon) on a bed of LDEV-free MatriGel (Corning) in RPMI supplemented with 5% FBS, 1% penicillin/streptomycin and 2% MatriGel. Then, media was replaced every 24 hours in all conditions. Images were taken on a Nikon Eclipse TE2000-U Microscope after 5-8 days. Sphere area was quantified using ImageJ on approximately 100 colonies per replicate. Cells were harvested after incubation in Cell


Recovery Solution (Corning: 354253) for one hour at 4 °C and counted with a Beckman Coulter Counter.

**Proliferation Assays:** 25,000 cells were seeded in triplicate in 6-well dishes and counted daily for one week on a Beckman Coulter Counter. Points were fit to the exponential growth equation \( Y = Y_0 e^{(kX)} \) to obtain the rate constant \( (k) \). Then, the equation \( DT = \ln(2)/k \) was used to calculate doubling time (DT).

**Transmission Electron Microscopy:** Cells were seeded to 70% confluency in 6-well dishes and treated with respective conditions. Cells were fixed directly to the 6-well dish by incubating for 1 hour at 37°C in a 1:2 mixture of fixative buffer (1.25% formaldehyde, 2.5 % glutaraldehyde and 0.03% picric acid in 0.1 M Sodium cacodylate buffer, pH 7.4) and cell culture media. Transmission Electron Microscopy was performed on the Tecnai™ G² Spirit BioTWIN microscope. Images were taken by the staff at Harvard Medical School Electron Microscopy facility.

**Generation of Stable Cell Lines:** shRNAs against GDH, GOT1/2, and SLC25A22, were subcloned into the pLKO.1 puro vector (Addgene Plasmid #8453) at EcoRI and AgeI sites. Subcloned plasmids were transfected into HEK293T cells with lentiviral packaging vectors. After 48 hours, lentivirus was harvested and target cells were infected in the presence of 8 ug/mL polybrene. Following infection, cells were selected with puromycin and knockdowns validated by western blot.

For re-expression studies, GOT1 and GOT2 were cloned into the pLenti-puro vector (Addgene Plasmid #39481) at EcoRI and Xhol sites. Silent mutations were generated to the target sequence of GOT1 shRNA #1 and GOT2 shRNA #1 and confirmed with sequencing. Stable knockdown cells were transfected with their respective overexpression plasmids and experiments were seeded after 24 hours.
Sequencing reactions were carried out with an ABI3730xl DNA analyzer at the DNA Resource Core of Dana-Farber/Harvard Cancer Center (funded in part by NCI Cancer Center support grant 2P30CA006516-48).

**Production of Cells with Epitope-Tagged Mitochondria:** Cells (MCF7 and 293T) containing epitope-tagged mitochondria were generated using an established protocol.\(^{509}\) Cells expressing the epitope tags HA-MITO (Addgene Plasmid #83356) and Control-MITO (Addgene Plasmid # 83355) were selected with blasticidin (4 mg/mL) and then FACS-sorted to enrich for EGFP signal.

**Western Blots:** Adherent cells were lysed directly on the cell culture dish with NP40 lysis buffer (1% NP40, 1 mM DTT, 0.2% phosphatase inhibitor cocktail #2 & #3(Sigma), 1 cOmplete protease inhibitor tablet (Sigma)) or RIPA lysis buffer (50 mM HEPES, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 1 mM DTT, 1 cOmplete protease inhibitor tablet (Sigma), 0.2% phosphatase inhibitor #2 & #3 (Sigma)) for experiments involving mitochondrial proteins. Protein content was quantified using a BCA Assay (Thermo Scientific) and equal protein was run on 4-20% Tris-HCl Gel (BioRad). Protein was transferred for 2 hours (4 °C) to a nitrocellulose membrane (BioRad). Membranes were incubated in primary antibodies overnight (4 °C) with the following dilutions: GDH (1:1000), α-Tubulin (1:1000), β-Actin (1:1000), GOT2 (1:500), GOT1 (1:1000), SLC25A22 (1:1000), TOMM20 (1:1000), SDHA (1:1000), UQCRC2 (1:1000), MT-CO1 (1:500), MT-ND6 (1:500), MT-ND1 (1:500), MT-ATP6 (1:500), NDUFS3 (1:1000), ATP5O (1:1000), S6 Ribosomal Protein (1:1000), Phospho-S6 Ribosomal Protein (Ser240/244) (1:1000), CAD (1:1000), Phospho-CAD (1:1000). Secondary antibodies: Anti-rabbit IgG HRP-linked Antibody (1:5000, Cell Signaling), Anti-mouse IgG HRP-linked Antibody (1:5000, Cell Signaling). Blots were developed using Pierce ECL Western Blotting Substrate (Thermo Scientific) and were exposed using autoradiography film.

