Liver-Type Glutaminase GLS2 Is a Druggable Metabolic Node in Luminal-Subtype Breast Cancer

Graphical Abstract

Basal-subtype Breast Cancers

- c-Myc
- c-Jun

GLS dependence

Luminal-subtype Breast Cancers

- GATA3
- p73

GLS2 dependence

CB-839
BPTES
968

Glu → Cys → Biosynthesis → Glu → TCA

GLS

Gln

Gln

Glu

Glu

TCA

Highlights

- Basal- and luminal-subtype breast cancers express GLS and GLS2, respectively
- GLS2 expression in breast cancer is regulated by promoter methylation and GATA3
- GLS2 supports proliferation and tumorigenesis in luminal-subtype breast cancers
- 968 inhibits GLS2 to suppress luminal-subtype breast cancer growth

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In Brief

Lukey et al. report that basal- and luminal-subtype breast cancers employ different strategies for glutamine catabolism, impacting their sensitivity profiles to glutaminase inhibitors. Elevated GLS2 expression in luminal-subtype cancers is driven in part by GATA3. Targeting GLS2 with the pan-glutaminase inhibitor 968 inhibits luminal-subtype breast cancer cell proliferation and tumorigenesis.
Liver-Type Glutaminase GLS2 Is a Druggable Metabolic Node in Luminal-Subtype Breast Cancer

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SUMMARY

Efforts to target glutamine metabolism for cancer therapy have focused on the glutaminase isozyme GLS. The importance of the other isozyme, GLS2, in cancer has remained unclear, and it has been described as a tumor suppressor in some contexts. Here, we report that GLS2 is upregulated and essential in luminal-subtype breast tumors, which account for >70% of breast cancer incidence. We show that GLS2 expression is elevated by GATA3 in luminal-subtype cells but suppressed by promoter methylation in basal-subtype cells. Although luminal breast cancers resist GLS-selective inhibitors, we find that they can be targeted with a dual-GLS/GLS2 inhibitor. These results establish a critical role for GLS2 in mammary tumorigenesis and advance our understanding of how to target glutamine metabolism in cancer.

SIGNIFICANCE

Humans have two genes encoding glutaminase enzymes, GLS and GLS2. Efforts to target glutamine catabolism for cancer therapy have focused on GLS, an inhibitor of which (CB-839) is currently in clinical trials. The GLS2 isozyme has previously been described as a tumor suppressor, with downregulated expression in liver cancer. We report here that GLS2 is overexpressed and essential for growth in the most prevalent subtypes of breast cancer, luminal A and B. Although GLS2 is insensitive to CB-839-class drugs, it is inhibited by the small molecule 968, which suppresses breast tumor growth in vivo. These findings establish a critical role for GLS2 in breast cancer and advance our understanding of how to target aberrant glutamine metabolism for cancer therapy.

INTRODUCTION

Sustained biomass accumulation in tumors depends on cancer cells acquiring nutrients from the environment and processing them to meet the biosynthetic, bioenergetic, and redox demands of proliferation (Pavlova and Thompson, 2016). Many oncogenic signaling pathways regulate the expression, activity, or localization of nutrient transporters and metabolic enzymes, and extrinsic factors such as O2 availability also influence cellular metabolism (Vander Heiden and DeBerardinis, 2017). These variables cause cancer cell metabolism to be highly heterogeneous in nature, although certain metabolic alterations are consistently observed in diverse tumor types. For example, most tumors exhibit elevated glucose uptake coupled to lactate secretion regardless of O2 availability (the Warburg effect), and cancer cells also frequently depend on an exogenous supply of glutamine (Pavlova and Thompson, 2016). Glutamine is the most abundant amino acid in blood serum and is a major source of carbon and nitrogen for tumor cells. Its uptake into cells is facilitated by plasma membrane transporters, which in some cases are essential for tumorigenesis (van Geldermalsen et al., 2016). Once in the cytosol, there are several possible fates for glutamine in addition to its role as a proteinogenic amino acid. In mitochondria, glutamine catabolism is initiated by glutaminase, which releases the amide nitrogen as ammonia to generate glutamate. In turn, glutamate can be incorporated into the glutathione and proline biosynthesis pathways, or deaminated to produce the tricarboxylic acid (TCA) cycle intermediate α-ketoglutarate (α-KG).

This metabolic pathway is widely upregulated in cancer cells, with glutamine serving as a key anaplerotic substrate for the TCA cycle (Cluntun et al., 2017). Two genes encode glutaminases in mammals, GLS and GLS2, and different isoforms of each enzyme arise from alternative splicing and surrogate promoter mechanisms (Katt et al., 2017). The GLS isozyme is ubiquitous in healthy tissues, whereas liver-type glutaminase (GLS2) is restricted primarily to the liver,
pancreas, and brain (Altman et al., 2016). Expression of GLS is regulated by oncogenic transcription factors, and its role in cancer is well characterized and supportive of tumorigenesis (Gao et al., 2009; Lukay et al., 2016). Consequently, GLS has been investigated as a possible drug target for cancer therapy, and an allosteric inhibitor, CB-839, is currently being evaluated in clinical trials (Gross et al., 2014). The function of GLS2 in cancer is less well defined and appears to be context dependent. The GLS2 gene is a transcriptional target of p53 (Hu et al., 2010; Suzuki et al., 2010), and in glioblastoma and liver cancer GLS2 has been described as a tumor suppressor (Mates et al., 2018). However, GLS2 expression is also regulated by oncoproteins including N-myc (Xiao et al., 2015) and was identified as one of only 16 essential metabolic genes for tumorigenesis in a functional genomics screen (Possemato et al., 2011).

Here, we describe a critical onco-supportive role for GLS2 in breast cancer. We demonstrate that expression of the GLS2 gene is regulated by GATA3 and that the gene product is essential for cell proliferation and tumorigenesis in luminal-subtype breast cancers, which account for ~75% of total breast cancer incidence (Table S1) (Dai et al., 2015). Moreover, we show that GLS2 can be targeted with the small-molecule inhibitor 968 to suppress tumorigenesis and overcome resistance to GLS-selective inhibitors. These findings establish a previously unappreciated essential role for GLS2 in breast cancer biology and provide important insights regarding how to target glutamine metabolism for cancer therapy.

**RESULTS**

**Luminal Breast Cancers Use Glutamine Anaplerosis but Resist GLS Inhibitors**

The most extensively studied inhibitors of GLS are based on the bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-y) ethyl sulfide (BPTES) molecular scaffold, with the potent analog CB-839 currently in clinical trials for a number of malignancies. CB-839 was originally reported to be effective against triple-negative breast cancer (TNBC) cells (Gross et al., 2014), which is regulated by oncogenic transcription factors, and its role in cancer is well characterized and supportive of tumorigenesis (Gao et al., 2009; Lukay et al., 2016). Consequently, GLS has been investigated as a possible drug target for cancer therapy, and an allosteric inhibitor, CB-839, is currently being evaluated in clinical trials (Gross et al., 2014). The function of GLS2 in cancer is less well defined and appears to be context dependent. The GLS2 gene is a transcriptional target of p53 (Hu et al., 2010; Suzuki et al., 2010), and in glioblastoma and liver cancer GLS2 has been described as a tumor suppressor (Mates et al., 2018). However, GLS2 expression is also regulated by oncoproteins including N-myc (Xiao et al., 2015) and was identified as one of only 16 essential metabolic genes for tumorigenesis in a functional genomics screen (Possemato et al., 2011).

Here, we describe a critical onco-supportive role for GLS2 in breast cancer. We demonstrate that expression of the GLS2 gene is regulated by GATA3 and that the gene product is essential for cell proliferation and tumorigenesis in luminal-subtype breast cancers, which account for ~75% of total breast cancer incidence (Table S1) (Dai et al., 2015). Moreover, we show that GLS2 can be targeted with the small-molecule inhibitor 968 to suppress tumorigenesis and overcome resistance to GLS-selective inhibitors. These findings establish a previously unappreciated essential role for GLS2 in breast cancer biology and provide important insights regarding how to target glutamine metabolism for cancer therapy.

**Expression of GLS2 Is Elevated in Luminal-Subtype Breast Cancers**

Previously, the glutaminase isozyme GLS2 has been described as a tumor suppressor in some contexts, with downregulated expression in liver and brain cancers (Matés et al., 2018). However, since luminal-subtype breast cancer cells resist GLS inhibitors yet still exhibit glutamine-mediated anaplerosis, we hypothesized that they might instead be dependent on GLS2. We used The Cancer Genome Atlas (TCGA) invasive breast cancer dataset (Koboldt et al., 2012) to examine GLS2 transcript levels in the breast cancer molecular subtypes luminal A (LumA), luminal B (LumB), HER2+, and basal. Expression of GLS2 is indeed substantially higher in LumA and LumB tumors than in basal-subtype tumors, which instead have high levels of the GLS transcript (Figure 2A).

To compare protein levels of GLS2 in breast tumors and normal mammary tissue, we probed a tissue microarray (Figures 2B, 2C, and S2A). This revealed that GLS2 levels are significantly upregulated in receptor-positive breast tumors (the majority of which are luminal subtype) relative to normal tissue but are elevated only in a small number of receptor-negative breast tumors (generally basal subtype), with the mean level not differing significantly from normal tissue (Figure 2B). Microscopic examination of the stained tissue slices revealed that in receptor-positive tumors, GLS2 is much more abundant in carcinoma cells than in neighboring connective tissue (Figure 2C). We then confirmed that the expression patterns for GLS and GLS2 are conserved in breast cancer cell lines. Quantitative real-time PCR analysis showed that the GLS2 transcript is up to ~2,000-fold more abundant in luminal-subtype than in basal-subtype cells, which predominantly express GLS (Figures 2D and 2E). These differences are conserved at the protein level (Figure 2F). Consistent with other reports (Muir et al., 2017; Shin et al., 2017), high levels of GLS in basal-subtype cells are associated with expression of the glutamate/cystine antiport system “xCT” and corresponding rapid glutamate efflux (Figures S2B and S2C).

**GLS2 Is Localized to Mitochondria in Breast Cancer Cells**

Although GLS2 contains a predicted mitochondrial localization signal (Katt et al., 2017), several subcellular localizations have been reported, including the nucleus in neurons and astrocytes...
and as a binding partner of the plasma membrane/cytosolic protein Rac1 in liver cancer cells (Cardona et al., 2015; Zhang et al., 2016). To establish the localization of GLS2 in breast cancer cells, we fractionated both MDA-MB-453 (high-GLS2) and MDA-MB-231 cells (high-GLS) and performed western blot analysis on the whole-cell lysates along with the cytosolic, mitochondrial, and nuclear fractions. As markers for these fractions, we also probed for the cytosolic enzyme asparagine synthetase (ASNS), the mitochondrial voltage-dependent anion channel (VDAC), and the nuclear envelope protein lamin A. Both GLS2 and GLS, along with VDAC, were detected almost exclusively in the mitochondrial fractions of both cell lines (Figure 2G). A small proportion of each glutaminase was present in the nuclear fractions, but the similar pattern for VDAC suggests that these signals arise from small quantities of mitochondria co-pelleting with nuclei (Figure 2G). As expected, ASNS was detected in the cytosolic fraction, and lamin A in the nuclear fraction (Figure 2G). We also examined the subcellular localization of ectopically expressed GLS2 using immunofluorescence. Supporting a mitochondrial localization for GLS2 in breast cancer cells, GLS2-HA co-localizes with the endogenous mitochondrial marker protein Hsp60 in SK-BR-3 cells, as well as with ectopically expressed GLS-myc (Figure S2D).

