



# SIRT3 Suppresses Rewiring of Glutamine Metabolism in Cancer

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SIRT3 suppresses rewiring of glutamine metabolism in cancer

A dissertation presented

by

Karina Noemi Gonzalez Herrera

to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

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in the subject of

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**SIRT3 suppresses rewiring of glutamine metabolism in cancer****Abstract**

In the process of transformation, a cancer cell selects for a strategy to enhance its survival, growth, and proliferation. Metabolic rewiring is a hallmark of cancer cells and is at the heart of such a mechanism. Although oncogenes and tumor suppressors contribute to metabolic reprogramming in cancer, the exact mechanisms underlying deregulated nutrient metabolism continue to be elucidated. The mitochondrial sirtuin, SIRT3 has tumor suppressive function in various types of human cancer and represses the Warburg effect in breast cancer cells. Despite what is known about SIRT3 in the regulation of metabolism, the challenge in translating our findings into development of therapies for treatment of cancer remains. Thus, the goal of this dissertation was to develop a better understanding of the role of SIRT3 in tumorigenesis and to identify metabolic vulnerabilities associated with loss of SIRT3 in cancer.

In this dissertation, I identify deregulated glutamine metabolism as a vulnerability in tumor-like cells with SIRT3 deletion. First, I utilized immortalized WT and SIRT3 null (KO) cells and performed a small molecule screen to identify compounds that selectively decreased proliferation of cells lacking SIRT3. The top hit from the screen was a glutamine analog, azaserine. Like glucose, glutamine is crucial for synthesis of building blocks to support tumorigenesis. In combination with metabolomics analysis, RNA sequencing (RNA Seq), and bioinformatics analysis, I revealed that azaserine significantly represses a dependency on elevated *de novo* nucleotide metabolism, driven in part by increased mTORC1 signaling, to suppress growth of cells with decreased SIRT3 levels. Additionally, in human breast tumors,



SIRT3 expression is lower in the more aggressive basal-like tumors compared to luminal breast tumors, and is associated with increased mTORC1 signaling. Moreover, I demonstrate that SIRT3 represses mTORC1 signaling to suppress tumorigenesis.

Given that azaserine is a glutamine analog and that SIRT3 loss results in enhanced glutamine consumption, I further examined the effect of SIRT3 loss on glutamine metabolism. Using a combination of glutamine tracing and RNA Seq, I uncover alterations in various nodes of glutamine metabolism in the absence of SIRT3. More specifically, I identify increased distribution of glutamine to glutathione and the tricarboxylic acid (TCA) cycle, which is coupled with production of nonessential amino acids (NEAAs) in SIRT3 KO cells. Lastly, I find that SIRT3 represses glutaminolysis in breast cancer cells.

Taken together, my findings reveal a pathway through which glutamine contributes to tumor growth and identifies previously undescribed nodes of tumor metabolism deregulated in the absence of SIRT3. My findings shed light on potential metabolic pathways that may be targeted in cancers with decreased SIRT3 expression to suppress tumorigenesis.

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## CHAPTER I

### Introduction

Sections of this chapter have been adapted from:

**Intersections between mitochondrial sirtuin signaling and tumor cell metabolism**

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## **1.1 Overview of the dissertation**

During the process of transformation, a tumor cell selects for a mechanism to enhance its survival, growth, and proliferation. Many years of research have shed light on metabolic reprogramming as a key component of such a strategy. Additionally, the role of oncogenes and tumor suppressors and their contribution to metabolic rewiring has been elucidated. The mitochondrial sirtuin 3 (SIRT3) is a key regulator of many facets of biology and has been shown to have tumor suppressive function in part by repressing a major arm of cancer metabolism, glycolysis. Despite what is known about SIRT3 and metabolic rewiring in cancer, it continues to be a challenge to transfer our findings into development of therapies to treat these cancers. Acquiring a better understanding of the mechanism(s) employed by tumor cells with SIRT3 loss could reveal underlying vulnerabilities that could be targeted to treat cancer. Moving towards a future of personalized medicine could allow us to develop therapies based on the characteristics or strategies adapted by an individual's tumors. With this landscape in mind, the goal of my dissertation was to develop a better understanding of the mechanism by which SIRT3 loss promotes tumorigenesis with the goal of identifying an Achilles' heel for cancer therapy.

## **1.2 Overview of breast cancer**

Despite many years of research, cancer continues to be one of the top leading causes of death [1,2]. In 2012, it was estimated that 14.1 million people were diagnosed with cancer resulting in 8.2 million deaths worldwide. Cancer occurrence is expected to increase due to a growing and aging population, in addition to modern lifestyle choices, which may increase risk of developing this disease. Breast cancer is one of the most frequently diagnosed types of cancer and is the leading cause of death in women [1]. Breast cancers are subdivided into four subtypes: luminal A, luminal B, triple negative/basal like, and HER2-type, based on transcription profile,

and these are categorized into three therapeutic groups [3]. Luminal breast cancers are the most prevalent and tend to express the estrogen receptor (ER) and progesterone receptor (PR) and have a high survival rate [4-6]. Less prevalent are the HER2-type breast cancers, which exhibit amplification of the human epidermal growth factor receptor 2 (HER2) and tend to lack expression of the ER and PR. Until the development of trastuzumab (also known as Herceptin), an antibody that targets HER2-expressing breast cancer cells, this subtype of breast cancer had a poor prognosis [6]. As the name implies, triple-negative breast cancers, which encompass basal-like breast cancers, lack expression of the three receptors. Although this type of breast cancer is the least common, it has a poor prognosis as these cancers are the most aggressive and no targeted therapy is available for treatment [7]. Treatment for these types of cancer consists of DNA damaging agents, such as cisplatin [8,9]. However, basal-like breast cancers tend to develop resistance to this and other types of chemotherapies. Thus, identifying additional vulnerabilities in these types of cancer may provide insight into developing better therapies or combination therapies to treat these cancers.

### **1.3 Cellular metabolism and metabolic reprogramming in cancer**

#### **1.3 A. Overview**

A major goal in cancer biology is to identify molecular mechanisms that contribute to cancer growth and survival in order to provide insight into new biomarkers for precision medicine, as well as novel targeted therapies. Toward this end, recent studies have elucidated a new promising area of cancer cell biology – metabolic rewiring or reprogramming, which refers to altered metabolism in tumor cells [10-16]. Unlike differentiated or quiescent cells, tumor cells proliferate rapidly and rewire metabolism of glucose, amino acids, and fats to produce macromolecules, such as nucleic acids, proteins, and lipids, required for generation of new cells



[12]. In addition, metabolic reprogramming stimulates production of reducing equivalents (NADPH) and energy required for synthesis of these macromolecules (anabolism), as well as synthesis of antioxidants to provide growth and survival advantages.

### **1.3 B. Cancer cells and the Warburg effect**

Non-proliferating cells oxidize glucose via glycolysis and the tricarboxylic acid (TCA) cycle, coupled with the electron transport chain (ETC) to generate energy in the form of adenosine triphosphate (ATP) [11]. Unlike non-proliferating cells, cancer cells increase uptake of nutrients beyond what is needed to support bioenergetic needs [17]. In the 1920s, Otto Warburg made the first observation that cancer cells display aberrant metabolism, compared to normal cells. Warburg noted that cancer cells utilized glucose at higher rates than a normal cell and diverted its entry into the mitochondria to produce and secrete lactate in the presence of oxygen – a phenomenon termed the Warburg effect [18]. He hypothesized that this process was a result of a rapidly dividing tumor cell's requirement for energy in the presence of defective mitochondria [19]. However, this conclusion has proven to be untrue for many tumors. In fact, it is evident that although mutations in mitochondrial genes can promote the Warburg effect, mitochondria are intact and functional in many cancer cells with Warburg metabolism.

Studies have revealed that tumor cells rewire metabolism of glucose since various glycolytic intermediates can be shunted into alternate pathways to generate macromolecules to fuel increased biomass, which is necessary for rapid proliferation (Figure 1.1) [20]. For example, 3-phosphoglycerate (3PG) is diverted from glycolysis and is utilized for synthesis of nonessential amino acids (NEAAs). In a series of three steps, 3PG is converted to serine, which is further converted to glycine and cysteine [21,22]. The first enzyme involved in redirecting glucose to

serine is phosphoglycerate dehydrogenase (PHGDH), and expression of this enzyme is high in a number of cancers, including breast and melanoma cancers [22,23].

In addition to NEAA production, glucose oxidation supports biosynthesis via the pentose phosphate pathway (PPP), which provides reducing equivalents for anabolic reactions and 5-carbon sugars for nucleotide synthesis (Figure 1.1) [17,24,25]. The PPP is composed of 2 phases – the oxidative and non-oxidative phase [10,26]. In the oxidative phase of the PPP, glucose-6-phosphate dehydrogenase utilizes G6P in the first step of the PPP to divert it from glycolysis and support production of NADPH from NADP<sup>+</sup>. G6PD expression is elevated in tumors and expression of this protein has also been shown to increase NADPH levels [27-29]. In the non-oxidative phase of the PPP, fructose-6-phosphate and glyceraldehyde-3-phosphate (G3P) contribute to generation of ribose-5-phosphate, which is utilized for nucleotide synthesis. In addition, G3P is converted to dihydroxyacetone phosphate, which is utilized for synthesis of triglycerides – key components of cell membranes [14].

Another major regulatory node for glycolysis is the last step of glycolysis catalyzed by pyruvate kinase (PK). Cancer cells and proliferating cells express the PKM2 isoform, which has low enzymatic activity in its dimeric form, and thus, contributes to decreased entry of pyruvate to the mitochondria to allow diversion of glycolytic metabolites to other pathways to support biomass production [30]. In sum, tumors adjust glucose metabolism through coordinated rewiring of multiple nodes to maximize production of metabolites used for nucleotide, amino acid and fatty acid synthesis.

**Figure 1.1. Alterations in glucose and glutamine metabolism contribute to tumor growth and proliferation.** Rewiring of glucose and glutamine metabolism contribute to synthesis of macromolecules, antioxidants, and reducing equivalents. Major enzymes involved in each pathway are highlighted above, and expression or activity of these enzymes tends to be altered in tumor cells to contribute to metabolic reprogramming, as described in the text. NEAA, nonessential amino acids; HK, hexokinase; GFAT, glutamine fructose-6-phosphate amidotransferase; PHGDH, phosphoglycerate dehydrogenase; SHMT, serine hydroxymethyltransferase; PK, pyruvate kinase; PDH, pyruvate dehydrogenase; GCL, glutamate-cysteine ligase; GS, glutathione synthase; ME, malic enzyme; ACL, ATP citrate lyase; LDHA, lactate dehydrogenase; GLS, glutaminase; GDH, glutamate dehydrogenase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GLUT1, glucose transporter type 1; ASCT2, sodium-dependent neutral amino acid transporter type 2; SN2, system N transporter 2; MCT4, monocarboxylate transporter type 4; xCT, cystine/glutamate transporter.

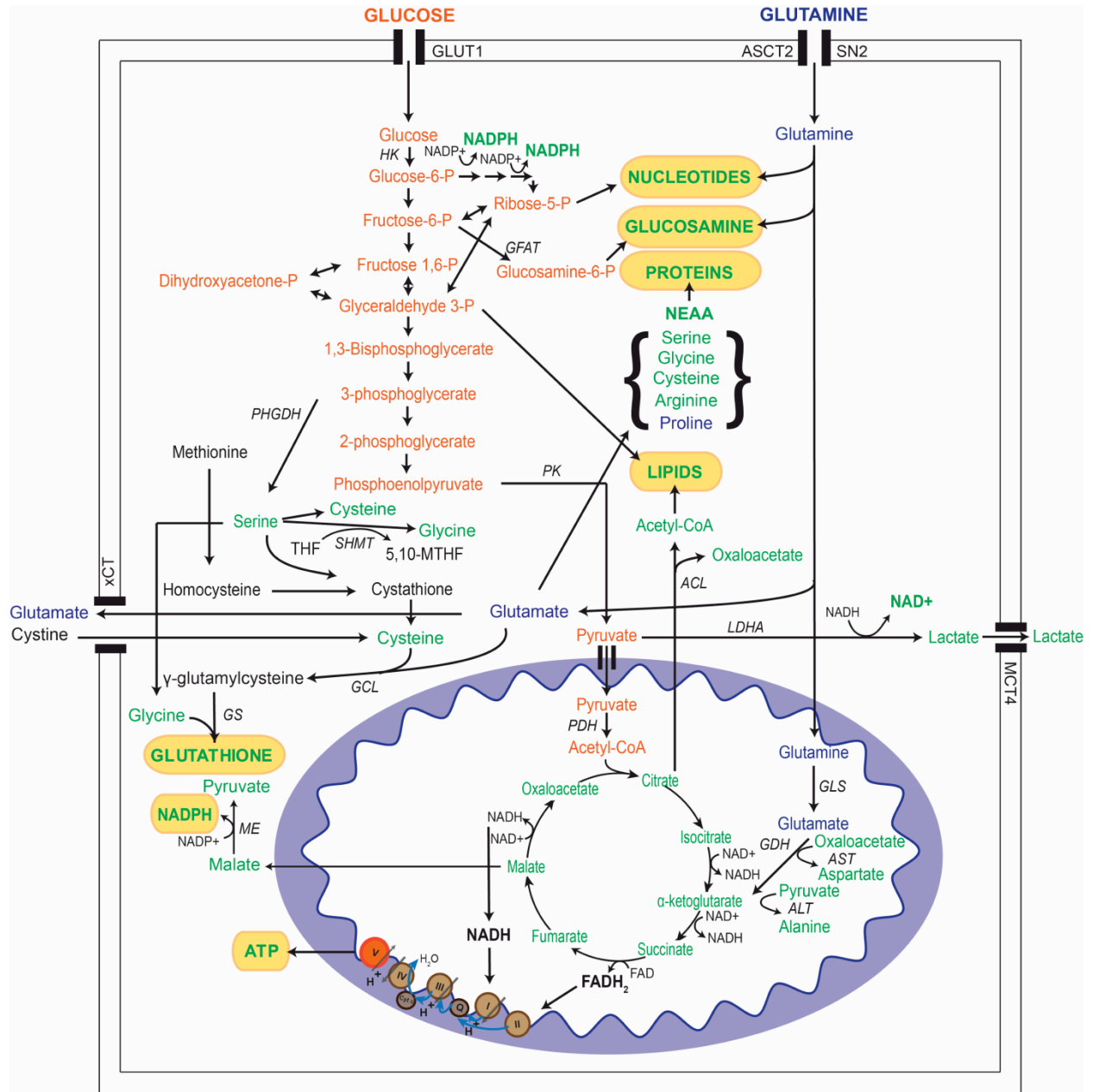


Figure 1.1 (Continued)

### **1.3 D. Glutamine metabolism and its importance to cancer cells**

For many years, cancer metabolism research focused on developing a better understanding of the Warburg effect and the importance of glucose metabolism to a cancer cell. An equally important nutrient to cancer cells is glutamine, the most abundant amino acid in human plasma [31]. Although glutamine is usually categorized as a nonessential amino acid for many resting cells, it can become an essential amino acid in a rapidly dividing cell as it complements glucose usage by providing its nitrogen and carbon atoms for synthesis of macromolecules, such as nucleic acids, proteins, and lipids to support cellular growth and proliferation, as well as for regulation of redox homeostasis (Figure 1.1 and 1.2) [32,33].

Glutamine metabolism varies across organs, with some organs utilizing glutamine while other organs play a key role in synthesizing glutamine [34,35]. Skeletal muscle is the main site of glutamine production in the body [36,37]. In addition, the lung, where glutamine synthetase (GS) is induced, is another major generator of glutamine [35,38-40]. The lung organ has the ability to produce large amounts of glutamine under stress conditions. Adipose tissue can also contribute to glutamine production. Part of the liver (perivenous cells) displaying high GS expression has the ability to generate glutamine, while another region of the liver (periportal cells) utilize glutamine [41,42]. Other organs, such as the kidney, the small intestine, the gut, neurons, pancreatic beta cells, and immune system cells are major glutamine consumers [34,43-45]. Compared to non-proliferating cells, various types of cancer cells display elevated glutamine consumption accompanied by reprogramming of glutamine metabolism [15,32,46-51].

### *Glutaminolysis and nonessential amino acid (NEAA) production*

Metabolism of glutamine contributes to various metabolic pathways, one of them being the TCA cycle where glutamine-derived carbon is metabolized to contribute to production of energy, reducing equivalents, and lipids to generate a new cell. Cells take up glutamine via high affinity glutamine transporters, such as Slc1a5 (ASCT2) or Slc38a5 (SN2), which are overexpressed in various types of tumors [49,52]. Inside the cell, glutamine is utilized for anaplerosis, which refers to fueling of the TCA cycle, via glutaminolysis, a canonical glutamine metabolism pathway. Glutaminolysis refers to the conversion of glutamine to glutamate by glutaminase (GLS), which is followed by deamination of glutamate via glutamate dehydrogenase (GDH) to produce  $\alpha$ -ketoglutarate [49,53,54]. Ammonium is a byproduct of these steps and increased ammonium production is observed in cancer cells that utilize glutamine at high rates [55]. Glutamine-derived carbon in the form of  $\alpha$ -ketoglutarate is then oxidized in the TCA cycle and converted to malate and citrate, which are important for NADPH and lipid synthesis respectively [56-58]. As part of the malate-aspartate shuttle, glutamine-derived malate can be exported to the cytosol where it is converted to pyruvate, by malic enzyme, resulting in the generation of NADPH, which is utilized in anabolic reactions as well as to maintain redox balance [59-61]. Alternatively, malate is converted to oxaloacetate (OAA), which is condensed with acetyl-coA to form citrate. Citrate can be exported to the cytosol, where it is converted to OAA and acetyl CoA by ATP citrate lyase [62]. The acetyl CoA derived from citrate may be used for epigenetic and post-translational modification, or synthesis of fatty acid and cholesterol – key components of cellular membranes [63]. Additionally, under low oxygen (hypoxia) conditions or stabilization of hypoxia inducible factor 1 (HIF1), glutamine-derived  $\alpha$ -ketoglutarate is reductively carboxylated to contribute to the citrate pool for lipid synthesis

[64,65]. Thus, mitochondrial glutamine metabolism is essential for production of lipids, as well as ATP and NADPH, which are necessary to support anabolic reactions and proliferation.

In addition to its anaplerotic role, glutamine is utilized as a nitrogen source for synthesis of NEAAs (Figure 1.1 and 1.2) [17,51,58,66]. A recent study has shown that quiescent cells have increased expression of GDH to allow entry of glutamine-derived carbon into the TCA cycle. In contrast, proliferating cells have increased expression of transaminases and decreased GDH expression to couple the conversion of glutamine-derived glutamate to  $\alpha$ -ketoglutarate with production of NEAAs [67]. Multiple transaminases transfer nitrogen from glutamate to carbon backbones to generate NEAAs. For example, aspartate transaminase (GOT) and alanine transaminase (GPT) transfer nitrogen from glutamate to oxaloacetate and pyruvate to generate aspartate and alanine, respectively [58,68]. In addition, phosphoserine aminotransferase (PSAT1) combines 3-phospho-hydroxypyruvate with glutamate-derived nitrogen to generate phosphoserine, a precursor to serine. Expression of these genes is regulated by the K-Ras and mTORC1 signaling pathways, detailed below, which downregulate expression of GDH and stimulate transaminase expression to promote NEAA production and proliferation [58,67,69].

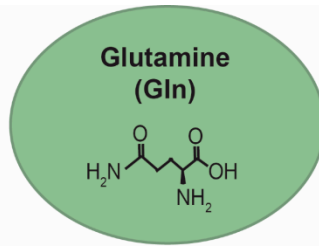
### *Nucleotide synthesis*

Glutamine is key to *de novo* nucleotide synthesis [17]. Glutamine donates its amide group in three steps of purine synthesis and two steps of pyrimidine synthesis to contribute to the pool of nucleotides for RNA and DNA synthesis, which is essential to generate the genetic material of a new cell [17]. In addition to glutamine, the PPP, one carbon metabolism, and NEAAs contribute to *de novo* nucleotide synthesis [70].

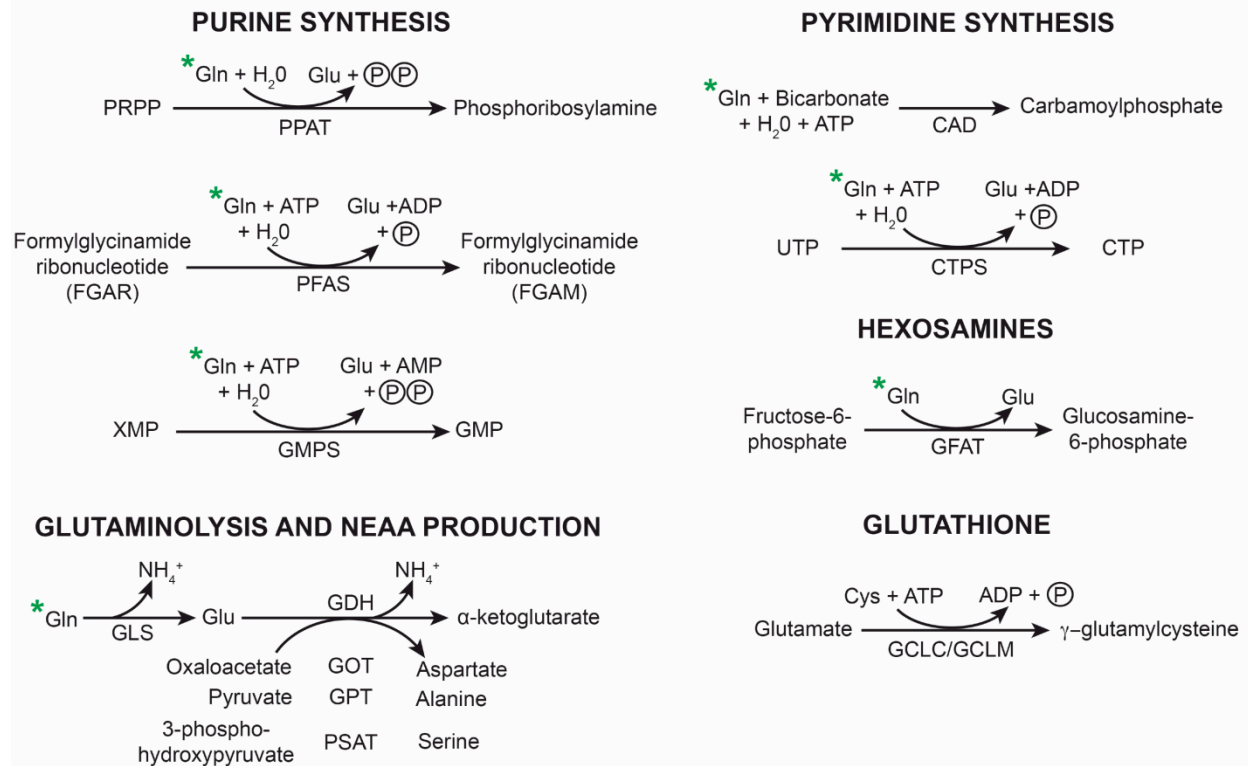
*De novo* production of purines, guanine and adenine, begins with ribose-5-phosphate, a PPP intermediate, which is phosphorylated to generate 5-phosphoribosyl- $\gamma$ -pyrophosphate (PRPP) (Figure 1.2). The second step of *de novo* purine synthesis is the rate-limiting step of the pathway and is catalyzed by phosphoribosyl pyrophosphate amidotransferase (PPAT), a glutamine-dependent enzyme. Two additional enzymes, FGAM synthetase (PFAS) and guanosine monophosphate synthase (GMPS) also utilize glutamine in the production of purines. Additionally, folate generated via one carbon metabolism, in the cytosol or the mitochondria, is utilized in two steps of purine synthesis. Like PPAT, folate metabolism is also rate limiting for nucleotide synthesis. Lastly, NEAAs, such as glycine and aspartate also contribute to purine synthesis. As previously mentioned, glucose metabolism contributes to synthesis of glycine, and both glucose and glutamine contribute to aspartate synthesis.

Synthesis of pyrimidines, cytosine, thymine, and uracil, relies on glutamine, aspartate, and the PPP (Figure 1.2). The first step of pyrimidine synthesis is catalyzed by the rate-limiting enzyme carbamoyl-phosphate synthetase 2, aspartate transcarbamoylase, and dihydroorotase (CAD). CAD utilizes glutamine, bicarbonate, energy, water, and aspartate to generate dihydroorotate. Dihydroorotate is then oxidized in the mitochondria by dihydroorotate dehydrogenase to generate orotate, which is then combined with PRPP (generated via the PPP) to produce oritidine 5'-monophosphate (OMP). OMP is then converted to uridine monophosphate (UMP) via OMP decarboxylase. Lastly, glutamine is utilized for generation of cytidine triphosphate (CTP) from uridine triphosphate (UTP).

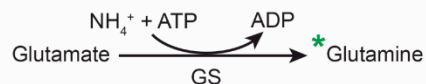




**GLUTAMINE UTILIZATION PATHWAYS**



**GLUTAMINE SYNTHESIS**



**Figure 1.2. Glutamine contributes to multiple nodes of metabolism.** Metabolism of glutamine contributes to synthesis of key biosynthetic precursors, and the antioxidant glutathione, which are necessary for growth and proliferation. Glutamine can also be synthesized by combining glutamate and ammonium. NEAA, nonessential amino acids; Glu, glutamate; Cys, cysteine;  $\text{NH}_4^+$ , ammonium; PPAT, phosphoribosyl pyrophosphate amidotransferase; PFAS, phosphoribosylformylglycinamide synthase; GMPS, guanosine monophosphate synthase; GLS, glutaminase; GDH, glutamate dehydrogenase; GOT, aspartate transaminase; GPT, alanine transaminase; PSAT1, phosphoserine aminotransferase; CAD, carbamoyl-phosphate synthetase 2, aspartate transcarbamoylase, and dihydroorotase; CTPS, CTP synthase; GFAT, glutamine:fructose-6-phosphate amidotransferase; GCLC/GCLM, gamma-glutamylcysteine synthetase; GS, glutamine synthetase.

Nucleic acid production requires a lot of energy [12,71-73]. Compared to a non-proliferating cell, cancer cells elevate nucleotide synthesis to meet the demands of proliferation. Nucleic acid synthesis requires nucleotide triphosphates or deoxyribonucleotides. The nucleotide monophosphates generated via purine and pyrimidine synthesis are converted to nucleotide triphosphates by nucleotide kinases, which are used for RNA synthesis. DNA synthesis requires deoxyribonucleotides, which are generated from ribonucleoside diphosphates by ribonucleotide reductase, which relies on NADPH and thioredoxin. In addition, because nucleic acids, like RNA, are often synthesized and degraded, salvage pathways exist to recover purine and pyrimidine bases and convert them back to nucleotides [73]. Salvage reactions are catalyzed by phosphoryl transferases and require less energy than *de novo* nucleotide synthesis.

### *Glutathione*

Glutamine regulates redox homeostasis by contributing to production of glutathione and NADPH [74,75]. Glutathione is the most abundant antioxidant in the cell, and is composed of three amino acids: glutamate, cysteine, and glycine [61]. Glutamine is the main contributor to the pool of glutamate, which is incorporated into glutathione. Glutamate is also important as it can be exported from the cell via the Slc7a11 (xCT) transporter for import of cysteine, the rate-limiting amino acid for glutathione synthesis [76,77]. In addition to glutamine, glucose contributes to glutathione synthesis via production of serine, which contributes to the pool of cysteine and glycine [22,78,79]. The antioxidant function of glutathione is mediated by glutathione peroxidases, which oxidize glutathione to reduce hydrogen peroxide or lipid peroxidases [80]. Regeneration of reduced glutathione is catalyzed by glutathione reductase, a reaction requiring NADPH, to which glutamine contributes via the malate-aspartate shuttle

[58,81]. Thus, glutamine plays a crucial role in protecting cells from oxidative stress. In addition, glutathione synthesis is required for cellular proliferation [82,83].