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**RNA Isolation and RT-PCR:** RNA was extracted with the Direct-zol RNA Miniprep Kit (Genesee Scientific) directly from adherent cells. cDNA was synthesized from 100 ng of RNA using the iScript cDNA synthesis kit (Bio-Rad). Real-time qPCR was performed on a Light Cycler 480 (Roche) using PerfeCta SYBR Green Fast Mix (Quanta BioSciences).

**MT-DNA Quantification:** Mitochondrial DNA was extracted from cells and analyzed by real time quantitative PCR as described in Baughman (2011). Briefly, 50,000 cells were pelleted and re-suspended in 50 uL of MT-DNA lysis buffer (25 mM NaOH, 0.2 mM EDTA). Lysates were heated to 95°C for 15 minutes and neutralized with 50 µL MT-DNA neutralization buffer (40 mM Tris-HCl). PCR reactions were performed on 5 uL of 1:50 diluted lysate. MT-DNA quantification was performed with mitochondrial markers MT-CO2: and D-loop. MT-DNA was normalized to nuclear DNA markers β-Actin: and β-Globin.

**Mitochondrial Mass Quantification:** 50,000 cells were adapted to their respective media conditions for 24 hours prior to the experiment. Cells were incubated with 75 nM Mitotracker Green (Invitrogen) for 1 hour, washed twice with PBS and analyzed on an LSR II Flow Cytometer (BD Biosciences), gated for single cells and assessed for fluorescence intensity.

**Metabolite Profiling and Tracing Studies:**

**Whole Cell Metabolite Extraction:** Prior to experiments, cells were adapted to 0.0 mM NH₄Cl, 1.0 mM NH₄Cl, or 3.0 mM NH₄Cl for four days. Samples were plated in quadruplicate for metabolite extraction and in quadruplicate for cell count normalization. For metabolite extraction cells were washed once with ice-cold PBS, and polar metabolites were extracted directly on the dish using 1 mL ice-cold 80% methanol.

**Metabolite Isolation from Purified Mitochondria:** Mitochondria were purified from 15 million 293T cells expressing epitope tagged (HA and Control) mitochondria using a previously optimized
protocol for mitochondrial metabolite extraction. Briefly, cells were washed twice with ice-cold PBS, scraped using 1 mL of KPBS (136mM KCl, 10mM KH$_2$PO$_4$, pH 7.25 adjusted with KOH) and centrifuged at 1000 x g for 2 minutes. Pelleted cells were homogenized in 1 mL KPBS with 25 strokes and centrifuged at 1000 x g for 2 minutes. The resulting supernatant was incubated with pre-washed Anti-HA magnetic beads (Thermo 88837) for 3.5 minutes on an end-over rotator. Beads were harvested using a DynaMag Spin Magnet, washed twice with KPBS and re-suspended in 1 mL KPBS. 250 uL is saved for detergent lysis (using RIPA buffer) and western blot is used to determine purity. Metabolites are extracted from the remaining beads in 100 uL of ice-cold 80% methanol and analyzed by LC-MS.

$^{15}$NH$_4$Cl Tracing: For cell culture experiments, cells were seeded in 6-cm plates for whole cell extractions and in 15-cm dishes for isolation of mitochondrial metabolites. Cells were incubated with 3.0 mM $^{15}$NH$_4$Cl (Sigma, 98% isotopic purity) supplemented in the previously described media conditions. Metabolites were isolated after 8 hours of incubation, which was previously determined to be the in the steady-state window for metabolic tracing of ammonia.

$^{15}$NH$_4$Cl Tracing into protein: 5 x 10$^8$ cells containing epitope-tagged (HA and MYC) mitochondria were adapted to control media and media containing 3 mM NH$_4$Cl for two days. Media was changed every 24 hours during this adaptation period. Then, cells were treated with control media or media with 3 mM $^{15}$NH$_4$Cl for an additional 48 hours with media change every 24 hours. After 48 hours, mitochondria were purified using the rapid immunoprecipitation protocol described above. Purified mitochondria (still bound to the HA-beads) were incubated at 95°C with 100 mL 1.0 M NaOH for 24 hours. 10 mL of the lysate was vortexed with 100 mL 80:20 MeOH:water and centrifuged at 13,000 x g for 10 minutes. Supernatants were moved into a new tube and dried down in a Speedvac concentrator (Thermo Fisher). Pellets were re-

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suspended in mobile phase and run on mass spectrometry to assess $^{15}$N-isotopologues. $^{15}$N-isotopologues detected in control (MYC) IP samples were subtracted out of both conditions.