GLS2 Expression Is Regulated by GATA3 and Promoter Methylation

To understand the upregulation of GLS2 in luminal-subtype breast cancers, we next investigated the mechanisms influencing GLS2 gene expression. Data from TCGA show a high frequency of copy number gains at the GLS2 gene locus in

![Figure 1. Luminal-Subtype Breast Cancer Cells Use Glutamine to Supply the TCA Cycle, but Resist GLS Inhibitors](image-url)
Figure 2. GLS2 Is Upregulated in Luminal-Subtype Breast Cancers

(A) Box and whisker plots showing transcript levels of GLS2 (left panel) and GLS (right panel) in the molecular subtypes of breast cancer. RNA-seq V2 RSEM data are from The Cancer Genome Atlas invasive breast cancer dataset. The mean expression in each group is indicated by a cross, and the box and whiskers indicate the minimum, first quartile, median, third quartile, and maximum values. **p < 0.01.

(B) Relative GLS2 protein levels in tissue microarray slices of normal mammary tissue, receptor-positive, and receptor-negative breast tumors. **p < 0.01.

(C) Microscopy images of breast tissue microarray slices stained brown for GLS2. Representative images are shown for normal breast tissue along with receptor-positive and receptor-negative breast tumors. Scale bars, 200 μm.

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Lamin A serve as mitochondrial, cytosolic, and nuclear marker proteins, respectively.

(F) Western blots showing relative levels of GLS and GLS2 in breast cancer cell lines. A non-specific band from the GLS2 antibody, clearly visible for MDA-MB-231 indicates the RQmax and RQmin values.

(E) Quantitative real-time PCR data showing relative levels of GLS2 transcript in breast cancer cell lines. Reactions were carried out in triplicate, and error bars indicate the RQmax and RQmin values.

(D) Quantitative real-time PCR data showing relative levels of GLS transcript in breast cancer cell lines. Reactions were carried out in triplicate, and error bars indicate the RQmax and RQmin values.

(G) Western blots of whole-cell lysates (WCLs), and cytosolic, mitochondrial, and nuclear fractions from MDA-MB-453 and MDA-MB-231 cells. VDAC, ASNS, and Hs 578T lysates, is labeled N/S.

See also Figure S2.

GLS2 Mediates Glutamine Anaplerosis in Luminal-Subtype Cells

To probe the role of GLS2 in luminal-subtype cells, we asked whether GLS2 is involved in the observed supply of glutamine-derived carbon to the TCA cycle (Figure 1D). For comparison, we also quantified the anaplerotic role of GLS in basal-subtype breast cancer cells. We transduced MDA-MB-453 (luminal, high-GLS2) or MDA-MB-231 (basal, high-GLS) cells with constructs for expressing a control short hairpin RNA (shRNA) or shRNAs targeting GLS2 or GLS. After 48 h, we confirmed knockdowns using western blot analysis, and observed that within this time frame there was no compensatory upregulation of the other glutaminase isozyme (Figure 4A). We then performed metabolomic experiments to assess the effects of the knockdowns on TCA cycle anaplerosis. Importantly, knockdown of GLS2 strongly inhibited glutamine-mediated TCA cycle anaplerosis in MDA-MB-453 cells (Figure 4B). As expected, knockdown of GLS in MDA-MB-231 cells also suppressed the delivery of carbon from [U-13C]-glutamine into the TCA cycle (Figure 4B). The glutaminase knockdowns also decreased the total abundance of glutamate and TCA cycle intermediates (Figure S4A). As a control, we carried out the reciprocal experiment, treating MDA-MB-453 cells with GLS-targeted shRNAs, and MDA-MB-231 cells with GLS2-targeted shRNAs. In each case, the abundance of glutamate and TCA cycle metabolites was minimally perturbed (Figure S4B), further demonstrating that GLS2 is the predominant glutaminase in MDA-MB-453 cells and that GLS is the primary isozyme in MDA-MB-231 cells. Collectively, these findings establish that GLS2 is a metabolically active mitochondrial enzyme, critical for glutamine-mediated anaplerosis in luminal-subtype breast cancer cells.

Across the cell lines, there is no clear association between GLS2 levels and any one of the receptors ER, PR, or HER2, with MDA-MB-453 cells, which have low expression of all three receptors, having the highest GLS2 levels (Figure 3B). Since GLS2 is so abundant in LumA and LumB breast cancers relative to other subtypes, we wondered whether its expression is intrinsically associated with luminal cell status. Levels of GLS2 in breast cancer cell lines correlate closely with that of full-length GATA3 (Figure 3A), a “master regulator” of luminal differentiation (Asselin-Labat et al., 2007). There is also a strong positive correlation between GATA3 and GLS2 transcript levels in human breast tumors (Figure 3C). We therefore used PROMO (Farré et al., 2003; Messeguer et al., 2002) to analyze the human GLS2 gene promoter, and identified a match to the GATA3 consensus motif at position −1,259 base pairs (bp) relative to the transcription start site (TSS) (Figure S3B). The probability of this sequence occurring randomly within 1,500 bp of the TSS is predicted by PROMO to be only 2.8%. We next carried out a chromatin immunoprecipitation (ChIP), pulling down GATA3 from cross-linked chromatin prepared from MDA-MB-453 cell nuclei and using quantitative real-time PCR to quantify a 176-bp stretch of the GLS2 promoter centered on the GATA3 consensus motif. The GATA3 pull-down yielded a ~6-fold higher signal relative to input than a negative-control IgG IP, consistent with direct binding of GATA3 to this region of the promoter (Figure 3D). Finally, we knocked down GATA3 in MDA-MB-453 and T-47D cells and found that, after 48 h, this resulted in a corresponding decrease in GLS2 levels in each cell line (Figures 3E and S3D). Thus, GATA3 contributes to the elevated expression of GLS2 in luminal-subtype breast cancers.

Because GLS2 levels are so low in basal-subtype breast cancers, we also tested whether expression is epigenetically silenced in these cells via methylation of the GLS2 gene promoter. After isolating genomic DNA from the basal-subtype cell lines with the lowest levels of GLS2 transcript (TSE and Hs 578T) and the luminal-subtype cell lines with the highest levels (T-47D and MDA-MB-453), we used the MassARRAY system to quantify methylation levels at sites in the CpG island centered on the TSS of the GLS2 gene (Figures S3B and S3C). In MDA-MB-453 cells, which have the highest levels of GLS2, there is minimal promoter methylation, whereas in TSE cells, which have the lowest levels of GLS2 transcript, almost every site within the CpG island has a high methylation ratio (Figure 3F). Treatment of TSE cells with the DNA hypomethylating agent aza-citidine for 48 h resulted in increased GLS2 protein levels (Figure 3G). Collectively, these results show that GLS2 expression is regulated by GATA3 in luminal-subtype breast cancers, and potentially also by copy number gains, whereas expression in basal-subtype breast cancers is repressed by promoter methylation.
Figure 3. GLS2 Gene Expression Is Regulated by GATA3 and Promoter Methylation

(A) Western blots showing relative levels of GLS2, and previously identified transcription factors for the GLS2 gene, in breast cancer cell lines.

(B) Western blots showing relative levels of GLS2, the luminal-transcription factor GATA3, and the receptors ERx, PR, and HER2 in breast cancer cell lines.

(C) Plot of GLS2 and GATA3 transcript levels in human breast tumors, with the Pearson correlation coefficient r indicated. RNA-seq V2 RSEM data from TCGA invasive breast cancer dataset.

(D) Quantitative real-time PCR data showing relative levels of a 176-bp fragment of the GLS2 gene promoter, centered on the putative GATA3 binding site, in chromatin immunoprecipitations (ChIPs) using negative control IgG or a GATA3-targeted antibody. Mean ± SD of biological triplicate samples.

(E) Western blots showing GLS2 and GATA3 levels in MDA-MB-453 and T-47D cells transfected with either a control siRNA (labeled C) or with two independent GATA3-targeted siRNAs. Bands in the GLS2 blot were quantified by densitometry using ImageJ, and relative band intensities are indicated above the blot. Since GATA3 regulates expression of Tubulin and several other cytoskeletal proteins, a non-specific (N/S) band is shown to demonstrate equal loading.

(F) Heatmaps showing relative methylation levels at CpG sites within the CpG island of the GLS2 gene promoter in breast cancer cell lines. Labels for the CpG sites refer to the amplicon in which the sites are located (amplicons 0 to 9) and then the position of the site within that amplicon (i.e., first CpG site, second CpG)

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GLS2 Is Essential in Luminal-Subtype Breast Cancer Cells

Because various functions have been reported for GLS2 in cancer, including tumor-suppressive activity in liver cancer and glioblastoma (Matés et al., 2018), we next investigated the importance of GLS2 for breast cancer cell proliferation and tumorigenesis. We transfected luminal- and basal-subtype cell lines with either a control small interfering RNA (siRNA) or siRNAs selectively targeting GLS2, GLS, or both isozymes simultaneously. Western blot analysis 48 h after transfection confirmed that potent and selective knockdowns had been achieved (Figure 4C, lower panels). We then measured cell proliferation over 6 days. Knockdown of GLS2 strongly suppressed proliferation of luminal-subtype cells (MDA-MB-453 and T-47D), but only modestly impacted proliferation of basal-subtype cells (MDA-MB-231 and TSE) (Figure 4C, upper panels). Knockdown of GLS had no effect on luminal-subtype cells but did inhibit basal-subtype cells, consistent with the results using GLS-selective inhibitors (Figure 1A). In all cases, the effects of GLS2 and/or GLS knockdowns could be rescued by supplementation of the culture medium with 2 mM dimethyl α-KG (dm-α-KG) (Figure S4C), confirming that suppressed proliferation was a result of impaired TCA cycle anaplerosis.

