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Myc induces expression of glutamine synthetase through promoter demethylation

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Running Title: Myc upregulates GS via promoter demethylation
Summary

The proto-oncoprotein Myc is known to promote glutamine usage by up-regulating glutaminase (GLS), which converts glutamine to glutamate that is catabolized in the tricarboxylic acid (TCA) cycle. Here we report that in a number of human and murine cells and cancers, Myc overexpression leads to elevated expression of glutamate-ammonia ligase (GLUL), also termed glutamine synthetase (GS), which catalyzes the de novo synthesis of glutamine from glutamate and ammonia. Elevated expression of GS promotes cell survival under glutamine limitation, while silencing of GS leads to decreased cell proliferation and xenograft tumor growth. Stable isotope based metabolite tracing shows that GS overexpression increases glutamine synthesis, cataplerotic flux at the $\alpha$-ketoglutarate ($\alpha$KG) step of the TCA cycle, and contributes to nucleotide synthesis and amino acid transport. Mechanistically, Myc binds to the promoter of thymine DNA glycosylase (TDG) and upregulates its expression, which leads to active demethylation of the GS promoter and its increased expression. These results demonstrate an unexpected role of Myc in promoting glutamine synthesis, and suggest a previously uncovered molecular connection between DNA demethylation and glutamine metabolism in Myc-driven cancers.
Introduction

Cancer cells rewire their metabolic programs to benefit their growth, proliferation, and survival (DeBerardinis et al., 2008). Abnormal glutamine metabolism is a defining hallmark of metabolic reprogramming. Some cancer cell lines display increased glutamine uptake and catabolism that render cells addicted to glutamine, which plays a critical role in malignant transformation by contributing to energy production by fueling the TCA cycle. Nonetheless, in addition to being a nutrient substrate, glutamine is involved in other biological processes including serving as the obligate nitrogen donor for the synthesis of nucleotides and non-essential amino acids, as an exchanger for the import of essential amino acids, and as a means to detoxifying intracellular ammonia and glutamate (Dang, 2012; DeBerardinis and Cheng, 2010; Hensley et al., 2013; Wise and Thompson, 2010).

While the metabolic changes in cancer cells can be promoted by a passive cell adaptation to environmental conditions such as hypoxia and redox stress, they are often actively regulated by genetic alterations such as activation of oncoproteins and loss of tumor suppressors (DeBerardinis et al., 2008; Kroemer and Pouyssegur, 2008; Vander Heiden et al., 2009). Two proto-oncoproteins, Akt and c-Myc (hereafter referred to as Myc), have been intensively studied for their functions in regulating cell metabolism. Both Akt and Myc can promote aerobic glycolysis, also termed the Warburg effect. With regard to glutamine metabolism the specific role of Akt is not yet well understood, but oncogenic Myc has been shown to promote glutamine uptake by directly transactivating the expression of glutamine transporters SLC1A5 and SLC7A5/SLC3A2 (Nicklin et al.,
2009), and to promote glutaminolysis by increasing the expression of glutaminase (GLS) via transcriptional suppression of the GLS repressor micro RNAs (miR)-23a/b (Gao et al., 2009). In a previous study (Fan et al., 2010), we established an isogenic dual-regulatable FL5.12 pre-B cell line in which myrAkt is expressed under the control of doxycycline (DOX), and Myc, fused to the hormone-binding domain of the human estrogen receptor (ER), is activated by 4-hydroxytamoxifen (4-OHT). Using this system, we have shown that Akt and Myc have differential effects on mitochondrial function which render cancer cells susceptible to the perturbation of different metabolic programs (Fan et al., 2010). We continued our study to compare their effects on gene expression using the Affymetrix DNA array analysis. To our surprise, we found that Myc but not Akt can upregulate the expression of glutamine synthetase (GS), the enzyme that catalyzes the synthesis of glutamine from glutamate and ammonia, which is the reverse reaction of glutaminolysis that is catalyzed by GLS. Here we studied Myc-mediated upregulation of GS and its biological relevance.

Results

Myc upregulates GS expression and activity

To compare the transcriptional regulation controlled by Akt and Myc, we used the FL5.12 Akt/Myc (AM) clones that we previously established where myrAkt and Myc can be induced individually or simultaneously in an isogenic background (Fan et al., 2010). An Affymetrix mouse cDNA array showed that GS expression was increased upon Myc but not Akt activation (Fig. 1A). This induction of GS expression was confirmed in two individual clones (AM10 and AM32) at both the transcription (Fig. 1B) and protein levels
The increased GS expression was accompanied by an increased GS enzymatic activity (Fig. 1D), using the GS activity assay whose specificity was validated using the GS-specific inhibitor methionine sulfoximine (MSO) and two independent GS shRNAs (Fig. 1E and 1F). Under a physiological setting, introduction of Myc into the Pdx1-Cre; LSL-KRasG12D model (Hingorani et al., 2003) by breeding it to the Rosa26-LSL-Myc mice (Murphy et al., 2008) led to a massive increase of GS expression in the pancreatic ductal neoplasia compared with the age-matched control KRasG12D mice (Fig. 1G). In the lung tumor cells isolated from the LSL-KRasG12D; tp53flox/flox mice (Jackson et al., 2001), induction of Myc using the MycER system also led to elevated GS mRNA level as seen by RNA-sequencing (RNA-Seq) analysis (Fig. 1H). In the immortalized yet non-transformed human mammary epithelial cell line MCF10A, expression of Myc (Fig. 1I), which stimulated cell proliferation (Fig. 1J), also led to elevated GS expression at both protein (Fig. 1I) and transcript levels (Fig. 1K) that corresponded with increased enzymatic activity (Fig. 1L). It is interesting to note that GLS protein level did not increase in this system (Fig. 1I). Using the Cancer Genome Atlas (TCGA) data of human T-lymphoma with Myc amplification and with sizable samples, a strong correlation between Myc amplification and GS expression was observed (Fig. 1M). Conversely, in a number of human cancer cell lines, silencing of endogenous Myc led to decreased GS expression, yet variable changes in the GLS level (Fig. 1N). These data indicate that Myc upregulation can promote GS expression in numerous mouse and human cell lines and cancers.

**Myc directly upregulates TDG that leads to GS expression via promoter demethylation**
We next studied how Myc induces GS expression. As Myc amplification is a major oncogenic event in human breast cancer, we focused on human breast epithelial cell lines for the following studies. Five out of the 6 breast cancer cell lines that we examined showed strong GS expression, excluding Hs578T cells (Fig. 2A). Therefore, we utilized Hs578T cells for studying the effect of GS upregulation in cancerous cells. We also chose to use MCF10A cells which have low levels of GS expression (Fig. 1G) to study GS regulation upon Myc activation in non-transformed cells. Myc is a helix-loop-helix leucine zipper family transcriptional regulator that dimerizes with Max family proteins to bind the CAC(G/A)TG (E-box) sequence and directly activates transcription of its target genes. However, analysis of the putative GS promoter regions using the Genomatix Genome Analyzer (http://www.genomatix.de) and the SABiosciences Transcription Factor Search Portal (http://www.sabiosciences.com) showed no canonical Myc binding sites in human, mouse, or rat GS promoters, consistent with previous analysis of the GS promoter (Kung et al., 2011). Interestingly, we noticed G/C rich regions spanning the transcriptional starting site in the GS gene, which was predicted to be a CpG island by the CpG Island Searcher (http://www.cpgislands.com) (Fig. 2B). This suggested a possible mechanism of transcriptional regulation by DNA methylation of the GS promoter. Indeed, treatment with 5-azacytidine, a DNA methyltransferase inhibitor, enhanced GS expression in both MCF10A and Hs578T cells (Fig. 2C). Bisulfite sequencing showed that the GS promoter was indeed methylated and that Myc expression led to its decreased methylation (Fig. 2D). To determine how Myc affects GS promoter methylation, we examined the expression of the major DNA methyltransferases and demethylases upon Myc expression. While Myc did not cause significant changes in the expression level of
DNA methyltransferases DNMT1, DNMT3A, and DNMT3B, or that of the DNA demethylases TET1, TET2, and MBD4 (Suppl. Fig. S1A), it induced a significant increase in the expression of TET3 (Suppl. Fig. S1A) and TDG (Fig. 2E). This suggests that Myc can induce “active demethylation” by up-regulating TET3 which oxidizes 5-methylcytosine (5mC) and TDG which then removes the oxidized products via excision repair (He et al., 2011; Ito et al., 2011). Supporting the active demethylation theory, increased DNA demethylase activity was observed in Myc-expressing cells (Fig. 2F). Gemcitabine, a pharmacological inhibitor of DNA demethylation, suppressed Myc-induced GS expression in both MCF10A-Myc (Fig. 2G) and in FL5.12-AM32 cells (Suppl. Fig. S1B).