**Mass Spectrometry**: Metabolites were extracted using 80% MeOH and analyzed using two liquid chromatography mass spectrometry (LC-MS) methods to profile ~300 metabolites in negative and positive ionization modes. Metabolites were analyzed on positive-ion mode using a Nexera UHPLC (Shimadzu) coupled to a Q Exactive Plus hybrid quadrupole orbitrap mass spectrometer (Thermo Fisher Scientific) as previously described (Spinelli et al., 2017b). Peaks were integrated using the TraceFinder 3.0 software (Thermo Fisher Scientific). Metabolites were analyzed on negative-ion mode with reverse phase ion-pairing chromatography on a ZORBAX Extend-C18, 2.1 × 150 mm, 1.8 µm column (Agilent) coupled to an Agilent 6460 Triple Quadrupole mass spectrometer (Agilent) as previously described. Peaks were integrated using the Mass Hunter software (Agilent).

**Respiration**. Respiration was assessed using the Seahorse XFe-96 Analyzer (Seahorse Bioscience). For experiments that measure the role of ammonia on oxygen consumption rate, cells were incubated with 1 mM NH$_4$Cl for 24 hours prior to the experiment. Following this incubation, media was changed to a non-buffered, serum-free Seahorse Media (Seahorse Bioscience, Catalog #102353) supplemented with 5 mM glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM NH$_4$Cl. Values were normalized to cell number.

**Mitochondrial Translation Assay**. Mitochondrial translation was assessed using an assay that measures the rate of incorporation of $^{13}$C-methionine (Sigma) into newly synthesized mitochondrial proteins. 2 x $10^6$ cells harboring epitope-tagged (HA) mitochondria were seeded 24 hours prior to the experiment. 1 hour prior to the start of the experiment, a subset of cells that were used as a negative control for mitochondrial translation were treated with 50 mg/mL chloramphenicol. At the start of the experiment, cells are washed twice with PBS and then media is changed to methionine-free RPMI supplemented with 2 mM $^{13}$C-methionine. The

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media for ammonia-treated conditions was also supplemented with 1 mM NH₄Cl and the media for chloramphenicol-treated samples (negative controls) was also supplemented with 50 mg/mL chloramphenicol. After the designated incubation time, mitochondria were purified as previously described using rapid immunoprecipitation. Mitochondrial amino acids were isolated from proteins as previously described, and profiled on mass spectrometry for % ¹³C-methionine incorporation.

**Oncomine Database Analysis.** Breast cancer patient data from The Cancer Genome Atlas (TCGA) was analyzed using the “Cancer Versus Normal” analytical tool on the public database Oncomine (www.oncomine.org). Relative levels of GOT1, GOT2, and SLC25A22 mRNA were measured with a Human Genome U133A Array. These results are based upon data generated by the TCGA Research Network: http://cancergenome.nih.gov/.

**In Vivo and Ex vivo Experiments.**

**Primary Patient Tissue:** Healthy and tumor tissue was resected from breast cancer patients at Massachusetts General Hospital under the Dana Farber Cancer Institute IRB protocol # 93-085 and assessed by the MGH pathology department for Estrogen Receptor status. Experiments were performed on primary tissue within 2-4 hours of surgery.

**Mouse Xenograft Model:** 7 week old mice were injected subcutaneously with 1 million MCF7 cells in 50% RPMI media and 50% LDEV-free MatriGel (Corning). Once tumors were ~100 mm³, tumor growth was measured every two days and volume was quantified using the equation: Volume = (π/6)(S²)(L) where S represents the shorter length, and L represents longer length.

**Ex vivo Metabolic Tracing:** For ex vivo tracing of primary patient tumors, 50% of the resected tumors were incubated in previously described media conditions supplemented with 3.0 mM ¹⁵NH₄Cl for 24 hours. Following incubation, tumors were flash-frozen and powderized. Metabolites were extracted from the powderized tissues and analyzed by LC-MS. For ex vivo tracing of mouse xenograft tumors ~150 mm³ tumors were excised and washed with 1 mL PBS.
Tumors were cut in half and incubated in previously described media conditions supplemented with 3.0 mM $^{15}$NH$_4$Cl for 24 hours. After incubation, tumors were powderized and metabolites were extracted as previously described.

**Isolation of Interstitial Fluids:** Interstitial fluids from tissue (healthy and tumor from patients and mouse models) were isolated using a published protocol (Wiig et al., 2003). Tissue was washed with 1 mL PBS and blotted dry with a kim wipe. Then, tissue was cut in half and centrifuged at 400 x g on top of a Nylon Mesh filter with 20 µm pores (EMB catalog #NY2004700) for 15 minutes. 2-10 uL interstitial fluid was vortexed in ice-cold 80% methanol at a 1:10 ratio and analyzed for ammonia and metabolites as described.

