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Oncogenic PI3K Promotes Methionine Dependency in Breast Cancer Cells Through the Cystine-Glutamate Antiporter xCT

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Abstract

The precursor homocysteine is metabolized either through the methionine cycle to produce methionine or through the transsulfuration pathway to synthesize cysteine. Alternatively, cysteine can be obtained through uptake of its oxidized form, cystine. Many cancer cells exhibit methionine dependency such that their proliferation is impaired in growth media in which methionine is replaced by homocysteine. Here, we showed that oncogenic *PIK3CA* and decreased expression of *SLC7A11*, a gene that encodes a cystine transporter also known as xCT, correlated with increased methionine dependency in breast cancer cells. Oncogenic *PIK3CA* was sufficient to confer methionine dependency to mammary epithelial cells, in part by decreasing cystine uptake through the transcriptional and posttranslational inhibition of xCT. Manipulation of xCT activity altered the proliferation of breast cancer cells in methionine-deficient, homocysteine-containing media, suggesting that it functionally contributed to methionine dependency. We propose that concurrent with decreased cystine uptake through xCT, *PIK3CA* mutant cells use homocysteine through the transsulfuration pathway to synthesize cysteine. Consequently, less homocysteine is available to produce methionine, contributing to methionine dependency. These results indicate that oncogenic *PIK3CA* alters methionine and cysteine utilization, in part by inhibiting xCT, to contribute to the methionine dependency phenotype in breast cancer cells.

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Introduction

In the past decade, there has been a resurgence of interest in elucidating how metabolism is altered in cancer cells, with the goal of identifying cancer-associated metabolic dependencies that can be exploited for cancer therapy (1). Metabolic differences between cancerous and normal cells often involve differential utilization of key ‘junction’ metabolites. For example, one aspect of the Warburg effect is the preferential usage of glycolysis-derived pyruvate to generate lactate in cancer cells, whereas in normal cells pyruvate is primarily directed towards the tricarboxylic acid (TCA) cycle. From a therapeutic standpoint, differences in how cancer cells regulate the fate of key metabolites may potentially provide a means of targeting these metabolic junctions for treatment.

Homocysteine (Hcy) is a key junction metabolite that lies at the nexus of two pathways involved in methionine (Met) and cysteine (Cys) metabolism. High concentrations of Hcy are toxic to cells, and medical disorders known as hyperhomocysteinemia and homocystinuria are characterized by the accumulation of Hcy in the blood, leading to various symptoms such as stroke, vascular diseases, and intellectual disabilities (2). Therefore, cells must metabolize Hcy primarily through two different pathways: the methionine cycle and the transsulfuration pathway (Fig. 1A). In the methionine cycle, Hcy is methylated to produce Met, an essential amino acid that is critical for cell growth and function. In addition to contributing to protein synthesis, Met is a precursor for the generation of S-adenosylmethionine (SAM), which as the principal methyl group donor is critical for various cellular methylation reactions (3). S-adenosylhomocysteine (SAH) is generated in the process and subsequently converted into Hcy, which is then used to regenerate Met to complete the cycle. Alternatively, Hcy can be metabolized through the transsulfuration pathway to synthesize the amino acid Cys, which is involved in multiple cellular antioxidant systems such as the synthesis of glutathione (4). Depending on cellular demand, Hcy can be directed toward either the methionine cycle to increase methylation potential or through the transsulfuration pathway to contribute to antioxidant metabolism.