Glutathione is synthesized by glutamate cysteine ligase, which is composed of a catalytic (GCLC) and a modifier (GCLM) subunit, and by glutathione synthetase (GSS) [84]. Increases in glutathione levels are associated with increased expression of glutathione synthesis genes in liver where glutathione promotes growth [85]. Expression of these genes is stimulated by various stimuli, including oxidative stress and growth factors [86,87]. Signaling pathways, such as the phosphoinositide-3 kinase (PI3K) signaling pathway and the transcription factor Myc, discussed below, regulate expression of glutathione synthesis genes [88-92]. Moreover, the transcription factor, nuclear factor erythroid 2-related factor (NRF2), is the master regulator of the antioxidant stress response and induces expression of genes involved in ROS detoxification, including glutathione synthesis genes [91,93].

### *Hexosamines*

Like glucose, glutamine contributes to hexosamine biosynthesis. Hexosamine biosynthesis generates precursors for glycosylation of proteins [94,95]. Hexosamine synthesis also relies on nitrogen from glutamine in the conversion of fructose-6-phosphate to glucosamine-6-phosphate by glutamine fructose-6-phosphate amidotransferase (GFAT) (Figure 1.2) [96,97]. Synthesis of hexosamines, such as uridine diphosphate *N*-acetylglucosamine, plays a crucial role in cellular growth and proliferation, as glycosylation of cell surface receptors is necessary for nutrient uptake [97].

### *Alterations in glutamine metabolism in breast cancer*

Based on the realization that cancers rely on metabolism of glucose and glutamine, for proliferation, multiple attempts have been made to identify metabolic vulnerabilities that could be targeted for treatment of basal breast cancer. Thus, multiple studies have performed metabolomics analysis of human breast tumors and normal breast tissue with the goal of identifying metabolic alterations or target for cancer therapy [98,99]. Basal and luminal breast cancers display differences in metabolism – especially glutamine metabolism, *in vitro* and *in vivo*. For instance, basal breast cancer cells have been shown to take up more glutamine compared to luminal breast cancer cells, and these studies suggest that glutamine is utilized for glutaminolysis [100,101]. Consistent with this finding, Myc amplification and increased glutaminase expression is observed frequently in basal breast cancer cells and is associated with increased glutaminolysis [102,103]. In addition, suppression of anaplerosis with CB-839, a glutaminase inhibitor, or aminooxyacetate, a transaminase inhibitor, has a greater effect on basal-like breast cancer cells [103,104]. Lastly, the same effect was seen *in vivo* in a xenograft model of triple negative breast cancer. These findings suggest that targeting glutamine metabolism may be a viable option to treat the more aggressive basal-like breast cancers.

## **1.4 Oncogenic signaling contributes to metabolic reprogramming in cancer**

### **1.4 A. Overview**

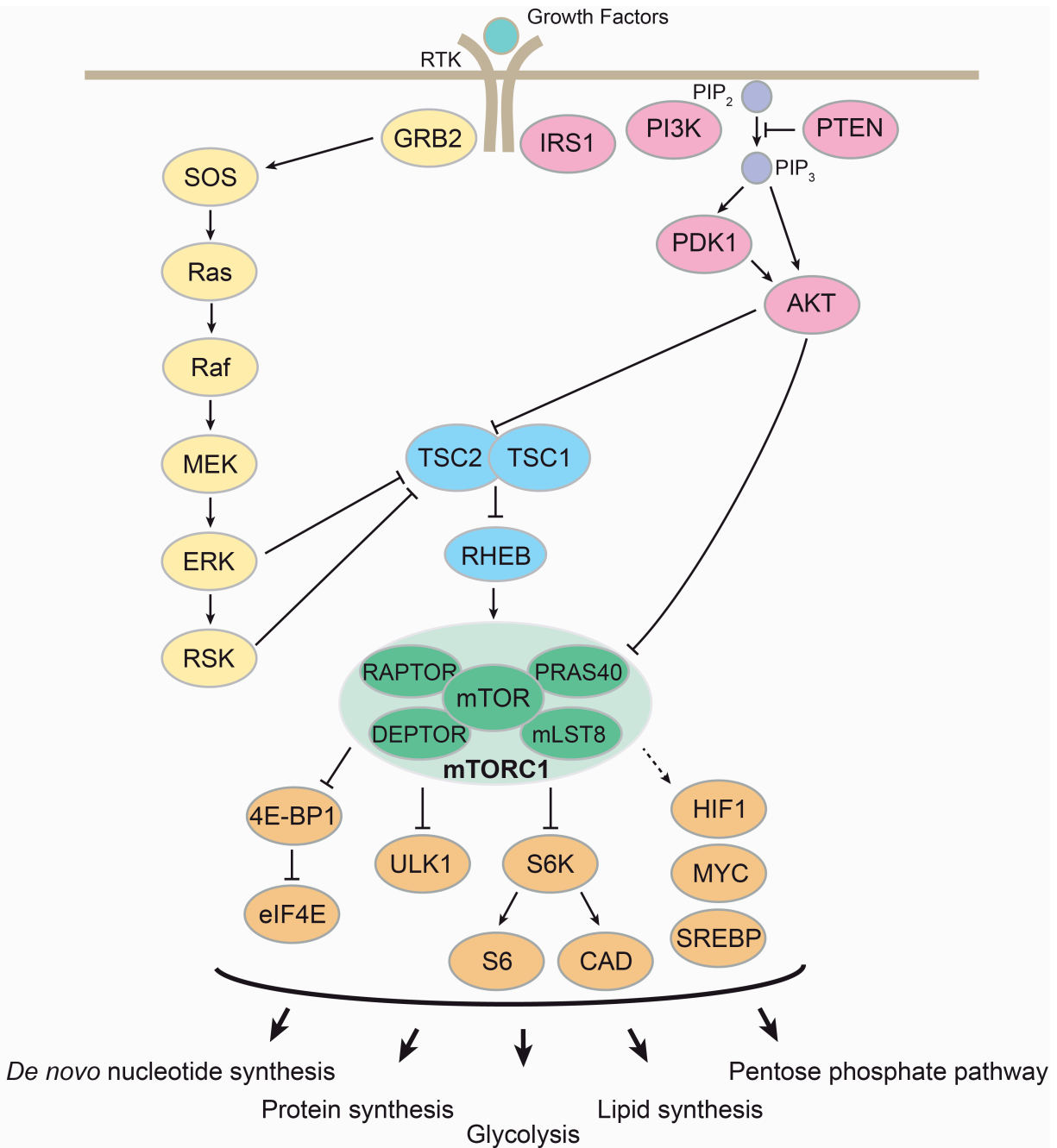
Metabolic rewiring allows a cancer cell to synthesize the biomass necessary to generate new cells. In a non-proliferating cell, metabolism is highly regulated to suppress proliferation. Many of the metabolic alterations in cancer cells mirror metabolism in rapidly proliferating cells, such as immune cells [105,106]. However, rapidly proliferating cells adjust metabolism in response to growth signals, whereas cancer cells have evolved mechanisms that allow them to

evade checkpoints to inhibit proliferation. Numerous studies have uncovered the regulation of various metabolic nodes by signaling cascades, such as the Ras, PI3K, and mechanistic target of rapamycin (mTOR) signaling pathways (Figure 1.3). Additionally, aberrant activation of these signaling pathways is common in cancer, and studies have revealed a new facet of oncogenic signaling that mediates metabolic reprogramming in cancer [26,107,108]. Oncogenic signaling by Ras, PI3K, and mTOR contributes to altered tumor metabolism, partly by regulating HIF1 or Myc [69,107,109-111]. Additionally, tumor suppressors, such as p53 and NRF2, are not discussed here but also rewire the metabolic landscape of a cancer cell [112,113]. Interestingly, all of these signaling pathways have been linked to sirtuins, which function to rewire metabolism in response to nutritional status.

#### **1.4 B. Ras signaling and cancer metabolism**

The Ras family of proteins controls differentiation, growth, and proliferation. Ras is a small GTPase, active in its GTP-bound form and inactive when bound to GDP [114]. Hyperactivation of this pathway, due to oncogenic mutations in the Ras protein, is common in cancer. The most common mutation is a point mutation in the Ras gene, resulting in a constitutively active Ras protein. Ras activates Raf, which phosphorylates and activates mitogen-activated protein kinases 1 and 2 (MEK1 and MEK2). MEK1 and MEK2 phosphorylate ERK1 and ERK2, which activate PI3K and Myc to alter cancer metabolism [114,115].

Ras signaling contributes to metabolic reprogramming in cancer. Cells transformed with oncogenic K-Ras depend on glucose and glutamine for survival and proliferation [58,110,116]. Oncogenic K-Ras signaling promotes increased glucose uptake and metabolism where glucose is diverted away from the TCA cycle [116]. Moreover, glutamine-tracing experiments have shown



### GROWTH AND PROLIFERATION

**Figure 1.3. The mTORC1 signaling pathway promotes growth and proliferation.** The Ras and phosphoinositide-3 kinase (PI3K) signaling cascades activate the mTORC1 signaling pathway. mTORC1 represses catabolism (autophagy) and promotes anabolism by phosphorylating its targets or stabilizing transcription factors known to stimulate production of biosynthetic intermediates to promote cellular growth and proliferation. RTK, receptor tyrosine kinase; PIP<sub>2</sub>, phosphatidylinositol (3,4)-bisphosphate; PIP<sub>3</sub>, phosphatidylinositol (3,4,5)-trisphosphate.

increased anaplerosis from glutamine with K-Ras transformation, highlighting how these fuels complement each other to promote cellular growth and proliferation [58,116]. Mechanistically, microarray data comparing non-transformed and K-Ras transformed cells showed increased expression of genes in glycolysis, nucleotide synthesis, and glutamine metabolism further supporting these findings [110]. A mouse model of oncogenic K-Ras driven pancreatic ductal adenocarcinoma supported the role of K-Ras in metabolic reprogramming [110]. In this mouse model, inactivation of K-Ras resulted in tumor regression, which was accompanied by a decrease in glucose uptake and lactate secretion, as well as a decrease in glycolytic intermediates known to be precursors for various biosynthetic pathways. Indeed, other anabolic pathways, such as the hexosamine biosynthesis and the nonoxidative arm of the PPP, are also activated by oncogenic K-Ras to promote protein glycosylation and ribose production for DNA and RNA synthesis. Knockdown of enzymes in the hexosamine and PPP pathway resulted in decreased xenograft tumor growth, supporting the regulation of these metabolic pathways by K-Ras to promote tumorigenesis. Lastly, metabolic gene expression decreases with K-Ras inactivation, which is also the case with MAPK inhibition and Myc knockdown, suggesting that regulation of these pathways by K-Ras contributes to metabolic reprogramming in cancer [110].

#### **1.4 C. Regulation of metabolism by Phosphoinositide-3 Kinase (PI3K) signaling**

In a normal cell, growth factors stimulate receptor tyrosine kinases to activate the PI3K signaling cascade [117]. PI3K is recruited to phosphorylated receptors where it is phosphorylated and activated. In addition, Ras signaling can stimulate PI3K signaling [114]. Activation of PI3K results in its localization to the plasma membrane where it converts phosphatidylinositol -4,5-biphosphate (PIP<sub>2</sub>) to phosphatidylinositol -3,4,5-triphosphate (PIP<sub>3</sub>) (Figure 1.3).

Phosphoinositide-dependent kinase 1 (PDK1) and protein kinase B (also known as AKT) are recruited to the plasma membrane by PIP<sub>3</sub>, which results in phosphorylation and activation of AKT [52,118]. As a result, AKT promotes various processes important to a cancer cell, such as survival, growth, proliferation, angiogenesis, metastasis, and metabolism [119,120].

Deregulation of the PI3K/AKT signaling pathway is common across various cancers. Activation of this pathway in cancer is often due to an activating mutation in the subunits of the PI3K complex, or inactivation/loss of phosphatase and tensin homolog (PTEN), a tumor suppressor [117]. Hyperactivation of this pathway increases glucose metabolism, via AKT, in cancer. In addition to promoting the localization of the glucose transporter GLUT1 to the plasma membrane, AKT increases the expression of and activates hexokinase 2 (HK2) [14]. Moreover, AKT phosphorylates phosphofructokinase 2 (PFK2) resulting in production of fructose-2,6-bisphosphate, which allosterically activates PFK1 to increase glycolysis [75]. AKT also stimulates *de novo* fatty acid synthesis by activating sterol regulatory element binding protein (SREBP) [121]. Lastly, AKT promotes Myc stabilization by repressing glycogen synthase kinase 3 (GSK-3), which phosphorylates Myc to target it for degradation [115].

#### **1.4 D. The mTORC1 signaling pathway promotes anabolism**

The mTOR signaling pathway regulates cellular growth, proliferation, and metabolism [122]. Like the Ras and PI3K/AKT pathways, mTOR signaling is deregulated in cancer [123,124]. mTOR is a highly conserved serine/threonine kinase found in two complexes in the cell, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Of the two complexes, more is known about the regulation of metabolism by mTORC1.



### *Upstream regulation of mTORC1 signaling*

mTORC1 is regulated by signals from growth factors, energy status, amino acids, and oxygen [125]. Most of these signals are integrated by the tuberous sclerosis complex (TSC), composed of TSC1 and TSC2, which functions as a GTPase activating protein (GAP) for the GTPase Rheb. In turn, in its GTP-bound state, Rheb activates mTORC1. mTORC1 is activated under nutrient rich conditions in the presence of growth factors and amino acids to promote growth and proliferation. As already mentioned, growth factors activate the PI3K and Ras signaling cascades, and signals from these pathways are sensed by the TSC complex to regulate mTORC1 activity [125]. Activated AKT phosphorylates and inhibits TSC2 to activate mTORC1. Likewise, ERK1/2 and RSK1, activated by the Ras signaling cascade, activate mTORC1 in the same manner [115,126,127]. In addition, amino acids, such as leucine and glutamine, are required for mTORC1 activation [128]. Import of leucine relies on export of glutamine via the Slc7a5 (LAT1) transporter, and inhibition of the glutamine transporter ASCT2 or LAT1 suppresses mTORC1 signaling. Lastly, under nutrient rich conditions, ATP is produced at a level necessary to support cell growth and proliferation. However, under nutrient-poor conditions, or under hypoxia, energy levels decline resulting in an increase in the AMP:ATP ratio. AMP activated kinase (AMPK), the energy status sensor of the cell, is activated when energy levels decrease, and AMPK negatively regulates mTORC1 by phosphorylating and activating TSC2 [129].

### *mTORC1 promotes growth and proliferation*

mTORC1 stimulation supports anabolic metabolism and represses catabolic processes to promote growth and proliferation (Figure 1.3) [130]. mTORC1 phosphorylates downstream

targets to regulate transcription, ribosome biogenesis, translation, and autophagy – processes important for biomass generation. Two well-known downstream effectors of mTORC1 are p70 ribosomal S6 kinase (S6K) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1), and phosphorylation of these targets promotes protein synthesis by activating mRNA and ribosome synthesis, as well as translation elongation. Moreover, mTORC1 phosphorylates and inactivates ULK1 and ATG13 to inhibit autophagy [131]. In addition, mTORC1 has been shown to regulate several metabolic pathways. Through S6K, mTORC1 stabilizes HIF1, SREBP1, and SREBP2 to increase expression of their metabolic targets and promote glucose uptake, glycolysis, *de novo* lipid synthesis, and the oxidative arm of the PPP. Recently, mTORC1 was shown to promote *de novo* synthesis of nucleotides necessary for nucleic acid synthesis. By activating S6K, mTORC1 stimulates *de novo* pyrimidine synthesis by activating carbamoyl-phosphate synthetase 2, aspartate transcarbamoylase, and dihydroorotase (CAD), the enzyme that catalyzes the first 3 steps of pyrimidine synthesis [132]. Additionally, mTORC1 stimulates activating transcription factor 4 (ATF4) to activate the mitochondrial tetrahydrofolate cycle via increased expression of methylenetetrahydrofolate dehydrogenase 2 (MTHFD2), which is the most frequently overexpressed enzyme in cancers [133,134]. Altogether, under nutrient-rich conditions mTORC1 stimulates biosynthetic pathways to generate crucial building blocks for growth and proliferation.

#### **1.4 E. Transcription factors downstream of signaling pathways mediate metabolic reprogramming**

Transcription factors have the ability to rewire metabolism by affecting expression of various genes, including metabolic genes. Although various transcription factors may be capable of altering gene expression to rewire tumor metabolism, it is well established that the

transcription factors: HIF1 and Myc play key roles in metabolic reprogramming of cancer cells [26,135-138].

### *HIF1 supports the Warburg effect*

The transcription factor HIF1 is composed of two subunits: HIF1 $\alpha$  and HIF1 $\beta$  (also known as the arylhydrocarbon receptor nuclear translocator, ARNT) [136,139]. The HIF1 $\alpha$  subunit is stabilized under low oxygen conditions (hypoxia). In the presence of oxygen, HIF1 $\alpha$  is hydroxylated by prolyl hydroxylases (PHD), a family of dioxygenases, activated by  $\alpha$ -ketoglutarate and inhibited by succinate [140,141]. Hydroxylation results in recruitment of the von Hippel-Lindau (VHL) tumor suppressor protein, an E3-ubiquitin ligase, which ubiquitinates HIF1 $\alpha$  and targets it for destruction by the proteasome [142]. Increased HIF1 protein levels are found in human tumors, such as pancreatic, breast, colon, and lung cancer [143]. Loss of VHL is observed in cancers, leading to HIF1 stability [144]. In addition, under normoxic conditions, mutations in other tumor suppressors, such as the mitochondrial enzymes succinate dehydrogenase or fumarate hydratase, also stabilize HIF1 potentially via increased  $\alpha$ -ketoglutarate levels [145,146]. HIF1 regulates cellular metabolism by inducing the expression of several glycolytic genes [136]. HIF1 contributes to increased glucose uptake by increasing the expression of the glucose transporter GLUT1. In addition, HIF1 induces glycolysis by stimulating expression of hexokinase (HK2), and an isoform of phosphofructokinase (PFK1), one of the three key regulatory enzymes in glycolysis. HIF1 also upregulates expression of pyruvate dehydrogenase kinase (PDK1) which inhibits PDH, and thus, HIF1 represses oxidative phosphorylation and promotes reliance on glycolysis [147,148]. Moreover, HIF1 supports regeneration of NAD<sup>+</sup> (required to maintain glycolysis) by increasing LDHA expression. Lastly,

HIF1 stimulates expression of the monocarboxylate transporter MCT4 to allow excretion of the high levels of lactate generated by high rates of glycolysis [135,137].

### *Myc promotes glucose and glutamine metabolism*

Elevated levels of the Myc transcription factor are observed in many tumors, such as Burkitt's lymphoma, breast cancer, and neuroblastoma [138,149,150]. Myc regulates a wide variety of biological processes, such as mitochondrial biogenesis, protein synthesis, and metabolism [107,151]. Recent studies show that Myc coordinates this wide range of cellular processes by amplifying transcription of already active genes in the cell [150,152]. Myc activates various facets of metabolism, including glucose and glutamine metabolism by directly binding to the promoters and increasing the expression of glycolytic and glutaminolytic genes. For instance, like HIF1, Myc upregulates transcription of glycolytic genes to increase glycolysis [58,110,138,151,153]. Additionally, Myc targets other metabolic enzymes, such as serine hydroxymethyltransferase (SHMT), which converts serine to glycine and is important for glutathione and nucleotide synthesis [151]. Thus, Myc supports the Warburg effect and promotes biogenesis.

Myc is a major driver of glutamine metabolism, and many cancer cells expressing high levels of Myc rely on glutamine for survival [154]. Myc regulates expression of genes involved in various nodes of glutamine-dependent metabolism, including glutaminolysis, nucleotide synthesis, and glutathione production. Myc promotes glutaminolysis in part by upregulating expression of the glutamine transporter ASCT2 contributing to increased glutamine uptake in cancer cells [49]. In addition, Myc stimulates glutaminolysis by increasing GLS1 expression in a mechanism that involves inhibition of a miRNA that targets a sequence in the GLS 3'

untranslated region, miR23a/b [155]. Myc directly binds to and induces expression of various genes along the *de novo* purine and pyrimidine synthesis pathways, such as the rate limiting enzymes, PPAT and CAD to generate nucleotides necessary for DNA and RNA synthesis. Thus, Myc may also play a role in regulating nucleic acid synthesis for a rapidly proliferating cell [149]. Moreover, Myc stimulates the expression of glutathione synthesis genes and is key for protection against oxidative stress [149]. By stimulating multiple glucose and glutamine-dependent metabolic pathways, Myc promotes growth and proliferation.

#### **1.4 F. Sirtuins as regulators of cancer metabolism**

##### *Sirtuins as metabolic sensors*

The sirtuin family of proteins has been implicated in numerous biological processes, as well as longevity and diseases of aging, such as diabetes and cancer [156-159]. Mammals express seven sirtuins (SIRT1-7), first identified by their homology to the yeast Sir2 protein [160]. Several protein targets through which this family of proteins mediates their function have been identified. The first two enzymatic activities identified for this group of proteins were ADP-ribosylation and deacetylation; however, more recently other enzymatic activities, such as demalonylation, desuccinylation, and decrotonylation have been identified [161,162]. Sirtuins depend on NAD<sup>+</sup> as a cofactor for enzymatic activity. As these proteins catalyze their reactions, they convert NAD<sup>+</sup> to nicotinamide and *O*-acetyl-ADP-ribose – of which nicotinamide functions as a competitive inhibitor of sirtuins. Levels of NAD<sup>+</sup> vary depending on the metabolic state of the cell. Under conditions, such as caloric restriction, fasting, or exercise, NAD<sup>+</sup> levels increase resulting in sirtuin activation and posttranslational modification of their target proteins. Thus sirtuins sense the metabolic status of the cell allowing them to adjust to stress conditions, such as a low energetic state [163,164].

### *Sirtuin subcellular localization*

Sirtuins localize to various cellular compartments, such as the nucleus (SIRT1, SIRT6, SIRT7), cytosol (SIRT2), and mitochondria (SIRT3, SIRT4, SIRT5), where several targets and the effect of their function have been identified. This localization endows this family of proteins the potential to coordinate cancer metabolism in various ways, from direct regulation of metabolic enzymes, in the mitochondria or cytosol, to transcriptional regulation of metabolic gene expression in the nucleus. Indeed, studies uncovered sirtuins to play critical roles in tumor metabolism. Of the seven sirtuins, the best studied sirtuins in tumor metabolism are two mitochondrial sirtuins (SIRT3 and SIRT4), as well as a nuclear sirtuin (SIRT6).

## **1.5 The role of mitochondrial sirtuin 3 (SIRT3) in physiology and metabolic reprogramming**

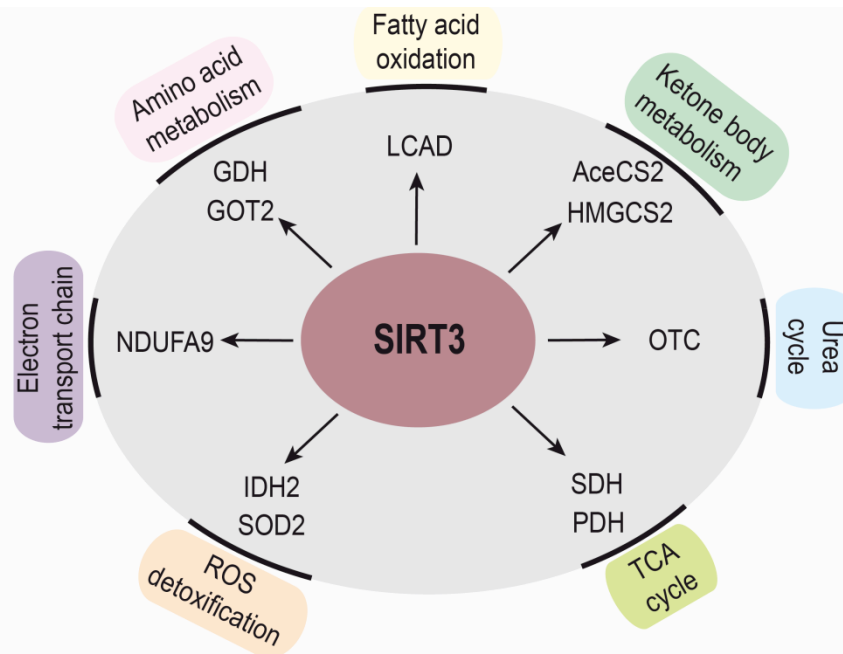
### **1.5 A. Overview**

The mitochondrion is a key metabolic hub that orchestrates multiple nodes of metabolism deregulated in cancer. Thus, in addition to oncogenes and tumor suppressors, mitochondrial enzymes are well poised to regulate tumor metabolism. Many mitochondrial enzymes are regulated by post-translational modifications. For instance, activity of GDH, succinate dehydrogenase (SDH), and isocitrate dehydrogenase (IDH) is regulated by lysine acetylation. Thus, determining how deacetylation of mitochondrial proteins regulates cancer metabolism is a growing area of interest. The mitochondrial sirtuin 3 (SIRT3) is a robust deacetylase that coordinates multiple facets of mitochondrial biology to maintain cellular homeostasis and protect from age-related diseases, including cancer.

### **1.5 B. SIRT3 regulates mitochondrial metabolism**

SIRT3 has multi-faceted control of metabolism. SIRT3 activates a mitochondrial program to promote energy production, oxidative metabolism, and redox homeostasis (Figure 1.4) [165]. First, SIRT3 mediates this metabolic control by deacetylating a number of mitochondrial proteins to boost their activity. Secondly, SIRT3 can affect cellular signaling pathways to control metabolism. The direct role of SIRT3 on cellular metabolism is clear from a wide-range of studies.

SIRT3 is a potent deacetylase, and in line with this, tissues from knockout (KO) mice display increased acetylation of mitochondrial proteins [166]. Several SIRT3 substrates have been identified and validated, and these enzymes are involved in mitochondrial metabolic pathways, such as the electron transport chain, TCA cycle, fatty acid oxidation, amino acid metabolism, and in maintenance of cellular redox homeostasis [167]. For example, SIRT3 stimulates energy production by deacetylating and activating pyruvate dehydrogenase (PDH) and components of the electron transport chain (NDUFA9 and SDH) [168-171]. Thus, low ATP levels have been observed in tissues with low SIRT3 levels [168,169]. Additionally, SIRT3 deacetylates long chain acyl coenzyme A dehydrogenase (LCAD) to promote fatty acid oxidation in the liver [172,173]. SIRT3 is also involved in the regulation of amino acid catabolism by activating GDH and promotes ammonia detoxification by deacetylating ornithine transcarbamoylase (OTC) and inducing the urea cycle [166,174,175]. Additionally, SIRT3 has recently been shown to deacetylate mitochondrial aspartate transaminase (GOT2) to destabilize its interaction with malate dehydrogenase and decrease activity of the malate aspartate shuttle and suppress pancreatic cancer [176]. By regulating these metabolic pathways, SIRT3 protects from development of various diseases [177].



**Figure 1.4. SIRT3 regulates several mitochondrial pathways to stimulate oxidative metabolism, energy production, and protection from oxidative stress.** In the mitochondria, SIRT3 directly deacetylates and activates multiple targets (within the gray circle) that play a role in amino acid metabolism, fatty acid oxidation, ketone body metabolism, urea cycle, the tricarboxylic acid (TCA) cycle, the electron transport chain, and reactive oxygen species (ROS) detoxification. NDUF9, NADH:Ubiquinone Oxidoreductase Subunit A9; GDH, glutamate dehydrogenase; GOT2, mitochondrial aspartate transaminase; LCAD, long-chain acyl-CoA dehydrogenase; AceCS2, acetyl-CoA synthetase 2; HMGCS2, 3-hydroxy-3-methylglutaryl-CoA synthase 2; OTC, ornithine transcarbamylase; SDH, succinate dehydrogenase; PDH, pyruvate dehydrogenase; IDH2, isocitrate dehydrogenase 2; SOD2, superoxide dismutase 2; ROS, reactive oxygen species.