**Ammonia Measurement:** Ammonia was measured using the MS-Berthelot assay for LC-MS quantification of ammonia in metabolite isolates. Briefly, lysates (isolated in 80% methanol) were reacted with Solution #1 and Solution #2 for 1 hour at 37°C to convert ammonia into indophenol. Solution #1 is composed of 100 mM Phenol (Sigma) and 50 mg/L sodium nitroprusside (Sigma). Solution #2 is composed of 0.38 M Dibasic Sodium Phosphate (Sigma), 125 mM NaOH, 1% sodium hypochlorite, available chlorine 10–15% (Sigma). Indophenol was measured on an Agilent 6460 Triple Quadrupole mass spectrometer (Agilent LC-MS). Ammonia levels were quantified based on a standard curve of ammonia concentrations reacted under the same conditions and run with the same LC-MS parameters.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Details regarding the specific statistical tests, definition of center, and number of replicates (n), can be found for each experiment in the Figure legends. GraphPad Prism and excel were used for all quantifications and statistical analyses.

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Chapter Six: Conclusions and Future Directions
I. Summary

In this dissertation, we examined the role of the metabolic waste product ammonia in breast cancer (Figure 6.1). Prior to this work, it was shown that ammonia accumulates in the microenvironment of solid tumors to levels approximately 100-fold greater than what is considered toxic to healthy tissue. This leads us to the hypothesis that tumors are uniquely poised to tolerate ammonia, and that its accumulation may serve functions beyond a mere waste product of tumor metabolism. Therefore, we investigated the role of ammonia in breast cancer growth, survival, and metabolism.

A major hurdle in studying the role of ammonia in breast cancer metabolism was the ability to detect and distinguish $^{14/15}$N-isotopologues of ammonia. This technology is required for metabolic tracing studies, but could not be performed with metabolic lysates because ammonia is undetectable by mass spectrometry. To address this, we developed a method that enabled LC-MS quantification of $^{14/15}$N-isotopologues of ammonia in metabolite lysates. This assay exploits a reaction discovered by Pierre Berthelot in the late 1800’s called the Berthelot Reaction, which was used at the time as a colorimetric method to detect ammonia contamination in water. Using this oxidative phenolic coupling reaction, we converted ammonia into an LC-MS compatible compound, indophenol. This assay not only provides the most sensitive method for ammonia detection available (detecting a lower limit of sub-nanomolar levels of ammonia), but also enabled our investigation of the metabolic utilization of ammonia in breast cancer.

Taking advantage of the MS-Berthelot assay, we probed the capacity for breast cancer cells to utilize ammonia as a nitrogen source through the three assimilating enzymes CPS1, GDH, and GS. Using an unbiased analysis of the potential $^{15}$N-isotopologues that are generated from treatment with $^{15}$N-(amide)-glutamine, we identified many metabolites that have no direct biosynthetic connection to this labeled nitrogen, leading to the hypothesis that nitrogen liberated in glutaminolysis (generating $^{15}$NH$_3$) can be recycled to facilitate these labeling events.
Using pharmacological inhibition and genetic perturbations in combination with metabolic tracing of glutamine, we identified that ammonia liberated in glutamine catabolism is recycled with 60% efficiency as a nitrogen source for glutamate and downstream non-essential amino acids. Importantly, we demonstrated that this recycling is catalyzed by the “reverse” activity of glutamate dehydrogenase (GDH), and accounts for approximately 20% of the nitrogen in the non-essential amino acid pool. Next, we sought to elucidate the role of ammonia recycling in breast cancer cell survival and proliferation. Surprisingly, we found that ammonia was not toxic to breast cancer cells, even up to 50 mM concentration. Moreover, treatment with concentrations of ammonia that are physiological in the TME lead to elevated proliferation rate in 3D culture models of breast cancer. Elevated proliferation was abrogated upon genetic depletion of GDH, demonstrating that ammonia assimilation was requisite for the stimulatory effect on proliferation. Finally, using in vivo tumor xenograft models, we demonstrated that ammonia is a nitrogen source for amino acids that are critical for tumor growth. Thus, metabolic recycling of ammonia through glutamate dehydrogenase provides a critical nitrogen source for amino acid synthesis in breast cancer.