In the context of cancer, the Hcy junction has been implicated in a cancer-associated metabolic vulnerability known as ‘methionine dependency’, in which a majority of cancer cells cannot proliferate in growth media in which Met is replaced by its precursor Hcy (Met⁻Hcy⁺ media). In contrast, most normal, non-tumorigenic cells such as fibroblasts and epithelial cells are not methionine dependent and can proliferate in Met⁻Hcy⁺ media (5–7). This phenotype has been demonstrated for various malignant cell lines and for patient tumors grown in primary culture from multiple cancers, including breast, bladder, colon, glioma, kidney, melanoma, and prostate cancer (8–13). Given these observations, methionine restriction has been proposed as a strategy to treat cancer, a notion that is supported by several pre-clinical models. For example, in animal models of various cancers, including rhabdomyosarcoma, Yoshida’s sarcoma, hepatoma, and colorectal cancer, methionine-restricted diets inhibit tumor growth, prevent metastases, and extend survival (14–17). Other studies suggest that the enzyme methioninase, which degrades methionine, may be used pharmacologically to systemically deplete methionine levels to exert anti-tumor effects (18–21). Finally, pre-clinical and clinical studies have indicated that methionine

restriction can act synergistically with chemotherapeutic agents to effectively treat tumors (22–26).

Despite these promising results, one critical concern is that complete and prolonged systemic depletion of methionine may be toxic and even lethal in humans (14, 27, 28). One challenge in more effectively exploiting methionine dependency in cancer treatment is that the mechanisms underlying this phenotype remain elusive. A few studies have evaluated the role of certain metabolic enzymes involved in methionine metabolism, especially methionine synthase (MTR), which converts Hcy to Met, and methylthioadenosine phosphorylase (MTAP), which is involved in the methionine salvage pathway (29–35). Although these enzymes have been associated with methionine dependency, the exact metabolic alterations that are causally responsible for conferring methionine dependency have not been determined. Here, we provide evidence that oncogenic *PIK3CA* is sufficient to confer methionine dependency to mammary epithelial cells, in part by decreasing cystine (Cys₂) uptake through the transcriptional and post-translational inhibition of xCT, a Cys₂ transporter encoded by *SLC7A11*.

Results

Methionine dependency varies across different breast cancer cell lines

To study methionine dependency in breast cancer, we screened 13 breast cancer cell lines for their ability to proliferate in Met[−]Hcy⁺ media (Fig. 1B). Based on their proliferative capacities, we segregated the cell lines into three groups: Group A cells proliferated; Group B cells did not proliferate, at least over the four days of the experiment; and Group C cells died when cultured in Met[−]Hcy⁺ media. To quantify the differences in methionine dependencies across the cell lines, we used these proliferation curves to calculate a Met[−]Hcy⁺ growth rate for each cell line, again observing a segregation into the three groups (Fig. 1C).

Differences in basal proliferation rates may potentially explain the observed range of methionine dependencies, since more proliferative cells may require more methionine for growth and therefore be more methionine-dependent. However, we did not find a significant correlation between the Met[−]Hcy⁺ growth rates of the cell lines and their doubling times in regular growth media (Fig. 1D). Therefore, the range of methionine dependencies across the cell lines was not likely to be simply due to differences in basal proliferative capacity.

Oncogenic *PIK3CA* is sufficient to confer methionine dependency to mammary epithelial cells

We noticed that many of the most methionine dependent breast cancer cell lines harbor a *PIK3CA* genomic mutation (Table 1), in particular two of the three Group C cell lines (MCF7 and T47D) and the most methionine dependent Group B cell line (SUM-159). Of note, *PTEN* genomic mutations also tended to cluster towards the Group B and C cell lines with negative Met[−]Hcy⁺ growth rates (Fig. 1C, Table 1). However, drawing a correlation between PTEN protein abundance and methionine dependency will require a quantitative analysis of PTEN protein abundance in these cell lines. All Group A cell lines were negative