We further characterized how Myc induces TET3 and TDG expression. Interestingly, two recent independent genome-wide chromatin immunoprecipitation-sequencing (ChIP-Seq) analyses suggested that Myc can directly bind to the promoter of TDG but not of TET3 (Perna et al., 2012; Walz et al., 2014). Promoter analysis identified two putative E-boxes in TDG promoter. We then focused on exploring TDG as a direct Myc target. ChIP analysis was performed in the Myc-expressing MCF10A cells using an anti-Myc antibody. PCR analysis using two specific sequences containing the E-boxes revealed that Myc was recruited to the TDG promoter (Fig. 2H). We then generated luciferase reporter constructs driven by wild-type TDG promoter or promoters mutated at E-box consensus sites. While the wild-type promoter drove a significantly higher luciferase activity in Myc-expressing cells, both single E-box mutants and the double mutant lost the Myc-mediated inducibility (Fig. 2I). Using the FL5.12-MycER cells and MCF10A-
MycER cells where Myc can be acutely induced, we observed a rapid induction of GS and TDG as early as 4 h and 8 h respectively upon Myc activation (Fig. 2J and Suppl. Fig. S1C), which correlated with decreased GS promoter methylation (Suppl. Fig. S1D and S1E) and is consistent with the active demethylation mechanism which is replication-independent. Lastly, TDG silencing in Myc-expressing MCF10A cells abrogated Myc-induced GS expression (Fig. 2K). Therefore, we identified a previously undescribed mechanism whereby Myc activates GS expression via the demethylation of its promoter.

**Myc-induced GS promotes glutamine metabolism**

We then studied the effect of GS in cancer cells. GS was ectopically expressed in Hs578T cells which have low endogenous levels of GS (Fig. 3A). GS catalyzes the condensation of glutamate and ammonia into glutamine. Therefore, cells were incubated with $^{15}$N-labeled ammonia (NH$_4$Cl) and subjected to gas chromatography-mass spectrometry (GC-MS) analysis. In vector control cells ~ 30% of the intracellular glutamine was m+1 ($^{15}$N$_1$) labeled, which is produced by the condensation of m+0 glutamate and NH$_4$Cl. The expression of GS led to a significant increase in $^{15}$N incorporation into glutamine with over 40% of the glutamine m+1 labeled (Fig. 3B), demonstrating that GS overexpression promotes glutamine synthesis.

Myc is known to promote glutaminolysis which leads to increased anaplerosis at the $\alpha$KG step of the TCA cycle (DeBerardinis et al., 2007; Wise et al., 2008). Hence GS-promoted glutamine synthesis might be expected to consume TCA cycle intermediates, of which a major source is glucose. Therefore, $^{13}$C-glucose was traced in GS-expressing Hs578T cells. Consistent with the $^{15}$N-NH$_4$Cl labeling, tracing of the $^{13}$C-labeled
glucose showed increased glutamine in GS-expressing Hs578T cells (Fig. 3C), accompanied by a reduced relative abundance of glutamate (Fig. 3D) and αKG (Fig. 3E). We did not observe significant changes in 13C incorporation into pyruvate and lactate, two of the glycolytic metabolites most proximal to the TCA cycle (Fig. 3F), nor did we observe a significant difference in the TCA cycle intermediate citrate (Fig. 3F). Moreover, the abundance of succinate, fumarate, and malate were significantly reduced (Fig. 3F). These data suggest that enforced expression of GS promotes cataplerotic efflux of the TCA cycle from the point of αKG.

Based on these data we sought to determine the fate of newly synthesized glutamine in both the context of GS and Myc overexpression. Aside from the anapleurotic role of glutamine in contributing carbons to the TCA, glutamine plays a major role in a variety of cellular anabolic processes. This contribution is predominately via the donation of the terminal nitrogen group which can be assayed via 15N-NH4Cl labeling which can be monitored by both GC-MS and LC-MS. To directly determine the fate of glutamine, we utilized the 15N-NH4Cl labeling followed by methanol extraction of metabolites then tracing via liquid chromatography-mass spectrometry (LC-MS). Consistent with the GC-MS data (Fig. 3B), LC-MS also revealed significant incorporation of 15N into glutamine accompanied by increased asparagine, the recipient of the terminal 15N-amine group donated by glutamine (Fig. 3G). Interestingly, labeling patterns revealed that while the incorporation of 15N into glutathione, which is synthesized from glutamate, was reduced, the 15N incorporation into ribonucleosides and monophosphate nucleotides was markedly increased in GS-expressing cells (Fig. 3G).
Importantly, similar to the GS-overexpressing cells (Fig. 3B), an increase in the fraction of m+1 (\(^{15}\text{N}_1\)) glutamine was observed in Myc-expressing MCF10A cells by GC-MS using the \(^{15}\text{N}\)-NH\(_4\)Cl labeling (Fig. 3H). As GS promoted nucleotide synthesis (Fig. 3G) and Myc stimulated MCF10A cell proliferation (Fig. 1J), we examined whether GS plays a role in Myc-induced nucleotide synthesis, which would correlate with increased DNA synthesis and cell proliferation. To this end, GS was silenced in Myc-expressing MCF10A cells (Fig. 3I), and the cells were subjected to \(^{15}\text{N}\)-NH\(_4\)Cl labeling followed by LC-MS. Similar to the result obtained by GC-MS (Fig. 3H), Myc activation led to increased \(^{15}\text{N}\)-glutamine and \(^{15}\text{N}\)-asparagine, which was markedly reduced upon GS silencing (Fig. 3J). The \(^{15}\text{N}\) incorporation into glutathione, NADH, and N-acetyl-glucosamine-6-phosphate was not drastically affected upon GS silencing, suggesting GS-independent regulation mechanisms for these metabolites in Myc-active cells (Fig. 3J). Importantly, in Myc-overexpressing cells, although they did not show drastically increased steady-state levels of methanol-soluble free ribonucleosides, likely due to the faster incorporation of free nucleotides into DNA or RNA strands as indicated by faster proliferation of the Myc cells, GS silencing led to a marked decrease of the steady-state level of \(^{15}\text{N}\) incorporation into ribonucleosides (Fig. 3J). Together, these results strongly indicate that GS promotes glutamine production which can be used for anabolic processes such as synthesis of asparagine and nucleotides in Myc active cells.