Inspired by the fact that ammonia-assimilating enzymes are all localized to the mitochondria, we reviewed the multifaceted contributions of mitochondria in cellular metabolism. Mitochondria are classically appreciated for their role as the powerhouse of the cell, harnessing the potential energy of the electron transport chain to generate the energy storage molecule adenosine triphosphate (ATP). Mitochondria catabolize fatty acids, pyruvate, and amino acids to support ETC function and subsequently ATP synthesis (among other things). In addition to this role, mitochondria are biosynthetic hubs in a cell, contributing to the production of nucleotides, fatty acids, amino acids, heme, and glucose. Mitochondria are also critical for compartmentalization of cofactors such as the NAD/NADH and NADP/NADPH reducing equivalents. Pathways that facilitate this include the malate-aspartate shuttle, citrate-malate shuttle, glycerophosphate shuttle, and one-carbon metabolism. Finally, we outline the ways in
which mitochondria participate in maintenance of metabolic waste. Mitochondria are the hub of ammonia metabolism, hydrogen sulfide clearance, and reactive oxygen species production and maintenance. Taken together, we demonstrate that mitochondrial metabolism is an integral component of cellular metabolism. It is critical that we continue to understand the ways in which metabolic pathways are compartmentalized to the mitochondria, and how localization of these pathways affects their function in cellular biology.

Finally, we investigated how mitochondrial compartmentalization of ammonia metabolism affects breast cancer growth and proliferation. We first performed metabolic tracing with $^{15}$NH$_4$Cl and utilized a rapid mitochondrial purification protocol to determine what ammonia-derivatives are generated in the mitochondria, and which are generated outside of the mitochondria. This tracing revealed that most derivatives of ammonia are generated inside of the mitochondria, except alanine, which was only detected in whole cell lysates. Glutamate is the nitrogen donor to alanine, and therefore, these data suggest that ammonia assimilation originates in the mitochondria, and that nitrogen is eventually distributed to cytosolic reactions. We next identified that GOT2 mediates the secretion of this nitrogen from the mitochondria. Interestingly, depletion of GOT2 in breast cancer cells lead to increased proliferation rate, corresponding with the induction in proliferation observed with ammonia treatment. Mitochondrial metabolomics revealed that GOT2 depletion and ammonia treatment both caused an increase in the mitochondrial glutamate pool, leading to the hypothesis that mitochondrial glutamate is limiting for proliferation in breast cancer cells. This hypothesis was tested through inhibition of the mitochondrial glutamate importer to decrease mitochondrial glutamate, causing decreased proliferation, and subsequent rescue with ammonia treatment, which restored mitochondrial glutamate levels and proliferation rate. Finally, we assessed the mechanism in which mitochondrial glutamate is limiting for breast cancer proliferation. A major function of mitochondrial glutamate is in local protein synthesis, so we therefore investigated whether mitochondrial glutamate was limiting for the rate of local protein synthesis. Treatment with
ammonia increased the levels of MT-encoded proteins in a manner that is dependent on GDH. Moreover, genetic increase (with GOT2 knockdown) or decrease (with 25A22 knockdown) of mitochondrial glutamate levels toggled the translation rate in this compartment. Thus, ammonia stimulates breast cancer proliferation through elevating the mitochondrial glutamate pool to enhance local translation rate.
Figure 6.1. Summary of the Thesis Work. This thesis, focused on the fate of the metabolic waste product, ammonia (NH$_3$), has discovered that NH$_3$ generated by tumor metabolism is recycled by glutamate dehydrogenase (GDH) to generate glutamate and other non-essential amino acids. Ammonia recycling is localized to the mitochondria, and glutamate oxaloacetate transaminase 2 (GOT2) is the interface for nitrogen secretion from the mitochondria in the form of aspartate. Importantly, we uncovered that localized assimilation generates a pool of glutamate that is a limiting factor for local translation rate. Mitochondrial protein translation generates members of the complexes in the electron transport chain, enabling respiration (O$_2$) consumption. The connection between mitochondrial glutamate levels and accelerated translation rate are still being explored, but we hypothesize that mitochondrial glutamate is limiting for the rate of local tRNA charging.
II. Discussion