We next addressed the importance of GLS2 for luminal-subtype breast tumor growth in vivo. This experiment required stable rather than transient depletion of GLS2, but potent shRNA-mediated knockdowns severely impacted luminal-subtype cell viability after several days. We therefore used a low titer of virus for transducing MDA-MB-453 cells and generated stable GLS2 partial-knockdown cell lines (Figure 4D). Proliferation of the GLS2 partial-knockdown cells in culture was 40%–50% slower than that of cells expressing a control shRNA (Figure 4E, left panel), and could be rescued with 2 mM dm-α-KG (Figure 4E, right panel). We then injected 3 × 10⁶ control or GLS2 partial-knockdown cells, in Matrigel suspension, into each flank of female NOD.Cg-PkdcdScr /SzJ (NSG) mice, and measured tumor growth over 42 days (n = 6 tumors per condition). Both tumor initiation time and the rate of tumor growth were strongly inhibited in the GLS2 partial-knockdown samples relative to the control, providing a proof of principle that targeting GLS2 can inhibit luminal-subtype breast cancer tumorigenesis in vivo as well as cell proliferation ex vivo (Figure 4F).

GLS2 Mediates Resistance to GLS Inhibitors

The BPTES class of inhibitors has been extensively studied and is highly selective for GLS over GLS2 (Figures S5A and S5B) (Robinson et al., 2007). Since GLS2 is capable of mediating TCA cycle anaplerosis in breast cancer cells, we tested whether it is sufficient to confer resistance to these inhibitors. We began by looking at the inhibition of glutaminase activity in isolated mitochondria. In mitochondria from luminal-subtype breast cancer cells, glutaminase activity was only partially inhibited by addition of 10 μM BPTES (Figure 5A). In the case of MDA-MB-453 cells, BPTES treatment had no effect on activity. As expected, for mitochondria isolated from basal-subtype cells, glutaminase activity was almost completely abolished by 10 μM BPTES (Figure 5A).

To gain further insight into the glutaminase expression profiles of breast cancer cells, we performed western blot analyses using known amounts of purified, recombinant GLS and GLS2 to estimate absolute levels of each glutaminase in cells (Figure S5C). We found that MDA-MB-453 cells contain ~275 pg of GLS2 per μg of total cellular protein and ~75 pg/μg GLS. In contrast, MDA-MB-231 cells contain ~500 pg/μg GLS and <75 pg/μg GLS2 (Figure S5C). In T-47D cells, the absolute levels of GLS and GLS2 are approximately equal, at ~200 pg/μg (Figure S5C). However, GLS requires much higher concentrations of inorganic phosphate (Pi) to reach maximal activity (Figure 5B), and in contrast to GLS2, its enzymatic activity is inhibited by its product, glutamate (Watford, 1993). At physiological mitochondrial Pi concentrations of ~10 mM (Hutson et al., 1992; Rauch et al., 1994), only GLS2 is fully activated (Figure 5B).

We next treated cells with 10 μM BPTES in culture medium containing [U-13C]-glutamine and extracted and analyzed cellular metabolites. Consistent with the data above, BPTES treatment potently inhibited the supply of glutamine-derived carbon to glutamate and the TCA cycle in the basal-subtype cell lines Hs 578T and MDA-MB-231 (Figure 5C). In luminal-subtype MCF7 cells, which express GLS2 as well as GLS, BPTES treatment partially blocked glutamine anaplerosis (Figure 5C). In MDA-MB-453 cells, which express primarily GLS2, BPTES treatment had no impact on glutamine-mediated anaplerosis (Figure 5C). A similar pattern can be seen in the total abundances of TCA cycle intermediates (Figure S5D).

Using MDA-MB-231 and TSE cells, which express almost exclusively GLS, we generated derivative cell lines that ectopically express GLS2 (Figure 5D). In both cases, the clone with highest GLS2 expression showed a moderate decrease in the level of GLS. Although forced overexpression of GLS2 hinders the growth of liver cancer cells (Hu et al., 2010; Suzuki et al., 2010), in basal-subtype breast cancer cells each of the GLS2-overexpressing clones proliferated slightly more rapidly than control cells (Figure S5E). Ectopic expression of GLS2 greatly decreased sensitivity to BPTES treatment, with the EC₅₀ shifting to >20 μM for both cell lines, which matches the aqueous solubility limit of the inhibitor (Figure 5E; Table S4).

We then searched for basal-subtype breast cancer cells that are intrinsically resistant to GLS-selective inhibitors, to test whether resistance can be overcome by simultaneously targeting GLS2. The triple-negative, basal-subtype breast cancer cell line DU4475, which was originally derived from a metastatic lesion, is highly resistant to BPTES treatment, with an EC₅₀ value of >20 μM (Figure 5F). Western blot analysis showed that...
Figure 4. GLS2 Is Essential in Luminal-Subtype Breast Cancers

(A) Western blots showing GLS and GLS2 levels in MDA-MB-453 cells and MDA-MB-231 cells expressing either a control shRNA or two independent GLS2-targeted (MDA-MB-453 cells) or GLS-targeted (MDA-MB-231 cells) shRNAs.

(B) Fold changes in fully labeled glutamate and TCA cycle metabolites (i.e., derived directly from [U-13C]-glutamine) in MDA-MB-453 cells following knockdown of GLS2, or in MDA-MB-231 cells following knockdown of GLS. Mean ± SEM of biological triplicates.

(C) The effect of knocking down either GLS, GLS2, or both GLS and GLS2 simultaneously, on the proliferation of breast cancer cell lines over 6 days. For each condition, two independent siRNAs were used, and the effects were compared with those of a control siRNA (labeled C). Mean ± SD of triplicate assays. Lower panels show western blots for GLS and GLS2 in each sample. *p < 0.05; **p < 0.01; ns, not significant.

(D) Western blot showing partial knockdown of GLS2 in MDA-MB-453 stably expressing two independent GLS2-targeted shRNAs, relative to cells stably expressing a control shRNA.
DU4475 cells express higher levels of GLS than MDA-MB-231 cells, but simultaneously express higher levels of GLS2 than MDA-MB-453 cells (~750 pg/μg of cellular protein for GLS, and ~625 pg/μg for GLS2) (Figures 5G and S5C). Knockdown of either glutaminase isozyme alone did not impact DU4475 cell proliferation, but simultaneous knockdown of both GLS and GLS2 significantly inhibited growth (Figures 5H and S5F). Proliferation was again restored by supplementation of the culture medium with 2 mM dimethyl-a-ketoglutarate (dm-a-KG) (Figure S5G).

968 Inhibits GLS2 and Suppresses BPTES-Resistant Breast Cancer Growth

Previously, we reported that the small molecule 968 binds and inhibits the GLS splice variant GAC (Wang et al., 2010). This inhibitor has a much higher affinity for monomeric GAC than for the active tetramer and is proposed to bind to newly synthesized enzyme monomers and prevent the formation of activated tetramers (Stalnecker et al., 2015). However, the sensitivity of GLS2 to 968 has not previously been tested. We therefore measured the effect of 968 on the activity of purified, recombinant, glutaminase. In contrast to BPTES-class inhibitors, which are highly selective for GLS, 968 inhibited both GLS and GLS2, with a moderate (~3-fold) selectivity for GLS2 (Figure 6A). Compound 26, a quinoline derivative of 968 that differs by only a single carbon-to-nitrogen substitution, has greatly weakened affinity for GLS (Katt et al., 2012; Stalnecker et al., 2013). Similarly, we found that compound 26 minimally inhibits recombinant GLS2 at 50 μM, whereas 968 completely blocks activity at this concentration (Figure S6A).

Treatment of breast cancer cells with 968 inhibited proliferation with EC50 values of 3–5 μM (Figure S6B; Table S4). For the cells with highest expression of GLS, maximal inhibition was ~70%–75%. The inactive analog compound 26 did not inhibit the proliferation of any of the cell lines we tested it against. Despite their intrinsic resistance to BPTES, DU4475 cells responded to 968 treatment with an EC50 value of 4 μM (Figure 6B). We also measured the response of the GLS2-overexpressing derivatives of MDA-MB-231 and TSE cells, described above. Consistent with the ability of 968 to inhibit both glutaminase iso-zymes, overexpression of GLS2 had little effect on the EC50 values for 968 (Table S4).

We next treated basal- and luminal-subtype breast cancer cells with 10 μM 968 and extracted metabolites for analysis at different time points. Consistent with the mechanism of 968 binding to monomeric glutaminase and preventing the formation of active tetramers, we observed a time-dependent inhibition of glutamine-mediated TCA cycle anaplerosis (Figure 6C). Matching the partial selectivity of 968 for GLS2 over GLS, the effects were more pronounced in MDA-MB-453 cells than MDA-MB-231 cells (Figure 6C). Treatment with 968 also decreased the total abundance of TCA cycle intermediates (Figure S6C).

Since 968 inhibits the proliferation of breast cancer cells ex vivo but does not affect the growth of primary human mammary epithelial cells or fibroblasts (Wang et al., 2010), we tested whether it can be used to treat luminal-subtype breast tumor growth in vivo. We injected 3 x 10^6 MDA-MB-453 cells in Matrigel suspension into each flank of NSG mice (n = 6 xenografts per condition) and waited until palpable tumors 1–2 mm in diameter were present (14 days). At this point, mice were separated into two groups, with one group receiving subcutaneous injections of 968 (10 mg/kg body weight) 3 times per week, and the other group receiving carrier solution only. We continued to monitor tumor growth for 3 weeks after initiating treatment and found that it was robustly inhibited in 968-treated animals (Figure 6D). In contrast, treatment of animals with 10 mg/kg BPTES did not significantly impact tumor growth (Figure 6E). Thus, 968 inhibits GLS2 in vitro, suppresses GLS2-mediated anaplerosis, and is effective against BPTES-resistant breast cancer cell proliferation and tumorigenesis. Taken together, our results reveal an essential and potentially druggable role for GLS2 in luminal-subtype breast cancers.

DISCUSSION

Efforts to target glutamine catabolism for cancer therapy have focused on inhibiting the glutaminase isozyme GLS, which is highly expressed and onco-supportive in diverse malignancies (Cluntun et al., 2017). The importance of the other mammalian glutaminase, GLS2, in tumorigenesis has remained less clear, and various subcellular localizations and functions have been described, including tumor suppressor activity (Maté et al., 2018). We report here that GLS2 is upregulated in luminal-subtype/receptor-positive breast cancers, where it is essential for glutamine-mediated TCA cycle anaplerosis, cell proliferation, and tumorigenesis. These findings explain the identification of GLS2 as one of only 16 metabolic enzymes required for tumorigenesis in an earlier functional genomics screen (Possemato et al., 2011).

Previous studies found that triple-negative, but not receptor-positive, breast cancer cell lines often express high levels of the GLS splice variant GAC (Gross et al., 2017). The importance of the other mammalian glutaminase, GLS2, in tumorigenesis has remained less clear, and various subcellular localizations and functions have been described, including tumor suppressor activity (Maté et al., 2018). We report here that GLS2 is upregulated in luminal-subtype/receptor-positive breast cancers, where it is essential for glutamine-mediated TCA cycle anaplerosis, cell proliferation, and tumorigenesis. These findings explain the identification of GLS2 as one of only 16 metabolic enzymes required for tumorigenesis in an earlier functional genomics screen (Possemato et al., 2011).