**GS promotes amino acid transport, cell survival, and oncogenesis**

To assay another downstream function of this glutamine anabolic program, we also tested
the possibility of glutamine acting as an exchange factor in the bidirectional transport of leucine (Nicklin et al., 2009). Overexpression of GS alone was sufficient to increase leucine uptake by approximately 20%, in a System L-dependent manner as the uptake was abrogated by the System L inhibitor BCH (Fig. 4A, left and right panels). The importance of intracellular amino acids for system L-mediated leucine uptake in both cell lines was also demonstrated by the inhibition of leucine uptake when cells were incubated in amino acid depleted media (Fig 4A, KRB). Additionally, high level of GS provided a sustained ability to uptake leucine even in the absence of extracellular glutamine (Fig. 4A), in agreement with GS contributing to increased intracellular levels of glutamine that act as an efflux substrate for the uptake of leucine (Krokowski et al., 2013; Nicklin et al., 2009). Consistent with the increased leucine uptake and glutamine efflux, increased levels of extracellular glutamine was detected in GS-expressing cells (Fig. 4B).

We then went on to determine the biological consequences of GS expression. In Hs578T cells, overexpression of GS enhanced cell viability upon glutamine deprivation (Fig. 4C and 4D). Conversely, silencing GS in Myc-expressing MCF10A cells led to decreased cell proliferation in complete medium (Fig. 4E) and sensitized cells to glutamine deprivation (Fig. 4F). The generality of this phenomenon was further illustrated by silencing GS with two independent shRNAs in MCF10A-Myc and the more aggressive breast cancer cell lines MDA-MB-231 and MDA-MB-468 (Fig. 4G), which led to decreased proliferation (Fig. 4H). Silencing of GS did not have an obvious effect on the non-transformed MCF10A parental cells (Fig. 4G and 4H). GS silencing also led
to spontaneous cell death in MDA-MB-231 cells (Fig. 4I). The pro-survival role of GS was further demonstrated using the GS inhibitor MSO that enhanced cell sensitivity to glutamine deprivation in Myc-expressing cells (Fig. 4J). Interestingly, unlike MSO, BPTES, the inhibitor of GLS, did not confer sensitivity to glutamine deprivation but rather suppressed cell death in the MCF10A-Myc cells (Fig. 4K and 4L), consistent with an anabolism-promoting function of glutamine. Furthermore, to test the effect of GS suppression in vivo, we introduced a Tet-inducible GS shRNA in MDA-MB-468 cells that have high level of endogenous GS. Addition of doxycycline led to a progressive loss of GS (Fig. 5A) as well as decreased cell growth (Fig. 5B) and xenograft tumor growth (Fig. 5C). These data indicate that GS promotes cell proliferation and survival, and may be a viable therapeutic target in Myc-driven cancers.

Discussion

Here we report that Myc upregulates expression of GS, the enzyme that catalyzes the formation of glutamine from glutamate and ammonia. This Myc-induced GS expression promotes glutamine anabolism and is associated with cell proliferation, survival, and xenograft tumor growth, suggesting an important role of GS in Myc-driven cancers. In addition to being a nutrient substrate, glutamine is involved in many important biological processes including serving as the obligate nitrogen donor for multiple steps in both purine and pyrimidine synthesis and can contribute to the production of non-essential amino acids (DeBerardinis and Cheng, 2010; Wise and Thompson, 2010). A recent report showed that cell death induced by glutamine deprivation is not mediated by lowered TCA cycle intermediates, but rather through a mechanism dependent on
asparagine, which is produced by aspartate receiving nitrogen from glutamine (Zhang et al., 2014). Indeed, our data indicate that several fates elucidated for newly synthesized glutamine, namely nucleotide and asparagine synthesis as well as amino acid transport (Fig. 3F and 4A), fit into the anabolism-promoting role of glutamine.

The connection between Myc and numerous essential cellular processes (macromolecule biosynthesis, ribosome biogenesis, and DNA replication) has previously been established and offers an explanation for the enhanced glutamine anabolism we report. GS-driven glutamine synthesis is the only known reaction for endogenous glutamine production. In mammals, nearly 90% of glutamine production originates from endogenous sources (Biolo et al., 2005; Kuhn et al., 1999). While muscle tissue accounts for the majority of synthesized glutamine that can be released into the circulating system and taken up by other tissues and tumors, many tissues and cell types have the ability to upregulate GS and glutamine synthesis upon glutamine shortage (He et al., 2010; Newsholme et al., 2003). In cells with Myc amplification, increased biosynthetic processes may increase the demand for glutamine to serve as a basis for many anabolic processes. This is supported by a recent report that de novo glutamine synthesis was found to be elevated in human glioblastoma (Maher et al., 2012). Therefore, oncogenic Myc may hijack the glutamine synthesis system to ensure glutamine supply for autonomous need or perhaps in a symbiotic fashion that benefits tumor tissue heterogeneity and microenvironment, especially under the condition of glutamine shortage such as in poorly vascularized tumors.
While our findings are seemingly paradoxical to Myc’s role in promoting glutaminolysis to facilitate energy production via the entry of glutamate into the TCA cycle, it is not entirely surprising. Indeed, recent reports highlighted glucose oxidation as a predominant fuel source in patient samples and human orthotopic tumor models which show glucose as a means of anaplerosis and accumulation of glutamine in tumors (Maher et al., 2012; Marin-Valencia et al., 2012). Myc may preferentially activate either glutaminolysis or glutamine synthesis in a context-dependent manner. This notion is supported by the observations that Myc often does not lead to simultaneous upregulation of GLS and GS (Yuneva et al., 2012), and that a mutual suppressive mechanism may even exist between GS and GLS in various breast cancer subtypes (Kung et al., 2011).

In addition, within a single cell, the two reactions may be tightly regulated as they occur at different subcellular compartments: glutaminolysis predominantly in mitochondria and glutamine synthesis in the cytosol (Svenneby and Torgner, 1987). The precise regulation and biological relevance of glutaminolysis and glutamine synthesis requires further study.

We also show that Myc-induced GS expression is mediated by a novel mechanism that involves active promoter demethylation. Although we cannot completely rule out the possibility that Myc may activate GS expression via other mechanisms, our data indicate that Myc can induce GS expression by directly activating the expression of the DNA demethylase TDG. This is consistent with recent genome-wide analyses that identified Myc-binding in the TDG promoter (Perna et al., 2012; Walz et al., 2014). DNA methylation has been regarded as a critical factor in oncogenesis yet its precise role and
regulation remain largely elusive. Promoter hypermethylation leading to repression of
tumor suppressor genes is a well-recognized tumor-promoting mechanism. Along this
direction, TET inactivation or suppression, via genetic alterations or biochemical
inhibitions by cell metabolites such as 2-hydroxyglutarate (2-HG), has been implicated in
cancer development. Nevertheless, global hypomethylation has also been prevalently
detected and recognized as a cause for cancer, although the regulation of specific genes is
yet to be illustrated (Das and Singal, 2004; Kulis and Esteller, 2010). Our current data
provides a novel example that Myc can induce DNA demethylation and alters cell
metabolism to promote its oncogenic function. This is consistent with the evidence that
active DNA demethylation plays an important role in the development of germ cells and
pluripotent stem cells (Wu and Zhang, 2014), which share many common signaling
pathways and metabolic alterations with Myc-driven oncogenesis (Goding et al., 2014).
Author contributions

AJB, ICP, YF, and WXZ conceived the ideas and designed the experiments. All authors performed experiments or data analysis. AJB, ICP, and WXZ wrote the paper.