A. Ammonia provides a critical nitrogen source for cancer cells in the nutrient-limited tumor microenvironment

Although cancer metabolism has been studied for almost 100 years, the role of the metabolic waste product ammonia had been largely overlooked. Aside from the simple observation that ammonia accumulated in the tumor microenvironment, very little was known about the implications of ammonia in tumor biology. Our findings demonstrate that the elevated levels of ammonia in the TME initiate assimilation to generate amino acids via GDH. Importantly, GDH is a bi-directional reaction; it can catalyze an oxidative deamination of glutamate to make α-ketoglutarate and ammonia, and it can catalyze a reductive amination of α-ketoglutarate and ammonia to generate glutamate. Importantly, the directionality of GDH is dictated by ammonia levels, since the $K_m$ of ammonia is approximately 9 mM. Thus, in most tissues in the human body, ammonia levels are very low (approximately 50 µM) and therefore GDH favors oxidative deamination (ammonia production). Typically, healthy tissues are well perfused and have access to abundant nutrients, including glutamine and alanine, the major nitrogen carriers in the body. Therefore, in well-perfused tissue, ammonia is not a requisite nitrogen source for biomass. Conversely, in poorly perfused tissue such as solid tumors, biosynthetic precursors such as glucose and glutamine become limiting and metabolic waste products such as lactate and ammonia are trapped, leading to their accumulation. This accumulation of ammonia activates a switch, leading to GDH operating in the reductive amination direction in which it assimilates ammonia to generate glutamate. The bi-directional nature of GDH is likely a sensor for cellular nutrient availability, since ammonia will only accumulate when tissues are poorly perfused. The reversibility of GDH enables cell survival.


in the context of limiting nitrogen sources by capturing the accumulating nitrogen waste and recycling it. Thus, GDH reversibility is critical for cancer cell survival in the tumor microenvironment.

B. How ubiquitous is ammonia biology among tumor subtypes?

We demonstrated that glutamate dehydrogenase is ubiquitously expressed at high levels in all tissue (Figure 2.S1) and is even further induced in a number of cancer subtypes (Figure 2.1). Since ammonia assimilation through GDH is dictated by ammonia concentration, theoretically, any given cell that is in an environment with high enough ammonia levels should assimilate it through this pathway. Therefore, ammonia assimilation through GDH is likely a common feature of most solid tumors due to poor vascularization and ammonia accumulation. Hematological malignancies are less likely to rely on ammonia recycling as a consequence of their microenvironment, however, whether intracellular ammonia levels in these cancer subtypes are sufficient to reverse GDH activity to promote ammonia recycling is unclear.

Although we anticipate ammonia assimilation to be a ubiquitous feature of solid tumors, it is likely that ammonia-stimulated proliferation is dependent on cancer subtype, genetic drivers, and subsequently metabolic dependencies. Our studies demonstrate that in Estrogen Receptor positive breast cancer, ammonia can stimulate proliferation through supporting a limitation in the mitochondrial glutamate pool (Chapter 5). Many metabolic pathways can contribute to the mitochondrial glutamate pool beyond GDH, such as glutamine catabolism through GLS, branched chain amino acid catabolism through BCAT2, and proline catabolism through PRODH.\textsuperscript{516} Many cancer subtypes rely on elevated flux through these alternative pathways for mitochondrial glutamate, such as triple negative breast cancer, which elevate c-MYC and therefore enhance expression of GLS,\textsuperscript{517} Kras mutant non-small cell lung carcinoma, which rely


on elevated branched chain amino acid catabolism for growth, and in metastatic lung lesions that rely on proline catabolism. Thus, the extent that GDH-mediated ammonia assimilation affects the mitochondrial glutamate depends on what metabolic pathways are already activated and feeding into that pool. We anticipate that ammonia will enhance proliferation in cancer cells that do not have access to many other sources of mitochondrial glutamate. Beyond ER positive breast cancer, we hypothesize that ammonia will support proliferation of pancreatic ductal carcinoma, which do not catabolize BCAAs or proline and are highly dependent on nitrogen from their microenvironment. Moreover, liver cancer cells suppress the urea cycle, and likely rely on ammonia assimilation as a major source of glutamine and glutamate pools. Additionally, we anticipate that colorectal cancers will be particular dependent on ammonia-derived mitochondrial glutamate because the tissue of origin has very high levels of ammonia (due to the microbiome) and colorectal cancers require high levels of mitochondrial respiration to support growth and proliferation. Thus, while ammonia assimilation is likely a ubiquitous feature of solid tumors, we anticipate that the extent to which this source affects mitochondrial glutamate levels will vary depending on cancer subtype.

C. Mitochondrial respiration in tumors

Mitochondrial respiration has classically been misinterpreted to be suppressed in cancer cells due to elevated aerobic glycolysis (The Warburg Effect). However, many studies have pointed to a critical role for mitochondrial respiration in tumor biology, including for the

production of ATP, biosynthesis of aspartate, production of nucleotides, and replenishing cytosolic NAD⁺ through the glycerophosphate shuttle. As a proof of principal, many studies have investigated the efficacy of respiratory chain inhibitors such as Metformin, Rotenone, Piericidin, and Oligomycin in a multitude of cancer subtypes (see Chapter 1 for overview). In our studies, chloramphenicol, which inhibits mitochondrial translation and consequently halts the respiratory chain, caused significant inhibition of proliferation in breast cancer.