In normal, primary tissues, SIRT3 has been implicated in the regulation of cellular signaling in heart and skeletal muscle, brown adipose tissue, and liver [169,178-180]. SIRT3 plays a role in response to cardiac hypertrophy as its expression increases in hearts of mice treated with hypertrophy agonists in part by activating the transcription factor Foxo3a to induce expression of antioxidant genes [178,181]. Induction of signaling cascades, such as the PI3K/Akt and MAPK/ERK pathways, also may contribute to the pathogenesis of cardiac hypertrophy, as these pathways were repressed in transgenic mice overexpressing SIRT3 [182]. In addition, SIRT3 repressed activation of Ras, which is upstream of PI3K and MAPK/ERK signaling. Finally, the mTOR signaling pathway was activated in hearts of SIRT3 null mice and this was abolished by overexpression of SIRT3 [178]. The mTORC1 signaling pathway is negatively regulated by AMP kinase (AMPK), and its regulator LKB1 [183]. In concordance with increased mTOR signaling, SIRT3 null mouse hearts have decreased phosphorylation and activity of AMPK and LKB1 [178]. Thus, SIRT3 plays a protective role during the response to cardiac hypertrophy.

In addition to regulating signaling in heart, SIRT3 plays a role in insulin signaling in skeletal muscle, which is important as SIRT3 KO mice have impaired insulin signaling, which is an early feature of type 2 diabetes [180]. However, cell signaling differs between skeletal muscle and heart. Skeletal muscle from SIRT3 null mice displays decreased insulin signaling evidenced by decreased phosphorylation of tyrosine residues in the insulin receptor (IR). In addition, SIRT3 loss decreases phosphorylation of IR targets, such as PI3K and AKT, which is accompanied by decreased glucose uptake [180]. Decreased ERK phosphorylation is also evident in skeletal muscle from SIRT3 null mice stimulated with insulin. Like hearts from SIRT3 null mice, skeletal muscle from these mice displays increased oxidative stress and increased expression of genes

involved in oxidative stress response, suggesting that increased ROS in the absence of SIRT3 may play a role in regulating signaling cascades, such as PI3K. Various kinases, such as the Jun N-terminal kinase (JNK), protein kinase C, and S6K, are activated by oxidative stress and phosphorylate the IR and IRS-1 to decrease PI3K activation [180,184]. Upon closer examination, skeletal muscle from SIRT3 null mice have increased phosphorylation of JNK, which is in agreement with observations in mouse embryonic fibroblasts from these mice. The study proposes increased ROS in SIRT3 null mouse tissue as a mechanism for activation of JNK and deregulation of insulin signaling [170,180]. As discussed above, these signaling pathways regulate tumor metabolism. However, whether SIRT3 controls signaling pathways, such as PI3K or mTOR signaling, remains unknown.

### **1.5 C. SIRT3 promotes protection from oxidative stress**

SIRT3 controls redox homeostasis by promoting reactive oxygen species (ROS) detoxification via activation of manganese superoxide dismutase 2 (SOD2) and isocitrate dehydrogenase 2 (IDH2) [185-189]. SOD2 scavenges superoxide and converts it to hydrogen peroxide, a substrate for catalase, which generates water and oxygen from hydrogen peroxide [188]. IDH2, a TCA cycle enzyme, decarboxylates isocitrate to produce  $\alpha$ -ketoglutarate under normal conditions [187]. This reaction generates reducing equivalents as it uses  $\text{NADP}^+$  to generate NADPH, which is essential for anabolic reactions and is used by glutathione reductase for regeneration of a major antioxidant, glutathione. Taken together, numerous studies suggest that SIRT3 coordinates a program to protect a cell from oxidative stress and cellular damage.

#### **1.5 D. SIRT3 represses the Warburg effect**

The importance of SIRT3 in human cancers is beginning to crystallize. First, decreased SIRT3 levels are observed across a wide range of human tumors, and particularly in breast cancer where SIRT3 gene deletions are also observed [190-197]. Compared to normal tissue, breast cancers have decreased SIRT3 protein expression [192]. Lastly, SIRT3 is undetectable in metastatic samples, suggesting that SIRT3 may play a role in repressing metastasis [191]. It is important to note that many tumors also display increased SIRT3 expression, which may indicate a pro-survival role for SIRT3 in these cancers [198,199]. It will be critical for future studies to elucidate the importance of SIRT3 deletion versus amplification based on cancer type. Moreover, it will also be important to determine if and whether SIRT3 suppresses tumorigenesis by inhibiting cancer initiation or cancer progression.

Cellular and mouse models have provided insight into the role of SIRT3 in repressing tumorigenesis, in part by suppressing metabolic rewiring [191,192,200]. First, compared to WT MEFs, SIRT3 null MEFs display features of cancer cells, such as rapid proliferation, aneuploidy, anchorage-independent colony formation, and allograft tumor formation in nude mice [191,192]. Moreover, SIRT3 null MEFs have higher superoxide levels under stress conditions – consistent with the role of SIRT3 in deacetylating and activating SOD2 [185,192]. Second, SIRT3 null mice spontaneously develop breast tumors at 12 months. Mammary tumors from SIRT3 null mice have increased protein damage, suggesting that these mice have increased levels of ROS [191]. Additionally, SIRT3 overexpression can inhibit growth of breast and pancreatic cell lines *in vitro* [192,196]. Together, these studies indicate that SIRT3 inhibits tumorigenesis of certain types of cancers.

### *How does SIRT3 regulate tumor growth?*

One mechanism is that SIRT3 deacetylates a specific target with strong tumor suppressive activity. One such target has not been identified. Based on published data thus far, it is more likely that SIRT3 deacetylates an array of targets that either change the metabolic status or redox of the cell. For example, SIRT3 plays a pivotal role in diminishing oxidative stress, in part, by regulating SOD2 and IDH2, but it may also control ROS production by regulating the efficiency of the ETC [81,185,186,201]. This particular arm of SIRT3 activity may have important roles in cancer, as many cancer cells have increased ROS levels in comparison with non-tumorigenic cells [202]. Paradoxically, chronic elevation of ROS in tumors may trigger important signaling cascades that promote cellular proliferation and survival (Figure 1.3) [203,204]. Indeed, numerous studies have observed that SIRT3 suppresses ROS. It was proposed that elevated ROS in SIRT3 null cells and mice contributes to increased genomic instability, leading to increased tumorigenesis.

### *How does SIRT3 regulate signaling?*

Our lab and the Guarente lab independently discovered that increased ROS in SIRT3 null cells results in HIF1 $\alpha$  stabilization. HIF1 $\alpha$  stability is regulated by prolyl hydroxylases (PHDs), which hydroxylate HIF1 $\alpha$  under normoxia to allow the von Hippel-Lindau protein (vHL) to ubiquitinate HIF1 $\alpha$ , leading to proteosomal degradation [141]. SIRT3 null MEFs demonstrated decreased HIF1 $\alpha$  hydroxylation, and overexpression of SIRT3 decreased hydroxylation in breast cancer cells. Importantly, although PHDs function as oxygen sensors, the regulation of PHD activity by SIRT3 occurred during normoxia.

As discussed above, elevated HIF1 activity has important consequences in the metabolism of human cancers and this fact is supported by studies of SIRT3. First, SIRT3 null MEFs display elevated glycolytic metabolism, which can be inhibited with antioxidants or by knockdown of HIF1 $\alpha$  [192,200]. Steady-state metabolomics analysis shows that the absence of SIRT3 alters glucose metabolism by increasing several intermediates in the glycolytic pathway and the PPP, suggesting rewiring of glucose metabolism and the potential for glucose to contribute to additional pathways to support synthesis of macromolecules for cellular proliferation. Glucose flux analysis yielded similar results [167]. Cells and tissues from SIRT3 KO mice demonstrate a signature of elevated HIF1 gene expression. Finally, expression of SIRT3 decreases HIF1 $\alpha$  levels, represses the Warburg effect, and decreases cell growth in breast cancer cells [192]. While these studies highlight HIF1 as a signaling pathway regulated by SIRT3, many other signaling pathways are controlled by mitochondria or ROS. Thus, it is important to examine: 1) whether SIRT3 affects other mitochondrial- rooted signaling pathways, and 2) if this control of other signaling pathways contribute to the tumor suppressive activity of SIRT3.

## **1.6 Scope of the dissertation**

The main goal of my dissertation was to identify mechanism(s) by which SIRT3 depletion promotes tumorigenesis to uncover novel vulnerabilities that may underlie in cancers with SIRT3 loss. In Chapter II, I uncover nucleotide metabolism as a vulnerability in breast cancer cells with low SIRT3. I collaborated with a visiting postdoctoral fellow and performed a small molecule screen to identify vulnerabilities in cells with SIRT3 deletion. The top hit from the screen was azaserine, a glutamine analog, which selectively decreased growth in the absence

of SIRT3. Steady-state metabolomics analysis revealed that azaserine significantly repressed elevated *de novo* purine synthesis in cells with SIRT3 loss. In agreement with the role of mTORC1 signaling in stimulating *de novo* nucleotide synthesis, I identify hyperactive mTORC1 signaling as part of the mechanism by which SIRT3 loss induces nucleotide synthesis. Moreover, in breast tumors, low SIRT3 expression is inversely associated with increased mTORC1 signaling. I show that SIRT3 represses growth of breast cancer cells in 3-dimensional culture and xenograft tumors. Lastly, treatment with azaserine or rapamycin strongly reduces growth of breast cancer cells with low SIRT3 levels. In conclusion, I utilized a chemical biology approach to identify a new metabolic vulnerability in tumors that lack SIRT3. My findings suggest that SIRT3 may be used as a biomarker for to identify cancer patients who may benefit from mTORC1 or nucleotide synthesis targeted therapies.

In Chapter III, I reveal deregulation of multiple nodes of glutamine metabolism in cells lacking SIRT3. Together with glucose, glutamine contributes for synthesis of key macromolecules necessary for proliferation. Although SIRT3 has been shown to suppress rewiring of glucose metabolism in breast cancer cells, whether SIRT3 played a role in regulating glutamine metabolism remained unknown. Given that SIRT3 loss results in elevated glutamine consumption (Chapter II), I utilized glutamine tracing and metabolomics to determine the effect of SIRT3 deletion on metabolism of glutamine. SIRT3 loss promotes increased entry of glutamine-derived carbon into the tricarboxylic acid (TCA) cycle, which is coupled with increased contribution of glutamine-derived nitrogen to production of nonessential amino acids. Moreover, my data demonstrate that SIRT3 represses glutaminolysis in breast cancer cells and that SIRT3 catalytic activity is necessary for this effect. Lastly, the increased glutamine taken up by the cell is used to support increased glutathione production, which promotes proliferation.

Altogether, my data show that SIRT3 represses multiple nodes of glutamine metabolism previously shown to be deregulated in cancer. These findings highlight additional glutamine-dependent pathways that may be altered in tumors with decreased expression or deletion of SIRT3.

Altogether, my findings further underscore the role of SIRT3 as a tumor suppressor in breast cancer via repression of glutamine metabolism. My work unveils novel potential vulnerabilities that may be targeted to suppress tumorigenesis of cancers with loss or decreased SIRT3 levels. I conclude by discussing the significance and implications of my work and propose future directions based on my findings.

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## CHAPTER II

### **Small molecule screen identifies nucleotide metabolism as a vulnerability in breast cancers with low SIRT3**

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## **2.1 Abstract**

Oncogenes and tumor suppressors underlie metabolic reprogramming to enhance tumor survival, growth, and proliferation. The mitochondrial deacetylase sirtuin 3 (SIRT3) has tumor suppressive function and represses the Warburg effect. Despite what is known about the role of SIRT3 in tumorigenesis, the challenge in translating our findings into developing better therapies for treatment of cancer remains. Here, we performed a small molecule screen and identified nucleotide metabolism as vulnerability in cells with SIRT3 loss. Deletion of SIRT3 results in increased *de novo* nucleotide synthesis, driven, in part, by hyperactive mechanistic target of rapamycin complex 1 (mTORC1) signaling. In breast cancer cells, we show that SIRT3 represses 3-dimensional (3D) sphere growth and mTORC1 signaling. Moreover, low SIRT3 expression is associated with increased mTORC1 activation and nucleotide synthesis gene expression in breast tumors. These findings reveal insights into mechanisms by which SIRT3 represses tumor metabolism and growth.

## **2.2 Introduction**

Tumors cells rely on metabolism of crucial nutrients, such as glucose and glutamine for growth, proliferation, and survival [1-4]. In fact, deregulated metabolism of these nutrients is a hallmark of cancer cells and is key to production of building blocks, such as nucleotides, amino acids, and lipids, necessary to generate new cells during proliferation [5-7]. Oncogenes, tumor suppressors, and aberrant signaling have been shown to underlie the metabolic switch observed in cancer [4,8-10].

Mitochondria are the metabolic hub of the cell, and many of the metabolic pathways reprogrammed in cancer are coordinated by this key organelle. The mitochondrial sirtuin 3 (SIRT3) is a potent deacetylase and a major regulator of multiple facets of biology [11-14].

SIRT3 resides in the mitochondria where it maintains cellular homeostasis by deacetylating and activating its targets to promote activation of mitochondrial metabolic pathways, energy generation, and protection against oxidative stress. For instance, SIRT3 induces fatty acid oxidation, amino acid catabolism, and ammonia detoxification by deacetylating and activating enzymes in these pathways [15-18]. Additionally, SIRT3 promotes ATP production by activating multiple components of the electron transport chain [19-23]. Lastly, SIRT3 protects against oxidative stress by stimulating clearance of reactive oxygen species (ROS) via activation of manganese superoxide dismutase 2 (SOD2) and isocitrate dehydrogenase 2 (IDH2) [24-31].

Numerous studies have unveiled the role of SIRT3 as a tumor suppressor in various types of cancer – especially in breast cancer [1,28,32-41]. Many cancer cells with loss or decreased expression of SIRT3 exhibit tumor-like phenotypes, such as metabolic rewiring, increased proliferation, and increased xenograft tumor size [28,32,36,42]. In fact, SIRT3 represses tumorigenesis by suppressing a major arm of tumor metabolism, glycolysis. For example in breast cancer cells, SIRT3 represses glycolysis by decreasing ROS to destabilize hypoxia inducible factor 1 (HIF1), which contributes to reprogramming of this metabolic pathway [28,36,42]. Deletion of the SIRT3 locus has been observed across multiple types of human tumors where SIRT3 overexpression suppresses tumorigenesis.

However, beyond glycolysis, other nodes of metabolism regulated by SIRT3 in cancers are poorly defined. Moreover, although low SIRT3 activity is a feature of many breast cancers, there is no pharmacological intervention to target tumors with low SIRT3 levels. Therefore, with the push for personalized medicine, developing a better understanding of the mechanism(s) employed by tumor cells with decreased levels of SIRT3 is key to identifying new vulnerabilities and potential therapeutic targets to treat these cancers. Given that SIRT3 loss mirrors phenotypes



of cancer cells, in this study, we took a chemical biology approach and performed a small molecule screen to identify targetable pathways to selectively inhibit SIRT3 KO cell proliferation. Our goal was to identify novel vulnerabilities in tumors with low SIRT3, as well as to gain basic mechanistic insights into additional pathways regulated by this sirtuin.

## **2.3 Results**

### *Small molecule screen identifies increased dependency on glutamine in SIRT3 KO cells*

In order to identify novel vulnerabilities in cells with low SIRT3, we performed a high throughput small molecule screen using immortalized wild type (WT) and SIRT3 KO MEFs, which we have previously shown to recapitulate the metabolic reprogramming observed in tumor cells lacking SIRT3 [28,43-45]. We screened the known bioactives library at the Harvard Institute of Chemistry and Cell Biology (ICCB) Longwood screening facility, in order to identify compounds which selectively inhibited the growth of SIRT3 null cells, compared with WT cells (Figure 2.1A). Of approximately 8,000 compounds tested, 108 passed our screening criteria to inhibit the growth of SIRT3 KO MEFs by 50% more than WT MEFs, without decreasing WT cell viability below 20% (Figure 2.1B, Table S2.1). From these, 50 compounds were validated in a dose response curve (Figure S2.1A-D). Notably, the top scoring compound was azaserine, which inhibited the growth of SIRT3 KO cells with an IC<sub>50</sub> of 2.9  $\mu$ M, which was 10 fold lower than WT MEFs. As azaserine is structurally similar to glutamine, and SIRT3 loss is associated with fuel reprogramming, we hypothesized that the identification of azaserine in this screen may highlight a dependency in glutamine metabolism in SIRT3 KO MEFs (Figure 2.1C). We tested this idea using a multifaceted approach. First, we treated cells with another glutamine analog, 6-diazo-5-oxo-L-norleucine (DON), and found that DON likewise inhibits proliferation in SIRT3

**Figure 2.1. Chemical screen identifies glutamine metabolism as a vulnerability in SIRT3 KO cells.** (A) Layout of experimental design for the small molecule screen. (B) Primary screen data highlighting the top 5 compounds inhibiting SIRT3 KO cell growth. (C) Schematic of the structures of glutamine and its analogs, azaserine and 6-Diazo-5-oxo-L-norleucine (DON). (D) Dose response curves of WT and SIRT3 KO MEF growth after treatment with azaserine or (E) DON for 72 hours. (F) Cell viability measured by propidium iodide incorporation in WT and SIRT3 KO MEFs after 72 hours of glutamine deprivation. (G) Relative growth of WT and SIRT3 KO MEFs treated with 30  $\mu$ M azaserine compared to control for 4 days. (H) Clonogenic growth assay of Kras (G12V) transformed WT and SIRT3 KO MEFs treated with DMSO (as a control) or 30  $\mu$ M azaserine for 8 days. (I) Quantification of colony formation assay using ImageJ to calculate the area of each well covered by WT or SIRT3 KO MEF colonies (n = 3). (J) Glutamine uptake and (K) ammonium production in WT or SIRT3 KO MEFs after 24 hours.

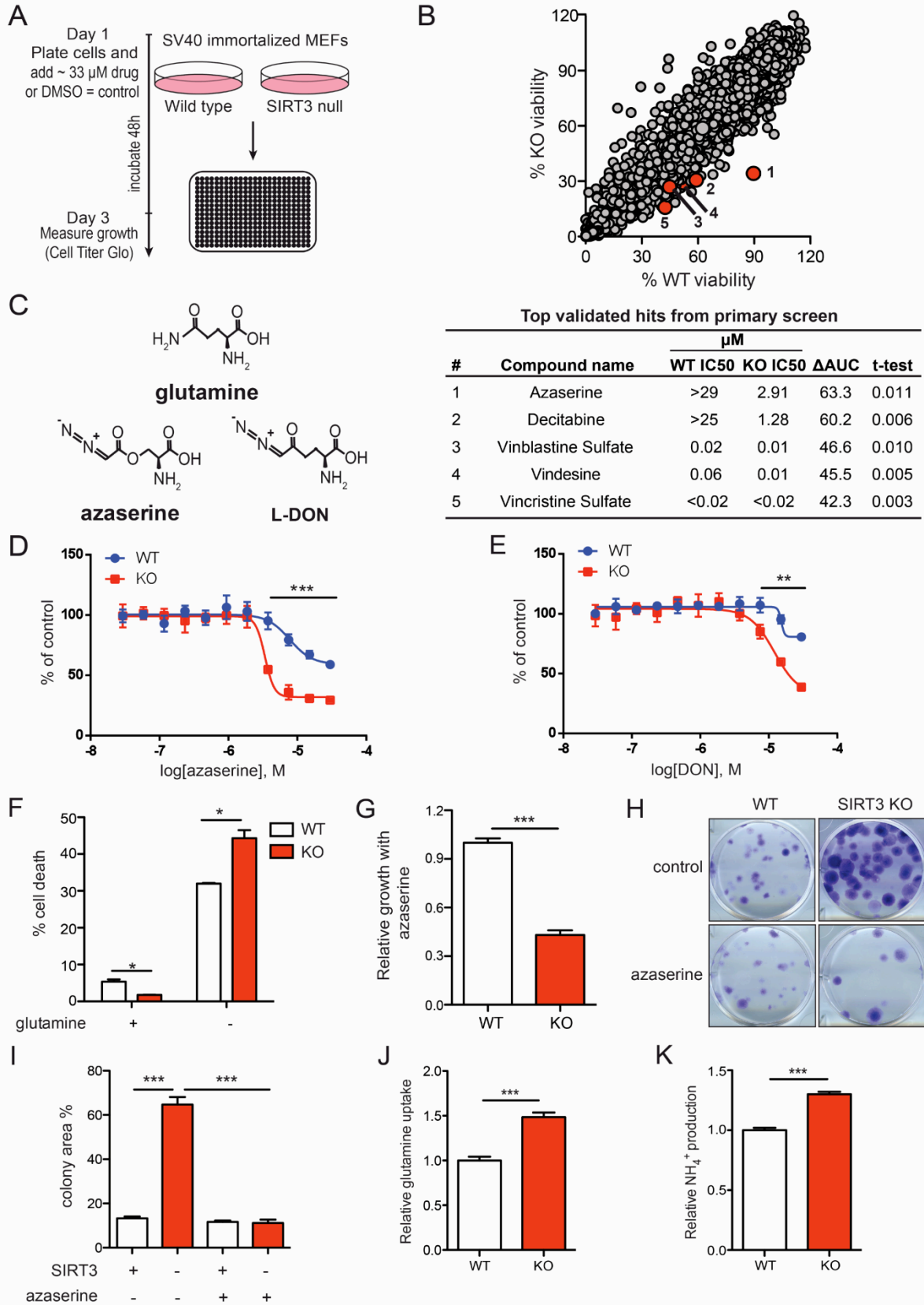


Figure 2.1 (Continued)

KO MEFs to a greater extent compared to WT MEFs (Figure 2.1D-E). Next, we probed whether SIRT3 null cells were more susceptible to glutamine deprivation. Indeed, SIRT3 KO MEFs died 15% more compared to WT cells when deprived of glutamine (Figure 2.1F). To assess the role of azaserine in regulating proliferation, we examined growth in the presence of azaserine, which preferentially inhibited SIRT3 KO MEF proliferation (Figure 2.1G). Last, to determine the effect of azaserine on tumorigenic potential, we performed colony formation assays using K-Ras transformed SIRT3 WT and KO MEFs. Consistent with previous findings, SIRT3 KO MEFs formed more colonies than WT MEFs [1,44]. Although azaserine slightly decreased the number of colonies formed by WT cells, it was apparent that azaserine significantly decreased the number of colonies formed by SIRT3 KO cells (Figure 2.1H-I). These findings suggested an increased reliance of SIRT3 KO cells on glutamine for survival and proliferation and a potential deregulation of glutamine usage.

#### *SIRT3 loss results in altered glutamine usage*

To probe whether glutamine utilization was altered by SIRT3, we examined fuel uptake. Indeed, we observed an increase in glutamine consumption in SIRT3 KO MEFs compared to WT MEFs (Figure 2.1J). Glutamine can be metabolized to fuel the TCA cycle where glutaminase (GLS) converts glutamine to glutamate, the substrate of glutamate dehydrogenase (GDH) to generate  $\alpha$ -ketoglutarate, both steps which generate ammonium. In addition, SIRT3 KO MEFs secreted higher amounts of ammonia compared to WT MEFs, consistent with the idea of increased glutamine metabolism (Figure 2.1K). The same effect was observed upon silencing of SIRT3 with lentiviral shRNA in an immortalized breast cell line, MCF10A (Figure S2.1E-F). The effect of SIRT3 loss on glutamine consumption is similar to that observed in cells with

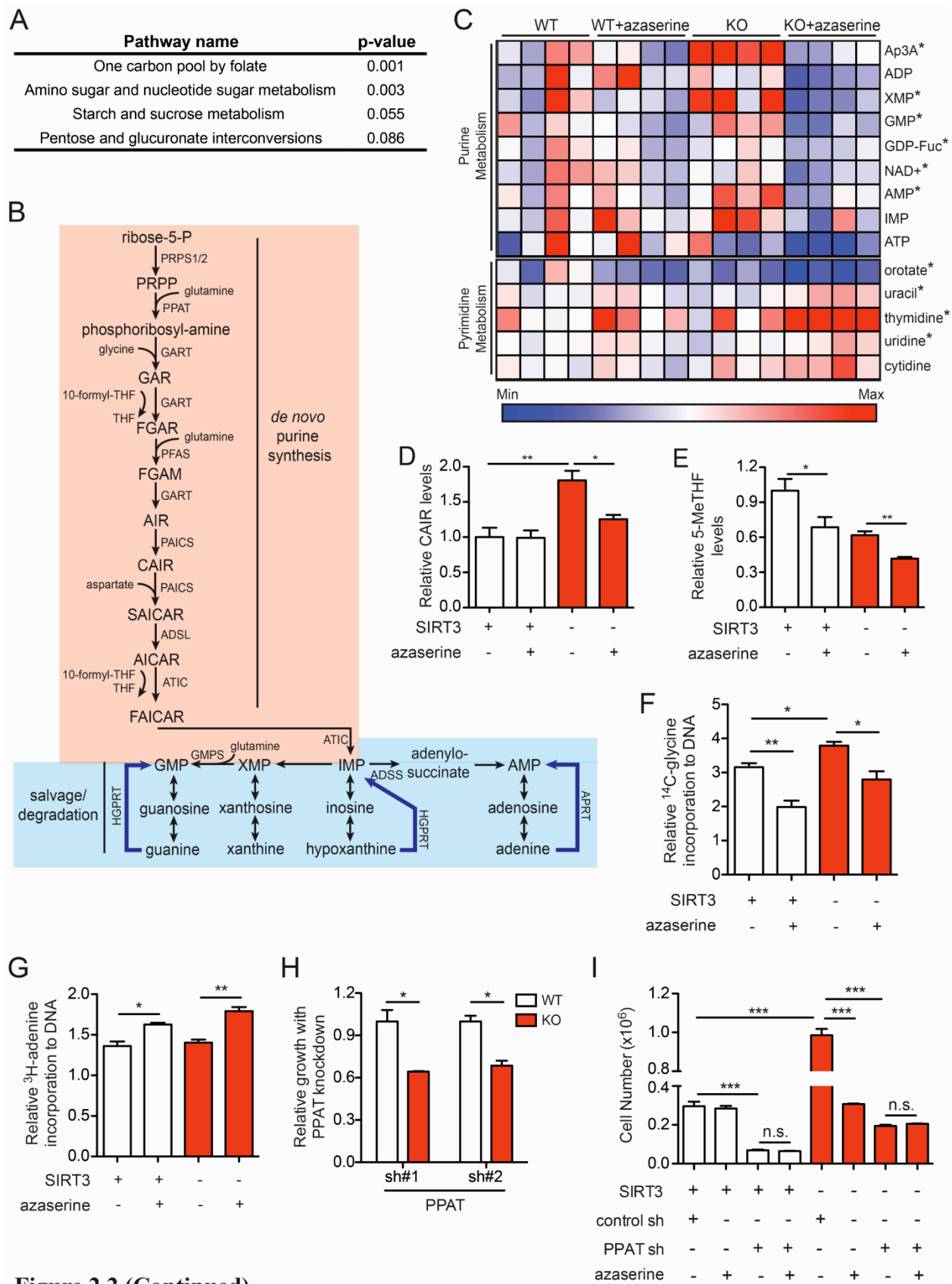
hyperactive mTOR signaling [1,5,28]. Moreover, stable overexpression of SIRT3 in SIRT3 null cells resulted in decreased glutamine uptake and ammonium secretion (Figure S2.1G-H). In sum, an unbiased, small molecule screen of sensitivity in SIRT3 KO cells revealed glutamine metabolism as a novel metabolic vulnerability in cells with low SIRT3.