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Experimental Procedures

Antibodies and reagents

The following antibodies were used: GS (G2781; 1:2,000 for WB from Sigma-Aldrich; BD 610517 1:1,000 for IHC from Becton-Dickinson), tubulin (T4026; 1:5,000 for WB) from Sigma-Aldrich; Myc (N-262; 1:750 for WB, N-262X; 10 μg per 100 μg DNA for ChIP) from Santa Cruz Biotechnology; GLS (WH00027441M1; 1:1,000 for WB) from Sigma-Aldrich; TDG (ab154192; 1:2,000 for WB) from Abcam. The shRNAs were from Sigma-Aldrich. Gemcitabine was from Enzo Life Sciences, Inc. 5-azacytidine was from Sigma-Aldrich. U-13C glucose and 15N-NH4Cl were purchased from Cambridge Isotopes (CLM-1396). BPTES (SML0601) and MSO (M5379) were purchased from Sigma-Aldrich.

Cell lines and culture

The dual-regulatable FL5.12 Tet-myrAkt/MycER (AM) clones were established previously (Fan et al., 2010). Cells were maintained in complete medium (RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 20 mM HEPES, pH 7.5, 50 μM 2-mercaptoethanol, and 0.4 ng/ml IL-3). The Myc-expressing MCF10A cells were established by retroviral infection of the pBabe-c-Myc construct and selected in 8 μg/ml puromycin for 4 days and used as a stable cell pool. The cells were maintained in MCF10A complete medium (DMEM and F-12 medium supplemented with 5% horse serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 20 ng/ml EGF, 0.5 μg/ml
hydrocortisone, 0.1 \( \mu g/ml \) cholera toxin, and 10 \( \mu g/ml \) insulin). MCF7, Hs578T, and HEK293T cells were cultured in complete medium (DMEM with 10% FBS, 100 units/ml penicillin, 100 \( \mu g/ml \) streptomycin, and 10 \( \mu g/ml \) insulin). MDA-MB-231 and MDA-MB-468 cells were cultured in complete medium (MEM with 10% FBS, 100 units/ml penicillin, 100 \( \mu g/ml \) streptomycin, 1 mM sodium pyruvate, 1% MEM NEAA and 1% Hyclone vitamin mix). SKBR3 cells were cultured in complete medium (McCoy’s 5A with 10% FBS, 100 units/ml penicillin, 100 \( \mu g/ml \) streptomycin). T47D cells were cultured in complete medium (RPMI 1640 medium supplemented with 10% FBS, 100 units/ml penicillin, 100 \( \mu g/ml \) streptomycin, and 0.2 units/ml insulin).

**Cell death assay**

Propidium iodide (PI) was added to each sample at 1 \( \mu g/ml \). Cell viability was measured by plasma membrane permeability indicated by PI exclusion using a flow cytometer (FACSCalibur, BD Biosciences).

**Cell growth assay**

Cell growth rate was measured by crystal violet staining. Briefly, cells were fixed with 4% PFA then stained with 0.1% crystal violet. After washing, crystal violet was extracted with 10% acetic acid and absorbance was measured at 590 nm. Readings for subsequent days were normalized to the reading of day zero.

**Immunoblotting**

Cell lysates were prepared in RIPA buffer (1% sodium deoxycholate, 0.1% SDS, 1%
Triton X-100, 0.01M Tris pH 8.0, 0.14M NaCl). All primary antibodies were incubated overnight at 4 °C. Horseradish peroxidase (Rockland) or Alexafluor-conjugated goat anti-rabbit (IRDye 800 - Rockland) or goat anti-mouse (Alexafluor-680 - Life Technologies) antibodies were used as secondary antibodies (1:2,000-1:4,000). Western blots were developed using an ECL detection kit (Thermo Scientific) or an Odyssey Imager (LI-COR).

**GS expression in mouse tumor models examined by RNA-seq**

Murine lung tumor cells derived from viral-Cre induced LSL-KRas\(^{G12D}\); tp53\(^{flox/flox}\) mice, generously provided by Tyler Jacks (Winslow et al., 2011), were infected with pBabe-MycER\(^{T2}\) (Murphy et al., 2008), selected on puromycin and treated for 24 h with 100 nM 4-OHT or ethanol vehicle. Purified mRNA was subjected to Illumina sequencing. Sequence alignment was performed using TopHat (Kim et al., 2013) and analyzed using R (Anders et al., 2013).

**Immunohistochemistry**

5 μM sections of FFPE archived tumor-bearing pancreata from Pdx1-CRE; LSL-KRas\(^{G12D}\); Rosa26-LSL-Myc mice (Nathiya Muthalagu and DJ Murphy, unpublished results) were de-paraffinized and rehydrated through graded alcohols. After standard antigen retrieval (microwaving in 10mM Sodium Citrate pH6.0), endogenous peroxide was quenched in 3% H\(_2\)O\(_2\) and sections were blocked for 1hr in 5% BSA. Primary antibody against GS (BD 610517; (Meabon et al., 2012)) was added at 1:1,000 in blocking buffer and incubated o/n at 4 °C. Bound antibody was detected with biotin-
conjugated anti-mouse IgG, followed by avidin/biotin-HRP binding (Vector Labs),
stained with stable DAB (Invitrogen) and counterstained with Gil 1 Hematoxylin.

**Quantitative RT-PCR**

Total RNA was isolated with RNeasy kit (Qiagen). Reverse transcription was carried out with 2 μg of total RNA using the SuperscriptIII First Strand Synthesis system (Invitrogen). The synthesized cDNA was used for real-time quantitative PCR (qPCR) with the PerfeCTa SYBR Green Super mix (Quanta Bioscience 95055) on the StepOnePlus (Applied Biosystems). The sequences of primer sets were: hGLUL, ACTTCGCAAGCGGCACCA and TTGGCTACACCAGCAGAAAA; mGLUL, CGTTTTATCTTGCATCGGGT and CCTCAATGCACTTCAGACCA; hDNMT1, GCACAAACTGACCTGCTTCA and GCCTTTTCACCTCCATCAA; hDNMT3a, GACAAGAATGCCACCAACAGC and CGTCTCCGAACCACATGAC; hDNMT3b, CCAGCTGAAGCCCATGTT and ATTTGTCTTGAGGCCTTGT; hTET1, GCCTGGACTTCTGTGCAT and CTCGATGGCCAGATTTGA; hTET2, CTCGCAATGCAAGTCCAGTCT and ATGTGGCCAGCCTCTCTTT; hTET3, CTGAGAACCCACTCACACCC and TCCATACCGCCTCATGAC; mTET3, GCCTCAATGATGACCGGCC and AGTGGCCAGATCCTGAAAGC; hTDG, TGGACGTCCAAGAGGGGAGG and CCTTGCTCCAGAGAATAGCG; hMBD4, ACCATCCCACGAACACAGAT and CCATTTTTGCTGAGGTCCGA; hGAPDH, AAGGTCGGAGTCAAGGCAGTG and CCATGGGTGAATCATATTTG; mACTIN, CTGTCGAGTGCACTCCA and CATCACAACCCCTGGCGCTC.
relative abundance of specific mRNAs was normalized to human GAPDH or mouse ACTIN mRNA as the invariant control.

**Measurement of glutamine synthetase activity**

Glutamine synthetase activity was assessed using a previously described method to measure $\gamma$-glutamylhydroxamate synthesized from glutamine and hydroxylamine (Deuel et al., 1978). Briefly, the cells were lysed in 50 mM imidazole-HCl, pH 6.8. After removing debris by centrifugation, lysates were incubated with an equal amount of buffer containing 50 mM imidazole-HCl (pH 6.8), 50 mM L-glutamine, 25 mM hydroxylamine, 25 mM sodium arsenate, 2 mM MnCl$_2$, and 0.16 mM ADP, for 30 min at 37°C. Reaction was stopped by the addition of a buffer containing 90 mM FeCl$_3$, 1.45% trichloroacetic acid, and 1.8 M HCl. The solution was cleared by centrifugation at 16,000 x g for 10 min at 4°C, and $\gamma$-glutamylhydroxamate was measured in the supernatant at 560 nm.