Importantly, we observed the effects of chloramphenicol on breast cancer cells that were grown in 3D culture, which is a more physiological model of solid tumors because they have the nutrient/waste gradients, oxygen gradients, and cells in a broad range of proliferative capacities. Although 3D culture models (like solid tumors) are prone to hypoxia, studies have shown that growing cells in 3D culture increases respiration. Moreover, tumors with mitochondrial DNA mutations are more likely to be benign and depletion of MT-DNA (p⁰ cells) impairs tumor growth, which can only be restored when respiratory complexes are re-introduced into the cells. When genes in MT-DNA acquire truncation mutations, they are almost

always negatively selected against in tumors. Some examples of detrimental MT-DNA mutations include renal oncocytomas (benign) with inactivating complex I mutations, and in numerous tumor subtypes, a notably strong negative selection against mutations in the complex IV gene MT-CO1. Opposite to benign oncocytomas are the malignant oncocytic renal Birt-Hogg-Dube tumors, which accumulate highly functional mitochondria that respire more than healthy tissue. It is important to note that some mutations to MT-DNA, especially those in D-LOOP (transcriptional regulatory region), are associated with poor prognosis in various cancer subtypes. Nonetheless, paradoxically, although tumors are fairly hypoxic (0.3 - 2.0% O2) compared to normal tissue (5.0 – 7.0% O2), they are highly dependent on mitochondrial translation and respiration.

One potential reason for increased reliance on mitochondrial protein synthesis in tumors is that low oxygen levels cause tumors to accumulate high levels of ROS, leading to heightened damage to the respiratory chain. This damage may in turn activate a dependency on the production of new MT-encoded proteins to enable repair of the respiratory chain for continued respiration. An alternative hypothesis is that ROS generated from hypoxia causes MT-DNA damage and mutagenesis, leading to heteroplasmy among copies of the MT-DNA. Since some copies of MT-DNA are mutated in this scenario, efficient translation of the remaining (wild-type)
copies may be very critical, rendering cancer cells sensitive to inhibition of mitochondrial translation. This would also cause cancer cells to increase proliferation when MT-protein translation rate is elevated (such as with ammonia treatment). Finally, reliance on mitochondrial respiration may be heterogeneous within a tumor, such that cells on the periphery with sufficient oxygen levels are utilizing mitochondrial respiration more than those residing in hypoxic environments. Nonetheless, natural selection within the TME demonstrates that there is a critical requirement for tumors to engage mitochondrial respiration. Ammonia, through raising the mitochondrial glutamate pools, enables survival in the TME by accelerating the rate of mitochondrial translation.

III. Future Directions

A. Depletion of mitochondrial glutamate in cancer therapeutics

A major takeaway from this dissertation work is that mitochondrial glutamate has a critical role in breast cancer growth and proliferation. An important future direction will be to develop small molecules that impede the described contributors to mitochondrial glutamate such as glutamate dehydrogenase (ammonia assimilation) and the mitochondrial glutamate importer SLC25A22. Furthermore, it is critical to avoid targeting pathways that could cause mitochondrial glutamate to rise, such as inhibition of GOT2. In fact, when considering the cancer metabolism therapies that have demonstrated efficacy clinically, many of them have direct effects on mitochondrial glutamate. For example, small molecules targeting glutaminase (GLS) have been developed and tested clinically in cancers including triple negative breast cancer and IDH mutant glioma cells.\textsuperscript{539 540} Glutaminase catalyzes the conversion of mitochondrial glutamine to mitochondrial glutamate, and is elevated in many cancer subtypes due to transcriptional activation by c-MYC (see Chapter 1). However, resistance to the GLS


inhibitors has impeded the continued clinical use of these therapies, inspiring studies on the metabolic adaptations that confer resistance to GLS inhibition.\textsuperscript{541}

Given that our studies demonstrate a critical role for mitochondrial glutamate in proliferation, we tested the hypothesis that ammonia metabolism may be an adaptive node for assimilation of mitochondrial glutamate when GLS is inhibited. Solid tumors accumulate ammonia in the TME, causing GDH to reverse flux and generate mitochondrial glutamate (Chapter 3). We hypothesized that the GLS inhibitor fails \textit{in vivo} in part due to the capacity for ammonia to rescue mitochondrial glutamate levels. Thus, we hypothesized that combined inhibition of GLS with GDH will improve the efficacy of the GLS inhibitor (Figure 6.2a).