for PTEN abundance at the protein level (Table 1), weakening the association of *PTEN* mutational status with methionine dependency. Given that two of the three Group C cell lines harbored a *PIK3CA* mutation, we focused on assessing whether oncogenic *PIK3CA* might contribute to the methionine dependency phenotype. To test this hypothesis, we evaluated whether expression of oncogenic *PIK3CA* mutations might render the non-tumorigenic mammary epithelial MCF10A cell line sensitive to the Met⁻Hcy⁺ media. Wild-type *PIK3CA*, *PIK3CA*(E545K), or *PIK3CA*(H1047R) were stably expressed at similar abundances in MCF10A cells (Fig. S1A). While these cell lines proliferated well in normal media containing Met (Fig. S1B), they behaved differently in Met⁻Hcy⁺ media (Fig. 2A). Empty vector-transfected MCF10A cells proliferated in Met⁻Hcy⁺ media, as might be expected for a relatively normal cell line. Expression of wild-type *PIK3CA* inhibited the growth of MCF10A cells, whereas cells expressing *PIK3CA*(E545K) or *PIK3CA*(H1047R) died when cultured in the Met⁻Hcy⁺ media. Consistently, the *PIK3CA* mutant cells underwent a significantly greater degree of cell death in the Met⁻Hcy⁺ media, as measured by a propidium iodide (PI) assay (Fig. 2B) and by immunoblotting for cleaved PARP (Fig. 2C). Together, these results indicate that oncogenic *PIK3CA* is sufficient to confer methionine dependency to mammary epithelial cells.

Oncogenic *PIK3CA* inhibits cystine uptake through xCT

We reasoned that oncogenic *PIK3CA* may re-wire cellular metabolism to confer methionine dependency, perhaps by altering the expression of genes involved in methionine and cysteine metabolism. To identify these candidate genes, we used published RNA expression data (36) to correlate the expression of a selected set of methionine and cysteine metabolism genes (listed in the Materials and Methods) to the Met⁻Hcy⁺ growth rates of our panel of cell lines (Supplemental Data File 1). Within this analysis, two genes were significantly correlated: Increased methionine dependency was associated with high *MAT1A* (which encodes Methionine Adenosyltransferase 1A) expression and low *SLC7A11* (which encodes Solute Carrier Family 7 Member 11) expression (Fig. 3A, S2A–B). Next, we asked whether these transcriptional changes were also observed in the methionine dependent *PIK3CA* mutant MCF10A cells. *SLC7A11* expression was significantly lower in the *PIK3CA* mutant cells, whereas there was no significant difference in *MAT1A* expression (Fig. 3B). Consistently, *SLC7A11* expression trended towards lower expression in the methionine dependent Group C breast cancer cell lines, relative to the Group A cell lines (Fig. 3C). Immunoblotting for endogenous SLC7A11 in two cell lines each from Groups A, B, and C revealed that the Group C cell lines MDA-MB-468 and MCF7 had lower SLC7A11 protein abundance compared to the Group A cell lines HCC38 and SUM-149 (Fig. 3D). SLC7A11, also known as xCT, is a cystine-glutamate antiporter that exchanges intracellular glutamate to take up extracellular cystine (Cys₂), which is the oxidized form of Cys (37). Consistent with reduced xCT expression, *PIK3CA* mutant cells exhibited a significantly decreased rate of Cys₂ uptake (Fig. 3E).

In addition to transcriptional regulation, we also noted that multiple mass spectrometry-based studies have identified xCT to be phosphorylated on Ser²⁶, and the amino acid sequence surrounding this phospho-site is similar to the AKT substrate motif RxRxxS/T, with an Arg at the -3 position (Fig. 4A, www.phosphosite.org). To determine whether xCT