*Azaserine has a drastic effect on de novo nucleotide synthesis in SIRT3 KO cells*

Glutamine is an anaplerotic substrate that contributes to various nodes of metabolism to generate key building blocks, such as nucleotides, nonessential amino acids (NEAAs), and lipids, to support proliferation. Considering that glutamine fuels various metabolic pathways, we sought to identify the metabolic vulnerability that renders SIRT3 KO cells susceptible to azaserine by performing a steady-state metabolomics analysis in WT and SIRT3 KO MEFs. Thus, we first examined the acute metabolic response to azaserine after 6 hours to determine the most immediate effect of the drug on the cells. The effect of azaserine on the metabolic profile of SIRT3 KO cells was dramatic; the levels of 22 metabolites were significantly affected compared to only 3 metabolites in WT cells (Table S2.2). Next, to identify the metabolic node most significantly affected by azaserine, we utilized MetaboAnalyst 3.0 to perform an unbiased metabolic pathway enrichment analysis and identified a profound effect of azaserine on nucleotide metabolism, a glutamine-dependent pathway, in SIRT3 KO cells (Figure 2.2A-B and S2.2A-B). This was evidenced by the fact that half of the differentially affected metabolites in SIRT3 KO MEFs play a role in purine or pyrimidine metabolism (Table S2.2). Treatment with

**Figure 2.2. Azaserine inhibits increased de novo purine synthesis in SIRT3 KO MEFs.**

(A) Table of the metabolic pathways significantly affected by a 6 hour treatment with 30  $\mu$ M azaserine in SIRT3 KO MEFs. The pathway analysis module in MetaboAnalyst 3.0 was utilized for the analysis. (B) Schematic of purine metabolism. Shaded in pink is the *de novo* purine synthesis pathway, and shaded in blue are intermediates in purine degradation or the salvage pathway. Blue arrows represent purine salvage pathways. (C) Heat map comparing metabolite levels in the presence or absence of azaserine in WT and SIRT3 KO MEFs (n = 4) treated with DMSO (control) or 30  $\mu$ M azaserine for 6 hours. Red indicates upregulation and blue indicates downregulation. (D) Relative levels of carboxyaminoimidazole ribonucleotide (CAIR) and (E) 5-MeTHF in WT and SIRT3 KO MEFs after treatment with 30  $\mu$ M azaserine for 6 hours. (F) Incorporation of  $^{14}$ C-glycine, or (G)  $^3$ H-adenine into DNA in WT and SIRT3 KO cells in the presence or absence of 30  $\mu$ M azaserine for 8 hours. (H) Growth of WT and SIRT3 KO MEFs with PPAT knockdown relative to control cells and to WT cells with the corresponding PPAT shRNA. (I) Relative growth of WT and SIRT3 KO MEFs treated as specified.



azaserine resulted in a significant decrease in the products of *de novo* purine synthesis, such as AMP, ADP, XMP, and 5'-GMP, in SIRT3 KO MEFs (Figure 2.2C). Additionally, levels of a *de novo* purine synthesis intermediate, carboxyaminoimidazole ribonucleotide (CAIR), were significantly higher in SIRT3 KO MEFs compared to WT MEFs and significantly decreased in SIRT3 KO cells upon treatment with azaserine (Figure 2.2D). These findings are consistent with previous studies showing that azaserine inhibits *de novo* purine synthesis [8,46,47].

As pyrimidines complement purines for DNA synthesis, we examined the effect of azaserine on pyrimidine metabolism. In addition to reducing *de novo* purine synthesis intermediates, azaserine significantly decreased *de novo* pyrimidine synthesis intermediates. N-carbamoyl aspartate, a key intermediate in *de novo* pyrimidine synthesis was significantly increased in SIRT3 KO cells compared to WT cells and significantly depleted by azaserine treatment in both WT and SIRT3 KO cells (Figure S2.2B-C). Orotate, another *de novo* pyrimidine synthesis intermediate, was reduced by 50% with azaserine treatment (Figure S2.2D). Moreover, folate metabolism, which plays an important role in *de novo* purine and pyrimidine synthesis by contributing to the formation of the purine ring and synthesis of dTMP for DNA synthesis, was likewise significantly decreased by azaserine in WT and SIRT3 KO cells (Figure 2.2E). Surprisingly, in contrast to *de novo* nucleotide synthesis, pyrimidine salvage intermediates were significantly increased by azaserine treatment in SIRT3 KO cells (Figure 2.2C). Taken together, our results indicate that azaserine significantly inhibits nucleotide synthesis in SIRT3 KO cells, with its major effect being suppression of the *de novo* purine synthesis pathway.



### *SIRT3 loss stimulates de novo nucleotide synthesis*

Given that azaserine suppresses the levels of metabolites in *de novo* nucleotide synthesis in SIRT3 KO MEFs, we hypothesized that SIRT3 may play a role in regulating this metabolic pathway. Glycine and aspartate contribute to formation of the purine and pyrimidine rings during *de novo* nucleotide synthesis. Thus, to directly examine whether SIRT3 had an effect on *de novo* purine or pyrimidine synthesis, we measured incorporation of radiolabeled carbon from  $^{14}\text{C}$ -glycine or  $^{14}\text{C}$ -aspartate into DNA, respectively. Consistent with increases in *de novo* purine synthesis intermediates, SIRT3 KO cells displayed increased incorporation of radiolabeled glycine into DNA (Figure 2.2F). As a control, we measured the incorporation of radiolabeled adenine into DNA to assess the purine salvage pathway and global DNA synthesis. No difference in the salvage pathway was observed upon stimulation of *de novo* purine synthesis between WT and SIRT3 KO MEFs (Figure 2.2G). Additionally, upon stimulation of *de novo* pyrimidine synthesis, SIRT3 KO MEFs displayed increased incorporation of radiolabeled aspartate into DNA compared to WT MEFs (Figure S2.2E). The increase in *de novo* pyrimidine synthesis was accompanied by a slight increase in the pyrimidine salvage pathway based on incorporation of radiolabeled thymidine (Figure S2.2F). Moreover, given that azaserine decreased *de novo* purine synthesis intermediates, we probed the effect of azaserine using this assay. Azaserine significantly dampened *de novo* purine synthesis evidenced by decreased incorporation of glycine into DNA in both WT and SIRT3 KO cells (Figure 2.2F-G). These results show that loss of SIRT3 promotes *de novo* synthesis of nucleotides – key building blocks for DNA and RNA synthesis, which are crucial for proliferation.

### *Inhibition of de novo purine synthesis significantly decreases SIRT3 KO cell proliferation*

Because the major effect of azaserine was to decrease purine synthesis products, we next tested the idea that elevated purine synthesis would provide a proliferative advantage to SIRT3 KO cells. The first rate-limiting enzyme in *de novo* purine synthesis is phosphoribosyl pyrophosphate amidotransferase (PPAT) [11,13,48]. To determine if SIRT3 KO cells are susceptible to inhibition of *de novo* purine synthesis, we examined the role of PPAT knockdown on WT and SIRT3 KO cell proliferation (Figure S2.2G). PPAT knockdown significantly decreased proliferation of SIRT3 KO cells compared to WT cells (Figure 2.2H-I). Additionally, PPAT knockdown combined with azaserine treatment did not have a further effect on WT or SIRT3 KO cells suggesting that a major effect of azaserine on proliferation is via inhibition of purine synthesis. Altogether, these findings show that elevated *de novo* purine synthesis contributes to the increased proliferation, especially in the absence of SIRT3.

### *SIRT3 KO cells have hyperactive mTORC1 signaling*

To better understand the sensitivity of SIRT3 KO cells to azaserine and identify pathways significantly altered in SIRT3 KO cells that could be the underlying mechanism of increased susceptibility to glutamine metabolism targeted drugs we performed RNA sequencing (RNA Seq) of WT and SIRT3 KO MEFs. Using the DESeq2 differential expression algorithm, we found differential expression of 2,650 between WT and SIRT3 KO MEFs [1,15,28,36]. We utilized MotifADE, a previously described approach to perform an unbiased transcription factor binding motif enrichment analysis using the genes that were significantly upregulated or downregulated in SIRT3 KO MEFs [19,21,41]. Using this approach we found enrichment of binding sites for 11 transcription factors in SIRT3 KO MEFs compared to WT MEFs with an

adjusted p-value of less than 0.05 (Table 2.1). Given that SIRT3 loss has been shown to result in stabilization of hypoxia inducible factor 1 (HIF1) known to contribute to increased glycolytic gene expression, we expected to find enrichment of the HIF1 binding motif, as well as additional transcription factors known to contribute to metabolic reprogramming in cancer. In agreement with preliminary findings, we found enrichment of Myc and the hypoxia response element to which HIF1 $\beta$  binds [1,24,26,28,36]. Furthermore, we identified enrichment of the SREBP binding motif in SIRT3 KO cells. These transcription factors have been shown to regulate metabolic rewiring in cancer cells and are known to operate downstream of mTORC1 to regulate metabolism [32,34,35,49].

Given that, in addition to elevated glutamine consumption, nucleotide synthesis, and rapid proliferation, SIRT3 KO MEFs have increased glucose metabolism supported by stabilization of HIF1 – phenotypes consistent with active mTORC1, we hypothesized that SIRT3 loss could result in stimulation of the mTORC signaling pathway. To test this, we first examined the phosphorylation status of downstream targets of mTORC1 in WT and SIRT3 KO MEFs. As a control, we starved WT and SIRT3 KO cells of growth factors to dampen cellular signaling. As expected, WT MEFs displayed decreased phosphorylation of downstream mTORC1 targets, such as carbamoyl-phosphate synthetase 2, aspartate transcarbamoylase, dihydroorotase (CAD), p70 S6 kinase (S6K), and ribosomal protein S6 (S6) (Figure 2.3A). Strikingly, SIRT3 KO cells were insensitive to growth factor deprivation and had three fold higher mTORC1 signaling compared to WT cells under serum starvation (Figure 2.3A). As expected, under basal conditions, in the presence of growth factors, we observed phosphorylation of mTORC1 targets in WT cells. In contrast to WT cells, SIRT3 KO cells displayed even higher phosphorylation levels. Likewise,

Table 2.1. Enrichment of transcription factor binding sequences in promoters of differentially expressed genes in SIRT3 KO cells.

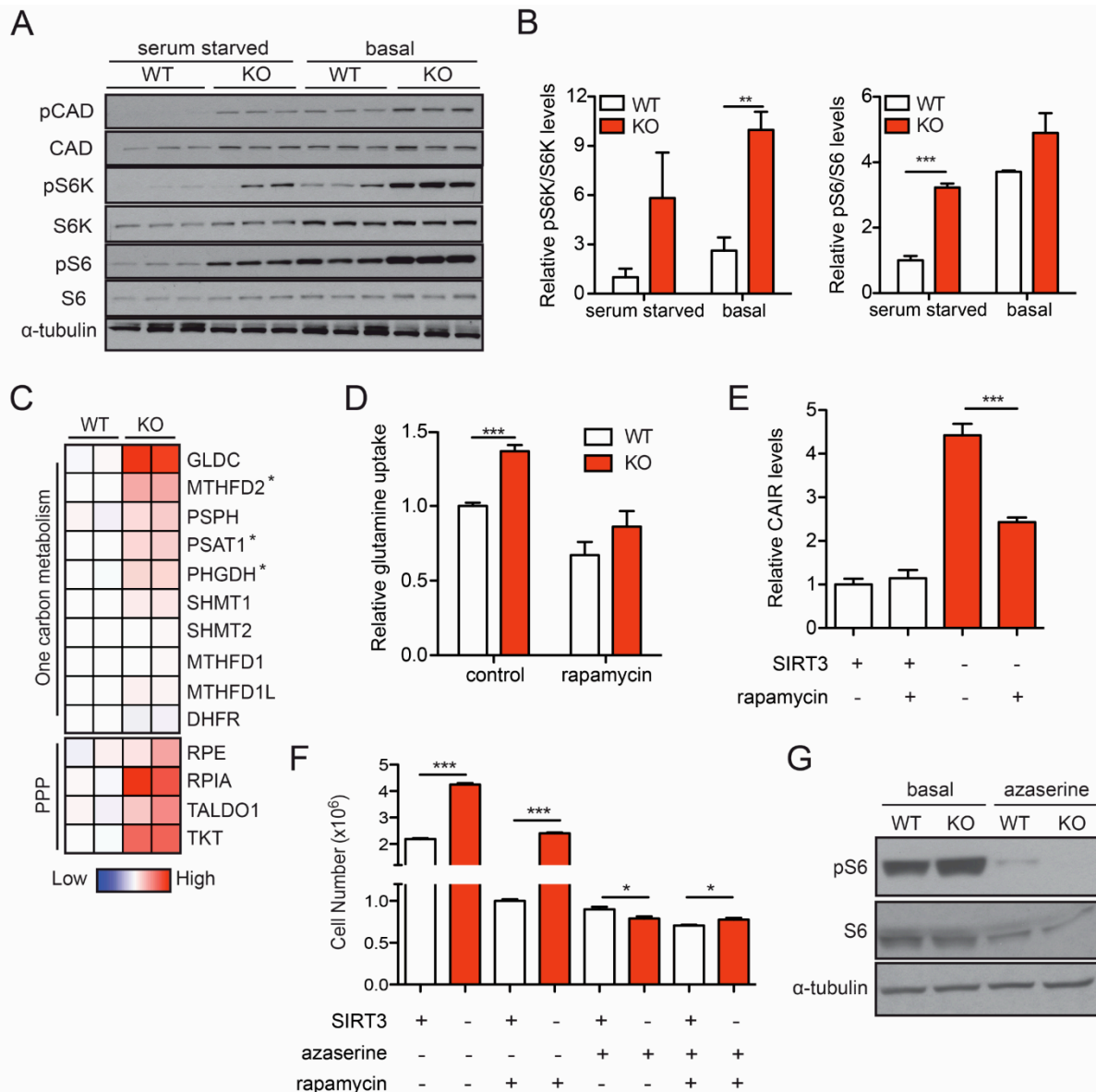
Transcription Factor:	Binding Motif:	Frequency:	P Value:	Adjusted P Value:
E2F	THVGC GCGGAAAAAHR	0.7551	3.48E-06	0.004
Pax-3	TCGTCACRCYYHM			
ATF3	NBSTGACGTCANCS			
HTF	VDHHWDNDMCACGTCABYNHWNDN	0.4488	2.61E-05	0.027
E4F1	SHTACGTCAC			
CREB	CGTCAB			
Myc	CACGTGB			
N-Myc	NNNCACGTGNNN			
c-Myc:Max	NNNNNNCACGTGNNNNNN / NDASCACGTGSTHN			
USF	NCACGTGN / NNRHCACGTGDYNN	0.5843	3.81E-05	0.039
SREBP-1	NATCACGTGAB			
ARNT	NDDDNACACGTGNNNN / NVNNNRWCACGTGWYNNNBN			
HTF	VDHHWDNDMCACGTCABYNHWNDN			

knockdown of SIRT3 in MCF10A mammary cells resulted in increased phosphorylation of ribosomal protein S6 (Figure S2.3A). Moreover, mTORC1 is known to promote cellular growth, and concordant with elevated signaling, SIRT3 KO cells were bigger than WT cells in the presence or absence of serum (Figure S3B). Taken together, these data demonstrate that SIRT3 loss results in hyperactive mTORC1 signaling.

The mTOR signaling pathway integrates cues from various inputs, such as growth factors which activate the phosphoinositide 3-kinase (PI3K) and Ras signaling cascades to activate mTORC1. Thus, we probed for substrates of PI3K and Ras signaling to examine whether these pathways were altered in the absence of SIRT3. Compared to WT cells, these pathways are hyperactivated to the same extent as the mTORC1 pathway in SIRT3 KO cells (Figure S2.3C and S2.3D). These results indicate that loss of SIRT3 results in stimulation of PI3K and Ras signaling, which converge on the mTORC1 signaling cascade.

#### *Hyperactive mTORC1 signaling in SIRT3 KO cells contributes to alterations in nucleotide metabolism*

Next, because the mTORC1 signaling cascade stimulates the expression of genes along multiple metabolic pathways that contribute to nucleotide synthesis, such as the pentose phosphate pathway (PPP) and one carbon metabolism, we examined the expression of genes in these pathways, as well as in the *de novo* nucleotide synthesis pathways in our RNA Seq data [32,42,50,51]. We found that expression of genes in the PPP and *de novo* purine, and pyrimidine synthesis was 1.4 to 3.5 fold higher in the absence of SIRT3 (Figure 2.3C and S2.3E). Strikingly, various genes in one carbon metabolism were significantly increased in SIRT3 KO cells compared to WT cells (Figure 2.3C and S2.3E). Of the genes in one carbon metabolism,



**Figure 2.3. Hyperactive mTORC1 signaling in the absence of SIRT3 contributes to increased de novo purine synthesis.** (A) Immunoblots of proteins phosphorylated upon mTORC1 activation in WT and SIRT3 KO MEFs after 16 hours of serum starvation or nutrient replete conditions. (B) Quantification of immunoblots in (A). (C) Heat map of expression of genes in one carbon metabolism and the pentose phosphate pathway (PPP) from RNA Seq data on WT and SIRT3 KO MEFs. Stars represent significant difference in gene expression between WT and SIRT3 KO cells. (D) Glutamine uptake in WT and SIRT3 KO MEFs treated with 100 nM rapamycin or DMSO for 24 hours (n = 6). (E) Carboxyaminoimidazole ribonucleotide (CAIR) levels in WT and SIRT3 KO MEFs treated with DMSO or rapamycin for 24 hours. (F) Proliferation of WT and SIRT3 KO MEFs treated with rapamycin (100 nM), azaserine (30 μM) or DMSO (as a control) for 4 days. (G) Western blot of WT and SIRT3 KO MEFs treated with 30 μM azaserine for 24 hours. Data are means ± SEM (n = 2-4). \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001.

methylenetetrahydrofolate dehydrogenase (MTHFD2), was recently shown to be regulated by mTORC1 to stimulate *de novo* purine synthesis, and in contrast to WT cells, expression of MTHFD2 was significantly increased in SIRT3 KO cells [42,52,53]. These results show that SIRT3 loss mirrors phenotypes observed in cells with hyperactive mTORC1 signaling.

Given that mTORC1 regulates *de novo* nucleotide synthesis, we hypothesized that hyperactive mTORC1 signaling in the absence of SIRT3 may contribute to altered nucleotide metabolism in these cells. To test this hypothesis, we examined whether elevated mTORC1 contributed to increased glutamine consumption and rewiring of nucleotide metabolism in the absence of SIRT3 by inhibiting mTORC1 signaling using rapamycin (Figure S2.3F). In agreement with the fact that mTORC1 promotes glycolysis, inhibition of mTORC1 decreased and normalized glucose uptake and decreased lactate production in WT and SIRT3 KO cells (Figure S2.3G and S2.3H). Moreover, rapamycin decreased glutamine uptake in WT and SIRT3 KO cells, thereby abolishing the difference in glutamine usage between these cells (Figure 2.3D). Importantly, rapamycin treatment specifically and significantly decreased levels of *de novo* purine and pyrimidine synthesis intermediates in SIRT3 null cells (Figure 2.3E and S2.3I). These results suggest that increased mTORC1 signaling in the absence of SIRT3 contributes to increased nucleotide synthesis and metabolic reprogramming.

Because SIRT3 loss stimulates mTORC1 signaling, which promotes anabolic growth and proliferation, we next examined whether mTORC1 signaling contributed to increased cell size or proliferation in SIRT3 KO cells. Treatment with rapamycin normalized cell size between WT and SIRT3 KO cells (Figure S2.3J). However, mTORC1 inhibition did not mitigate the difference in proliferation between WT and SIRT3 KO cells (Figure 2.3F). To test whether hyperactive mTORC1 contributed to increased susceptibility of SIRT3 KO cells to azaserine, we

treated WT and SIRT3 KO MEFs with rapamycin and azaserine and measured proliferation. To our surprise, the drug combination modestly decreased WT cell growth, but had no further effect on SIRT3 KO cell proliferation when compared to azaserine treatment alone (Figure 2.3F), indicating that azaserine may repress proliferation, in part by inhibiting mTORC1. Glutamine metabolism has been shown to be necessary for mTORC1 activation [43-45,54,55]. Because rapamycin had no further effect on repressing SIRT3 KO cell proliferation when combined with azaserine, we examined whether azaserine had an effect on mTORC1 signaling. Indeed, azaserine decreased mTORC1 signaling in WT and SIRT3 KO cells, but to a greater extent in SIRT3 KO cells (Figure 2.3G). This is consistent with previous findings showing that DON also inhibits mTORC1 signaling [28,44]. Taken together, these findings show that increased *de novo* nucleotide synthesis in the absence of SIRT3 is in part due to hyperactive mTORC1 signaling, which is suppressed by azaserine.

#### *SIRT3 levels are low in human basal breast tumors*

To determine the relevance of our findings in cancer, we focused on breast cancer where previous studies have shown decreased SIRT3 levels in human tumors [1,28,56,57]. Multiple studies have identified differences in glutamine metabolism between luminal and basal subtypes of breast cancers [46,47,56-58]. Basal-like breast tumors, which tend to be more aggressive and have poor prognosis, have decreased levels of SIRT3 compared to luminal breast tumors [43,48,59] (Figure 2.4A). Because SIRT3 expression was higher in luminal breast tumors, we analyzed the RNA Seq and the reverse phase protein array (RPPA) datasets in the TCGA breast tumor data to determine if there was a correlation between SIRT3 mRNA levels and protein expression of the receptors used to classify breast cancers. Luminal breast cancers tend to be



**Figure 2.4. SIRT3 represses mTORC1 signaling and glutamine metabolism in breast cancer *in vivo*.**

(A) SIRT3 mRNA levels in basal and luminal breast cancers. Red represents high expression and blue represents low expression. Expression was determined using the Oncomine cancer microarray database (<http://www.oncomine.org>). (B) Pearson correlations between SIRT3 mRNA levels and proteins levels of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), phosphatase and tensin homolog (PTEN) and the transcription factor GATA3, and (C) phospho-protein levels of mTORC1 targets, and (D) mRNA expression of one carbon metabolism genes in human breast tumors. Gene and protein expression data was obtained from the RNA sequencing (RNA Seq) and the reverse phase protein array (RPPA) datasets in The Cancer Genome Atlas (TCGA). Red represents a positive association and blue represents a negative association. \* represents significance. (E) SIRT3 and MTHFD2 mRNA expression in human breast tumors – from the RNA Seq data in TCGA. (F) SIRT3 and mitochondrial protein levels in a panel of basal and luminal breast cancer cells. (G) Relative fold change of the area of spheres formed by control or SIRT3 MDA-MB-231 cells in 3D culture. The circumference of 150-200 spheres per condition was measured using ImageJ. (H) Representative image of nude mice injected with control or SIRT3 overexpressing MDA-MB-231 cells. (I) Growth curve of xenograft tumors:  $2 \times 10^6$  control or SIRT3 overexpressing MDA-MB-231 cells were injected into the flanks of nude mice (n = 10). (J) Representative images and (K) quantification of the area of spheres formed by control or SIRT3 overexpressing MDA-MB-231 cells treated with empty vehicle or 30  $\mu$ M azaserine. (L) Representative images of spheroids formed by control or SIRT3 overexpressing MDA-MB-231 and T47D cells treated with DMSO or 100 nM rapamycin. Quantification of the area of spheroids formed by control and SIRT3 overexpressing (M) MDA-MB-231 cells treated with DMSO or 100 nM rapamycin in 3-dimensional (3D) culture.

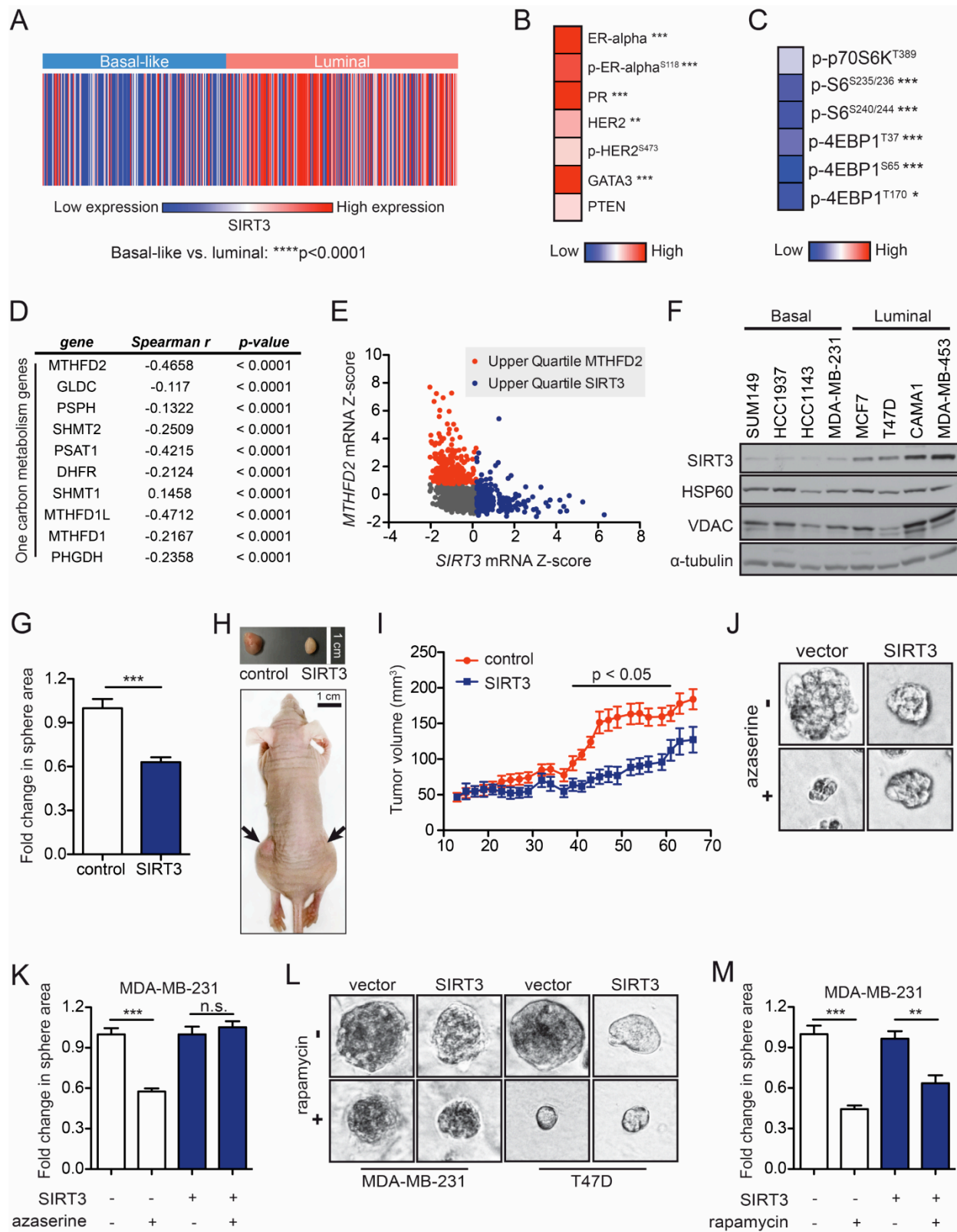


Figure 2.4 (Continued)

positive for the estrogen receptor (ER), and/or the progesterone receptor (PR), and sometimes the human epidermal growth factor receptor (HER2), while basal-like tumors tend to be triple negative and lack these receptors. We found a positive correlation between SIRT3 expression and expression of luminal breast cancer markers, suggesting that SIRT3 levels are higher in luminal breast tumors (Figure 2.4B).

*SIRT3 is inversely correlated with mTORC1 signaling in human breast tumors*

Because we observed hyperactive mTORC1 signaling in the absence of SIRT3, we next examined TCGA breast tumor data to determine if SIRT3 mRNA expression was associated with phosphorylation of proteins activated by mTORC1. We found a significant negative correlation between SIRT3 expression and various markers of overactive mTORC1 in human breast tumors (Figure 2.4C). Notably, expression of most of the genes in one carbon metabolism was inversely correlated with SIRT3 levels (Figure 2.4D). Given that mTORC1 stimulates expression of MTHFD2 and that SIRT3 loss results in increased expression of MTHFD2, we examined the interaction between SIRT3 and MTHFD2 in breast tumors. We found that tumors with the highest expression of MTHFD2 have lower SIRT3 levels, while tumors with higher expression of SIRT3 have decreased levels of MTHFD2 (Figure 2.4E).

Next we investigated the functional consequences of increased or decreased SIRT3 in breast cancers. We began by measuring SIRT3 expression in a panel of breast cancer cell lines, and in line with our findings in breast tumors, SIRT3 levels were higher in luminal breast cancer cell lines compared to basal breast cancer cell lines (Figure 2.4F). As SIRT3 is a mitochondrial protein, we assayed additional mitochondrial proteins, such as heat shock protein 60 (HSP60) and porin (VDAC), to determine if differences in SIRT3 levels were due to mitochondrial

content. Levels of mitochondrial proteins did not match the SIRT3 expression pattern across breast cancer subtypes, suggesting that the expression of SIRT3 is independent of mitochondrial content. Additionally, because SIRT3 loss resulted in stimulation of mTORC1 signaling, we examined the effect of SIRT3 on mTORC1 signaling in T47D and MDA-MB-231 cells (Figure S2.4A). SIRT3 overexpression resulted in decreased phospho-S6 signal compared to control cells, suggesting that SIRT3 may repress mTORC1 signaling (Figure S2.4B-C).