(B) Inhibition of proliferation over 6 days for MDA-MB-453 cells stably expressing GLS2-targeted shRNAs, relative to cells stably expressing a control shRNA (labeled C). Experiments were run either without (left panel) or with (right panel) supplementation of the culture medium with 2 mM dimethyl-a-ketoglutarate (dim-a-KG). Mean ± SD of triplicate assays. *p ≤ 0.05; **p ≤ 0.01; ns, not significant.

(F) Growth of xenograft tumors in mice by MDA-MB-453 cells stably expressing either a control shRNA or two independent GLS2-targeted shRNAs. Mean ± SD (n = 6 tumors per condition). *p ≤ 0.05; **p ≤ 0.01.

See also Figure S4.
Figure 5. GLS2 Expression Is Sufficient for Resistance to GLS Inhibitors

(A) Inhibition of glutaminase activity by 10 μM BPTES in mitochondria isolated from breast cancer cell lines. Plot shows activity in the presence of 10 μM BPTES relative to matched samples with no BPTES present. Mean ± SD of triplicate assays.

(B) The effect of increasing inorganic phosphate concentrations on the catalytic activity of recombinant full-length human GLS (GAC splice variant) and GLS2. Mean ± SD of triplicate assays.

(C) Fold changes in fully labeled intracellular metabolites (i.e., derived directly from [U-13C]-glutamine) in basal-subtype (top) and luminal-subtype (bottom) breast cancer cell lines, following treatment with 10 μM BPTES. Mean ± SEM of triplicates relative to the vehicle.

(D) Western blots showing levels of endogenous GLS and ectopically expressed V5-tagged GLS2 in MDA-MB-231 and TSE cells.

(E) Inhibition of proliferation over 6 days of MDA-MB-231 and TSE cells, stably expressing GLS2 (two clones for each cell line) or carrying the plasmid vector only, by treatment with different concentrations of BPTES. Mean ± SD of triplicate assays.

(F) The effect of different concentrations of BPTES on proliferation of DU4475 cells over 6 days. Mean ± SD of triplicate assays.

(G) Western blots showing relative levels of GLS and GLS2 in MDA-MB-453, MDA-MB-231, and DU4475 cells. A non-specific band from the GLS antibody is marked N/S.

(H) The effect of knocking down either GLS or GLS2, or both GLS and GLS2 simultaneously, on the proliferation of DU4475 cells over 6 days. For each condition, two independent siRNAs were used, and the effects were compared with those of a control siRNA (labeled C). Mean ± SD of triplicate assays. **p < 0.01; ns, not significant.

See also Figure S5 and Table S4.
Our findings highlight the importance of considering GLS2 when identifying target diseases for treatment with GLS-selective inhibitors. Gene expression data from TCGA show that GLS2 transcript levels are consistently upregulated relative to healthy tissue in colorectal tumors and also in a subset of lung tumors. With clinical trials underway to evaluate the efficacy of CB-839 against these cancers, it will be important to characterize further the function of GLS2 in these contexts. In pancreatic ductal adenocarcinoma (PDAC) cells, GLS inhibitors have only a temporary cytostatic effect, which is followed by metabolic adaptation and recovery of proliferation (Biancur et al., 2017). Notably, GLS2 is present in both healthy pancreas and PDAC cells (Altman et al., 2016; Biancur et al., 2017), and thus might provide a critical supply of glutamate following GLS inhibition. The sensitivity of some cancer cells to GLS inhibitors requires high expression of the xCT antiporter, which exchanges intracellular glutamate for extracellular cystine and can therefore deplete intracellular glutamate reserves (Muir et al., 2017). Thus, a signature of concurrent high expression of GLS and xCT, along with low levels of GLS2, might identify tumors that are most likely to respond to GLS-targeted therapy.

GLS2 as a Potential Therapeutic Target

In healthy tissues, GLS2 expression is highest in periportal regions of the liver, where it allows glutamine carbon to be directed via the TCA cycle into the gluconeogenic pathway in response to glucagon (Lacey et al., 1981; Watford and Smith, 1990). For GLS2 to be targeted for cancer therapy, any toxicity arising from inhibiting its normal physiological function must be within a tolerable range. We observed that mice treated with 968 at 10 mg/kg body weight, three times weekly for 3 weeks, showed no gross evidence of toxicity. Moreover, it was recently reported that GLS2 knockout mice are viable, albeit with a decreased ability to maintain plasma glucose levels during fasting (Miller et al., 2018). These results indicate that GLS2 could be safely targeted as a strategy for treating luminal-subtype breast cancers, most likely as part of a combination therapy designed to maximize cancer cell dependence on the glutaminase reaction. To date, drug discovery efforts for blocking glutamine catabolism in cancer have focused almost exclusively on the GLS isozyme. However, a small number of molecular scaffolds have now been reported to inhibit GLS2 (Wu et al., 2018), including 968, which we show targets both isozymes with a moderate (3-fold) selectivity for GLS2. Our finding that GLS2 is upregulated and essential in the most prevalent subtypes of breast cancer support the notion of building on these scaffolds to develop more potent inhibitors for selective targeting of GLS2-high cancers.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.08.076.

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AUTHOR CONTRIBUTIONS

Conceptualization, R.A.C., M.J.L., W.P.K., and A.A.C.; Methodology, R.A.C., M.J.L., W.P.K., and A.A.C.; Investigation, M.J.L., A.A.C., W.P.K., M.-c.J.L., J.E.D., B.B., J.W.E., S.R., J.W.E., H.H.L., Z.-E.W., B.K.S.G., and R.A.C.; Writing—Original Draft, M.J.L., A.A.C., W.P.K., and R.A.C.; Writing—Review and Editing, M.J.L., A.A.C., W.P.K., M.-c.J.L., J.E.D., S.R., J.W.E., H.H.L., Z.-E.W., B.K.S.G., and R.A.C.; Funding Acquisition, R.A.C., M.J.L., and A.A.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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# STAR METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| ASCT2 Antibody      | Cell Signaling Technology | Cat# 5345; RRID:AB_10821427 |
| α/β-Tubulin Antibody| Cell Signaling Technology | Cat# 2148; RRID:AB_2288042 |
| GLS2 antibody (C-term E513) | Abgent | Cat# AP6650D; RRID:AB_10818414 |
| GLS antibody (C-term) | Abgent | Cat# AP8809b; RRID:AB_10553123 |
| Anti-GLS2 antibody | Abcam | Cat# ab150474 |
| 4F2hc/CD98 Rabbit mAb | Cell Signaling Technology | Cat# 13180; RRID:AB_2687475 |
| xCT/SLC7A11 Rabbit mAb | Cell Signaling Technology | Cat# 12691; RRID:AB_2687474 |
| VDAC Rabbit mAb | Cell Signaling Technology | Cat# 4661; RRID:AB_10557420 |
| ASNS Antibody | Proteintech | Cat# 14681-1-AP; RRID:AB_2060119 |
| Lamin A/C Mouse mAb | Cell Signaling Technology | Cat# 7777; RRID:AB_10545756 |
| p53 Mouse mAb | Cell Signaling Technology | Cat# 2524; RRID:AB_331743 |
| p63 Rabbit mAb | Cell Signaling Technology | Cat# 39692; RRID:AB_2799159 |
| p73 Rabbit mAb | Cell Signaling Technology | Cat# 14620; RRID:AB_2798542 |
| N-Myc Rabbit mAb | Cell Signaling Technology | Cat# 51705; RRID:AB_2799400 |
| c-Myc Rabbit mAb | Cell Signaling Technology | Cat# 5605; RRID:AB_1903938 |
| GATA-3 Rabbit mAb | Cell Signaling Technology | Cat# 5852; RRID:AB_10835960 |
| HER2/ErbB2 Rabbit mAb | Cell Signaling Technology | Cat# 4290; RRID:AB_10828932 |
| Progesterone Receptor A/B Rabbit mAb | Cell Signaling Technology | Cat# 8757; RRID:AB_2797144 |
| Estrogen Receptor α Rabbit mAb | Cell Signaling Technology | Cat# 13258; RRID:AB_2632959 |
| HSP60 Rabbit mAb | Cell Signaling Technology | Cat# 12165; RRID:AB_2636800 |
| HA-Tag Mouse mAb | Cell Signaling Technology | Cat# 2367; RRID:AB_10691311 |
| Myc-Tag Rabbit mAb | Cell Signaling Technology | Cat# 2278; RRID:AB_1093332 |
| V5 Tag Monoclonal Antibody | Thermo Fisher Scientific | Cat# R960-25; RRID:AB_2556564 |
| Anti-rabbit IgG, HRP-linked Antibody | Cell Signaling Technology | Cat# 7074; RRID:AB_2099233 |
| Anti-mouse IgG, HRP-linked Antibody | Cell Signaling Technology | Cat# 7076; RRID:AB_330924 |
| **Bacterial and Virus Strains** |        |            |
| *Escherichia coli* BL21(DE3) | Thermo Fisher Scientific | Cat# C600003 |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| Recombinant human Glutaminase C | This paper | N/A |
| Recombinant human GLS2 | This paper | N/A |
| SuperScript III Reverse Transcriptase | Thermo Fisher Scientific | Cat# 18080044 |
| RNase One Ribonuclease | Promega | Cat# M4261 |
| CB-839 | Millipore-Sigma | Cat#533717 |
| BPTES | Dr. Scott Ulrich, Ithaca College | N/A |
| 968 | ChemBridge Corporation | N/A |
| Dimethyl 2-oxoglutarate | Sigma-Aldrich | Cat# 349631 |
| [(13C5)-L-Glutamine | Cambridge Isotope Laboratories | Item# CLM-1822-H-PK |
| **Critical Commercial Assays** |        |            |
| L-Glutamine/Ammonia Assay Kit (Rapid) | Megazyme | Cat# K-GLNAM |
| Protein Assay Dye Reagent Concentrate | Bio-Rad | Cat# 5000006 |

(Continued on next page)
Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Deposited Data      |        |            |
| TCGA Breast Invasive Carcinoma (BRCA) gene expression RNaseq | The Cancer Genome Atlas, UCSC Xena, cBioPortal | https://xena.ucsc.edu, http://www.cbioportal.org |
| Human reference genome GRCh38/hg38 | UCSC Genome Browser, NCBI | https://genome.ucsc.edu, https://www.ncbi.nlm.nih.gov/gene/27165 |

Experimental Models: Cell Lines

| Human: MCF7 | ATCC | Cat# HTB-22 |
| Human: BT-474 | ATCC | Cat# HTB-20 |
| Human: T-47D | ATCC | Cat# HTB-133 |
| Human: MDA-MB-453 | ATCC | Cat# HTB-131 |
| Human: MDA-MB-231 | ATCC | Cat# HTB-26 |
| Human: TSE | Dr. Steven Abcouwer, University of Michigan | N/A |
| Human: Hs 578T | ATCC | Cat# HTB-126 |
| Human: HCC38 | ATCC | Cat# CRL-2314 |
| Human: SK-BR-3 | ATCC | Cat# HTB-30 |
| Human: DU4475 | ATCC | Cat# HTB-123 |
| Human: 293T | ATCC | Cat# CRL-3216 |