**Affymetrix oligonucleotide array**

Total cellular RNA was isolated with RNeasy kit. Subsequent RNA processing procedures and Affymetrix Murine Genome Array U74Av2 application were followed according to the manufacturer's protocol (Affymetrix, Santa Clara, CA) by the Stony Brook University DNA Microarray Facility. The data sheet for Affymetrix Murine Genome Array for the U74Av2 chips can be found at http://www.affymetrix.com/products/arrays/specific/mgu74.affx. Data were analyzed using Affymetrix Genechip and GeneSpring software. The analysis of the data was
performed at the Stony Brook University DNA Microarray Center (http://www.osa.sunysb.edu/udmf/) with the help of the Bioinformatics Core Facility (http://www.osa.sunysb.edu/bioinformatics/index.html).

**5-azacytidine treatment**

Cells were treated with 20 μM 5-azacytidine (Sigma-Aldrich) every 24 h over a 96 h period. Total RNA was then isolated and analyzed by qRT-PCR.

**Bisulfite sequencing**

For the Myc-expressing MCF10A cells, single cell clones and vector control cells were established by limited dilution. Genomic DNA was isolated from each single cell clone. For sequencing, bisulfite-converted DNA was PCR amplified using methyl sequencing primers, GTTTTTTTTTTAAGTTAATTTTTG (-111~ -90) and ATACCCCTAATTACTCCCAAC (+343~+363). The PCR product was extracted from the gel and sequenced using the primer GTTTTTTTTTTAAGTTAATTTTTG (-111~ -90) or AAAAAACTAAAAACAAAAAAAAC (+151~+172). For MCF10A-MycER and FL5.12-AM32 cells, cells were left untreated or treated with 4-OHT (8 h for MCF10A-MycER and 4 h for FL5.12-AM32). Cells were collected and the genomic DNA was extracted with QIAamp DNA Mini Kit (Qiagen). The purified genomic DNA was subjected to bisulfite treatment with MethylEdge™ Bisulfite Conversion System (Promega). The bisulfite converted DNA was used as template for PCR with primer pairs, for MCF10A-MycER cells, 5’ CTCCCTCTCAAACTAACCCT3’ (-111~ -91) and 5’ GGAGGGTTTGGAGGTAGAGGG3’ (+153~+171); and for FL5.12-AM32,
5’GGATGGGTGAATAGTTAGGAGAG3’ (+85~+107) and
5’ CCTCCCCACTCCCTTTCTC 3’ (-189~ -168).   PCR fragments were purified and
inserted in to pGEM-T vector (Promega).    After the transformation, white colonies were
verified by colony PCR assay.   Positive colonies were amplified and plasmids extracted
for sequencing with T7 primers.

**DNA demethylase activity assay**

Nuclear proteins were isolated using the EpiQuik™ Nuclear Extraction Kit (Catalog #
OP-0002). DNA demethylase activity measurement and calculation were performed
according to the manufacturer’s protocols and formulas. DNA demethylase activity was
measured using 10 μg of nuclear protein extracts with the EpiQuik™ DNA Demethylase
Activity/Inhibition Assay Ultra Kit (Catalog # P-3008) (Epigentek, Farmingdale, NY,
USA).

**ChIP assay**

ChIP assays were performed according to standard protocols available at Abcam Inc.
Protein was cross-linked to DNA with the addition of formaldehyde. Cells were washed
two times with phosphate buffer saline (PBS) and then scraped into PBS. Cells were
collected after centrifugation and lysed in ChIP lysis buffer (50 mM HEPES-KOH, pH
7.5, 140 mM NaCl, 1 mM EDTA, pH 8.0, 1% Triton X-100, 0.1% sodium deoxycholate,
0.1% SDS and protease inhibitors). Resulting cell lysates were sonicated with
Ultrasonic Processor Sonicator W-380. Target proteins were immunoprecipitated
overnight at 4 °C with protein A conjugated Sepharose beads in the presence of desired
antibody. The beads were washed three times with wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, pH 8.0, 150 mM NaCl, 20 mM Tris-HCl, pH 8.0) and eluted in elution buffer (1% SDS, 100 mM NaHCO₃). All DNA samples were purified using phenol:chloroform followed by ethanol precipitation in the presence of glycogen before qPCR analysis. Primers used for qPCR analysis of immunoprecipitated DNA were as follows: TDG PCR #1: TTACTCAGAAAGCCTGGATC (−909~−889) and CATGGCTGCTGCTGTATTTG (−746~−726); TDG PCR #2: CTCCCTCAGATCTCTCATTACCTG (−318~−297) and GCTTTTGTCTCCAGTATTTCGG (−165~−143).

Luciferase assay

A 1.2 kb fragment containing the TDG 5’-flanking region was PCR amplified from genomic DNA of MCF10A cells. The PCR product was cloned into the pGL3 vector containing the firefly luciferase reporter. PCR based site-directed mutagenesis was performed to ablate the two putative E-boxes individually or simultaneously. All constructs were confirmed by DNA sequencing. To examine the promoter activity, MCF10A cells stably transduced with either vector or Myc were transiently transfected with the promoter constructs along with internal control plasmid pCMV-RL using Lipofectamine 2000. Cells were seeded at 5 x 10⁴ cells per well in 24-well plates. Transfections were comprised of either wild-type or mutant E-box TDG promoter luciferase vectors (1.0 µg) together with pCMV-RL (100 ng). Twenty-four hours post transfection, cells were washed with PBS and lysed in 100 µl passive lysis buffer for 10 minutes. Luciferase activity was determined following the manufacturer’s
recommended protocol with a SpectraMax M5 Microplate Reader. The ratios of firefly luciferase versus renilla luciferase were used as relative luciferase activities.

**Amino acid transport assays**

Amino acid uptake was performed as previously described (Krokowski et al., 2013). Briefly, cells were grown in 24-well plates to 70% confluency. Uptake assays were performed after wash out of growth media in EBSS with sodium chloride replaced with choline chloride. Leu uptake was performed at 10 μM (4 μCi/ml) for 1 min at 37°C. Ethanol extracted radioactivity was normalized over protein content assayed by the Lowry method. The inhibitor of system L-BCH (Sigma-Aldrich) was used at 5mM concentration. Amino acid depletion was performed after washing cells with warm PBS and incubation in amino acid free KRB (Sigma-aldrich) supplemented with 10% dialyzed FBS (Gibco) and insulin or DMEM without Gln supplemented with 10% dialyzed FBS and insulin.

**Measurement of extracellular glutamine**

5x10^5 cells were seeded in 6-cm plate overnight. Cell culture medium was replaced with 2.5 ml fresh medium and cultured for 48 h. 1.5 medium was collected and the concentration of glutamine was determined using an automated electrochemical analyzer (BioProfile 100 Plus analyzer; NOVA Biomedical, Waltham, MA, USA).

**Analysis of metabolites by GC-MS**

For ^13^C-glucose labeling, protocols have been outlined previously (Faubert et al., 2013).
Briefly, cells were cultured in glutamine-free medium for 16 h, then cultured with 12.5 mM U-\textsuperscript{13}C glucose. Cells were washed with ice-cold 0.9% saline solution, lysed with ice-cold 50% methanol on dry ice, supplemented with the internal standard adonitol, and subjected to three rounds of rapid freeze thaw between liquid nitrogen and 37 °C or sonication. Lysate was pelleted by centrifugation at 12,000 x g for 10 min at 4 °C and the supernatant was transferred to glass containers for sample concentration and derivatization. Dried samples were re-suspended in 30 μL anhydrous pyridine containing methoxyamine (10 mg/ml) and added to GC-MS autoinjector vials containing 70 μL N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) derivatization reagent. The samples were incubated at 70 °C for 1 h, following which aliquots of 1 μL were injected for analysis. GC-MS data were collected on an Agilent 5975C series GC/MSD system (Agilent Technologies) operating in electron ionization mode (70 eV). Data were corrected for natural abundance.