*SIRT3 represses breast cancer cell growth in 3-dimensional (3D) culture and in vivo*

SIRT3 has been shown to have tumor suppressive function. These findings are supported by the fact that SIRT3 null mice develop mammary tumors towards a later stage of life, and by xenograft studies where cells with loss or decreased SIRT3 expression form bigger tumors compared to their control counterparts [1,28,36,60]. Additionally, overexpression of SIRT3 in hepatocellular carcinoma cells decreased tumor growth *in vivo* [28,41]; however, whether SIRT3 represses growth of breast cancer cells *in vivo* has not been shown. To address this, we first examined proliferation of control or SIRT3 overexpressing MDA-MB-231 or T47D cells in two-dimensional culture and found no difference in proliferation between control and cells overexpressing SIRT3 (Figure S2.4D-E). However, in a three-dimensional (3D) culture system, where cells form spherical structures on a layer of Matrigel, SIRT3 overexpression repressed sphere growth in MDA-MB-231 and T47D cells (Figure 2.4G and S2.4F). We then performed xenograft experiments and found that tumors derived from MDA-MB-231 cells overexpressing SIRT3 grew at a slower rate and were 30% smaller than tumors derived from control cells (Figure 2.4H-I). These results highlight the role of SIRT3 as a tumor suppressor in breast cancer.

### *Low SIRT3 levels sensitize breast cancer cells to nucleotide synthesis and mTORC1 inhibition*

Our small molecule screen identified inhibition of nucleotide synthesis as a susceptibility in cells with SIRT3 loss. Thus, we tested whether this was true in breast cancer cells. Overexpression of SIRT3 in MDA-MB-231 and T47D cells represses growth of 3D spheres (Figure 2.4J and S2.4G). In agreement with our findings in SIRT3 KO cells, azaserine treatment significantly decreased the growth of 3D spheres formed by control cells without having an effect on SIRT3 overexpressing breast cancer cells (Figure 2.4J-K and S2.4G-H). Next, we examined the effect of mTORC1 inhibition on 3D sphere growth. Rapamycin decreased sphere size of control and SIRT3 overexpressing breast cancer cells. These results are in accord with the fact that mTORC1 regulates multiple processes to promote cell growth. Importantly, in contrast to SIRT3 overexpressing cells, mTORC1 inhibition had a much more drastic effect on repressing growth of spheres formed by control breast cancer cells (Figure 2.4L and S2.4I). Taken together, our results illustrate that SIRT3 functions as a tumor suppressor in part by repressing mTORC1 signaling and nucleotide synthesis and that tumors with low SIRT3 may be sensitive to inhibition of these pathways.

## **2.4 Discussion**

In this study, we demonstrate that SIRT3 represses nucleotide metabolism in part by dampening mTORC1 signaling to suppress tumor growth. Previous studies have shown that SIRT3 loss results in rewiring of glucose metabolism to promote tumorigenesis, and its overexpression represses breast cancer cell growth in part by suppressing the Warburg effect [1,28,36,61,62]. We show that SIRT3 loss renders cells susceptible to glutamine withdrawal or azaserine treatment (Figure 2.1), which decreases *de novo* purine synthesis, a pathway increased

in the absence of SIRT3 (Figure 2.2). SIRT3 inhibits nucleotide synthesis partly via repression of the mTORC1 signaling pathway (Figure 2.3). We reveal differences in SIRT3 expression across breast tumor subtypes with the more aggressive, basal-like tumors, which tend to have a poor prognosis, having decreased SIRT3 expression compared to luminal-like tumors (Figure 2.4). SIRT3 expression is inversely correlated with markers of mTORC1 activity in human breast tumors, and lastly, we demonstrate that SIRT3 represses mTORC1 signaling and tumor growth of breast cancer cells in a xenograft model (Figure 2.4). In sum, our findings uncover repression of mTORC1 signaling and nucleotide synthesis as additional mechanisms by which SIRT3 functions as a tumor suppressor. Moreover, our findings highlight the potential to use SIRT3 levels to aid in determining breast cancer subtype as well as sensitivity to mTORC1 or nucleotide synthesis inhibitors.

Metabolic rewiring of glutamine-dependent pathways contributes to biomass production to support growth and proliferation [49,63]. In this study we show deregulation of a glutamine-dependent pathway, *de novo* nucleotide synthesis, in the absence of SIRT3. Moreover, inhibition of *de novo* purine synthesis by a glutamine analog, azaserine, significantly decreases proliferation of SIRT3 null cells, suggesting that these cells rely on nucleotide synthesis for proliferation. Indeed, nucleotides are essential building blocks necessary for synthesis of nucleic acids (DNA and RNA), which code the genetic material of the cell and are crucial components for generation of new cells during proliferation. Thus, the strategy of inhibiting nucleic acid synthesis to suppress tumorigenesis is well known and several inhibitors of DNA synthesis or DNA-damaging agents that stall DNA replication are used to treat cancer [50,51,64]. Moreover, additional constituents, such as lipids, energy, and NEAAs, to which glutamine contributes, are indispensable to a rapidly dividing cell. Given that SIRT3 is localized to the mitochondria, a key

organelle known to contribute to production of such building blocks, SIRT3 is well poised to regulate these additional metabolic pathways necessary for proliferation. However, whether SIRT3 regulates additional glutamine-dependent metabolic pathways contributing to these cellular components remains to be shown.

Our results are consistent with previous findings that hearts of SIRT3 null mice exhibit hyperactive mTORC1 signaling compared to their wild type littermates, which is dampened by SIRT3 overexpression in transgenic mice [52,53,64,65]. How SIRT3 regulates mTORC1, or upstream signaling pathways that converge on mTORC1, is an area that remains to be elucidated; however, one potential mechanism by which SIRT3 may regulate this key nutrient sensing pathway is retrograde signaling via modulation of ROS [46,47,54,55]. The regulation of glycolysis by SIRT3 via repression of ROS to suppress HIF1 activity is an example of this mechanism [28,66]. Additionally, ROS has been shown to be a double-edged sword that can promote cell death at high levels or activate signaling pathways [28,56,57]. ROS oxidizes key cysteine residues in phosphatases, such as PTEN, which results in its inactivation to increase PI3K signaling [56-58,67]. Indeed, increased ROS in the absence of SIRT3 contributes to increased activation of mTORC1 signaling since treatment with the antioxidant N-acetylcysteine (NAC) decreases mTORC1 signaling in SIRT3 KO cells (data not shown). Additionally, mTORC1 is regulated by additional nutrients, such as glucose and amino acids, including glutamine; thus, by regulating glucose and potentially glutamine metabolism, SIRT3 may regulate mTORC1 signaling [43,59].

Although we show that hyperactive mTORC1 contributes to increased glutamine consumption and nucleotide synthesis in cells with SIRT3 loss, additional pathways or transcription factors may contribute to rewiring of nucleotide synthesis in these cells. SIRT3 may

regulate nucleotide metabolism by directly regulating enzymes along pathways that contribute to nucleotide synthesis. SIRT3 may regulate one carbon metabolism by deacetylating serine hydroxymethyltransferase (SHMT2), a mitochondrial enzyme known to modulate production of glycine and folate, both of which are required for nucleotide synthesis. Alternatively, SIRT3 may mitigate additional signaling pathways that contribute to metabolic reprogramming. For instance, the transcription factor Myc, is a key regulator of many aspects of cellular biology, one of them being metabolism, and has been shown to regulate glutamine usage by directly binding to promoters of glutamine transporters to increase their expression [60]. Moreover, the Myc binding motif was found to be enriched in our RNA seq data, as well as in SIRT3 null brown adipose tissue [28]. Myc regulates expression of various genes in purine and pyrimidine synthesis, including expression of the first rate-limiting enzymes in these pathways, PPAT and CAD [61,62]. Additionally, Myc regulates the expression of genes in the one carbon metabolism pathway, which are important for production of folate and glycine necessary for nucleotide synthesis. The PI3K pathway, also hyperactive in the absence of SIRT3, regulates Myc stability and has been shown to stimulate purine synthesis [63]. Thus, induction of PI3K or stimulation of Myc in the absence of SIRT3 provides additional mechanisms by which SIRT3 loss may promote elevated nucleotide synthesis.

Lastly, we show that SIRT3 expression is low in basal-like human tumors, which tend to be more aggressive and have poor prognosis [64]. The options for treatment of basal-like or triple-negative breast cancers are limited, and these tumors tend to develop resistance to currently used chemotherapeutic regimens [64,65]. Multiple studies point to elevated glutamine consumption in basal-like breast cancer cells as a target for treatment of basal-like breast cancers [46,47]. Here we report that loss of SIRT3 results in increased reliance on glutamine for survival,



suggesting that SIRT3 expression may be used as a marker to aid in determining breast cancer subtype and vulnerabilities in breast cancers. Altogether, our findings further highlight the importance of SIRT3 as a mediator of the metabolic switch observed in cancer.

## **2.5 Experimental Procedures**

### *Two-dimensional (2D) Cell Culture*

Immortalized SIRT3 WT and KO mouse embryonic fibroblasts (MEFs) were cultured in DMEM media supplemented with 10% FBS, penicillin/streptomycin, 2 mM L-glutamine, non-essential amino acids, and 0.1 mM  $\beta$ -mercaptoethanol. Breast cancer cell lines SUM149, HCC1937, HCC1143, MDA-MB-231, MCF7, T47D, CAMA-1, and MDA-MB453 were maintained in DMEM media supplemented with 10% FBS, penicillin/streptomycin, and 2 mM L-glutamine. MCF10A cells were cultured in 1:1 DMEM/F12 media supplemented with penicillin/streptomycin, 5% horse serum, 20 ng/mL EGF, 100 ng/mL cholera toxin, 0.5 mg/mL hydrocortisone, and 10  $\mu$ g/mL insulin.

K-Ras transformed WT and SIRT3 KO MEFs were generated by retroviral infection with pBabe vector containing K-Ras G12V. SIRT3-overexpressing cells were generated by retroviral infection with empty pBabe vector or pBabe vector containing human WT SIRT3 or catalytic mutant SIRT3 H248Y with a C-terminal FLAG tag [66]. Knockdown cell lines were generated by lentiviral infection with shRNAs obtained from The RNAi Consortium (TRC) at the Broad Institute. Clone TRCN0000038892 was used to silence SIRT3 as previously described [28]. Clone TRCN0000295367 (sh#1) and TRCN0000295429 (sh#2) were used to silence phosphoribosyl pyrophosphate amidotransferase (PPAT).

### *Three-dimensional (3D) Cell Culture*

Three-dimensional cultures using laminin-rich extracellular matrix were prepared as previously described with minor modifications [67]. Briefly, 4000-6000 single cells were seeded on top of a thin layer of Engelbreth-Holm-Swarm (EHS) tumor extract (Corning Matrigel) to which media containing 2% EHS was added. All 3D cultures were maintained for 10-16 days, and media was changed every 2 days after day 4. 3D cultures were slightly modified to for  $^{14}\text{C}$ -aspartate and  $^{14}\text{C}$ -glycine incorporation assays. Briefly, 5 - 50  $\mu\text{L}$  drops containing 6000 cells were plated in a well of a 6-well plate in triplicate. Cells were incubated at  $37^\circ\text{C}$  to allow the Matrigel to solidify. Media was added to each well to cover the Matrigel drops, and cells were incubated for 9 days.

### *Reagents*

The following antibodies were obtained from Cell Signaling (unless noted otherwise) and were used for immunoblotting: SIRT3 (#5490), phospho-MEK (#9121), MEK (# 4694), phospho-ERK (#9106), ERK (#9102), phospho-CAD (Ser1859; #12662), CAD (#11933), phospho-AKT (Thr308; #13038), AKT (#2920), phospho-S6K (#9234), S6K (#9202), phospho-S6 (#5364), S6 (#2317), phospho-PRAS40 (# 29975), PRAS40 (#26915),  $\alpha$ -tubulin (Santa Cruz sc-8035), actin (Sigma A5441), Hsp60 (Abcam ab3080), VDAC (Abcam ab14734), and FLAG (Sigma #1804). Cells were treated with Azaserine (Cayman Chemical 14834), insulin (Sigma I0516), or rapamycin (Sigma R0395). Radioactive metabolites were purchased from Perkin Elmer:  $\text{U}^{14}\text{C}$ -aspartic acid (NEC268E050UC), 5,6- $^3\text{H}$ -uridine (NET367250UC), 6- $^3\text{H}$ -thymidine (NET355250UC), and  $\text{U}^{14}\text{C}$ -glycine (NEC276E250UC), and 2,8- $^3\text{H}$ -adenine (NET063001MC).

### *Glutamine and Ammonium Measurements*

Cells were plated in 6-well plates and allowed to adhere. Cells were washed once with PBS prior to change of media. Glutamine and ammonium concentrations in fresh and culture media were measured after 8 to 24 hours using the BioProfile FLEX analyzer (NOVA Biomedical). Measurements were normalized to cell number.

### *Metabolite Profiling*

For metabolite profiling, cells were seeded and 24 hours later, media was removed and replaced with fresh media 4 hours prior to metabolite extraction. Polar metabolites were extracted on dry ice using 80% ice-cold methanol as previously described [28,68]. A duplicate experiment was seeded in the same manner as for metabolite profiling to extract protein using a BCA assay (Thermo Fisher Scientific). Protein content was used to normalize metabolite levels.

### *Aspartate and Glycine incorporation into nucleic acid*

Cells were plated in 6-well plates (in triplicate) and after 24 hours, cells were washed once with PBS and serum starved for 16 hours, as previously described [42,68]. Cells were treated with media containing 2  $\mu$ Ci U-<sup>14</sup>C-glycine, 2  $\mu$ Ci 2,8-<sup>3</sup>H-adenine, 2  $\mu$ Ci U-<sup>14</sup>C-aspartic acid, 1  $\mu$ Ci 6-<sup>3</sup>H-thymidine, or 1  $\mu$ Ci 5,6-<sup>3</sup>H-uridine and incubated for the specified time. DNA was extracted from cells using the DNeasy Blood and Tissue kit (Qiagen) based on manufacturer's instructions. Nucleic acid concentration was determined using a Nanodrop spectrophotometer. Isolated nucleic acid was added to scintillation vials and radioactivity was measured using a liquid scintillation counter. Data was normalized to total nucleic acid concentration.

Cells were treated as previously described for 3D culture. After incubation with radiomolecules, 3D cultures were harvested in Cell Recovery Solution (Corning #354253) and incubated on ice for 20 min to allow Matrigel to dissolve. Cultures were centrifuged at 400 x g for 3 min to pellet cells, and DNA was extracted as described above.

### *RNA Sequencing*

Total RNA was isolated from immortalized WT and SIRT3 KO MEFs by a TRIzol (Invitrogen) extraction followed by a Direct-zol RNA MiniPrep Kit (Zymo Research) purification following the manufacturer's instructions. Library preparation and RNA sequencing was performed as previously described [69]. Briefly, 2 µg RNA was incubated with poly-T oligo beads and unbound RNA was washed two times to remove ribosomal RNA and enrich for mRNA transcript pools. The Superscript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) was used to synthesize cDNA, and cDNA libraries were generated using Nextera XT DNA sample preparation kit. Libraries were sequenced on the Illumina NextSeq and data were processed as previously described [70]. Data were normalized to the total number of reads per kilobase of exon per million (RPKM), and reads were aligned to the mm10 mouse genome using Spliced Transcripts Alignment to a Reference (STAR) [71].

### *RNA Sequencing Data Analysis*

To identify differentially expressed genes between 2 wild type and 2 SIRT3 KO MEF samples we used the DESeq2 differential gene expression algorithm as previously described [15]. We filtered genes significantly differentially expressed based on false discovery rate of less than 0.05 and an absolute log<sub>2</sub> fold change greater than 0.05 (absolute fold change of 1.3). We

also filtered for genes that were expressed at a minimum of 1 RPKM. In addition, the list of differentially expressed genes was used to identify enrichment of transcription factor binding motifs using the previously described MotifADE method [19,21]. Lastly, the Database for Annotation, Visualization and Integrated Discovery (DAVID 6.7) was used to examine enrichment of molecular functions in genes that were significantly differentially expressed genes between WT and SIRT3 KO MEFs [39,72].

#### *Immunocytochemistry:*

Cells were washed once with PBS and fixed in 4% paraformaldehyde for 15 min at room temperature. Cells were permeabilized and blocked in PBS containing 0.3% Triton X-100 and 5% normal goat serum (Life Technologies) for 1 hour at room temperature. Cells were incubated with phospho-S6 primary antibody overnight at 4 °C. The cells were washed with PBS and incubated with Alexa Fluor 488 secondary antibody (Life Technologies) for 1 hour at room temperature. Cells were washed three times with PBS followed by DAPI stain for 1 min. PBS was used to wash cells two more times and Prolong Gold antifade reagent (Invitrogen) was used to mount slides. The Nikon A1R Laser Scanning Confocal Microscope was used to acquire images using a 488nm laser with 525/50 emission filter and a 404nm laser with 550/50 emission filter to image phospho-S6 and DAPI (nucleus), respectively. The Nikon Plan Apo 60x 1.4 NA objective lens was used to acquire images.

#### *Immunoblotting*

Cells were washed with cold PBS and lysed on ice for 15 min in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS)

supplemented with 1 mM DTT, protease inhibitor cocktail (Roche), and phosphatase inhibitors (Sigma). Lysates were cleared by centrifugation at 16,000 x g for 10 min at 4 °C. Protein was quantified by a BCA protein assay (Thermo Fisher Scientific) and equal amounts of protein were run on a 10-20% Tris-Glycine gel (Bio-Rad) and transferred to nitrocellulose membrane (Bio-Rad) for analysis by immunoblotting. Membranes were blocked in 5% BSA in TBS containing 0.1% Tween 20 for 1 hour at room temperature and incubated with primary antibody at 4°C overnight. Band intensities were quantified using Image J.

### *Immunohistochemistry*

Tumors were fixed in 10% buffered formalin and submitted to the Dana-Farber/Harvard Cancer Center facility for embedding in paraffin, sectioning, and hematoxylin and eosin staining. Cyclic immunofluorescence was performed as previously described [73], using the following antibodies: phospho-S6 (#5018 and #4851) and Ki67 (#11882) from Cell Signaling Technology.

### *Clonogenic growth assay*

Colony formation assays were performed with KRAS G12V transformed WT and SIRT3 KO MEFs. 100 cells were seeded in 2 mL of media in six well plates. Media was replaced every 2-3 days. Colonies were stained with crystal violet after 8-10 days as previously described [74]. For quantification, crystal violet from the colonies was solubilized using 10% acetic acid as previously described [75]. The absorbance of the extracted crystal violet solution at a wavelength of 590 nm was measured using a Varian Cary 50 Bio UV-visible spectrophotometer.

### *Animal studies*

All animal studies were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee, the Standing Committee on Animals at Harvard. For xenograft studies,  $1 \times 10^6$  or  $2 \times 10^7$  MDA-MB-231 or T47D cells (accordingly) were mixed with 1:1 HBSS:Matrigel (Corning #354234) and injected subcutaneously into the flanks of 8- to 10-week-old female nude mice (Charles River). Control cells were injected into the left flank and SIRT3-overexpressing cells were injected into the right flank of the same mouse. Tumors were measured every 2 to 3 days as indicated, and tumors were dissected and weighed at the end of the experiment. Tumor volume was calculated using the formula  $V = (w^2 \times l) / 2$ , where  $w$  represents width and  $l$  represents length.

### *Statistics*

Statistical significance between groups was determined using the unpaired Student's t test (unless otherwise noted). Significance is as follows: \*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ . All experiments were performed at least two to three times.

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## CHAPTER III

### **Glutamine tracing and metabolomics analysis reveals deregulation of multiple nodes of glutamine metabolism upon SIRT3 deletion**

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### **3.1 Abstract**

Metabolic reprogramming of glutamine contributes to production of building blocks for growth, proliferation, and survival in cancer. The mitochondrial deacetylase sirtuin 3 (SIRT3) regulates various metabolic pathways and suppresses metabolism of glucose – a key nutrient for tumors, in breast cancer cells. However, whether SIRT3 affects glutamine metabolism remains unknown. Here we perform glutamine labeling combined with metabolomic and RNA sequencing (RNA Seq) analysis to examine the role of SIRT3 in regulating glutamine usage. The absence of SIRT3 results in increased consumption of glutamine and its metabolism to fuel the tricarboxylic acid (TCA) cycle while contributing to production of nonessential amino acids (NEAAs). Additionally, overexpression of SIRT3 in a panel of breast cancer cells represses glutaminolysis. Lastly, increased glucose and glutamine uptake support elevated glutathione production in cells lacking SIRT3. These findings identify additional nodes of metabolism that may be repressed by SIRT3 to suppress tumorigenesis.

### **3.2 Introduction**

Glutamine metabolism supports anabolic growth, energy production, and antioxidant stress response, all of which are critical for cellular proliferation and survival. Deregulated metabolism is a hallmark of cancer cells, and increased consumption of glucose and glutamine, as well as deregulation of their metabolism, has been observed in cancer cells compared to non-malignant cells [1-5]. Additionally, various types of cancer cells rely on glutamine for survival underscoring glutamine metabolism as a potential target for development of therapies to treat cancer [6-9].

Glutamine metabolism consists of various nodes that generate crucial molecules, such as nucleotides, amino acids, hexosamines, TCA cycle intermediates, lipids, and glutathione, which

are essential for proliferation. Glutamine contributes its nitrogen backbone for synthesis of hexosamines, nucleotides, and nonessential amino acids – intermediates important for nutrient sensing, as well as for synthesis of nucleic acids and proteins, which are important to rapidly proliferating cells [1-5,10-13]. Like glucose, glutamine is an anaplerotic substrate and contributes its carbon backbone to replenish TCA cycle intermediates thereby contributing to generation of reducing equivalents necessary for energy production via the electron transport chain [14-16]. Additionally, under hypoxia, glutamine-derived  $\alpha$ -ketoglutarate is converted to citrate, via reductive carboxylation, and can contribute to production of acetyl-CoA utilized for fatty acid synthesis, which is crucial to a proliferating cell as it contributes to production of lipids necessary for generation of cell membranes [17,18]. Lastly, by contributing to production of glutathione and NADPH, glutamine supports the antioxidant stress response, which is implicated in resistance to chemotherapy [14,19]. Although glutamine is in many ways critical for a tumor cell, how a cell regulates distribution of glutamine to the various metabolic pathways is an area of research that remains to be fully elucidated. Thus, having a better understanding of the mechanisms by which tumor cells regulate glutamine usage will provide insight into targeting glutamine metabolism for treatment of cancer.

The mitochondrial sirtuin 3 (SIRT3), a robust deacetylase, regulates metabolism and has tumor suppressive function in breast cancer, lung adenocarcinoma, gastric cancer, hepatocellular carcinoma, and pancreatic cancer [6-9,20-27]. SIRT3 promotes fatty acid oxidation, amino acid catabolism, urea cycle, and energy production by deacetylating and activating key targets in these metabolic pathways [28-34]. Additionally, SIRT3 regulates a major arm of tumor metabolism, glycolysis, by decreasing oxidative stress via activation of superoxide dismutase (SOD2) and isocitrate dehydrogenase (IDH2), to destabilize hypoxia inducible factor 1 (HIF1),

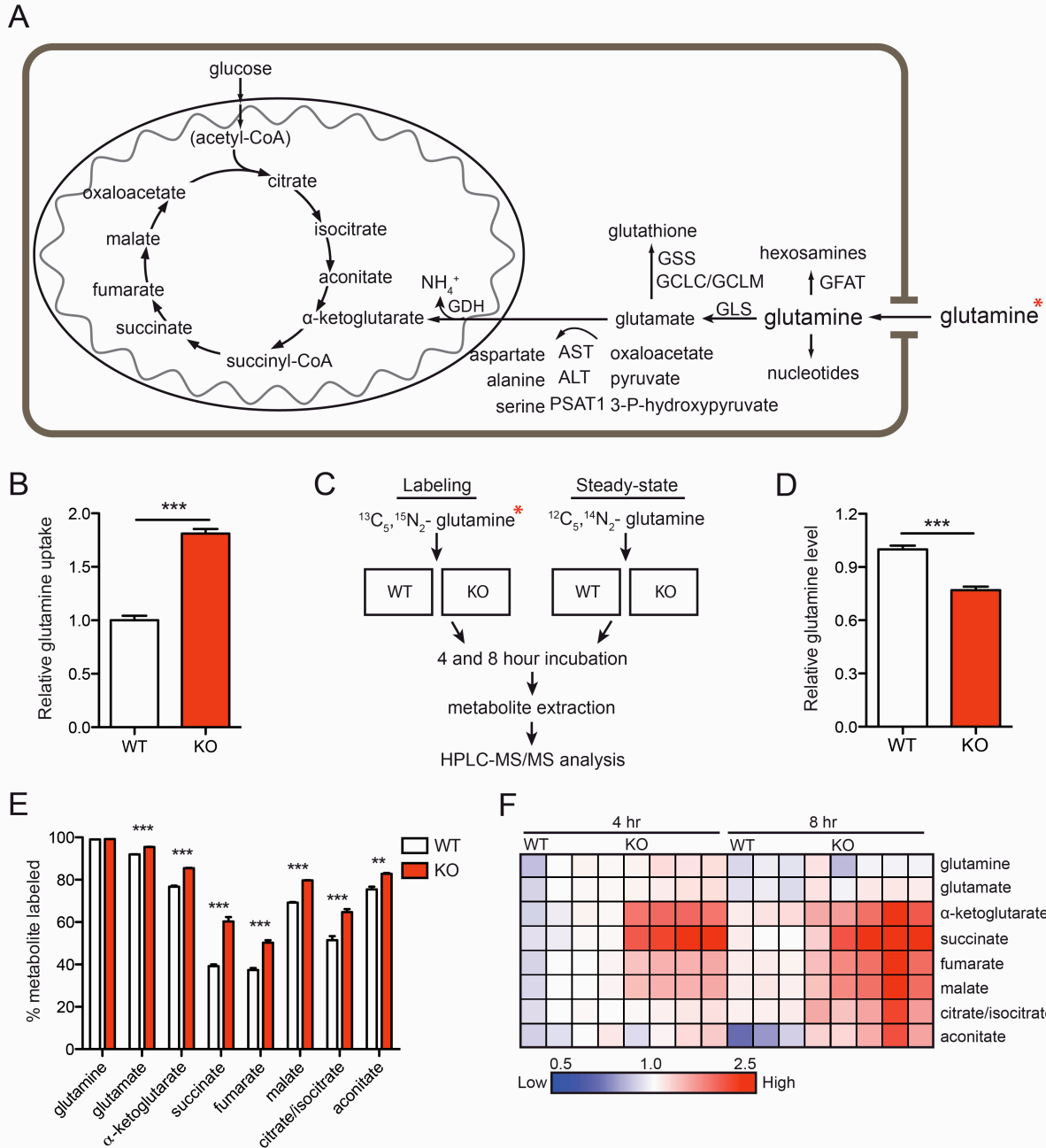
which is known to regulate glycolytic gene expression [21,22,35-39]. Although SIRT3 modulates various metabolic pathways, its role in regulating glutamine metabolism has not been examined.

In this study, we perform glutamine tracing and metabolomics analysis in wild type (WT) and SIRT3 null (KO) mouse embryonic fibroblasts (MEFs) to determine the effect of SIRT3 loss on glutamine metabolism. We show that loss of SIRT3 results in deregulation of multiple nodes of glutamine metabolism. Increased glutamine consumed in the absence of SIRT3 is utilized for anaplerosis, synthesis of aspartate and serine, and to support increased glutathione synthesis. Although SIRT3 loss results in increased levels of glutathione, inhibition of glutathione synthesis alone did not abolish the difference in proliferation between WT and SIRT3 KO cells (Figure 3). Taken together these data suggest that SIRT3 represses glutamine metabolism, and highlights additional alterations in glutamine-dependent pathways that may contribute to proliferation of tumors with deletion or decreased expression of SIRT3.