Experimental Models: Organisms/Strains

Mouse: NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG), female, 6-8 weeks | The Jackson Laboratory | Stock# 005557 |

Oligonucleotides

| Oligonucleotides | SOURCE | IDENTIFIER |
|------------------|--------|------------|
| Primers | Table S6 | N/A |
| Silencer Select Negative Control No. 1 siRNA | Invitrogen | N/A |
| Silencer Select GLS siRNA | Invitrogen | siRNA ID# s5838 |
| Silencer Select GLS2 siRNA | Invitrogen | siRNA ID# s5840 |
| Silencer Select GLS2 siRNA | Invitrogen | siRNA ID# s25940 |
| Silencer Select GATA3 siRNA | Invitrogen | siRNA ID# s5600 |
| Silencer Select GATA3 siRNA | Invitrogen | siRNA ID# s533216 |

Recombinant DNA

| Recombinant DNA | SOURCE | IDENTIFIER |
|-----------------|--------|------------|
| pCDNA3.1-GAC-myc | This paper | N/A |
| pCDNA3.1-GLS2-HA | This paper | N/A |
| pCDNA3.1-GLS2-V5 | This paper | N/A |
| pQE80-GAC-72-598 | Huang et al., 2018 | N/A |
| pQE80-GLS2-38-602 | Huang et al., 2018 | N/A |
| pCMV-dR8.2 | pCMV delta R8.2 was a gift from Didier Trono | Addgene plasmid #12263 |
| pMD2.G | pMD2. G was a gift from Didier Trono | Addgene plasmid #12259 |
| pLKO.1-puro shRNA control plasmid | Sigma-Aldrich | Cat# SHC002 |
| pLKO.1:TRCN0000051326 targeting GLS2 | Sigma-Aldrich | Clone ID: NM_013267.2-2053s1c1 |

Software and Algorithms

| Software and Algorithms | SOURCE | IDENTIFIER |
|-------------------------|--------|------------|
| ToxID 2.0 | Thermo Fisher Scientific | N/A |
| MetaboAnalyst | McGill University | https://www.metaboanalyst.ca/ |
LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Richard A. Cerione (rac1@cornell.edu). Plasmids generated in this study are available from the Lead Contact without restriction.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human Cell Lines

Human breast cancer cell lines MCF7, BT-474, T-47D, MDA-MB-453, MDA-MB-231, Hs 578T, HCC38, SK-BR-3, and DU4475 were purchased from the American Type Culture Collection (ATCC) and no additional cell authentication was performed. The TSE human breast cancer cell line was supplied by Dr. Steven Abcouwer (University of Michigan). All breast cancer cells were cultured at 37°C under a 5% CO₂ atmosphere in RPMI 1640 medium containing 2 mM glutamine (GIBCO) and supplemented with 10% fetal bovine serum (FBS) (GIBCO). The 293T cell line (ATCC) used for generating lentivirus particles was cultured as above but using DMEM, high glucose (GIBCO).

Animals

All experiments involving mice were carried out according to protocols approved by the Institutional Animal Care and Use Committee at Cornell University. In all cases, 6-8 week old female NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl</sup>/SzJ (NSG) mice (The Jackson Laboratory) were used. For xenograft experiments, a suspension of MDA-MB-453 breast cancer cells (or derivative cell lines stably expressing shRNAs) was mixed 1:1 with Matrigel Matrix (BD Biosciences) to give a final concentration of 3 x 10⁶ cells per 100 µl, and 3 x 10⁶ cells were immediately injected into each of the two flanks of 6-8 week old female NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl</sup>/SzJ (NSG) mice (n = 3 mice per condition). Tumor sizes were measured using calipers, and estimated volumes were calculated using the formula V = (π/6) x length x width², as described previously (Tomayko and Reynolds, 1989). For experiments using MDA-MB-453 cells stably expressing shRNAs, mice were randomly divided into two groups and intraperitoneal (IP) injections of 968 (10 mg/kg) solution or carrier solution were initiated and carried out three times weekly. The formulation was prepared immediately prior to injection and consisted of 70% PBS, 20% Cremophor EL, 10% DMSO, and 968 diluted from a 21 mM DMSO stock. Mice were sacrificed after 3 weeks of treatment.
METHOD DETAILS

Cell Lysis and Western Blot Analysis
Cells, or fractionated mitochondria and nuclei, were lysed with ice-cold lysis buffer (50 mM HEPES pH 8.0, 150 mM NaCl, 25 mM NaF, 1% (v/v) Triton X-100, 1 mM MgCl₂, 50 mM β-glycerophosphate, 30 μg/ml leupeptin, 5 μg/ml aprotinin), and insoluble debris cleared by centrifugation at 4°C. Protein concentrations were determined by Bradford assay (Bio-Rad), and lysate was then boiled for 10 min in reducing SDS-sample buffer. Lysate proteins (20 μg total protein/lane, except for the cell fractionation experiment in which 5 μg total protein/lane was used) were then resolved on Novex 12% Tris-Glycine Mini Gels (Thermo Fisher Scientific) and transferred to PVDF membrane (PerkinElmer). Membranes were blocked in 7% BSA in Tris-Buffered Saline plus 0.05% TWEEN 20 (TBST) for 1 h at room temperature and probed overnight at 4°C in primary antibody solution in TBST (see Table S5 for antibody dilutions). They were then washed with TBST and incubated in TBST containing 25% (v/v) non-fat dry milk powder and anti-Rabbit or anti-Mouse secondary antibody (1:2500) for 1 h. Finally, membranes were washed in TBST, and imaged using Western Lightning Plus-ECL (PerkinElmer) and HyBlot ES autoradiography film (Denville Scientific Inc.).

Breast Tumor Tissue Microarray
Tissue microarray BRC961 (US Biomax) was probed as follows, using the anti-GLS2 antibody (Abgent, AP6650D). Reagents were from Vector Laboratories VECTASTAIN Elite ABC HRP kit (Cat# PK-6200), Avidin/Biotin blocking kit (Cat# SP-2001), and ImmPACT DAB Peroxidase (HRP) Substrate (Cat# SK-4105). Deparaffinization was carried out by heating the slide to 60°C for 20 min and then immersing the slide in mixed xylene (2 × 10 min), 100% ethanol, 95% ethanol, 70% ethanol (5 min each) and finally H₂O (2 × 5 min). The antigen retrieval step involved immersing the slide in 10 mM sodium citrate buffer, pH 6.0 at ~95°C for 15 min and then cooling at room temperature for 20 min. To remove endogenous peroxidase the slide was washed with H₂O (2 × 5 min) and then incubated in 3% H₂O₂ in H₂O for 10 min. The slide was then incubated in H₂O for 5 min followed by PBS for 5 min. Blocking was carried out at room temperature using horse serum (20 min), PBS rinse, avidin solution (15 min), PBS rinse, biotin solution (15 min), PBS rinse, followed by PBS washes (2 × 5 min). The slide was then incubated overnight at 4°C with the anti-GLS2 primary antibody (1:100 in horse serum). Next, the slide was washed with PBS (4 × 5 min), incubated for 30 min at room temperature with biotinylated universal secondary antibody, and washed again with PBS (4 × 5 min). Then, the slide was incubated for 30 min at room temperature with VECTASTAIN ABC reagent and washed again with PBS (4 × 5 min). To develop, the slide was incubated with diluted ImmPACT DAB chromogen for ~2 min and then washed in H₂O (2 × 5 min). Finally, the stained slide was dehydrated by immersing in 70% ethanol, 95% ethanol, 100% ethanol (5 min each) and mixed xylene (2 × 5 min), mounted using Permout mounting medium (Fisher) and sealed. Signal intensity was quantified using ImageJ. Receptor staining intensity data were from US Biomax.

RNA Isolation and Quantitative Real-Time PCR
Total RNA was extracted from cells using the RNeasy Mini Kit (QIAGEN) and QIAshredder (QIAGEN), and cDNAs were prepared using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific). RT-PCR was carried out using the 7500 fast real-time PCR system (Applied Biosystems), using appropriate primers (Table S6) with cDNA as the template. In all cases, 18S rRNA served as the endogenous control. All primer sequences were obtained from PrimerBank (https://pga.mgh.harvard.edu/primerbank/), and primers were synthesized by Integrated DNA Technologies. Reactions were carried out using Power SYBR Green PCR Mix (Thermo Fisher Scientific).

Genomic DNA Isolation and DNA Methylation Analysis
High molecular weight genomic DNA was isolated from breast cancer cells using QIAamp DNA Mini Kit (QIAGEN), and contaminating RNA was digested using RNase One Ribonuclease (Promega) followed by re-purification of DNA, elution with water, and adjustment of DNA concentration to 50 ng/μl. To assess DNA purity, UV/visible absorption spectra were measured, and for all samples the A260/280 ratio was >1.7 and the A260/230 ratio was >2.0. Samples were then submitted to the Epigenomics Core at Weill Cornell Medical College, where bisulfite conversion was carried out followed by DNA methylation analysis using Mass ARRAY EpiTYPER1.2 Suite (Agena Bioscience). The sequence of the CpG island in the GLS2 gene promoter was obtained from human reference genome GRCh38/hg38 using the UCSC Genome Browser (https://genome.ucsc.edu). Primers for DNA methylation analysis were designed using EpIDesign (Agena Bioscience) and synthesized by Integrated DNA Technologies (Table S6). Relative methylation ratios at CpG sites are presented as a heatmap, generated using MORPHEUS (https://software.broadinstitute.org/morpheus).

Immunofluorescence
Cells were fixed with 3.7% formaldehyde in PBS for 20 min and then permeabilized with PBS containing 0.1% Triton X-100 (v/v) for 20 min. Samples were blocked with 10% BSA (w/v) in PBS for 1 h, rinsed with PBS and then incubated with primary antibody in PBS containing 5% BSA (w/v) for 2 h. Samples were washed 3 times with PBS, and then Texas Red- or Oregon Green 488-conjugated secondary antibody diluted 1:400 in PBS containing 5% BSA (w/v), along with DAPI counterstain, was added for 1 h. Samples were washed 3 times with PBS, mounting medium was applied, and slides were sealed with a coverslip prior to imaging with a ZEISS Axioscope.
Cell Fractionation and Mitochondrial Isolation
Breast cancer cells were fractionated into cytosolic, mitochondrial, and nuclear components via partial lysis and centrifugation using the Qproteome Mitochondria Isolation Kit (QIAGEN). For all samples, a portion of the starting material (i.e., whole cells) was retained for comparison with the isolated fractions. When cellular fractions were to be analyzed only by western blot, a single 10 cm dish of exponentially growing cells was used. When mitochondria were isolated for glutaminase assays, two 15 cm dishes of exponentially growing cells were used.