For \textsuperscript{15}N-NH\textsubscript{4}Cl labeling, cells (2 x 10\textsuperscript{6}) were plated and washed three times with pre-warmed PBS. Cells were overlaid with 5 mM \textsuperscript{15}N-NH\textsubscript{4}Cl in glutamine free DMEM for 6 h after which cells were washed with ice-cold 0.9% saline solution, lysed with ice-cold 50% methanol on dry ice, supplemented with the internal standard adonitol, and subjected to three rounds of rapid freeze thaw between liquid nitrogen and 37 °C. Lysate was pelleted by centrifugation at 12,000 x g for 10 min at 4 °C and the supernatant was transferred to glass containers for sample concentration and derivatization. Supernatants were dried under constant air flow for a minimum 4 h at 42 °C, then treated with Methoxamine Reagent (Thermo Scientific) overnight at room
temperature to prepare oximes of ketoacids before silylation. Samples were dried under constant air flow for a minimum of 2 h, then treated with TRI-SIL (Thermo Scientific) for 2 h at 42 °C to derivatize. GC-MS data was collected on an Agilent 7890B GC/5977A MS. Glutamine was monitored at 258, 259 and 260 m/z. Data were analyzed using Mass Hunter and corrected for natural abundance using ISOCOR. Quantified metabolites were normalized relative to protein content or cell number.

Measurement of $^{15}$N-labeling pattern of cellular metabolites using LC-MS

Hs578T and MCF10A cells were plated and cultured in DMEM with dialyzed serum for 48 h to reach 90% confluency. Cells were washed three times with pre-warmed PBS and overlaid with 5 mM $^{15}$N-NH$_4$Cl in glutamine replete media (2 mM) for 16 h after which media was aspirated and cells were overlaid with ice-cold 80% methanol. Plates were incubated at -80°C for 15 min, scraped, and transferred to polypropylene tubes. Cells were pelleted by centrifugation and supernatant was transferred to fresh tubes. Pellets were washed twice with 80% methanol and supernatants collected. The cell extracts were dried under nitrogen flowing, and re-dissolved in 500 μL HPLC-grade water, and analyzed via reverse-phase ion-pairing chromatography coupled to an Exactive orbitrap mass spectrometer (ThermoFisher Scientific, San Jose, CA). The mass spectrometer was operated in negative ion mode with a scan rate of 1 Hz and resolving power of 100,000, scanning range being $m/z$ 75-1000. The LC method has been described previously (Lu et al., 2010), using a Synergy Hydro-RP column (100 mm × 2 mm, 2.5 μm particle size, Phenomenex, Torrance, CA) with a flow rate of 200 μL/min. The LC gradient was 0 min, 0% B; 2.5 min, 0% B; 5 min, 20% B; 7.5 min,
20% B; 13 min, 55% B; 15.5 min, 95% B; 18.5 min, 95% B; 19 min, 0% B; 25 min, 0% B. Solvent A is 97:3 water:methanol with 10 mM tributylamine and 15 mM acetic acid; solvent B is methanol. Other LC parameters were autosampler temperature 5°C, injection volume 10 μL, and column temperature 25 °C. Data analyses were performed using MAVEN software (Melamud et al., 2010). The metabolites, both unlabeled and the 15N-labeled forms, were detected using the accurate mass with an 8 ppm m/z window. Labeling percentages were calculated from signal intensity using Excel.

**Xenograft experiments**

Female athymic nude mice, 6 to 8 weeks old, were obtained from Charles River Laboratories. Mice were housed and monitored at the Division of Laboratory Animal Resources at Stony Brook University. All experimental procedures and protocols were approved by the Stony Brook University institutional animal care and use committee (IACUC). Tumors were established by resuspending 1 x 10^6 MDA-MB-468 cells carrying a Tet-inducible shRNA for GS in 50 μl Matrigel and injecting in the mid-flanks of mice using a 26-gauge needle. Experimental mice received 5% sucrose water with doxycycline hyclate (1 mg/ml) changed every 48 h to induce shRNA. For each tumor, the tumor length (l) and the width (w) was measured every 3-4 days with an electronic caliper. Tumor volume (v) was calculated using the formula v = (l x w) x (l + w/2) x 0.56 and plotted in mm³.

**Statistical analyses**

The longitudinal data analyses were performed to assess the growth curves under
different treatments. The ANOVA procedures were used to evaluate differences among multiple groups with the Dunnett or Newman-Keuls test. The independent two-sample and one sample t-tests were used to make comparisons between two groups and to evaluate whether fold changes are different from one, respectively. The analyses were mainly carried out using PROC MIXED, PROC FREQ, PROC MEANS and PROC TTEST in the SAS 9.4 (SAS institute, Cary, NC). Some initial analyses were also carried out with GraphPad Prism 5 for Windows (GraphPad Software Inc, San Diego, CA). Different levels of statistical significance are indicated as: * for p<0.05, ** for p<0.01, *** for p<0.001, and **** for p<0.0001.
References


Figure Legends

Figure 1.  **Myc upregulates GS expression.**  (A) FL5.12 parental cells and the AM clones (AM10 and AM32) were cultured in the control medium, with Dox to activate Akt, or 4-OHT to activate Myc, or both, for 36 h. Mouse cDNA microarray analysis was performed. The fold change of GS expression is shown.  (B-D) FL5.12 parental and the AM clones were cultured without or with 4-OHT to activate Myc for 36 h. (B) GS transcript level was analyzed by qRT-PCR, and normalized to that of cells without 4-OHT treatment. Data shown are the mean of a representative experiment performed in triplicates.  \( ***p<0.001. \)  (C) Cell lysates were collected and analyzed by immunoblotting for indicated proteins. (D) GS activity was determined using the total cell lysates. Data shown are the mean plus SEM of at least 5 independent experiments.  \( *p<0.05. \)  (E) Hs578T cells overexpressing GS were treated with 100 µM MSO for 24 h then tested for GS activity. Data shown are mean of 2 samples.  (F) HEK293T cells with two independent shRNAs against GS were tested for GS activity. Data shown are mean plus SD of 3 individual samples.  (G) Pancreata from 2-month old Pdx1-Cre; LSL-KRasG12D (n = 4) and age-matched Pdx1-Cre; LSL-KRasG12D; R26-LSL-MYC (n = 4) mice were stained for GS by IHC. I: Islets; N: normal tissue; T: tumors. Note that tumors in all 4 KRasG12D mice are negative, whereas tumors in all 4 KRasG12D/Myc mice are positive for GS. Shown are representative micrographs of tumors from one mouse of each group.  (H) Murine lung tumor cells derived from viral-Cre induced LSL-KRas\(^{G12D} \); p53\(^{fl/fl} \) mice were infected with pBabe-MycER\(^{T2} \) and treated for 24 h with ethanol vehicle or 100 nM 4-OHT. Purified mRNA was subjected to
Illumina sequencing. Sequence alignment was performed using TopHat and analyzed using R. GS mRNA levels are expressed as number of sequencing reads. Shown is the mean value plus SD (n=4). **** p<0.0001. (I-L) MCF10A cells were stably transfected with vector or pBabe-c-Myc. (I) Cell lysates were probed for indicated proteins by immunoblotting. (J) Cell growth was measured by crystal violet staining and normalized to that of cells on day zero. Data shown are the mean plus SD of a representative experiment of three independent experiments performed in triplicates. (K) Total RNA was extracted and GS transcript level was analyzed by qRT-PCR, and normalized to that of vector control cells. Data shown are the mean plus SEM from 3 independent experiments performed in duplicates. *p<0.05. (L) GS activity was measured. Data shown are the mean plus SEM of 3 independent experiments. *p<0.05. (M) Differential expression levels of GS in Myc-high and Myc-low groups in T-cell lymphoma (ipbal, PMID: 19965671). Shown are box plots of expression levels of GS. The Myc high and Myc low groups were defined by Myc expression levels above or below the median level. Two-tailed t-tests were performed to test the significance of the differences in gene expression. **p<0.01. (N) Indicated cell lines were stably infected with control shRNA (shNTC) or Myc shRNA (shMyc). Indicated proteins were probed by immunoblotting.