### **3.3 Results**

#### *SIRT3 loss promotes glutamine anaplerosis*

Glutamine contributes to multiple nodes of metabolism in the cell (Figure 3.1A). Given that SIRT3 KO cells have increased glutamine consumption (Figure 3.1B), we utilized our steady-state metabolomics data and examined whether intracellular glutamine levels were different between WT and SIRT3 KO MEFs (Figure 3.1C). SIRT3 KO cells had 25% less intracellular glutamine than WT cells, suggesting that the increased glutamine taken up by



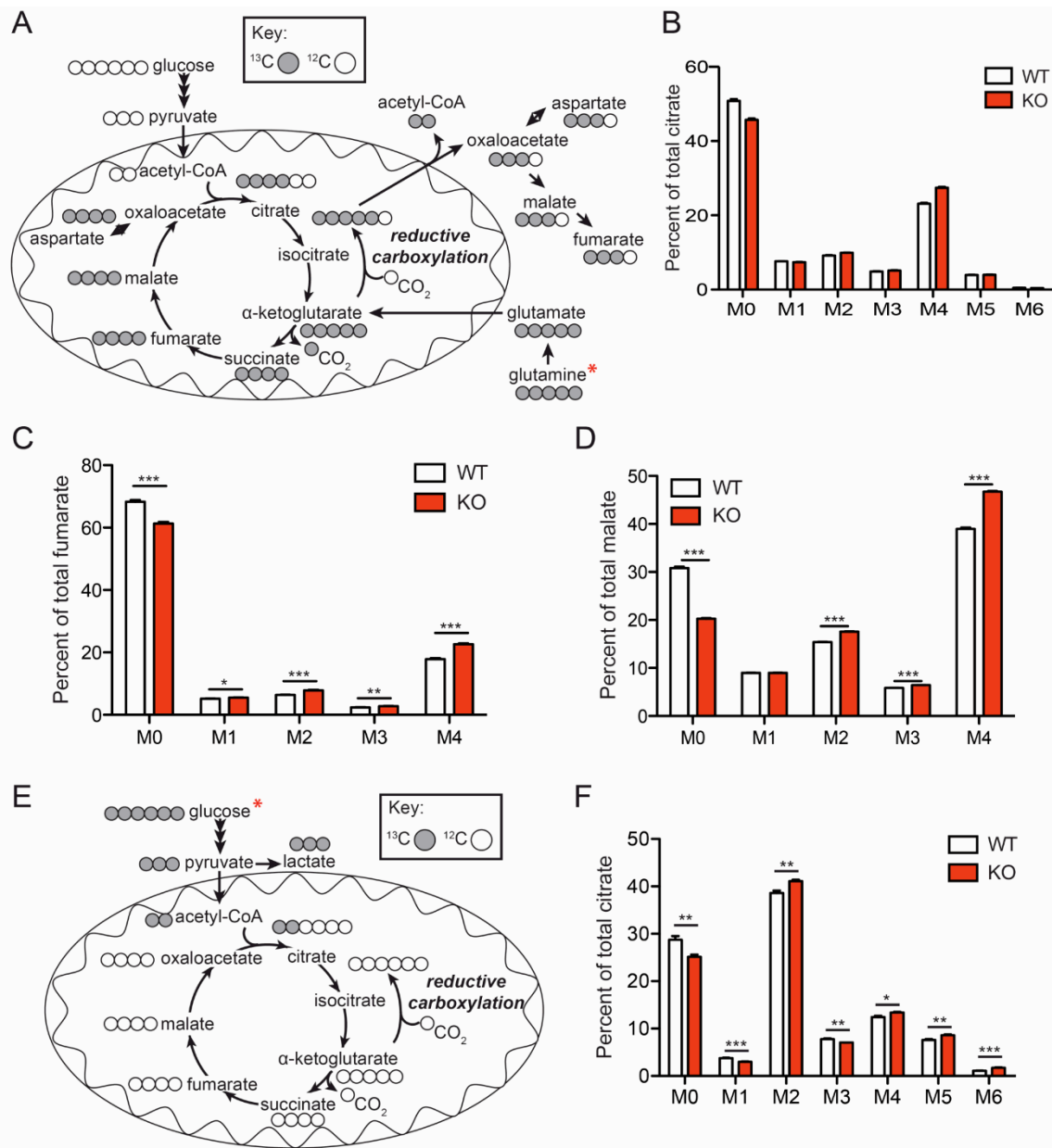
**Figure 3.1. SIRT3 KO MEFs have increased contribution of glutamine-derived carbon to the TCA cycle.** (A) Schematic of the contribution of glutamine to various metabolic pathways. (B) Glutamine consumption in WT and SIRT3 KO MEFs. (C) Layout of labeled glutamine and steady-state metabolomics experiment. (D) Steady-state glutamine levels. (E) Percentage of TCA cycle metabolites labeled by [U- $^{13}\text{C}_5$ ,  $^{15}\text{N}_2$ ]glutamine in WT and SIRT3 KO MEFs after 8 hours of labeling. (F) Heat map showing incorporation of [U- $^{13}\text{C}_5$ ,  $^{15}\text{N}_2$ ]glutamine to TCA cycle intermediates in WT and SIRT3 KO MEFs relative to WT samples at after 4 hours of labeling. Data are means  $\pm$  SEM (n = 4). \*\*p < 0.01; \*\*\*p < 0.001.

SIRT3 KO MEFs may be utilized at higher rates in the SIRT3 KO cells (Figure 3.1D). In addition to its role as an anaplerotic substrate, glutamine contributes to synthesis of hexosamines, nucleotides, amino acids, and glutathione. To examine how SIRT3 loss affects the contribution of glutamine to the various metabolic pathways, we performed a tracing time course experiment using carbon and nitrogen labeled glutamine (U-<sup>13</sup>C<sub>5</sub>, U-<sup>15</sup>N<sub>2</sub>-glutamine) in WT and SIRT3 KO MEFs and analyzed the incorporation of glutamine into TCA cycle intermediates, amino acids, and glutathione (Figure 3.1E).

Glutaminolysis is the process by which glutamine-carbon is utilized to fuel the TCA cycle. In this process, glutaminase (GLS) converts glutamine to glutamate, which is then converted to  $\alpha$ -ketoglutarate by glutamate dehydrogenase (GDH) or various transaminases, which include aspartate transaminases (GOT1/2), alanine transaminases (GPT1/2), and phosphoserine transaminase (PSAT1) to replenish the TCA cycle. We observed increased incorporation of carbon label from glutamine into various TCA cycle intermediates, such as  $\alpha$ -ketoglutarate, succinate, fumarate, malate, citrate, and aconitate, at 4 and 8 hours in SIRT3 KO MEFs compared to WT MEFs (Figure 3.1E-F). Although the majority of TCA cycle intermediates displayed increased levels of labeling at 4 hours in SIRT3 KO cells compared to WT cells,  $\alpha$ -ketoglutarate and succinate were labeled to a higher extent than fumarate and malate (Figure 3.1E-F). This is consistent with SIRT3 activating succinate dehydrogenase, which converts succinate to fumarate [32]. Moreover, at 8 hours all of the TCA cycle intermediates detected by our metabolomics analysis were labeled to a higher extent in SIRT3 KO cells compared to WT cells. These results suggest that SIRT3 KO MEFs have an increased rate of glutaminolysis to refill the TCA cycle, compared to WT cells.

*Glutamine-derived carbon undergoes oxidative metabolism in the TCA cycle in SIRT3 KO MEFs.*

Under hypoxia, cells reductively carboxylate glutamine-derived  $\alpha$ -ketoglutarate into isocitrate, which is converted to citrate and contributes to fatty acid synthesis [17,18]. Under normoxia, HIF1 activation is sufficient to promote reductive carboxylation of glutamine-derived  $\alpha$ -ketoglutarate [17]. Because SIRT3 stabilizes HIF1 $\alpha$ , we next examined whether under normoxia SIRT3 KO cells metabolized glutamine-derived carbon via oxidation or reductive carboxylation in the TCA cycle. In normoxia, the majority of the citrate pool is expected to contain 4 labeled carbons (M+4), generated from glutamine-labeled  $\alpha$ -ketoglutarate (M+5), which is oxidized to oxaloacetate (M+4) and is further condensed with 2 unlabeled carbons from acetyl-CoA (derived from glycolysis) to generate a 6 carbon citrate molecule (Figure 3.2.A). In hypoxia, however, glutamine-labeled  $\alpha$ -ketoglutarate (M+5) would be reductively carboxylated to isocitrate and converted to citrate, thereby producing a greater amount of citrate with 5 labeled carbons (M+5) than citrate with 4 labeled carbons (M+4). After 4 hours of labeling, most of the citrate pool in WT and SIRT3 KO cells was comprised of unlabeled citrate (M+0), followed by 4-carbon labeled citrate (M+4) (Figure 3.2.B). In fact, SIRT3 KO cells had lower levels of unlabeled citrate and higher levels of M+4 citrate. Equal, but low percentages of 5-carbon labeled citrate were observed in WT and SIRT3 KO cells. These findings are consistent with those of glioblastoma cells under normoxia suggesting that glutamine-derived carbon is oxidized in both WT and SIRT3 KO cells [17]. Next, because oxidation of citrate replenishes the TCA cycle, we examined the effect of SIRT3 loss on the contribution of glutamine to TCA cycle intermediates by labeling WT and SIRT3 KO cells with [U-<sup>13</sup>C,<sup>15</sup>N]glutamine for 4 hours and analyzing incorporation of glutamine to fumarate and malate by LC-MS. Under normoxia, the



**Figure 3.2. SIRT3 loss promotes oxidation of glutamine-derived  $\alpha$ -ketoglutarate in the TCA cycle.** (A) Schematic of the contribution of glutamine-derived carbon to TCA cycle intermediates and downstream metabolites. Labeling of citrate (B), fumarate (C), and malate (D) by  $[U-^{13}C_5, ^{15}N_2]$ glutamine in WT and SIRT3 KO MEFs. Cells were seeded and cultured with complete culture media for 24 hours under normoxia (21%  $O_2$ ), followed by 4 hours of labeling with glutamine-free media supplemented with  $[U-^{13}C, ^{15}N]$ glutamine prior to metabolite extraction. (E) Schematic of the contribution of glucose to citrate in the TCA cycle. (F) Labeling of citrate by  $[U-^{13}C]$ glucose in WT and SIRT3 KO MEFs. Cells were cultured as in B-D, but labeled in glucose-free media supplemented with  $[U-^{13}C]$ glucose for 4 hours prior to metabolite extraction. Data are means  $\pm$  SEM (n = 4). \*\*p < 0.01; \*\*\*p < 0.001.



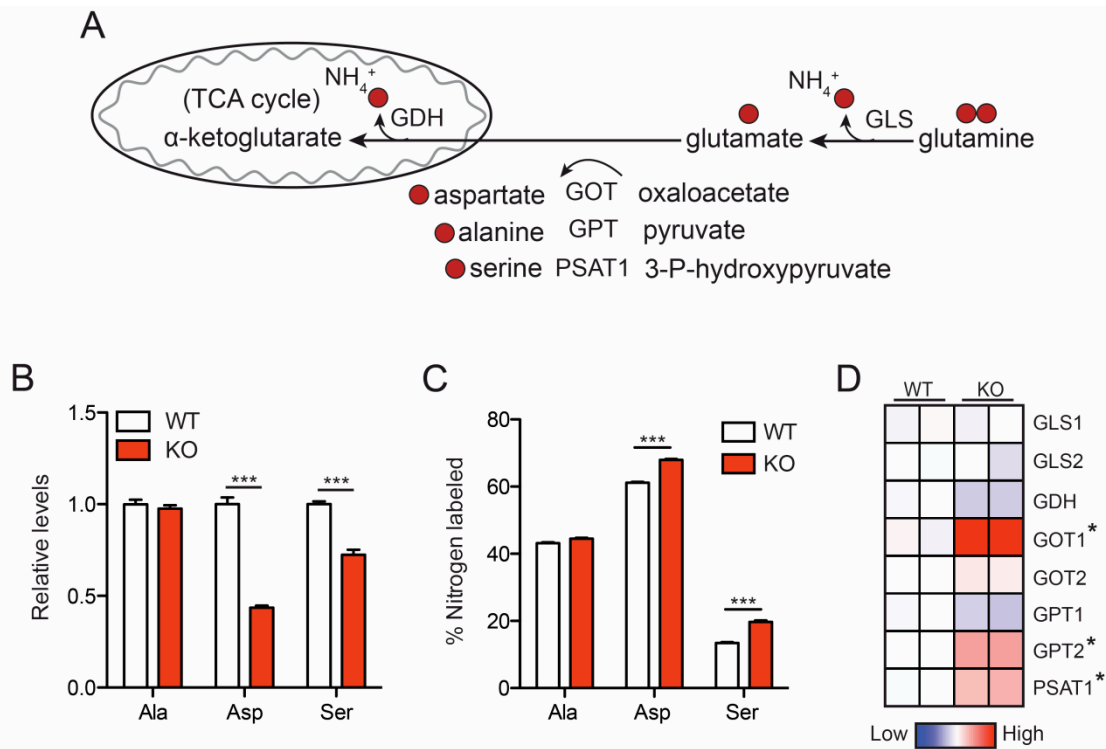
M+4 fumarate and malate pool should constitute the majority of labeled fumarate and malate since glutamine-derived  $\alpha$ -ketoglutarate (M+5) is oxidized 4-carbon labeled succinate, and further to fumarate and malate (Figure 3.2.A). In hypoxia, however, the 3-carbon labeled fumarate and malate species would be expected to predominate the pool of labeled fumarate and malate as 5-carbon labeled citrate (produced via oxidative carboxylation of glutamine-derived  $\alpha$ -ketoglutarate) would be exported out of the mitochondria and cleaved to generate 3-carbon labeled oxaloacetate, which is metabolized to malate and fumarate as part of the malate-aspartate shuttle. In WT and SIRT3 KO MEFs, the majority of labeled malate and fumarate contained 4 carbons labeled (M+4) and a small amount of malate and fumarate with 3 labeled carbons (M+3) (Figure 3.2.C-D). These findings suggest that despite increased HIF1 stability, glutamine-derived  $\alpha$ -ketoglutarate is not reductively carboxylated in SIRT3 KO MEFs. Instead, glutamine-derived carbon is oxidized in the TCA cycle to contribute to citrate in both WT and SIRT3 null MEFs.

Next, as SIRT3 KO MEFs have deregulated glucose metabolism, we evaluated the effect of SIRT3 on the contribution of glucose to the TCA cycle. Glucose-derived pyruvate is utilized by pyruvate dehydrogenase to produce acetyl-CoA, which is condensed with oxaloacetate to generate citrate (Figure 3.2.E). To assess the effect of SIRT3 on the contribution of glucose to the TCA cycle, we labeled WT and SIRT3 KO MEFs with [U-<sup>13</sup>C]glucose for 4 hours and determined the contribution of glucose to citrate. The majority of citrate pool (~40%) contained 2 labeled carbons (M+2) and this was followed by unlabeled carbon citrate (M+0) in WT and SIRT3 KO cells (Figure 3.2F). These data demonstrate that despite increased lactate production in SIRT3 KO MEFs, increased glucose taken up by the cell also contributes to the TCA cycle to the same extent as in WT cells. Thus, taken together, our results highlight the importance of the synergy between glucose and glutamine to fuel the TCA cycle.

*SIRT3 KO MEFs couple use of glutamine for the TCA cycle with NEAAs.*

In the process of fueling the TCA cycle, transaminases transfer nitrogen from glutamate onto carbon molecules, such as oxaloacetate and pyruvate, to generate NEAAs. Aspartate transaminases (GOT1/2) transfer nitrogen from glutamate to oxaloacetate to generate aspartate, while alanine transaminases (GPT1/2) transfer nitrogen to pyruvate to produce alanine, and phosphoserine transaminase (PSAT1) combines nitrogen from glutamate with 3-phospho-hydroxypyruvate to generate 3-phospho-serine (Figure 3.3A). To determine if the pool of NEAAs generated by transaminases is affected by SIRT3, we analyzed steady-state levels of alanine, aspartate, and serine in WT and SIRT3 KO cells. Alanine levels are similar between WT and SIRT3 KO MEFs; however, SIRT3 KO cells have 55% less aspartate and 25% less serine than WT cells (Figure 3.3B). Given that SIRT3 KO cells have increased nucleotide synthesis compared to WT cells, aspartate and serine (via glycine) may be diminished in the absence of SIRT3 to support nucleic acid production. To determine if SIRT3 affects the contribution of glutamine to NEAAs, we analyzed the amount of nitrogen labeling from glutamine into alanine, aspartate, and serine. In contrast to WT MEFs, SIRT3 KO MEFs have increased incorporation of nitrogen derived from glutamine (Figure 3.3C). These results suggest that SIRT3 KO cells may couple entry of glutamine-derived carbon into the TCA cycle with production of NEAAs.

Similar results have been observed in proliferating cells, as well as in cancer cells, and is supported by elevated expression of transaminases [13,40]. This prompted us to examine our RNA sequencing data to determine whether SIRT3 loss had an effect on expression of the genes involved in metabolism of glutamine into the TCA cycle. No difference in GLS expression was observed between WT and SIRT3 KO cells (Figure 3.3C). While GDH expression was slightly decreased in SIRT3 KO cells, the expression of 3 out of 5 transaminases (GOT1, GPT2, and

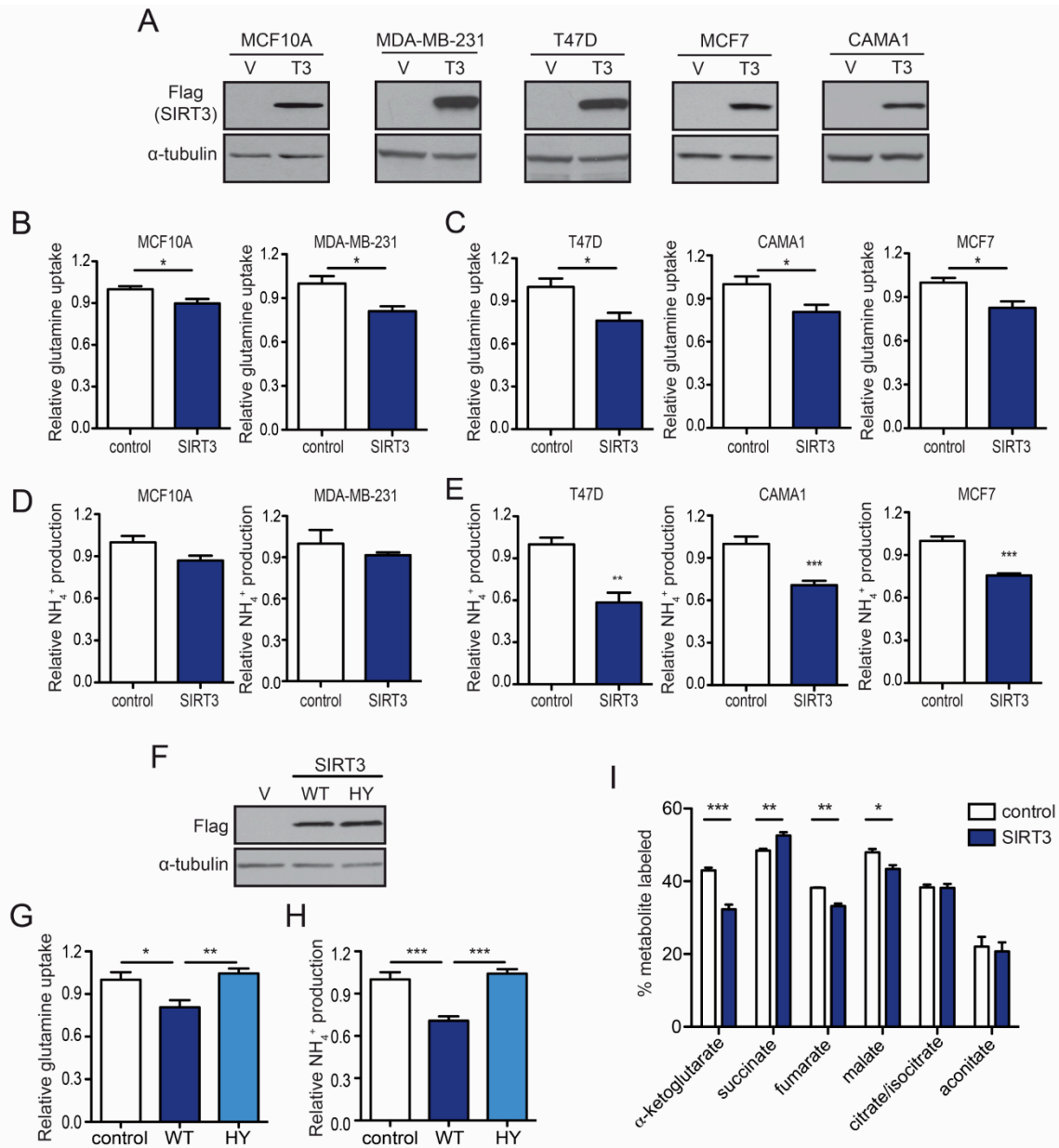


**Figure 3.3. Contribution of glutamine to TCA cycle is coupled with synthesis of aspartate and serine in SIRT3 KO MEFs.** (A) Schematic of the contribution of glutamine-derived nitrogen from [U-<sup>13</sup>C,<sup>15</sup>N]glutamine to non-essential amino acids (NEAAs). Red-filled circles represent labeled nitrogen. (B) Relative levels and (C) nitrogen labeling of NEAAs from [U-<sup>13</sup>C,<sup>15</sup>N]glutamine in WT and SIRT3 KO MEFs. Cells were cultured as described in figures 3.2 B-D, followed by addition of fresh culture media or glutamine-free media supplemented with [U-<sup>13</sup>C,<sup>15</sup>N]glutamine, incubation for 4 hours, and metabolite extraction. (D) Heat map representing expression of genes involved in glutamine anapleurosis in WT and SIRT3 KO MEFs. Data are means  $\pm$  SEM (n = 4). \*p < 0.05; \*\*\*p < 0.001.

PSAT1) was significantly higher in SIRT3 KO cells compared to WT cells (Figure 3.3D). Taken together, these results illustrate a plausible mechanism for the increased glutaminolysis observed in the absence of SIRT3.

### *SIRT3 represses glutamine metabolism in breast cancer*

Next, we tested the hypothesis that SIRT3 could repress glutaminolysis. Because SIRT3 expression is low in breast tumors, we stably overexpressed SIRT3 in a panel of breast cancer cells and examined its effect on glutaminolysis (Figure 3.4A). First, we measured glutamine consumption and ammonium secretion in two basal breast cancer cell lines, which have low endogenous SIRT3 expression (compared to luminal breast cancer cells) and found that SIRT3 overexpression significantly decreased glutamine uptake without affecting ammonium production in these cells (Figure 3.4B-C). However, SIRT3 suppressed glutamine consumption by 10-25% and ammonium production by 30-40% in luminal breast cancer cells (Figure 3.4D-E). To determine if the catalytic activity of SIRT3 is required for repression of glutamine metabolism, we overexpressed wild type (WT) or catalytic inactive (HY) SIRT3 in CAMA1 cells and measured glutamine uptake and ammonium production (Figure 3.4F). Overexpression of WT SIRT3, but not the catalytic mutant repressed glutamine uptake and ammonium production demonstrating that catalytic activity of SIRT3 is required to repress glutamine metabolism (Figure 3.4G-H). These findings suggest that SIRT3 may play a role in repressing glutaminolysis; thus, we examined the incorporation of labeled glutamine into TCA cycle intermediates in control and SIRT3-overexpressing T47D breast cancer cells. Compared to control cells, cells overexpressing SIRT3 had decreased labeling of 3 out of 6 measured TCA



**Figure 3.4. SIRT3 represses glutaminolysis in breast cancer cells.** (A) Western blots showing SIRT3 overexpression in a panel of breast cancer cells. Glutamine uptake in (B) basal and (C) luminal breast cancer cells. Ammonium production in (D) basal and (E) luminal breast cancer cells. (F) Western blot demonstrating the expression level of wild type (WT) SIRT3-Flag, or catalytic inactive (H248Y) SIRT3-Flag in CAMA1 breast cancer cells. (G) Glutamine uptake and (H) ammonium uptake in CAMA1 cells with SIRT3 WT or SIRT3 HY overexpressed. (I) Percentage of TCA cycle metabolites labeled with  $[\text{U-}^{13}\text{C}_5, ^{15}\text{N}_2]$  glutamine in control, WT SIRT3 overexpressing T47D cells labeled for 1 hour. Data are means  $\pm$  SEM (n = 4). \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001.

cycle intermediates (Figure 3.4I). These results show that SIRT3 represses glutaminolysis in breast cancer cells.

*The absence of SIRT3 promotes glutathione synthesis.*

Increased entry of glutamine carbon coupled with amino acids production has been shown to contribute to glutathione levels in the cell [13,41]. Glutathione is composed of three amino acids, glutamate, cysteine, and glycine (Figure 3.5A). Glutamine contributes to the pool of glutamate, which is utilized for glutathione synthesis. To determine if SIRT3 affects usage of glutamine for glutathione synthesis, we used a combination of glutamine tracing, metabolomics, and a kit to measure glutathione in WT and SIRT3 KO cells. Analysis of steady-state metabolic profiles showed that SIRT3 KO cells had 1.6 fold times higher levels of glutathione compared to WT cells (Figure 3.5B). In agreement with this result, measurement of total glutathione levels using another method also showed that SIRT3 KO cells had 50% more glutathione than WT cells (Figure 3.5C). Hydrogen peroxide is known to deplete glutathione, and indeed hydrogen peroxide decreased and normalized the difference in glutathione levels between WT and SIRT3 KO cells suggesting that SIRT3 KO cells are more sensitive to oxidative stress (Figure 3.5C).

Next, to determine whether glutamine affected glutathione levels, WT and SIRT3 KO cells were deprived of glutamine for 24 hours, after which glutathione was measured. Glutamine deprivation decreased glutathione levels in both WT and SIRT3 KO cells and normalized the difference in glutathione between these cells (Figure 3.5D). To directly test if glutamine contributes to glutathione synthesis, we examined labeling from glutamine in glutathione. Consistent with our findings, more glutathione was labeled by glutamine in SIRT3 KO MEFs

**Figure 3.5. Glutamine supports increased glutathione levels in SIRT3 KO MEFs.**

(A) Schematic of the contribution of glucose and glutamine to glutathione synthesis. (B) Steady-state glutathione levels in WT and SIRT3 KO MEFs. (C) Glutathione levels in WT and SIRT3 KO MEFs treated with 10 mM hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) or water as a control for 15 minutes. (D) Glutathione levels in WT and SIRT3 KO MEFs in complete medium (control) or media without glutamine (-Gln). Levels of (E) glutamine-labeled or (F) glucose-labeled glutathione in WT and SIRT3 KO MEFs. (G) Relative mRNA levels of genes involved in glutathione synthesis. (H) Schematic depicting the target of butathione sulfoximine (BSO). (I) Growth curve of WT and SIRT3 KO MEFs treated with 100 mM BSO or DMSO (control). Data are means  $\pm$  SEM (n = 4). n.s., not significant. \*\*p < 0.01; \*\*\*p < 0.001.