Mitochondrial Glutaminase Activity Assays
A two-reaction protocol was used to measure mitochondrial glutaminase activity. Mitochondria (5 μg total protein) were added to 105 μL of Reaction Mix 1 (20 mM glutamine, 0.2 mM EDTA, 50 mM Tris-acetate pH 8.6), supplemented with 10 μM BPTES when appropriate, and samples were incubated at 37°C for 40 min. The reaction was then quenched by addition of 10 μL of 2.4 M HCl, and samples placed on ice. Next, 20 μL of quenched Reaction Mix 1 was added to 200 μL of Reaction Mix 2 (1 unit bovine liver glutamate dehydrogenase (Sigma-Aldrich), 80 mM Tris-HCl pH 9.4, 200 mM hydrazine, 0.25 mM ADP, 2 mM NAD) and samples were incubated for 1 h at room temperature. The absorbance at 340 nm was then measured against a matched sample in which heat-inactivated mitochondria (immersed in boiling water for 5 min) were used. A standard curve was prepared using given concentrations of glutamate in Reaction Mix 2, allowing the amount of glutamate produced in Reaction 1 to be determined.

Real-Time Recombinant Glutaminase Activity Assays
Real-time monitoring of glutaminase activity through NADH production was performed on a Cary Eclipse fluorescence spectrometer. The excitation and emission wavelengths were set at 340 nm and 460 nm, respectively. To a 1 mL cuvette, 900 μL of assay buffer was added, followed by 10 μL of GDH, 40 μL of 50 mM NAD and 20 μL of either DMSO or various dilutions of 968. Then, 100 μL of either 100 nM GAC or 500 nM GLS2 was added to this mixture and the fluorescence emission was monitored in real time. After 30 s, 200 μL of a mixture of glutamine and K2HPO4 was added such that the final concentrations of K2HPO4 and glutamine were 100 mM and 20 mM, respectively. The initial velocity of glutamine hydrolysis was obtained from the slopes of the linear portion of the kinetic curve.

Glutamine Consumption and Glutamate Release Assays
To 6-well plates containing 2 mL phenol red-free culture medium/well, 2 x 10^5 cells/well were added and incubated overnight to attach. Wells were then rinsed twice with serum-free, phenol red-free culture medium, and 2 mL/well fresh serum-free/phenol red-free medium (containing 2 mM glutamine) was added, followed by incubation for 19 h. As a negative control, wells containing culture medium only were used. Medium was then collected, cellular debris removed by centrifugation at 4°C, and the supernatant retained and stored on ice. Meanwhile, cells attached to the wells were lysed and total protein was quantified using the Bradford assay. Glutamine concentrations were determined using the L-Glutamine/Ammonia Assay Kit (Rapid) (Megazyme) following the manufacturer’s instructions. Briefly, 50 μL sample was mixed with 100 μL Assay Buffer 1 and 10 μL Glutaminase Suspension and incubated at room temperature for 5 min. For all reactions, a blank containing 50 μL H2O was run in parallel. Then, 150 μL Assay Buffer 2, 100 μL NADPH Solution, and H2O to bring the final volume to 1160 μL was added, followed by incubation at room temperature for 4 min. Absorbance A1 was then measured at 340 nm. Next, 10 μL Glutamate Dehydrogenase Suspension was added, samples were mixed and incubated at room temperature for 5 min, and absorbance A2 was measured at 340 nm. Sample concentrations of glutamate were calculated using the extinction coefficient of NADPH at 340 nm. Changes in sample glutamine concentrations were measured relative to the culture medium samples which had been incubated in cell-free wells. To measure glutamate levels in culture medium, samples were analyzed by Reaction 2 described above for the mitochondrial glutaminase activity assays.

Cell Proliferation Assays
Culture medium was added to 12-well plates (1 ml/well) and wells were seeded with cells at Day 0 as follows. MCF7, T-47D, BT-474, HCC38 cells: 2 x 10^5 cells/well. MDA-MB-453 and MDA-MB-231 cells: 1 x 10^5 cells/well. TSE and Hs 578T cells: 0.3 x 10^5 cells/well. After 16 h, culture medium was replaced with fresh medium supplemented with appropriate concentrations of inhibitors and was subsequently replaced every 48 h. On Day 6 cells were trypsinized and suspended in an appropriate volume of culture medium, and the total number of cells per well was determined using a hemocytometer or a TC20 Automated Cell Counter (Bio-Rad).

DNA Constructs for Expressing GLS and GLS2
Vectors for expressing human GAC or GLS2 in breast cancer cell lines were based on pCDNA3.1/V5-His TOPO (Thermo Scientific), with the appropriate gene sub-cloned in and the tag switched to HA-tag or myc-tag for immunofluorescence experiments. Vectors pQE80-GAC-72-598 and pQE80-GLS2-38-602, for expressing the processed forms of human GAC (residues 72 to 598) or GLS2 (residues 38 to 602) in E. coli, were described in Huang et al. (2018). Briefly, E. coli strain BL21(DE3) (Thermo Fisher Scientific) were transformed with vector pQE80-GAC-72-598 or with pQE80-GLS2-38-602 to express the processed forms of GAC and GLS2, respectively. For both constructs, expression was induced with 0.3 mM IPTG for 20 h at 18°C, and cells were then
harvested by centrifugation and lysed by sonication in binding buffer (500 mM NaCl, 50 mM Tris-HCl pH 8.5, 10 mM imidazole, 5 mM β-mercaptoethanol, 1 mM benzamidine chloride). The lysate was centrifuged, and the supernatant applied to a Ni-NTA column which was then washed with 100 column volumes of binding buffer followed by 10 volumes of wash buffer (500 mM NaCl, 50 mM Tris-HCl pH 8.5, 40 mM imidazole, 5 mM β-mercaptoethanol, 1 mM benzamidine chloride). Protein was eluted with 5 column volumes of elution buffer (500 mM NaCl, 300 mM imidazole-HCl pH 7.5, 5 mM β-mercaptoethanol, 1 mM benzamidine chloride). The eluate was centrifugally concentrated and then further purified by FPLC using a HiLoad 16/600 Superdex 200 column (GE Healthcare) with 150 mM NaCl, 5 mM Tris-HCl pH 7.5.

**Transfection of Breast Cancer Cells with DNA Constructs**

For 60 mm dish format, 0.2 mL Opti-MEM (GIBCO) containing 1.5 μg of the appropriate DNA construct, along with 0.2 mL Opti-MEM containing 12 μL Lipofectamine 2000 (Invitrogen), were separately incubated at room temperature for 5 min. The two solutions were combined and incubated for an additional 20 min, mixed with 1.6 mL culture medium and added to cells. After 5 h incubation at 37°C the transfection mixture was replaced with fresh culture medium, and cells were then incubated for an additional 48 h to allow for ectopic expression of GLS or GLS2. To select for cells stably expressing the DNA construct, culture medium supplemented with 500 μg/ml G-418 disulfate (Research Products International) was added and replaced every 2 days for 2-3 weeks until isolated colonies ~2 mm in diameter were present. Individual colonies were transferred to a 12-well plate (1 colony per well) using sterile blotting paper soaked in trypsin solution and were then cultured in medium supplemented with 250 μg/ml G-418 disulfate. All colonies were screened by western blot for ectopic expression, and positive clones were maintained in medium supplemented with 250 μg/ml G-418 disulfate.

**Genetic Knockdowns using siRNAs**

Transient knockdowns of GATA3, GLS, and GLS2 were achieved using two rounds of transfection with Silencer Select pre-designed siRNAs (Invitrogen). For 60 mm dish format, 0.3 mL Opti-MEM (GIBCO) containing 100 nM of the appropriate siRNA (to give a final siRNA concentration of 10 nM when diluted as below), along with 0.3 mL Opti-MEM containing 12 μL Lipofectamine 2000 (Invitrogen), were incubated separately at room temperature for 5 min. The two solutions were then combined and incubated for an additional 20 min, mixed with 2.4 mL culture medium, and added to cells. After 5 h incubation at 37°C the transfection mixture was replaced with fresh culture medium. For all knockdowns, two independent siRNAs were used, along with a negative control siRNA.

**Genetic Knockdowns using shRNAs**

The MISSION RNAi system (Sigma-Aldrich) was used for shRNA-mediated knockdown of GLS and GLS2. Lentivirus particles for each shRNA construct were generated using exponentially growing 293T cells (ATCC) as follows. For 10 cm dish format, 570 μL DMEM was mixed with 33 μL FuGENE 6 (Promega) and incubated at room temperature for 5 min. Plasmids pLKO.1-shRNA (5 μg), pCMV-dr8.2 (packaging vector) (5 μg), and pMD2.G (envelope vector) (1 μg) were then added to the solution, incubated for an additional 15 min, mixed with 8 mL culture medium and added to cells. Cells were incubated at 37°C overnight and the transfection medium was then replaced with fresh culture medium, followed by an additional 24 h incubation to allow for production of virus particles. Virus-containing medium was then collected, and cellular debris removed by centrifugation. To transduce breast cancer cells, virus-containing supernatant was diluted 1:12 in fresh culture medium, and 6 μg/ml polybrene was added before applying to cells. After 6 h incubation at 37°C the transduction medium was replaced with fresh culture medium, and cells were incubated for an additional 48 h before knockdowns were validated. For both GLS and GLS2 knockdowns, two independent shRNA constructs were used, and for all experiments the effects of knockdown were compared with those of a control shRNA. To select for stable expression of the constructs, cells were cultured in medium containing 0.5 μg/ml puromycin.

**Data from The Cancer Genome Atlas**

Gene expression data (RNA-Seq V2, RSEM) from TCGA invasive breast cancer dataset (Koboldt et al., 2012) were accessed using UCSC Xena (https://xena.ucsc.edu) or cbioPortal (http://www.cbioportal.org). Breast tumor subtype calls made by UCSC Xena were based on RNA-Seq data. Outlier readings are not shown on box and whisker plots but are included in calculation of the mean. Copy-number analysis data was accessed using cbioPortal.

**Metabolite Extraction**

The procedures for metabolite extraction from cultured cells are described in previous studies (Cluntun et al., 2015; Liu et al., 2014). Briefly, adherent cells were grown in 6-well plates in biological triplicate to 80% confluence, medium was rapidly aspirated and cells were washed with cold PBS on ice. Then, 1 mL of extraction solvent (80% methanol/water) cooled to ~80°C was added to each well, and the dishes were transferred to ~80°C for 15 min. Cells were then scraped into the extraction solvent on dry ice. All metabolite extracts were centrifuged at 20,000 x g at 4°C for 10 min. Finally, the solvent in each sample was evaporated in a Speed Vacuum. The cell extracts were dissolved in 15 μL water and 15 μL methanol/acetone (1:1 v/v) (LC-MS optima grade, Thermo Fisher Scientific). Samples were centrifuged at 20,000 x g for 10 min at 4°C and the supernatants were transferred to Liquid Chromatography (LC) vials. The injection volume for polar metabolite analysis was 8 μL.
For metabolite abundances in Figure S4a slightly modified protocol was used, as follows. The cell extracts were dissolved in 50 μL water (LC-MS optima grade, Thermo Fisher Scientific) and sonicated to ensure analytes were completely dissolved. Samples were centrifuged at 18,000 × g for 30 min at 4°C and the supernatants were transferred to Liquid Chromatography (LC) vials. The injection volume for polar metabolite analysis was 1 μL.