We utilized ER positive breast cancer cells as a model to test this hypothesis because we have previously demonstrated that ammonia can be assimilated to generate glutamate in this system. First, we tested whether CB-839 treatment altered steady-state metabolite levels in MCF7 cells. We found that glutamate and $\alpha$-ketoglutarate levels were substantially decreased in response to low levels of CB-839 treatment, which is consistent with inhibition of glutamine anaplerosis of the TCA cycle (Figure 6.2b). Interestingly, concentrations over 1 nM CB-839 began to rescue (partially) the levels of $\alpha$-ketoglutarate and glutamate in the cells, suggesting that the depletion of these pools of metabolites can stimulate alternative (adaptive) pathways that are capable of restoring their levels. Importantly, an unbiased analysis of the metabolites altered by CB-839 treatment identified glutamate metabolism as the most significant pathway (Figure 6.2c). To test if ammonia assimilation contributes to the glutamate pool upon CB-839 treatment, we traced $^{15}$NH$_4$Cl in MCF7 cells treated in low versus high CB-839 (Figure 6.2d). Ammonia tracing revealed that ammonia contributed to a larger portion of the glutamate pool at high CB-839 concentration, suggesting that ammonia recycling could compensate for glutamate levels in CB-839 treatment. It is important to note that other pathways including BCAA

catabolism, proline catabolism, aspartate and alanine catabolism can also generate this mitochondrial glutamate pool. To test the importance of GDH in adaptation to GLS inhibition, we generated MCF7 cells that were depleted of GDH and treated them with the GLS inhibitor BPTES in our 3D culture model (Figure 6.2e-f). Although control cells did not change proliferation rate upon treatment with BPTES, cells depleted of GDH had significantly slower growth rate. Thus, these data suggest that ammonia assimilation can be an adaptive node of metabolism that supports the mitochondrial glutamate pool to sustain accelerated proliferation.

A number of critical experiments must be performed to assess whether combinatorial GDH and GLS inhibition could improve the efficacy of GLS inhibitors in vivo. First, the levels of glutamate in GDH-depleted cells before and after GLS inhibition must be assessed to determine that the effect of combined inhibition is through this pool. Moreover, glutamate supplementation should be tested in control, GDH-depleted, and GDH/GLS inhibited conditions to determine if this can rescue growth in 3D culture. Importantly, the combined efficacy of GDH and GLS inhibition must be tested in vivo. Since there is no specific inhibitor of GDH available, genetic depletion of GDH in tumor xenograft models will need to be used to test this model in vivo. Treatment of control and GDH-depleted tumors with CB-839 will inform whether this combination therapy could work in vivo. Finally, ammonia tracing in vivo in the presence and absence of CB-839 treatment is critical to demonstrate that ammonia provides an additional source of nitrogen for glutamate synthesis in vivo.
Figure 6.1. Ammonia assimilation is an adaptive source of glutamate upon GLS inhibition. 

A. Schematic of the interplay between GDH and GLS. 

B. LS-MS analysis of glutamate and α-KG levels in MCF7 cells treated on a dose with the CB-839 inhibitor for 24 hours. N=3 per condition. 

C. Metaboanalyst analysis of statistically significant metabolic pathways in MCF7 cells treated with 1 uM CB-839 for 24 hours. 

D. $^{15}$NH$_4$Cl tracing in MCF7 cells in low (0.0001 nM) and high (1 uM) CB-839 onto glutamate. 

E-F. Growth in 3D culture of MCF7 cells depleted of GDH and treated with or without 1 uM BPTES. N=3 per condition, cells grown for 6 days.
Our work has unveiled an unexpected role for the mitochondrial glutamate pool in regulation of mitochondrial translation. Beyond glutamate, the role of subcellular compartmentalization of metabolic pathways in tumor growth and progression has been little studied. Mitochondrial nutrient transporters are the gatekeepers of these pathways, and may be good targets for therapy development. One critical reason for the lack of studies focused on metabolic compartmentalization is that many of the mitochondrial nutrient transporters are uncharacterized. Among the 60 members of the Solute-like Carrier (SLC) family of nutrient transporters on the mitochondrial surface, 27 of them (45%) have no known function. Consistent with this disparity, there are many mechanisms of metabolic compartmentalization that remain undetermined and may be linked to these uncharacterized transporters. For example, it is unknown how glycine-serine are exchanged for 1C metabolism, how NH$_4^+$ enters the mitochondria for assimilation, and how 15 out of the 20 amino acids enter the mitochondria for local protein translation. Characterizing these transport mechanisms will not only reveal new therapeutic targets for treatment of cancer, but also advance our fundamental understanding of metabolic plasticity in cell biology.

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