Figure 2. Myc upregulates GS expression via TDG-mediated demethylation of GS promoter. (A) Total lysates of indicated breast cancer cell lines were probed for GS expression. (B) Schematic representation of the CpG distribution in the 5′-regulatory region of the GLUL gene from the CpG Island Searcher (http://www.cpgislands.com).
The CpG sites are represented by vertical tick marks, and the beginning of exon 1 is depicted as “+1”. (C) MCF10A cells and Hs578T cells were treated with 20 μM 5-azacytidine (5-Aza) for indicated times. Total RNA was extracted and GS transcript levels were analyzed via qRT-PCR, and normalized to that of untreated cells. Data shown are the mean plus SEM of a representative experiment performed in triplicates. *p<0.05; ****p<0.0001. (D) Single cell clones of vector control and Myc-expressing MCF10A cells were established by limited dilution. The GS promoter was PCR amplified in 5 clones of each cell type, and analyzed by bisulfate sequencing. The schematics of the sequencing results and the human GS promoter with the numbered CpGs are shown. Open and filled circles indicate unmethylated and methylated cytosines, respectively. (E) TDG transcript level was analyzed by qRT-PCR in vector control or Myc-expressing MCF10A cells, and normalized to the level in the vector control. Data shown are the mean plus SEM of 5 independent experiments performed in triplicates. *p<0.05. (F) DNA demethylase activity was determined using the EpiQuik™ DNA Demethylase Activity/Inhibition Assay Ultra Kit in vector control or Myc-expressing MCF10A cells, and normalized to that in vector control. Data shown are the mean plus SEM of 4 independent experiments. *p<0.05. (G) Vector control and Myc-expressing MCF10A cells were treated with vehicle control or gemcitabine at indicated concentrations for 24 h. GS expression was detected by immunoblotting. (H) The level of Myc binding to the TDG promoter in vector and Myc-expressing MCF10A cells was analyzed by ChIP assay using the Myc antibody followed by PCR of two specific regions within the promoter (PCR#1 and PCR#2). Fold enhancement represents the abundance of enriched DNA fragments over an IgG control. Data
represent mean plus SEM from 3 independent experiments performed in duplicates. **p<0.01.  (I) Vector control or Myc-expressing MCF10A cells were transfected with the luciferase reporters driven by the pGL3 control vector, the wild-type TDG promoter, or the TDG promoter mutants with the two E-boxes mutated individually or simultaneously, together with a renilla luciferase construct. Twenty-four hours post transfection, cells were lysed and luminescence was quantified. Luciferase activity was standardized based on renilla luciferase activity and normalized to that of the pGL3-transfected cells. Data shown are the mean plus SD of a representative experiment performed in triplicates. **p<0.01; ***p<0.001.  (J) FL5.12-AM32 cells were cultured in the absence or presence of 4-OHT for indicated time periods. Expression levels of GS and TDG were analyzed by qRT-PCR, and normalized to that of cells cultured in the absence of 4-OHT. Data shown are the mean plus SEM from 2 independent experiments performed in triplicates or duplicates. *p<0.05, **p<0.01, ****p<0.0001.  (K) Vector control or Myc-expressing MCF10A cells were stably infected with lentiviral shRNA control (shNTC) or hairpins targeting TDG (shTDG). TDG transcript level was analyzed by qRT-PCR, and normalized to that in vector control cells. Data shown are the mean plus SEM of an experiment performed in triplicates. *p<0.05; **p<0.01.

**Figure 3. GS leads to increased cataplerotic flux towards glutamine synthesis and promotes nucleotide synthesis.**  (A) Hs578T cells were stably transfected with vector control or GS. Indicated proteins were probed by immunoblotting.  (B) Hs578T cells were labeled with $^{15}$N-NH$_4$Cl for 6 h in glutamine deficient media and nitrogen incorporation into glutamine was determined by GC-MS. The total pool of glutamine
was normalized for stable isotope natural abundances. Fractional $^{15}$N glutamine is indicated as black bars and $^{14}$N as white bars. *$p<0.05$; **$p<0.01$. (C-F) Metabolic tracing analysis of the vector control or GS-expressing Hs578T cells. Cells were cultured in glutamine-free medium for 16 h before labeling with 12.5 mM $^{13}$C-glucose for 6 h in glutamine-free medium. Relative abundance of $^{12}$C and $^{13}$C in each metabolite pool was measured by GC-MS, and is expressed relative to the internal standard and protein content in each sample normalized to the value of $^{12}$C fraction in vector control cells (C: glutamine, D: glutamate, E: $\alpha$-KG, F: other indicated metabolites). All data represent total labeling, i.e. all isotopologues. Data represent mean plus SD from 3 (in some cases 2) samples. n.s. non-significant; *$p<0.05$, **$p<0.01$. (G) Hs578T cells were labeled for 16 h with $^{15}$N-$\text{NH}_4\text{Cl}$ in glutamine replete (2 mM) medium and subjected to LC-MS. Percent of each $^{15}$N labeled molecule is shown. All data represent total labeling, i.e. all isotopologues. Data shown are mean plus SD from 3 individual samples. n.s. non-significant; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$. (H) Vector control and Myc-expressing MCF10A cells were labeled with $^{15}$N-$\text{NH}_4\text{Cl}$ for 6 h in glutamine deficient media and nitrogen incorporation into glutamine was determined by GC-MS. The total pool of glutamine was normalized for stable isotope natural abundances. Fractional $^{15}$N glutamine is indicated as black bars and $^{14}$N as white bars. *$p<0.05$. (I and J) Myc-expressing MCF10A cells were stably infected with lentiviral control (shNTC) or GS (shGS) short hairpin. (I) Cell lysates were analyzed by immunoblotting to show successful GS silencing. (J) Parental, Myc-expressing, and Myc-expressing with GS silencing MCF10A cells were labeled for 16 h with $^{15}$N-$\text{NH}_4\text{Cl}$ in glutamine replete (2 mM) media and subjected to LC-MS. Percent of each $^{15}$N
labeled molecule is shown. All data represent total labeling, i.e. all isotopologues.

Data shown are mean plus SD from a representative of three independent experiments performed in triplicate. n.s. non-significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Figure 4. GS promotes amino acid uptake and tumor cell survival/growth. (A) Hs578T cells were tested for leucine uptake at basal conditions, under amino acid withdrawal (KRB), and under glutamine deprivation alone for 5 h. System L-dependent uptake of leucine was assayed in EBSS without sodium and confirmed by the use of the System L inhibitor BCH. The amount of Leu uptake was normalized to that of the vector control cells cultured in DMEM. Data shown are mean plus SD of 3 samples from a representative experiment of three independent experiments. **p<0.01. (B) Extracellular glutamine levels in media from HS578T cells was measured with a NOVA Bioanalyzer 100 Plus. Concentration was normalized to protein concentration and displayed as a fold change from vector cells. Data shown are mean plus SD of two experiments performed in triplicates. *p<0.05. (C) Vector control and GS-expressing Hs578T cells were cultured in glutamine-free medium for the indicated periods of time. Cell viability was determined by PI exclusion. Data shown are the mean plus SD of three independent experiments. ****p<0.0001. (D) The cells in (C) were stained with the cell permeable DNA dye Hoechst33342, and observed with a fluorescence microscope at day 5. Note more cell death in vector control cells upon glutamine deprivation indicated by cell detachment and condensed nuclei. (E) Myc-expressing MCF10A cells with shNTC or shGS were cultured in complete medium. Cell growth
was measured by crystal violet staining and normalized to that of cells on day zero. Data shown are the mean plus SD of a representative experiment of three independent experiments performed in triplicates. ****p<0.0001. (F) Myc-expressing MCF10A cells with shNTC or shGS were cultured in complete or glutamine-free media for indicated days. Cell viability was measured by PI exclusion. Data shown are the mean plus SD of a representative experiment performed in triplicates. *p<0.05, **p<0.01.