[U-13C]-Glutamine Labeling
Cells were grown to 80% confluence in 6-well plates with standard culture medium and washed with sterile PBS. Then, culture medium in which glutamine was replaced by [13C5]-L-glutamine (Cambridge Isotope Laboratories), supplemented with dialyzed FBS (GIBCO) and appropriate concentrations of inhibitors was added (1.5 ml/well). At the appropriate time-point, metabolites were extracted as described above.

Liquid Chromatography
A hydrophilic interaction liquid chromatography method (HILIC) with an Xbridge amide column (100 × 2.1 mm, 3.5 μm) (Waters) was employed on a Dionex (Ultimate 3000 UHPLC) for compound separation and detection at room temperature. The mobile phase A was 20 mM ammonium acetate and 15 mM ammonium hydroxide in water with 3% acetonitrile, pH 9.0, and the mobile phase B was acetonitrile. The linear gradient was as follows: 0 min, 85% B; 1.5 min, 85% B, 5.5 min, 35% B; 10 min, 35% B, 10.5 min, 35% B, 14.5 min, 35% B, 15 min, 85% B, and 20 min, 85% B. The flow rate was 0.15 ml/min from 0 to 10 min and 15 to 20 min, and 0.3 ml/min from 10.5 to 14.5 min. All solvents were LCMS grade and purchased from Thermo Fisher Scientific.

For metabolite abundances in Figure S4a slightly modified protocol was used, as follows. A hydrophilic interaction liquid chromatography method (HILIC) with an ZORBAX HILIC Plus column (150 × 2.1 mm, 1.8 μm) (Agilent) was employed on a Dionex (Ultimate 3000 UHPLC) for compound separation and detection at room temperature. The mobile phase Water with 0.1% formic acid was mobile phase A and acetonitrile with 0.1% formic acid was mobile phase B. The linear gradient was as follows: 0 min, 95% B; 1.5 min, 95% B, 15.5 min, 50% B; 16.5 min, 10% B, 18.5 min, 10% B, 18.6 min, 95% B, 21 min, 95% B, and total flow rate was 0.5 ml/min. All solvents were LCMS grade and purchased from Thermo Fisher Scientific.

Mass Spectrometry
The Q Exactive MS (Thermo Scientific) is equipped with a heated electrospray ionization probe (HESI), and the relevant parameters are as listed: evaporation temperature, 120°C; sheath gas, 30; auxiliary gas, 10; sweep gas, 3; spray voltage, 3.6 kV for positive mode and 2.5 kV for negative mode. Capillary temperature was set at 320°C, and S-lens was 55. A full scan range from 60 to 900 (m/z) was used. The resolution was set at 70,000. The maximum injection time was 200 ms. Automated gain control (AGC) was targeted at 3,000,000 ions.

For metabolite abundances in Figure S4a slightly modified protocol was used, as follows. The Q Exactive HF (Thermo Scientific) is equipped with a heated electrospray ionization probe (HESI), and the relevant parameters are as listed: evaporation temperature, 120°C; sheath gas, 60; auxiliary gas, 20; sweep gas, 1; spray voltage, 3.0 kV for negative mode. Capillary temperature was set at 380°C, and S-lens was 50. A full scan range from 80 to 300 (m/z) was used. The resolution was set at 240,000. The maximum injection time was 500 ms. Automated gain control (AGC) was targeted at 3,000,000 ions.

Metabolomics and Data Analysis
Raw data collected from LC-Q Exactive MS were processed on Sieve 2.0 (Thermo Scientific) and ToxID 2.0 (Thermo Scientific). Peak alignment and detection were performed according to the protocol described by Thermo Scientific. For targeted metabolite analysis, the method ‘peak alignment and frame extraction’ was applied. An input file of theoretical m/z and detected retention time of 204 known metabolites was used for targeted metabolites analysis with data collected in positive mode, while a separate input file of 278 metabolites was used for negative mode. M/Z width was set at 10 ppm. The output file including detected m/z and relative intensity in different samples was obtained after data processing. The quantity of the metabolite fraction analyzed was adjusted to the corresponding protein concentration and cell count upon processing a parallel 6-well plate. Quantitation and statistics were calculated and visualized with Microsoft Excel, MORPHEUS and MetaboAnalyst online software.

QUANTIFICATION AND STATISTICAL ANALYSIS
Data are shown as mean ± standard deviation (SD) or mean ± standard error of the mean (SEM), and the number of replicates is indicated in the figure legends. Statistical significance was determined using a two-tailed Student’s t-test. Significance: *p < 0.05, **p < 0.01.

DATA AND CODE AVAILABILITY
This manuscript did not generate new datasets or code.
Supplemental Information

Liver-Type Glutaminase GLS2

Is a Druggable Metabolic Node

in Luminal-Subtype Breast Cancer

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Figure S1 (related to Figure 1). TCA Cycle Anaplerosis in Breast Cancer Cells

(A) Schematic depicting labeling of TCA cycle intermediates by a [U-13C]-glutamine tracer. The first turn of the TCA cycle in the oxidative direction generates m+4 citrate/isocitrate. In contrast, reductive carboxylation of glutamine-derived α-ketoglutarate (α-KG) generates m+5 citrate/isocitrate.

(B) Relative intracellular abundance of the TCA cycle metabolites α-KG, fumarate, and citrate/isocitrate in breast cancer cell lines. Mean ± SD of biological triplicate samples.

(C) Abundance ratios of fully-labeled intracellular glutamate and α-KG in breast cancer cells supplied with [U-13C]-glutamine for 1 hour or 24 hours. Mean ± SEM of biological triplicate samples.
Figure S2 (related to Figure 2). Basal-Subtype Breast Cancer Cells Express xCT and Secrete Glutamate, whereas Luminal-Subtype Breast Cancers have Upregulated GLS2 which is Localized to Mitochondria

(A) Mammary tissue microarray, probed for GLS2. The label ‘N’ indicates normal mammary tissue, ‘+’ indicates receptor-positive breast tumor tissue, and ‘-’ indicates receptor-negative breast tumor tissue. Unlabeled slices are of hyperplasia, non-malignant tumors, or sarcoma.

(B) Western blots showing relative levels of SLC3A2 and SLC7A11, the heavy and light chains, respectively, of the xCT transporter in breast cancer cell lines. Both are integral membrane proteins subject to post-translational modifications including glycosylations, which cause them to run at a range of molecular weights on SDS-PAGE.

(C) The rate of glutamate secretion by breast cancer cell lines. Mean ± SD of biological triplicate samples.

(D) Immunofluorescence images of SK-BR-3 breast cancer cells ectopically expressing HA-tagged GLS2 and/or myc-tagged GLS. Hsp60 serves as a mitochondrial marker. Scale bars, 10 μm.
Figure S3

**A**

|          | GLS2 |GLS |
|----------|------|-----|
| % cases  | Amp  | Gain|
| Lum A    |      |     |
| Lum B    |      |     |
| HER1+    |      |     |
| Basal    |      |     |

**B**

**GLS2 gene promoter (1500 bp)**

- **Predicted GATA3 binding site**
  - Predicted site: (5'-CGAGATAGCGCC-3')
  - Consensus motif 1: (5'-CGAGATAGCGCC-3')
  - Consensus motif 2: (5'-CTAGATAGTCGC-3')

**C**

UCSC Genome Browser on Human Dec. 2013 (GRCh38/hg38) Assembly

**D**

- **MDA-MB-453**
  - Relative GLS2 mRNA
  - GATA3 siRNA
  - C 1 2

- **T-47D**
  - Relative GLS2 mRNA
  - GATA3 siRNA
  - C 1 2
**Figure S3 (related to Figure 3). Analysis of the GLS2 gene promoter**

(A) Copy-number analysis showing copy-number gains and gene amplifications for \textit{GLS2} and \textit{GLS} in the different subtypes of breast tumor. Data are from TCGA invasive breast cancer dataset.

(B) Approximately 3600 base-pairs (bp) of the \textit{GLS2} gene promoter region. The CpG island is indicated by bold text. The transcription start site (TSS) is highlighted in yellow and the start codon in green. A close match to the GATA3 consensus motif is highlighted in grey.

(C) Position of the CpG island (shown in green) in the \textit{GLS}2 gene promoter, centered around the TSS.

(D) Quantitative RT-PCR data showing relative levels of \textit{GLS2} transcript in breast cancer cell lines transfected with either a control siRNA (C) or two independent GATA3-targeted siRNAs. Reactions were carried out in triplicate, and error bars indicate the RQ\textsubscript{max} and RQ\textsubscript{min} values.
Figure S4

A

MDA-MB-453 GLS2
shRNAs

MDA-MB-231 GLS
shRNAs

Fold change

Glu  a-KG  Succ  Fum  Mal

Fold change

Glu  a-KG  Succ  Fum  Mal

B

MDA-MB-453 GLS
shRNAs

MDA-MB-231 GLS2
shRNAs

Fold change

Glu  a-KG  Succ  Fum  Mal

Fold change

Glu  a-KG  Succ  Fum  Mal

C

T-47D
(Luminal)

MDA-MB-453
(Luminal)

MDA-MB-231
(Basal)

TSE
(Basal)

% of control growth

C  1  2

% of control growth

C  1  2

% of control growth

C  1  2

% of control growth

C  1  2

GLS +GLS2

GLS +GLS2

GLS +GLS2

GLS +GLS2

+2 mM dm-α-KG
Figure S4 (related to Figure 4). Knockdown of GLS2 or GLS Disrupts Glutamine-Mediated Anaplerosis in Luminal- and Basal-Subtype Cells, Respectively

(A) Fold changes in total abundance of TCA cycle metabolites in MDA-MB-453 and MDA-MB-231 cells resulting from knockdown of GLS2 and GLS, respectively. Mean ± SEM of triplicates relative to the control.

(B) Fold changes in total abundance of TCA cycle metabolites in MDA-MB-453 and MDA-MB-231 cells caused by knockdown of GLS-targeted and GLS2-targeted shRNAs, respectively. Mean ± SEM of triplicates relative to the control.

(C) Rescue of the inhibitory effects of glutaminase-knockdowns on cell proliferation by supplementation of culture medium with 2 mM dimethyl-α-KG.
Figure S5 (related to Figure 5). GLS2 Mediates Resistance to BPTES

(A) Real-time assays showing inhibition of recombinant GLS by different concentrations of BPTES (left panel) and CB-839 (right panel).