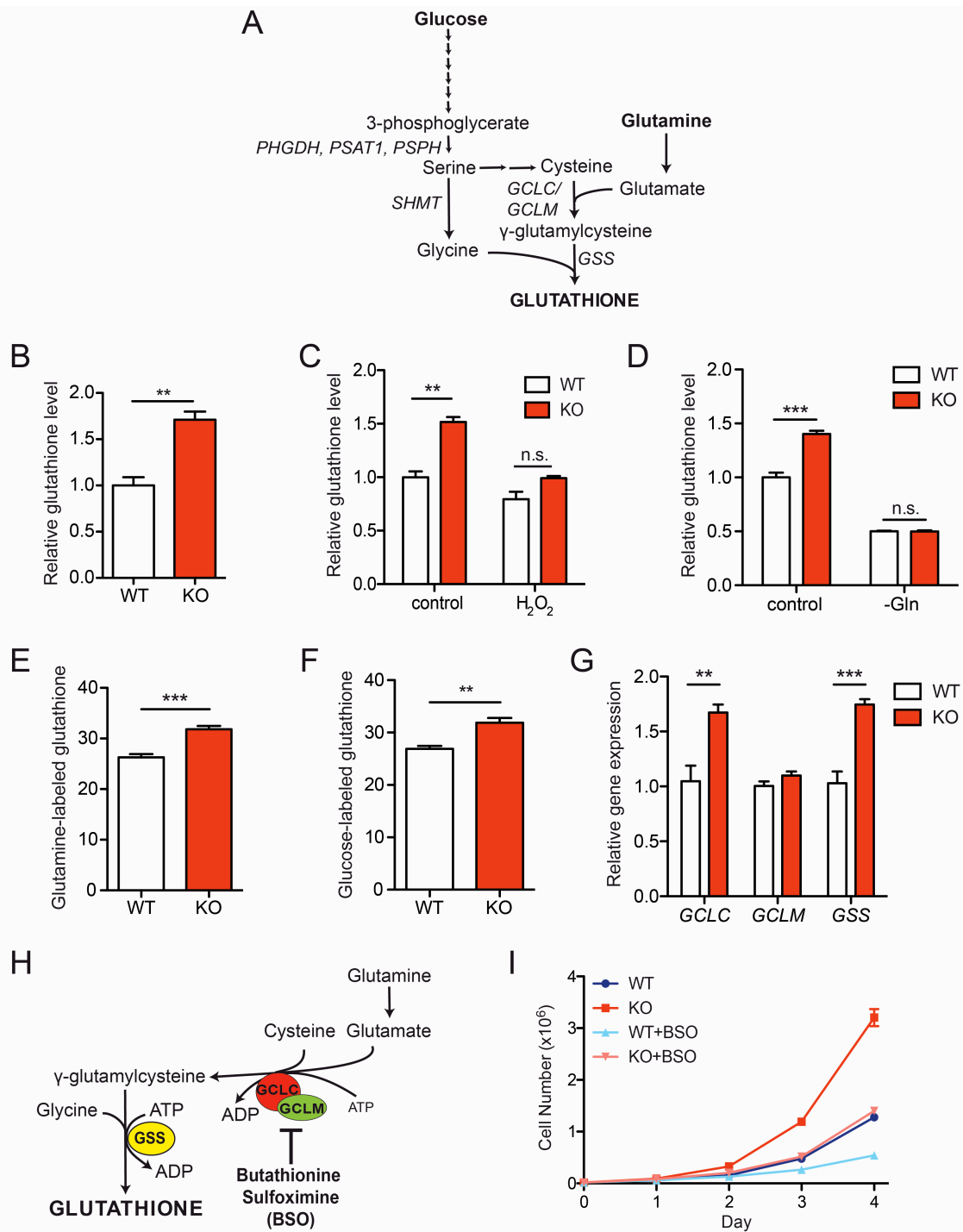


Figure 3.5 (Continued)



compared to WT MEFs (Figure 3.5E) suggesting that increased glutamine usage supports elevated glutathione levels in these cells.

In addition to glutamine, glucose also contributes to glutathione production. In glycolysis, glucose oxidation generates 3-phosphoglycerate, which can be used for synthesis of serine, which is metabolized to generate cysteine and glycine (Figure 3.5A). To examine whether increased glucose consumption in the absence of SIRT3 contributes to glutathione production, I performed a glucose tracing experiment and examined the levels of glucose-labeled glutathione. Indeed, loss of SIRT3 results in increased contribution of glucose to support increased glutathione levels (Figure 3.5F).

As depicted in Figure 3.5A, three enzymes (GCLC, GCLM, and GSS) catalyze the conversion of glutamate, cysteine, and glycine into glutathione. Given that glutathione levels are increased in SIRT3 KO cells, I examined whether expression of glutathione synthesis genes was altered between WT and SIRT3 KO cells. In the absence of SIRT3 we found increased expression of two of the three glutathione synthesis genes: GCLC and GSS (Figure 3.5G). Thus, increased expression of the glutathione biosynthetic machinery may contribute to increased glutathione production in SIRT3 KO cells. Taken together, our results demonstrate that SIRT3 loss leads to reprogramming of multiple nodes of glutamine metabolism known to be altered in cancer cells.

Given that SIRT3 KO cells have rewired usage of glutamine to support increased glutathione synthesis, a pathway shown to stimulate cancer cell proliferation, I used buthionine sulphoximine (BSO), an inhibitor of glutathione synthesis to examine if increased glutathione levels contribute to the rapid growth of SIRT3 KO cells (Figure 3.5H). Because SIRT3 loss results in increased levels of ROS and hydrogen peroxide has a greater effect in depleting

glutathione in these cells, we hypothesized that SIRT3 KO cells would be susceptible to glutathione synthesis inhibition. Although SIRT3 KO MEFs proliferated at about the same rate as control WT MEFs when treated with BSO, inhibition of glutathione synthesis did not ameliorate the difference in growth between these cells (Figure 3.5I). These data suggest that increased glutathione may contribute to, but is not the only factor, promoting increased proliferation of SIRT3 KO cells.

### **3.4 Discussion**

Here we show that deletion of SIRT3 results in rewired metabolism of glutamine, a crucial nutrient to cancer cells. The increased glutamine consumed in the absence of SIRT3 is utilized for anaplerosis while contributing to synthesis of amino acids and glutathione. Moreover, SIRT3 represses glutamine metabolism in breast cancer cells, suggesting that this may be an additional mechanism by which SIRT3 suppresses tumorigenesis.

How SIRT3 loss promotes usage of glutamine to fuel the TCA cycle is unknown. However, SIRT3 deacetylates and activates GDH; thus, in the absence of SIRT3, GDH activity may be decreased suggesting that an alternate pathway supports increased flux of glutamine into the TCA cycle [28,30]. The increased labeling of nitrogen on aspartate and serine suggests that there may be increased activity of transaminases in SIRT3 KO cells. Although SIRT3 deacetylates aspartate transaminase 2 (GOT2), it does not affect its activity [42]. However, elevated expression of transaminases could account for increased contribution of glutamine-derived nitrogen to NEAAs. Consistent with this idea, we found a significant increase in the expression of various transaminases in SIRT3 KO cells compared to wild type cells. This is consistent with pancreatic cancer cells where KRAS represses GDH levels while increasing

GOT1 expression to promote flux of glutamine to the TCA cycle [13]. Additionally, elevated transaminase expression is associated with rapidly proliferating human tumors, and the increase in expression of these genes is mediated in part via mTORC1 [40]. Given that the absence of SIRT3 results in activation of various signaling pathways, including KRAS and mTORC1, it is tempting to hypothesize that SIRT3 loss may result in coupling glutamine anaplerosis to synthesis of NEAAs via induction of these signaling pathways to regulate expression of genes encoding enzymes involved in glutamine metabolism.

How does SIRT3 regulate glutathione metabolism? SIRT3 loss results in increased oxidative stress due to decreased activity of SOD2 and IDH2, which promote clearance of reactive oxygen species (ROS). Glutathione is a key antioxidant that may be induced in the absence of SIRT3 to allow the cells to cope with increased oxidative stress. Glutathione is synthesized in the cytosol; thus, direct regulation of the enzymes that produce glutathione by SIRT3 seems unlikely [43]. However, elevated glutathione levels may be supported by increased glutathione synthesis gene expression in the absence of SIRT3. The transcription factors Myc and nuclear factor erythroid 2-related factor 2 (NRF2) activate expression of the genes encoding the glutathione biosynthetic machinery. Moreover, microarray data and RNA sequencing analysis show that SIRT3 loss induces a Myc signature, which may be a plausible mechanism by which SIRT3 loss induces glutathione production [22]. Alternatively, Myc and NRF2 are stabilized by phosphoinositide-3 kinase (PI3K) signaling, which is activated in the absence of SIRT3, and may also contribute to increased expression of glutathione synthesis genes to support elevated glutathione production [44-46].

Production of reducing equivalents, such as NADPH, is also important for glutathione metabolism, and may be regulated by SIRT3. Glutathione is oxidized in the process of reducing

ROS, and glutathione reductase utilizes NADPH to reduce oxidized glutathione. The oxidative arm of the pentose phosphate pathway (PPP) and the malate-aspartate shuttle contribute to generation of NADPH. In the malate-aspartate shuttle, glutamine-derived oxaloacetate can be converted to aspartate to exit the mitochondria and be converted back to oxaloacetate and to malate in the cytosol, where malic enzyme would convert malate to pyruvate while generating NADPH. Oxidative metabolism of glutamine in the absence of SIRT3 suggests that SIRT3 loss may promote the malate-aspartate shuttle. This is consistent with the idea that in pancreatic cancer cells SIRT3 loss results in increased acetylation of GOT2 to promote its interaction with malate dehydrogenase and stimulate the malate-aspartate shuttle [42]. Additionally, SIRT3 loss may also stimulate NADPH production via the oxidative arm of the PPP [22].

Here we identify two additional metabolic pathways altered upon loss of SIRT3 that may be targeted to decrease proliferation. Our results indicate that suppression of a single deregulated glutamine-dependent pathway, glutathione synthesis, does not ablate the difference in proliferation between control cells and cells with SIRT3 deletion. Additionally, recently it has been shown that inhibition of glutathione synthesis alone has no effect in cancer; however, inhibition of glutathione synthesis and a compensating antioxidant pathway, thioredoxin, synergize to kill cancer cells [47]. Because some types of cancer are refractory to currently used chemotherapies, the idea of treating cancers by targeting multiple deregulated pathways using combination therapies is becoming more and more accepted. Additionally, recent studies have shown that this is a feasible idea and have shed light on potential combination therapies for treatment of human cancer (Isaac, blenis, cichowski). In fact, inhibition of glutaminolysis combined with increased oxidative stress increases pancreatic cancer cell death *in vitro*, suggesting that this may be a possible combination to treat cancer cells with decreased SIRT3.

Thus, inhibition of multiple deregulated pathways in cells with loss of SIRT3 may be a better option to significantly inhibit the growth or survival of a cancer cell with SIRT3 loss.

Our findings underscore the importance of SIRT3 in repressing cancer cell metabolism. SIRT3 expression is low in various types of cancers where it functions as a tumor suppressor. We show that in addition to repressing the Warburg effect, SIRT3 overexpression suppresses glutamine metabolism in breast cancer cells. It is important for future studies to examine if our findings translate to tumors *in vivo* and to determine the importance of the identified metabolic alterations in the absence of SIRT3 to tumorigenesis. In sum, we reveal rewiring of various nodes of glutamine metabolism, similar to that observed in cancer cells, in the absence of SIRT3. These findings suggest that suppression of glutamine metabolism may be part of the mechanism by which SIRT3 suppresses tumorigenesis.

### **3.5 Experimental Procedures**

#### *Cell Culture*

SV40T immortalized WT and SIRT3 KO mouse embryonic fibroblasts (MEFs) were cultured in DMEM media supplemented with 10% FBS, penicillin/streptomycin, 2 mM L-glutamine, non-essential amino acids (NEAAs), and 0.1 mM β-mercaptoethanol. Breast cancer cell lines: MCF7, T47D, CAMA-1, and MDA-MB-231 were maintained in DMEM media supplemented with 10% FBS, penicillin/streptomycin, and 2 mM L-glutamine. MCF10A cells were cultured in 1:1 DMEM/F12 media supplemented with penicillin/streptomycin, 5% horse serum, 20 ng/mL EGF, 100 ng/mL cholera toxin, 0.5 mg/mL hydrocortisone, and 10 μg/mL insulin.

SIRT3-overexpressing cells were generated by retroviral infection with empty pBabe vector or pBabe vector containing human SIRT3 cDNA with a FLAG tag.

### *Reagents*

Labeled L-glutamine (U-<sup>13</sup>C<sub>5</sub>, 97-99%; U-<sup>15</sup>N<sub>2</sub>, 97-99%) and D-glucose (U-<sup>13</sup>C<sub>6</sub>, 99%) were obtained from Cambridge Isotope Laboratories. The HT glutathione assay was obtained from Trevigen (catalog #7511-100-K). Buthionine sulfoximine (BSO) was obtained from Sigma (catalog #B2515).

### *Glutamine and Ammonium Measurements*

Cells were plated in 6-well plates and media was changed 24 hours later. Culture media was collected after 8-24 hours, and the number of cells in each well was counted using a Beckman Coulter particle counter. Glutamine and ammonium concentrations in fresh and culture media were measured after 8 to 24 hours using the BioProfile FLEX analyzer (NOVA Biomedical), and the measurements were normalized to cell number.

### *Metabolite Profiling*

Metabolites were extracted from cells using 80% ice-cold methanol on dry ice [22,48]. Briefly, high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) was used to quantitatively profile polar metabolites. The QTRAP mass spectrometer (Thermo Fisher Scientific) was used to acquire data, and the TraceFinder software was utilized to analyze the data. An identical set of plates was seeded in the same way as for the experiment, and protein was extracted and quantified by a BCA assay (Thermo Fisher Scientific) to normalize metabolite levels. For tracing experiments, cells were seeded, and 24 hours later, media was removed and

cells were washed once with PBS. Cells were then incubated with glutamine-free media supplemented with 6 mM L-glutamine (U-<sup>13</sup>C<sub>5</sub>, 97-99%, <sup>15</sup>N<sub>2</sub>, 97-99%), or glucose-free media supplemented with 25 mM D-glucose (U-<sup>13</sup>C<sub>6</sub>, 99%), 10% dialyzed FBS, NEAAs, penicillin/streptomycin, and 0.1 mM β-mercaptoethanol for the specified time (1, 4, or 8 hours). Media was removed and cells were washed with cold PBS once prior to metabolite extraction.

### *Glutathione Measurements*

The HT Glutathione Assay Kit was utilized to measure total glutathione levels according to manufacturer instructions.

### *Quantitative real-time PCR:*

Total RNA was isolated by a TRIzol (Invitrogen) extraction followed by a Direct-zol RNA MiniPrep Kit (Zymo Research) purification following the manufacturer's instructions. Total RNA (1 μg) was used for reverse transcription with the iScript cDNA synthesis kit (Bio-Rad). cDNA was diluted and used for analysis by quantitative real-time PCR using Sybr Green I Mastermix on a Lightcycler 480 (Roche). Values were normalized to a reference gene, and the  $2^{-\Delta\Delta Ct}$  method was used for analysis. Primer sequences utilized in this study are listed below.

Primer sequences utilized in this study are listed below:

<b>Gene</b>	<b>Primer</b>
mouse GCLM	F GCCACCAGATTTGACTGCCTT
	R TGCTCTTCACGATGACCGAGT

mouse GCLC      F ACATCTACCACGCAGTCAAGG  
                         R CTCAAGAACATCGCCTCCATT

mouse GSS        F GAATGGAAGCTGCTTTGAGGC  
                         R AGGCACTAGAACCTGCTGGAA

mouse B2M        F GCTCGGTGACCCTGGTCTTT  
                         R TGTTCGGCTTCCCATTCTCC

Extracted RNA was also used for RNA sequencing as described in Chapter II.

### *Statistics*

Statistical significance between groups was determined using the unpaired Student's t test. Significance is as follows: \*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ . All experiments were performed at least two to three times.

### **3.6 Acknowledgements**

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## **CHAPTER IV**

### **Discussion**

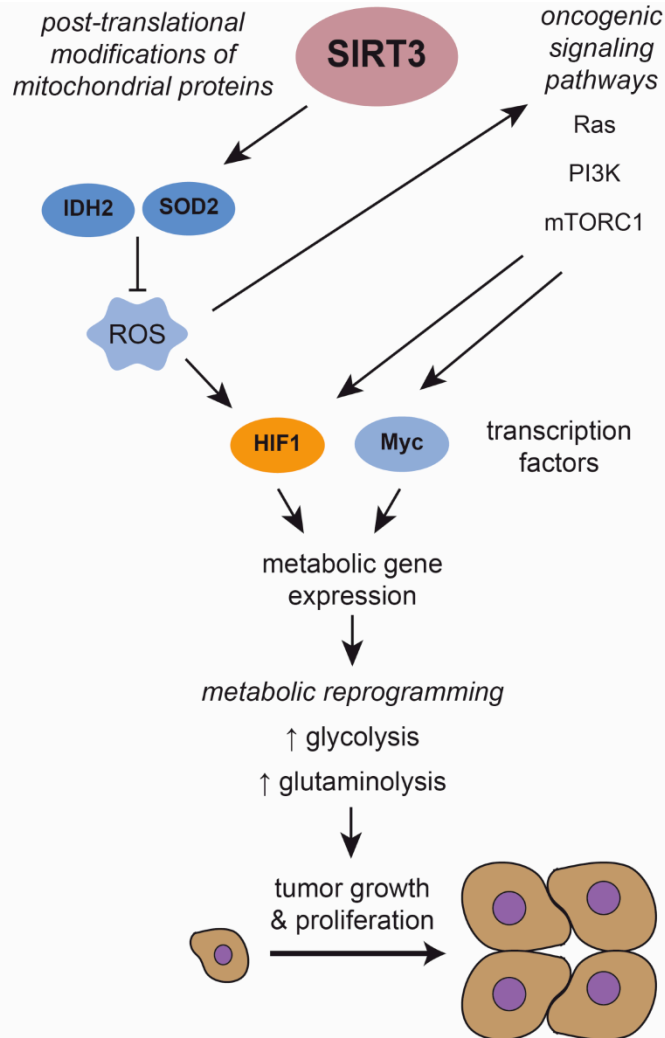
## **4.1 Overview**

Metabolic reprogramming is at the core of a cancer cell's strategy for enhancing its survival, growth, and proliferation. Oncogenes and tumor suppressors contribute to deregulated metabolism displayed by cancer cells. Decreased expression of SIRT3 is observed across multiple types of human cancer where SIRT3 has been shown to have tumor suppressive function. SIRT3 is a deacetylase and a key regulator of mitochondrial biology that stimulates metabolic targets to promote oxidative metabolism, energy production, and defense from oxidative stress. SIRT3 loss results in deregulation of numerous nodes of metabolism, and suppresses tumorigenesis in part by repressing the Warburg effect, via reduction of ROS (Figure 4.1). Although our understanding of the role that SIRT3 plays in cancer has grown, it continues to be a challenge to translate our findings into improving therapies for cancer treatment. Having a better understanding of the strategy adopted by cancer cells to sustain tumorigenesis could provide insight into potential targets for therapeutic intervention. Therefore, in this dissertation, I utilized a small molecule screen to identify new vulnerabilities that may provide mechanistic insight into how SIRT3 loss could promote tumorigenesis. First, I identified glutamine metabolism as a novel vulnerability in cells with SIRT3 deletion. Second, I systematically analyzed the effect of SIRT3 on multiple nodes of glutamine metabolism.

## **4.2 Small molecule screen**

We used a chemical biology approach to identify a new metabolic vulnerability in tumors with SIRT3 deletion. Chapter II described these findings where we identify a glutamine analog, azaserine, as a selective inhibitor of SIRT3 null cell growth. We show that SIRT3 deletion renders cells reliant on glutamine metabolism for growth and proliferation. Because glutamine





**Figure 4.1. Summary of the dissertation: oncogenic signaling pathways and tumor suppressors mediate metabolic reprogramming in cancer to increase tumor growth and proliferation.** Signaling cascades, such as Ras, phosphoinositide-3 kinase (PI3K), and mechanistic target of rapamycin complex 1 (mTORC1), regulate metabolism, and overexpression or mutations in components of these pathways contribute to altered metabolism in cancer. Additionally, signaling cascades can be activated by reactive oxygen species (ROS) and stabilize transcription factors, such as hypoxia inducible factor 1 (HIF1) and Myc, which control metabolic gene expression to increase glycolysis or glutaminolysis. Mitochondrial sirtuin 3 (SIRT3) represses ROS by deacetylating its targets, such as manganese superoxide dismutase 2 (SOD2) and isocitrate dehydrogenase 2 (IDH2), to destabilize HIF1 and repress the Warburg effect.

contributes to multiple nodes of metabolism, we utilized metabolomics to identify the metabolic node significantly affected by azaserine and found elevated nucleotide metabolism to be the pathway that SIRT3 null cells rely on for increased proliferation. Using bioinformatics and RNA sequencing analysis, we reveal mTORC1 signaling as part of the mechanism underlying alterations in nucleotide synthesis with SIRT3 loss. Most importantly, we reveal that decreased SIRT3 expression is lowest in basal-like breast cancers and is associated with increased mTORC1 signaling *in vitro* and in human breast tumor samples. Lastly, like SIRT3 overexpression, treatment with rapamycin or azaserine significantly suppressed the growth of breast cancer cell spheres in 3-dimensional culture with a stronger effect in control cells with low SIRT3 expression. Our findings suggest that SIRT3 may be used as a biomarker for determining susceptibility to nucleotide- or mTORC1-targeting therapies in breast cancer, and potentially as a biomarker for determining breast cancer subtype. Future study will be needed to examine the relevance of these findings in breast cancer patients. Moreover, whether these findings can be translated across other types of cancers would also be important to know.

Although the focus of Chapter II centered on azaserine as the top compound that selectively decreased growth of SIRT3 null cells, other top hits from the small molecule screen may reveal additional vulnerabilities in cells with SIRT3 loss. The second top hit from the screen was decitabine, a nucleoside analog known to incorporate into DNA to inhibit DNA methyltransferases to promote hypomethylation, and to induce the DNA damage response and result in cell cycle arrest [1-4]. Thus, SIRT3 null cells may also be susceptible to DNA damaging agents. The additional top 5 hits from the screen, vinblastine sulfate, vindesine, and vincristine sulfate, are part of a class of compounds known as alkaloids [5,6]. These compounds function by interfering with microtubule formation to inhibit cell division and are typically used as part of

combination therapies to treat cancer. Altogether these compounds suppress proliferation of cells with SIRT3 loss inhibiting cell cycle progression. Moreover, it would be interesting to explore if these compounds could synergize with azaserine to induce death of cells with SIRT3 deletion.

The approach of using a small molecule screen to identify vulnerabilities in cancer can be used to examine additional mechanisms underlying cancer. Moreover, this approach could be used to test combination therapies that could synergize to result in cancer cell death or tumor regression, as has been done recently [7]. In addition, because in some types of cancer, such as oral squamous cell carcinoma and clear cell renal carcinoma, SIRT3 promotes tumorigenesis, it would be interesting to analyze results from our small molecule screen to identify compounds that could selectively sensitize cells expressing SIRT3 [8,9]. Compounds identified in this kind of analysis could provide insight into pathways that could be targeted to treat cancers with increased SIRT3 levels.

#### **4.3 SIRT3 and glutamine metabolism**

Glutamine contributes to a cadre of metabolic pathways (Figure 1.1). Based on the observations that glutamine consumption was deregulated in the absence of SIRT3 and that glutamine analogs selectively decreased growth in cells with SIRT3 deletion, we examined the effect of SIRT3 on metabolism of glutamine, an important nutrient for many, but not all, cancer cells. In Chapter III, we describe rewiring of multiple facets of glutamine metabolism in cells lacking SIRT3. Strikingly, we demonstrate that SIRT3 is a major regulator of numerous facets of glutamine metabolism. First, we show that loss of SIRT3 stimulates entry of glutamine-derived carbon into the TCA cycle, and this is coupled with increased contribution of nitrogen to synthesis of nonessential amino acids. Additionally, overexpression of SIRT3 in breast cancer

cells suppresses glutamine anaplerosis. We also show that the increased glutamine consumption supports elevated glutathione levels in cells lacking SIRT3. These findings reveal new additional alterations, consistent with those seen in cancer cells, in the absence of SIRT3. A glutamine tracing experiment *in vivo* would be important to determine if SIRT3 null cells display the same pattern of deregulation in these nodes of metabolism. In addition, future studies will be crucial to determine the relevance of these findings in cancer, and to determine if these pathways may be targeted for therapy of patients with tumors lacking SIRT3.

#### **4.4 Mechanisms of action**

Our findings reveal alterations in multiple nodes of glutamine metabolism in the absence of SIRT3, and this along with published findings, suggest that SIRT3 loss results in induction of a program that contributes to reprogramming of glutamine metabolism to promote cancer. Therefore, although not impossible, it seems unlikely that a single SIRT3 target could account for the multiple alterations observed upon SIRT3 loss. Future studies could focus on examining the mechanism by which SIRT3 loss promotes glutamine anaplerosis. A major effort was made to identify a mechanism by which SIRT3 regulates glutamine metabolism. Two potential mechanisms by which SIRT3 could affect glutamine metabolism are: 1). Direct regulation of target enzymes involved in glutamine metabolism, and/or 2). Indirect regulation of pathways that control glutamine or nutrient utilization. Direct deacetylation of known SIRT3 targets involved in glutamine metabolism, such as glutamate dehydrogenase (GDH) does not seem logical as SIRT3 stimulates GDH activity and, thus, would be expected to be less active in the absence of SIRT3 [10,11]. Additionally, deacetylation of aspartate transaminase (GOT2) by SIRT3 does not have an effect on GOT2 activity [12]. However, consistent with recent publications, we show

that SIRT3 loss results in a significant increase of transaminase gene expression, which is observed during proliferation and in cancer cells [13,14]. This increase in gene expression, if also observed at the protein level, suggests that SIRT3 loss may result in increased transaminase activity and could be the mechanism underlying increased contribution of glutamine to TCA cycle intermediates and nonessential amino acids. Moreover, the use of glutaminase (BPTES or CB-839) or transaminase (aminooxyacetate, AOA) inhibitors would allow us to determine the importance of this pathway to SIRT3 null cells. Whether SIRT3 represses transaminase gene expression remains to be examined.

Another potential mechanism to explain our observations could be direct regulation of amino acid transporters by SIRT3. To my knowledge regulation of amino acid transporters by SIRT3 has been unexplored. However, unpublished findings from our laboratory reveal that SIRT3 interacts with various amino acid transporters. For instance, SIRT3 interacts with a glutamate/aspartate transporter, which exchanges mitochondrial aspartate for cytosolic glutamate. Given that SIRT3 tends to deacetylate and activate its targets, it may be possible that SIRT3 stimulates this amino acid transporter. If so, in the absence of SIRT3, decreased transporter activity could lead to decreased entry of glutamate into the mitochondria and result in increased glutaminolysis to generate glutamate upon SIRT3 loss.

Another plausible mechanism is that SIRT3 may indirectly affect glutamine metabolism by regulating glucose or pyruvate utilization. Glucose and glutamine complement each other for synthesis of biosynthetic intermediates, and it has been shown that usage of glutamine depends on the presence of glucose [15]. Thus, elevated glycolysis in the absence of SIRT3 could promote increased glutamine metabolism. SIRT3 stimulates pyruvate utilization by deacetylating and activating pyruvate dehydrogenase (PDH) [16]. Loss of SIRT3 could result in decreased

PDH activity shunting pyruvate away from the mitochondria, which may be compensated by stimulation of glutamine anaplerosis.

#### **4.5 Stress programs**

Indirect modulation of signaling pathways that regulate nutrient utilization could be an additional mechanism by which SIRT3 affects glutamine metabolism. Multiple signaling pathways, such as the Ras and mTORC1 signaling pathways, and transcription factors, such as Myc and nuclear factor erythroid 2-related factor 2 (NRF2), have been shown to regulate glutamine metabolism. These signaling pathways are regulated by reactive oxygen species (ROS). Our laboratory and numerous studies have highlighted the role of SIRT3 in promoting clearance of ROS by activating SOD2 and IDH2 [17-20]. SIRT3 may also regulate ROS generation via the electron transport chain and fatty acid oxidation. ROS is a double-edged sword. At high levels, ROS can cause significant cellular damage and lead to cell death; however, at low levels, ROS can function as a signaling molecule essential for induction of signaling cascades [21-24]. In fact, ROS is necessary for insulin or growth factor signaling and has been shown to activate signaling cascades by inactivating phosphatases (via oxidation of cysteine residues), such as PTEN [25]. Thus, increased ROS levels upon SIRT3 loss could activate these signaling pathways to regulate glutamine metabolism.