(G and H) Indicated cell lines were stably infected with two independent shRNAs against GS. Cell were analyzed by immunoblotting with indicated antibodies (G). Cells were cultured and growth rate was measured by crystal violet staining and normalized to the value at day zero. Data shown are mean plus SD of three individual samples (H). (I) MDA-MB-231 cells infected with two individual shGS hairpins were measured for spontaneous cell death by PI exclusion. Data shown are the mean plus SD of three individual samples. (J) Myc-expressing MCF10A cells were cultured in complete medium or glutamine-free medium, in the absence or presence of the GS inhibitor methionine sulfoximine (MSO) at indicated concentrations. Cell viability was measured 48 h later by PI exclusion. Data shown are the mean plus SD of a representative experiment performed in triplicates. ***p<0.001, ****p<0.0001. (K and L) MCF10A cells were cultured in complete or glutamine-free medium, in the absence or presence of the GS inhibitor MSO (D) or the GLS inhibitor BPTES (E). Cell viability was measured 48 h later by PI exclusion. Data shown are mean plus SD of a representative experiment of two independent experiments performed in triplicates. *p<0.05, ****p<0.0001.
Figure 5. GS promotes tumorigenesis in vivo. MDA-MB-468 cells were stably infected with a Tet-inducible shRNA for GS. (A) Cells were cultured with doxycycline for indicated days. Successful silencing of GS was detected by immunoblotting. (B) Cells were treated with or without doxycycline for indicated periods of time. Cell growth was measured by crystal violet staining and normalized to the value at day zero. Data shown are the mean plus SD of a representative experiment of three independent experiments performed in triplicates. ****p<0.0001. (C) Cells were injected subcutaneously into both flanks of athymic nude mice. Mice were fed with 5% sucrose water without (n = 8) or with (n = 10) doxycycline hyclate (1 mg/ml). Tumor growth was measured by caliper measurement. Shown is the average tumor size plus SEM. ****p<0.0001. Images of tumors are shown in the insert.
Bott et al. Figure 2

A

T47D  MCF7  MDA-MB-231  MDA-MB-468  Hs578T

GS

n.s.

B

-2000

-499

+1

GS Island

+528

C

CpG # 1  4  21

CpG # 23

-71  +1  +117

Vector

Myc

E-box1

E-box2

D

MCF10A

Hs578T

0  1  4

5-Aza

0  1  4  d

0  1  4

0.0  0.5  1.0  1.5  2.0

Relative GS expression

n.s.

****

0  1  4

5-Aza

Relative TDG expression

E

vector  Myc

Relative GS expression

1.0

2.0

3.0

vector  Myc

Relative demethylase activity

F

Relative TDG expression

vector  Myc

Relative demethylase activity

G

Gem  0  6.25  12.5  25  50  100  nM

0  1  4  8  16  24  h

0  1  4  8  16  24

0.0  0.5  1.0  1.5  2.0

Relative expression

GS

TDG

4-OHT

0  4  8  16  24

0  4  8  16  24

0  4  8  16  24

0  4  8  16  24

0  4  8  16  24

0  4  8  16  24

0  4  8  16  24

0  4  8  16  24

0  4  8  16  24

0  4  8  16  24

0  4  8  16  24

0  4  8  16  24
**A** Hs578T

Vector

GS

Tubulin

**B**

% intracellular glutamine

m+0

m+1

m+2

**C**

Glutamine

Relative abundance

V

GS

**D**

Glutamate

Relative abundance

V

GS

**E**

αKG

**F**

Pyruvate

Lactate

Citrate

Succinate

Fumarate

Malate

**G**

Glutamine

Asparagine

Glutathione

NADH

N-acetyl-glucosamine-6-phosphate

% intracellular glutamine

**H**

% intracellular glutamine

m+0

m+1

m+2

**I**

MCF10A-Myc

shNTC

shGS

**J**

Glutamine

Asparagine

Glutathione

NADH

N-acetyl-glucosamine-6-phosphate

Percent labeled

Inosine

Uridine

AMP

GMP

CMP

UMP
Bott et al. Figure 4

**A**

- Bar graph showing % Leu uptake for different conditions: DMEM, KRB, -Gln, -Na+ BCh, Vec, GS.
- Data points indicate significant differences between conditions.

**B**

- Bar graph showing extracellular glutamine levels for Vec and GS conditions over time.
- Data points indicate significant differences.

**C**

- Line graph showing % cell viability over Gln deprivation days for Vec and GS conditions.
- Data points indicate significant differences.

**D**

- Images of cells under different conditions: Untr, -Gln 5 days, Vec, GS.

**E**

- Line graph showing relative growth over time for shNTC and shGS conditions.
- Data points indicate significant differences.

**F**

- Bar graph showing % cell viability over time for different conditions: -Gln 0, 2, 3 d.
- Data points indicate significant differences.

**G**

- Western blot analysis for MCF10A, MCF10A Myc, MDA-MB-231, MDA-MB-468.
- Blots show expression levels for GS and Tubulin.

**H**

- Line graphs showing relative growth over time for MCF10A, MCF10A Myc, MDA-MB-231, MDA-MB-468.
- Data points indicate significant differences.

**I**

- Bar graph showing % cell viability for different conditions: shNTC, shGS#1, shGS#2.

**J**

- Bar graph showing % cell viability for different conditions: Untr, MSO 25 μM, 12.5 μM, 200 μM.

**K**

- Bar graph showing % cell viability for different conditions: Untr, MSO 25 μM.

**L**

- Bar graph showing % cell viability for different conditions: Untr, BPTES 5 μM.
Bott et al. Figure 5

A

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B

Relative growth

- Dox
- +Dox

C

Tumor volume (mm³)

- Dox
- +Dox

****
Inventory of Supplemental Information

Supplemental Figure Legend

Supplemental Figures S1
Supplemental Figure Legends

Figure S1. Related to Figure 2. (A) DNMT1, DNMT3A, DNMT3B, TET1, TET2, TET3, as well as MBD4 transcript levels were analyzed by qRT-PCR in vector control or Myc-expressing MCF10A cells, and normalized to the level in the vector control. Data shown are the mean ± SEM of 5 independent experiments performed in duplicates or triplicates. (B) FL5.12-AM32 clone was cultured without or with 4-OHT to activate Myc for 24 h. Cells were treated with vehicle control or gemcitabine at indicated concentrations for 24 h. GS expression was detected by immunoblotting. (C and D) MCF10A cells were stably infected with the retroviral MycER fusion construct and induced with 4-OHT for 8 h. (C) Cell lysates were analyzed by immunoblotting with indicated antibodies. (D and E) Bisulfite sequencing in MCF10A-MycER cells (D) and FL5.12-AM32 (E) cells. 5 representative sequencing results are shown for each condition. Open and filed circles indicate unmethylated or methylated cytosine of CpG site, respectively.