(B) Real-time assays showing resistance of full-length recombinant GLS2 to BPTES (left panel) and CB-839 (right panel).

(C) Western blot standard curves to estimate absolute levels of GLS and GLS2 in breast cancer cells. Estimated levels per µg of total cellular protein are as follows:
- MDA-MB-453 GLS: 1.5 ng per 20 µg = 75 pg/µg
- MDA-MB-453 GLS2: 5.5 ng per 20 µg = 275 pg/µg
- MDA-MB-231 GLS: 10 ng per 20 µg = 500 pg/µg
- MDA-MB-231 GLS2: <75 pg/µg
- T-47D GLS: 8 ng per 40 µg = 200 pg/µg
- T-47D GLS2: 8 ng per 40 µg = 200 pg/µg
- DU4475 GLS: 15 ng per 20 µg = 750 pg/µg
- DU4475 GLS2: 12.5 ng per 20 µg = 625 pg/µg

(D) Fold changes in total abundance of glutamine, glutamate, and TCA cycle metabolites in basal-subtype (upper panels) and luminal-subtype (lower panels) breast cancer cell lines following treatment with 10 µM BPTES. Mean ± SEM of triplicates relative to the vehicle.

(E) Relative proliferation rates of basal-subtype breast cancer cells stably overexpressing GLS2 or carrying the plasmid vector only. Proliferation over 6 days was measured. Mean ± SD of triplicate assays. All p values are slightly greater than 0.05, and changes are therefore classed as not significant (ns).

(F) Western blots showing levels of GLS and GLS2 in DU4475 cells transfected with either a control siRNA (labeled C), two independent GLS-targeted siRNAs, two independent GLS2-targeted siRNAs, or GLS- and GLS2-targeted siRNAs simultaneously.

(G) Rescue of the inhibitory effects of glutaminase-knockdowns on cell proliferation by supplementation of culture medium with 2 mM dimethyl-α-KG.
Figure S6

Anaplerosis.

(A) Real-time assay showing the effects of 968 and the inactive analog compound 26 on GLS2 activity.

(B) Dose curves showing inhibition of breast cancer cell proliferation over 6 days by different concentrations on 968. Mean ± SD of triplicate assays.

(C) Fold changes in total metabolite abundances in MDA-MB-231 (upper panel) and MDA-MB-453 (lower panel) cells following treatment with 10 μM 968 for different periods of time. Mean ± SEM of triplicates relative to the vehicle.

Figure S6 (related to Figure 6). The Glutaminase Inhibitor 968 Suppresses Proliferation and TCA Cycle
Table S1, related to Figure 1. The molecular subtypes of breast cancer.

| Molecular subtype | Typical receptor staining | Other markers | Relative incidence |
|-------------------|---------------------------|---------------|-------------------|
| Luminal A         | ER+ and/or PR+, HER2-     | Low Ki67 (<14%) | 50-60%            |
| Luminal B         | ER+ and/or PR+, HER2+/-   | High Ki67 (>14%) | 10-20%            |
| HER2+             | ER-, PR-, HER2+          |               | ~15%              |
| Basal             | ER-, PR-, HER2-          |               | ~15%              |
Table S2, related to Figure 1. Description of breast cancer cell lines.

| Cell line       | Reported subtype | Reported receptor status | Notes                                                                                     |
|-----------------|------------------|--------------------------|-------------------------------------------------------------------------------------------|
| MCF7            | Luminal A        | +                        | +                                           | +                                           | -                                           |
| BT-474          | Luminal B        | +                        | +                                           | +                                           | +                                           |
| T-47D           | Luminal A        | +                        | +                                           | -                                           | -                                           |
| MDA-MB-453      | Luminal (LAR)    | -                        | -                                           | +/-                                         | +                                           |
| MDA-MB-231      | Basal            | -                        | -                                           | -                                           | Basal B and Claudin-low subgroups.          |
| TSE             | Basal            | -                        | -                                           | -                                           | Basal B and Claudin-low subgroups.          |
| Hs 578T         | Basal            | -                        | -                                           | -                                           | Basal B and Claudin-low subgroups.          |
| HCC38           | Basal            | -                        | -                                           | -                                           | Basal B and Claudin-low subgroups.          |
| DU4475          | Basal            | -                        | -                                           | -                                           | Basal B and Claudin-low subgroups.          |
Table S3, related to Figure 1. EC<sub>50</sub> values for BPTES and CB-839 derived from 6-day cell proliferation assay.

| Cell line      | BPTES EC<sub>50</sub>/µM | CB-839 EC<sub>50</sub>/nM |
|----------------|--------------------------|---------------------------|
| MCF7           | ~20                      | >1000                     |
| BT-474         | ~20                      | >1000                     |
| T-47D          | ~20                      | >1000                     |
| MDA-MB-453     | ~20                      | >1000                     |
| MDA-MB-231     | 2.1                      | 19                        |
| TSE            | 3.1                      | 41                        |
| Hs 578T        | 3.7                      | 31                        |
| HCC38          | 4.7                      | 49                        |
Table S4, related to Figure 5. EC₅₀ values for BPTES and 968, derived from 6-day cell proliferation assay.

| Cell line                      | BPTES EC₅₀/µM | 968 EC₅₀/µM |
|--------------------------------|---------------|-------------|
| MDA-MB-231 (parental)          | 2             | 4           |
| MDA-MB-231 vector only         | 1.3           | 2           |
| MDA-MB-231 GLS2 clone 1        | >20           | 4           |
| MDA-MB-231 GLS2 clone 2        | >20           | 5           |
| TSE (parental)                 | 3             | 4           |
| TSE vector only                | 4             | 2           |
| TSE GLS2 clone 1               | >20           | 7           |
| TSE GLS2 clone 2               | >20           | 6           |
| MCF7                           | ~20           | 4           |
| BT-474                         | ~20           | 4           |
| T-47D                          | ~20           | 4           |
| MDA-MB-453                     | ~20           | 3           |
| MDA-MB-231                     | 2.1           | 5           |
| TSE                            | 3.1           | 5           |
| Hs 578T                        | 3.7           | 4*          |
| HCC38                          | 4.7           | 4**         |

*Maximal inhibition ~75%
**Maximal inhibition ~70%
Table S5, related to STAR Methods. Antibody dilutions used for western blot analysis.

| Antibody                                          | Dilution |
|---------------------------------------------------|----------|
| ASCT2 Antibody                                    | 1:2000   |
| α/β-Tubulin Antibody                              | 1:5000   |
| GLS antibody (C-term)                             | 1:10000  |
| Anti-GLS2 antibody                                | 1:8000   |
| 4F2hc/CD98 (SLC3A2) antibody                      | 1:2000   |
| xCT/SLC7A11 antibody                              | 1:2000   |
| VDAC Rabbit mAb                                   | 1:4000   |
| ASNS Antibody                                     | 1:4000   |
| Lamin A/C Mouse mAb                               | 1:5000   |
| p53 Mouse mAb                                     | 1:4000   |
| p63 Rabbit mAb                                    | 1:5000   |
| p73 Rabbit mAb                                    | 1:5000   |
| N-Myc Rabbit mAb                                  | 1:5000   |
| c-Myc Rabbit mAb                                  | 1:2000   |
| GATA-3 Rabbit mAb                                 | 1:32000  |
| HER2/ErbB2 Rabbit mAb                             | 1:4000   |
| Progesterone Receptor A/B Rabbit mAb              | 1:4000   |
| Estrogen Receptor α Rabbit mAb                    | 1:4000   |
| V5 Tag Monoclonal Antibody                        | 1:8000   |
**Table S6, related to STAR Methods.** List of primers.

| Name         | Sequence                                                                 |
|--------------|---------------------------------------------------------------------------|
| 18S rRNA FWD | CGCGAAGACGACCCATTCAAC                                                  |
| 18S rRNA REV | GAATCGAACCTGATCTCCCCGTC                                                  |
| GLS FWD      | TCTACAGGATGGCAGCTTCT                                                   |
| GLS REV      | CTTTGCTAGTCACAGCATCT                                                   |
| GLS2 FWD     | GCTTGGTGTAGTTGTCTTT                                                    |
| GLS2 REV     | CTTAGTCAGTGGGACTT                                                     |
| SLC7A11 FWD  | GTTCCTAACAGCTTTGTACG                                                   |
| SLC7A11 REV  | AATGTAGCGTCCTAAAATGCCAG                                                 |
| GLS2_000_10F | AGGAAGAGAGAGAGGTAGAGTTTGTATTGGT                                 |
| GLS2_000_T7R | CAGTAATAGACTCATATAGGGAGAGGCTCTTACCCTCCACTTCCACTTACTATACC           |
| GLS2_001_10F | AGGAAGAGAGATATTGATGGGAGGGAAGT                                 |
| GLS2_001_T7R | CAGTAATAGCTCATATAGGGAGAGGCTCTTACCCTCCACTTCCACTTACTATACC           |
| GLS2_002_10F | AGGAAGAGAGAGGTTTGAAGT                                  |
| GLS2_002_T7R | CAGTAATAGCTCATATAGGGAGAGGCTCTTACCCTCCACTTCCACTTACTATACC           |
| GLS2_003_10F | AGGAAGAGAGAGGTTTGAAGT                                  |
| GLS2_003_T7R | CAGTAATAGCTCATATAGGGAGAGGCTCTTACCCTCCACTTCCACTTACTATACC           |
| GLS2_004_10F | AGGAAGAGAGAGGTTTGAAGT                                  |
| GLS2_004_T7R | CAGTAATAGCTCATATAGGGAGAGGCTCTTACCCTCCACTTCCACTTACTATACC           |
| GLS2_005_10F | AGGAAGAGAGAGGTTTGAAGT                                  |
| GLS2_005_T7R | CAGTAATAGCTCATATAGGGAGAGGCTCTTACCCTCCACTTCCACTTACTATACC           |
| GLS2_006_10F | AGGAAGAGAGAGGTTTGAAGT                                  |
| GLS2_006_T7R | CAGTAATAGCTCATATAGGGAGAGGCTCTTACCCTCCACTTCCACTTACTATACC           |
| GLS2_007_10F | AGGAAGAGAGAGGTTTGAAGT                                  |
| GLS2_007_T7R | CAGTAATAGCTCATATAGGGAGAGGCTCTTACCCTCCACTTCCACTTACTATACC           |
| GLS2_008_10F | AGGAAGAGAGAGGTTTGAAGT                                  |
| GLS2_008_T7R | CAGTAATAGCTCATATAGGGAGAGGCTCTTACCCTCCACTTCCACTTACTATACC           |
| GLS2_009_10F | AGGAAGAGAGAGGTTTGAAGT                                  |
| GLS2_009_T7R | CAGTAATAGCTCATATAGGGAGAGGCTCTTACCCTCCACTTCCACTTACTATACC           |