In Chapter II we find that SIRT3 loss results in increased Ras, PI3K, and mTORC1 signaling. In fact, we show that elevation in nucleotide synthesis is due, in part, to hyperactive mTORC1 signaling. In addition to regulating *de novo* nucleotides synthesis, mTORC1 also stimulates glutaminolysis [14,26]. Whether deregulation of additional nodes of glutamine metabolism in the absence of SIRT3 is due to hyperactive mTORC1 signaling remains to be

examined. Moreover, the mechanism by which SIRT3 regulates mTORC1 signaling is unknown, but one possible model is that increased ROS may stimulate this and upstream signaling pathways.

The transcription factor Myc has been shown to stimulate glutaminolysis by inducing expression of glutamine transporters and glutaminase. Consistent with our RNA sequencing data, a previous study from our laboratory reported enrichment of the Myc signature from SIRT3 null brown adipose tissue. We validated increased expression of Myc targets, such as elongation factors (eIF4E and eIF2 $\alpha$ ), in SIRT3 null cells. However, expression of the Myc targets known to mediate glutaminolysis was not altered and knockdown of Myc had no effect on glutamine consumption in cells with SIRT3 deletion (data not shown).

A key finding in Chapter III is that SIRT3 loss stimulates glutathione production. Because SIRT3 loss has been shown to result in increased ROS, which is often cleared by glutathione, we anticipated a decrease in glutathione levels. However, we find increased glutathione levels in the absence of SIRT3. A potential explanation for this is that increased ROS induces the antioxidant stress response driven by NRF2, which is known to induce expression of genes encoding ROS detoxifying proteins, including glutathione synthesis genes [23,27]. Thus, given that SIRT3 null cells have increased expression of glutathione synthesis, it would be interesting for future studies to investigate whether the increased ROS in cells with SIRT3 deletion induces the antioxidant stress response via NRF2 to help these cells cope with increased oxidative stress.

In addition to playing an important role as an antioxidant, glutathione is important for proliferation and has been implicated in chemoresistance [28,29]. Along the line with the idea that glutathione may serve to protect SIRT3 null cells from increased ROS, glutathione inhibition

was hypothesized to have a significant effect on cells with SIRT3 deletion; however, that was not the case. We found that inhibition of glutathione synthesis decreased proliferation of control and SIRT3 null cells to the same extent. Although glutathione is thought to be the major antioxidant, a recent study has highlighted the fact that additional nodes of the antioxidant stress response can compensate in the absence of glutathione [30]. These findings highlight the plasticity of the cell and support the idea that combination therapies may be more efficient for treatment of cancer. Lastly, glutathione has been implicated in resistance to chemotherapy [23,31]. Thus, determining whether SIRT3 plays a role in mediating resistance to cancer therapies will also be important.

#### **4.6 Conclusion**

Cancer continues to be a leading cause of death worldwide. The impetus for this dissertation came from the hope of identifying an Achilles' heel for treatment of cancer. Moving towards a future of personalized medicine, a better understanding of the mechanisms harnessed by tumor cells may aid in determining the most advantageous therapy for a patient given the genetic background of their cancer. The work presented in this dissertation aimed to uncover mechanisms adopted by tumors with loss or decreased expression of SIRT3. SIRT3 is a master regulator of mitochondrial biology. By deacetylating its targets, SIRT3 orchestrates various nodes of metabolism to maintain cellular homeostasis. Our findings reveal alterations in multiple nodes of glutamine metabolism in the absence of SIRT3. This body of work contributes to the knowledge of how SIRT3 loss results in metabolic rewiring to promote tumorigenesis. We hope that these findings will provide insight into therapies for treatment of cancer.



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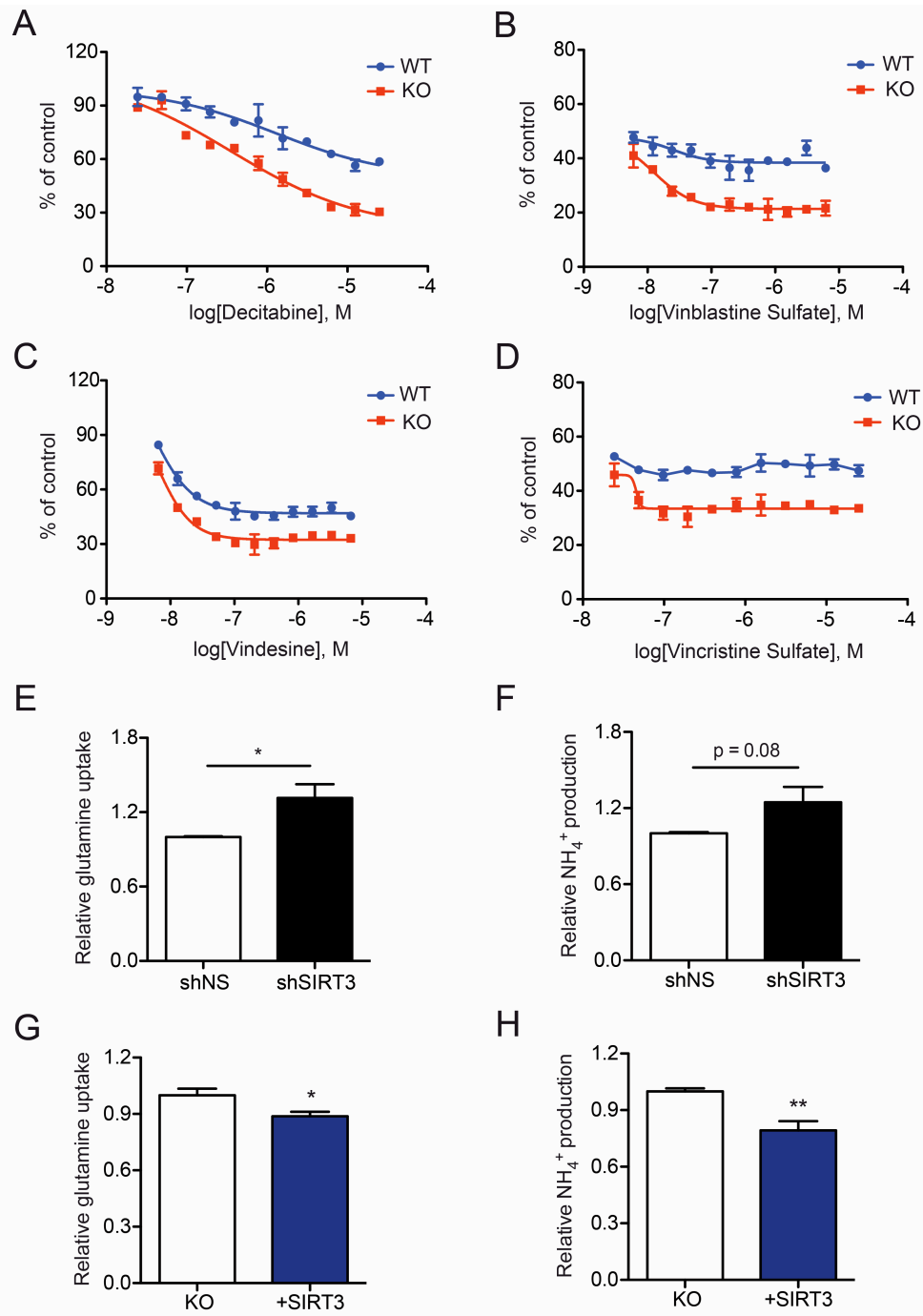
## **APPENDIX I**

**Supplemental data figures and tables to accompany Chapter II.**

**Table S2.1. Top 50 hits from small molecule screen to selectively inhibit SIRT3 KO MEF growth**

#	Compound name	WT IC50 (μM)	KO IC50 (μM)	ΔAUC	p-value
1	Azaserine	>29	2.91	63.3	0.011
2	Decitabine	>25	1.28	60.2	0.006
3	Vinblastine Sulfate	0.02	0.01	46.6	0.010
4	Vindesine	0.06	0.01	45.5	0.005
5	Vincristine Sulfate	<0.02	<0.02	42.3	0.003
6	Podofilox	0.05	0.02	41.5	0.002
7	Ch 55	>25	>25	39.9	0.018
8	Mebendazole	4.34	1.02	38.7	0.004
9	Nocodazole	0.22	0.06	35.1	0.004
10	Vinorelbine Bitartrate	0.17	0.15	34.0	0.009
11	Picropodophyllotoxin	5.81	2.53	30.4	0.006
12	Retinoic acid p-hydroxyanilide	10.75	4.56	28.9	0.052
13	Fenbendazole	>17	2.70	28.5	0.010
14	IC261	4.84	0.87	26.3	0.020
15	SB 225002	4.29	1.83	24.5	0.059
16	Albendazole	>19	4.30	23.4	0.036
17	cis-(Z)-Flupentixol dihydrochloride	15.92	12.18	23.0	0.097
18	JNK Inhibitor IX	2.24	1.20	22.7	0.002
19	Amiodarone-HCl	19.78	10.80	21.3	0.230
20	Fluphenazine Dihydrochloride	11.62	9.81	20.3	0.021
21	82640-04-8	26.57	15.21	18.8	0.101
22	Aclarubicin	0.03	0.02	18.3	0.091
23	Zucapsaicin	24.94	13.21	18.0	0.025
24	Prenylamine Lactate	10.56	9.95	17.5	0.079
25	LY 2183240	0.87	0.59	15.6	0.164
26	Suloctidil	7.48	5.70	15.1	0.530
27	Nortriptyline	45.33	35.75	12.6	0.168
28	Methiothepin Mesylate	15.92	10.60	12.6	0.279
29	Salmeterol	27.01	19.69	11.9	0.241
30	Duloxetine Hydrochloride	11.37	9.60	9.8	0.288
31	Itoraconazole	>25	>25	9.1	0.436
32	S 14506 Hydrochloride	56.76	25.02	8.7	0.238
33	Kenpauillone	29.95	15.87	8.5	0.259
34	Capsaicin	28.64	10.00	8.4	0.358
35	Spermidine Trihydrochloride	8.95	7.62	8.1	0.472
36	Perphenazine	15.07	11.91	7.6	0.345
37	AMN082	33.46	18.39	7.3	0.566
38	Plicamycin	1.07	0.82	5.2	0.797
39	TGF-β RI Inhibitor III	28.61	30.15	5.0	0.694
40	NNC 26-9100	17.88	13.05	4.6	0.771
41	Iodophenpropit Dihydrobromide	18.57	10.45	4.4	0.789
42	Fenretinide	>13	8.56	3.9	0.426
43	Triamterene; 396-01-0	>25	35.04	3.5	0.642
44	Flunarizine dihydrochloride	32.55	22.84	3.2	0.812
45	Furoxan	3.96	3.58	1.8	0.850
46	Bleomycin	17.21	17.09	0.2	0.971
47	AG 494	>25	>25	-1.3	0.964
48	WIN 55	24.72	19.02	-5.3	0.663
49	Dipyridamole	>25	>25	-6.2	0.343
50	24(S)-hydroxycholesterol	>31	>31	-12.2	0.108

**Figure S2.1, related to Figure 2.1.** (A) Dose response curves of WT and SIRT3 KO MEF growth after treatment with Decitabine, (B) Vinblastine Sulfate, (C) Vindesine, and (D) Vincristine Sulfate for 72 hours. (E) Glutamine uptake and (F) ammonium production in control (shNS) or SIRT3 knockdown (shSIRT3) MCF10A cells. (G) Glutamine uptake and (H) ammonium production in SIRT3 KO MEFs expressing empty vector or SIRT3.



**Figure S2.1 (Continued)**



**Table S2.2. Significantly affected metabolites by azaserine in SIRT3 KO MEFs**

Metabolite	Metabolic Pathway	Fold change relative to WT				p-value
		WT	WT + azaserine	KO	KO + azaserine	
glutamate	Glutamate Metabolism	1	0.12	1.66	0.17	0.000
diadenosine triphosphate	Purine Metabolism	1	0.78	1.48	0.80	0.001
N-acetylneuraminate	Aminosugar Metabolism	1	0.82	1.04	0.62	0.002
5-methyltetrahydrofolate	Folate Metabolism	1	0.69	0.62	0.42	0.002
fructose	Fructose Metabolism	1	1.43	0.92	2.74	0.002
ADP	Purine Metabolism	1	1.10	1.00	0.53	0.004
choline	Phospholipid Metabolism	1	0.99	1.14	1.55	0.005
uracil	Pyrimidine Metabolism	1	1.13	0.91	1.51	0.007
thymidine	Pyrimidine Metabolism	1	1.72	1.33	6.68	0.009
xanthosine 5'-monophosphate	Purine Metabolism	1	0.80	1.48	0.55	0.009
5'- GMP	Purine Metabolism	1	0.78	1.10	0.62	0.015
acetylcarnitine (C2)	Fatty Acid Metabolism	1	0.95	0.95	0.73	0.018
orotate	Pyrimidine Metabolism	1	0.52	0.57	0.28	0.020
CDP-choline	Phospholipid Metabolism	1	0.90	1.13	0.56	0.021
2'-deoxyuridine	Pyrimidine Metabolism	1	0.00	0.78	2.69	0.022
guanosine 5'-diphospho-fucose	Nucleotide Sugar	1	0.85	0.99	0.79	0.028
NAD+	Nicotinate Metabolism	1	0.94	1.00	0.70	0.028
leucylglutamate	Dipeptide	1	0.81	1.37	1.02	0.029
AMP	Purine Metabolism	1	0.91	1.21	0.81	0.029
3-hydroxydecanoate	Fatty Acid	1	0.94	1.18	0.90	0.039
uridine	Pyrimidine Metabolism	1	1.11	0.91	1.34	0.041
gamma-glutamylthreonine	Gamma-glutamyl Amino Acid	1	1.21	1.06	1.47	0.044

**Figure S2.2, related to Figure 2.2.** (A) Diagram generated by MetaboAnalyst 3.0 analysis of highlighting metabolic pathways significantly affected by azaserine in SIRT3 KO MEFs. (B) Schematic of pyrimidine metabolism. Shaded in pink is the *de novo* pyrimidine synthesis pathway, and shaded in blue are intermediates in pyrimidine degradation or the salvage pathway. Blue arrows represent purine salvage pathways. (C) N-carbamoyl aspartate, (D) orotate levels in WT and SIRT3 KO MEFs treated with 30  $\mu$ M azaserine (or DMSO as a control) for 6 hours. (E) Incorporation of  $^{14}$ C-aspartate, or (F)  $^3$ H-thymidine into DNA in WT and SIRT3 KO cells. WT and SIRT3 KO MEFs were serum starved for 16 hours and then labeled and stimulated by insulin for 8 hours. Induction with insulin compared to serum starved values were graphed. (G) Steady-state levels of metabolites in pyrimidine metabolism in WT and SIRT3 KO MEFs. (H) Immunoblot of control (shNS) and PPAT knockdown (sh#1 or sh#2) in WT and SIRT3 KO MEFs.

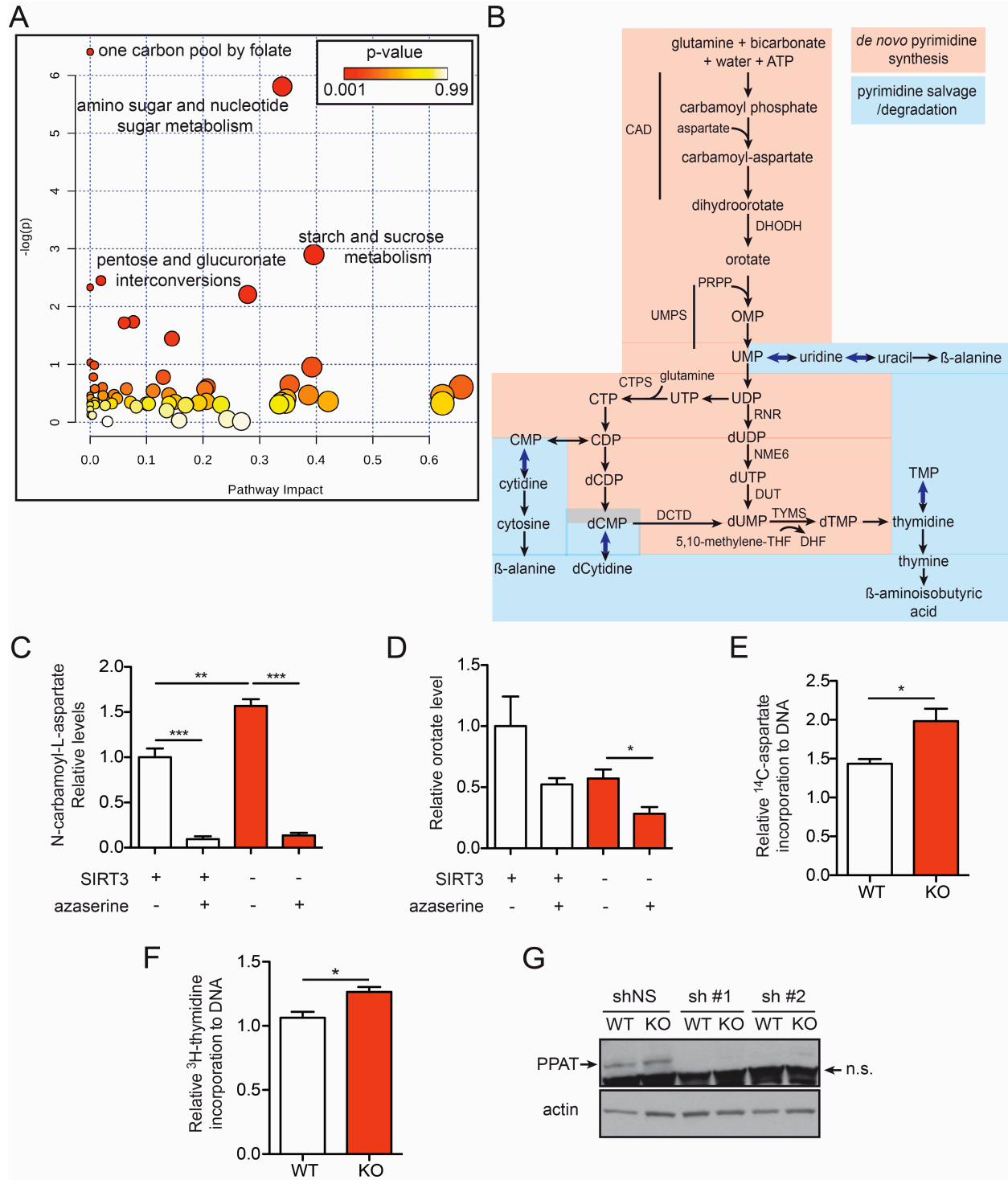
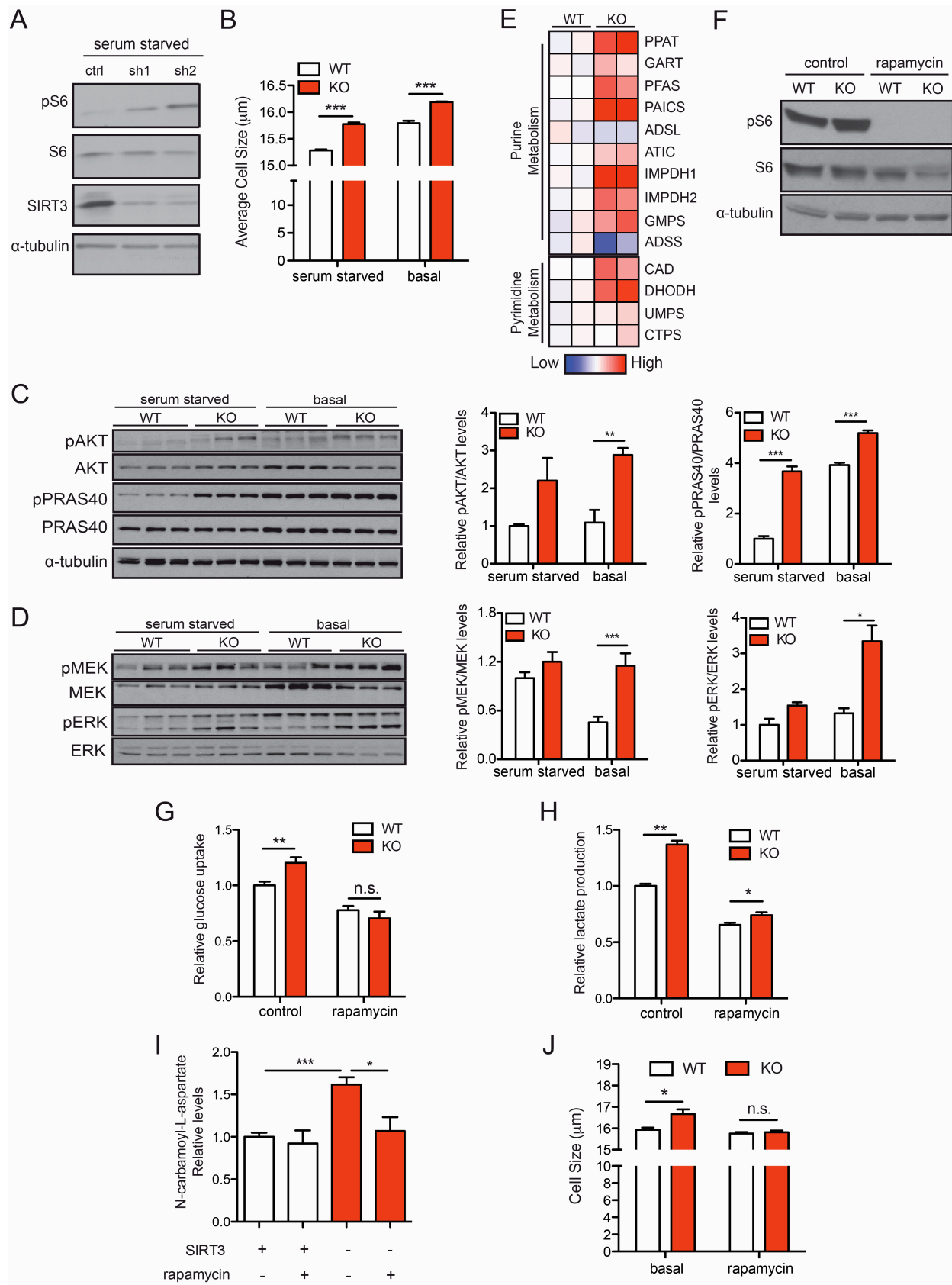
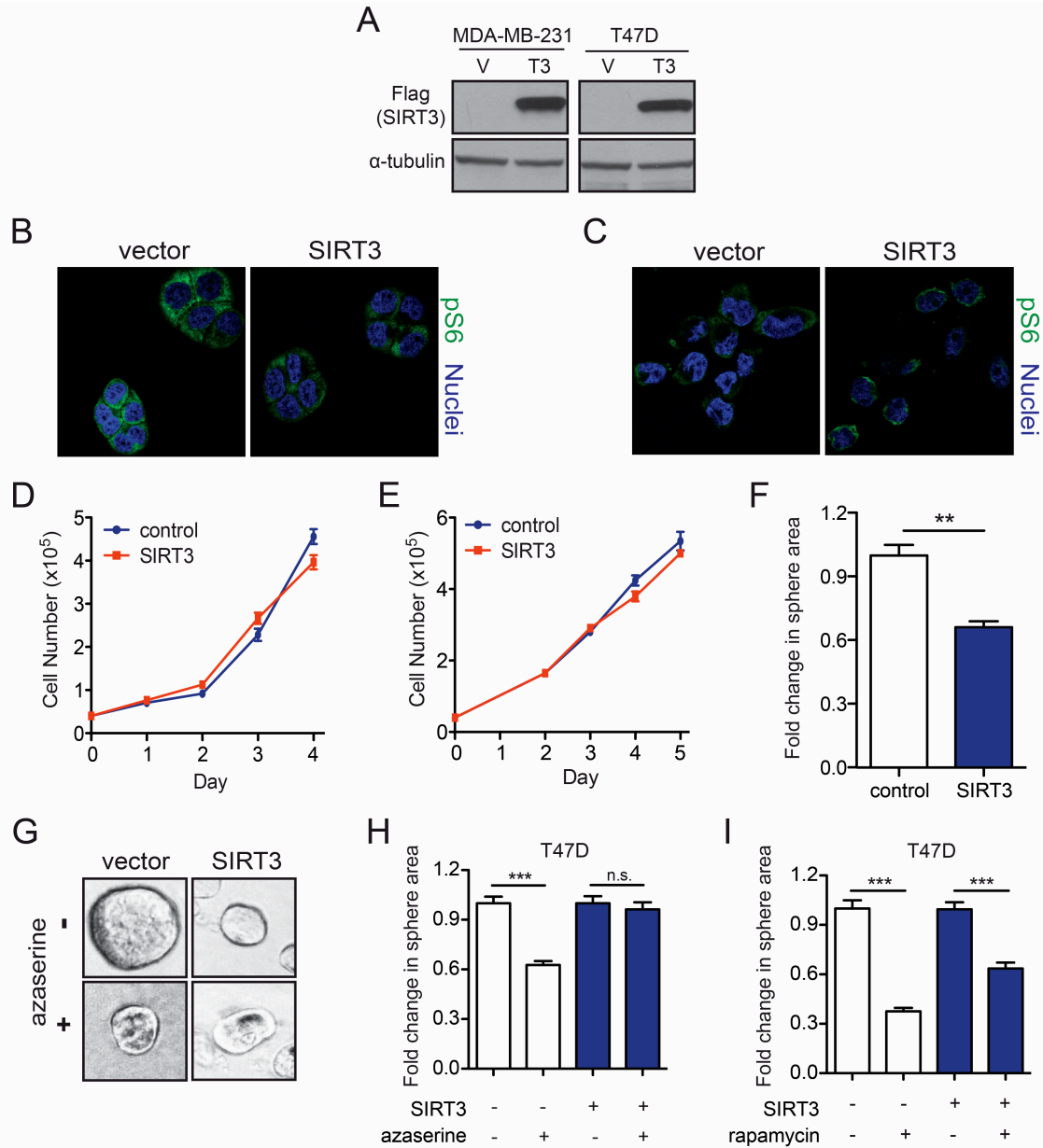


Figure S2.2 (Continued)

**Figure S2.3, related to Figure 2.3.** (A) Immunoblots of phospho- and total S6 in control (shNS) or SIRT3 knockdown (sh1 or sh2) MCF10A cells. (B) Cell size of WT and SIRT3 KO MEFs under serum starvation or basal conditions (n = 3). Cell size was measured using a Beckman Coulter Z2 cell counter. Immunoblots proteins phosphorylated upon PI3K (C), and Ras (D) activation in WT and SIRT3 KO MEFs after 16 hours of serum starvation or nutrient replete conditions. (E) Gene expression of genes in purine and pyrimidine synthesis from RNA Seq data from WT and SIRT3 KO MEFs. (F) Immunoblots of WT and SIRT3 KO MEFs treated with DMSO or 100 nM rapamycin for 24 hours. Glucose uptake (G) and lactate production (H) in WT and SIRT3 KO MEFs treated with 100 nM rapamycin or DMSO as a control. (I) N-carbamoyl aspartate levels in WT and SIRT3 KO MEFs treated with DMSO (control) or 100 nM rapamycin. (J) Cell size of WT and SIRT3 KO MEFs treated with DMSO as a control, or 100 nM rapamycin for 24 hours.



**Figure S2.3 (Continued)**



**Figure S2.4, related to Figure 2.4.** (A) Immunoblots depicting overexpression of SIRT3 in breast cancer cell lines. Representative immunofluorescence images of (B) MDA-MB-231 or (C) T47D control and SIRT3-overexpressing cells stained with phospho-S6 antibody. (D) Growth curve of control or SIRT3 overexpressing T47D or (E) MDA-MB-231 cells in 2D culture. (F) Quantification of the area of spheres formed by control or SIRT3 overexpressing T47D cells. (G) Representative image of spheres formed by control and SIRT3 overexpressing T47D cells treated with empty vehicle or 30  $\mu$ M azaserine in 3-dimensional (3D) culture, and (H) quantification of sphere area (normalized to the corresponding empty vehicle control). (I) Quantification of the area of spheres formed by control or SIRT3 overexpressing T47D cells treated with 100 nM rapamycin or empty vehicle (normalized to the corresponding empty vehicle control). Data are means  $\pm$  SEM (n = 3-4). n.s., not significant. \*\*p < 0.01; \*\*\*p < 0.001.