Ser²⁶ was phosphorylated downstream of PI3K, we stably expressed both wild-type and the S26A mutant of a C-terminally HA-FLAG-tagged xCT in MCF10A wild-type or *PIK3CA*(H1047R) cells. We then serum-starved these cells, and by immunoprecipitating xCT with an HA antibody and immunoblotting with an antibody recognizing the p-RxxS/T phospho-motif, we found that xCT was constitutively phosphorylated in the *PIK3CA*(H1047R) cells. The S26A mutation abolished phosphorylation, indicating that xCT was phosphorylated at Ser²⁶ downstream of the PI3K pathway (Fig. 4B). We also stimulated serum-starved wild-type MCF10A cells expressing xCT-HA-FLAG with insulin to activate PI3K. xCT phosphorylation was stimulated by insulin within 5–10 min and was attenuated by 30–60 min (Fig. 4C). The kinetics of xCT phosphorylation were similar to that of a canonical AKT substrate, PRAS40 (Fig. 4C), suggesting that AKT may be the kinase responsible for xCT phosphorylation. Indeed, phosphorylation of xCT in MCF10A *PIK3CA*(H1047R) mutant cells was abolished by the PI3K inhibitor GDC-0941, the AKT inhibitors GDC-0068 and MK2206, and the mTOR catalytic inhibitor Torin1. By contrast, the mTORC1/S6K inhibitor rapamycin did not affect xCT phosphorylation (Fig. 4D). Moreover, in MCF10A cells, we detected phosphorylation of endogenous xCT upon insulin stimulation, which was inhibited by both PI3K and AKT inhibitors (Fig. S3). These results suggest that xCT is phosphorylated either by AKT or a kinase downstream of AKT that is not mTORC1. To further confirm that AKT was the responsible kinase, we conducted in vitro kinase assays with immunoprecipitated xCT and recombinant AKT1 (Fig. 4E). In vitro assays indicated that AKT1 phosphorylated xCT, but not xCT(S26A), demonstrating that AKT can directly phosphorylate xCT at Ser²⁶ in vitro. Finally, to determine the functional consequence of xCT Ser²⁶ phosphorylation, we measured glutamate secretion to assay xCT activity. We found that MCF10A cells expressing xCT(S26A) secreted more glutamate than cells expressing wild-type xCT (Fig. 4F), suggesting that phosphorylation of xCT at Ser²⁶ inhibits its cystine transport activity. Together, the results above support a model in which oncogenic *PIK3CA* inhibits xCT activity through two mechanisms: (i) transcriptional inhibition of *SLC7A11* expression, and (ii) AKT-mediated phosphorylation of xCT at Ser²⁶.

xCT functionally contributes to the methionine dependency phenotype

The above results indicated that oncogenic *PIK3CA* decreased Cys₂ uptake through both the transcriptional and post-translational inhibition of xCT, and this metabolic phenotype was correlated with increased methionine dependency. Based on this model, we next explored the potential role of the inhibition of xCT and Cys₂ uptake in contributing to the methionine dependency induced by oncogenic *PIK3CA*.

We hypothesized that xCT overexpression may reverse methionine dependency because *PIK3CA* mutant MCF10A cells expressed lower amounts of xCT. Indeed, we found that MCF10A *PIK3CA*(H1047R) cells overexpressing xCT proliferated in both normal media containing Met and in Met[−]Hcy⁺ media (Fig. 5A, S4A). Similarly, xCT overexpression restored the viability of these *PIK3CA* mutant cells in Met[−]Hcy⁺ media, as measured with a PI-based cell death assay (Fig. 5B). Together, these results indicate that at least in the context of MCF10A cells, xCT is a mediator of the methionine dependency induced by oncogenic *PIK3CA*, since its expression was sufficient to rescue the methionine dependency of the *PIK3CA* mutant cells.

We also assessed whether manipulation of xCT expression and activity affects the methionine dependency of our panel of cell lines (Fig. 1B). Since Group A cells, which proliferated in Met⁻Hcy⁺ media, expressed higher amounts of xCT (Fig. 3A,C), then inhibition of Cys₂ uptake in these cells should confer the methionine dependency phenotype. Indeed, we found that removal of Cys₂ from the growth media to inhibit Cys₂ uptake led to proliferation of each of the Group A cell lines in either Met⁻Hcy⁺ media or Cys₂⁻Hcy⁺ media. However, if both Met and Cys₂ were removed (Met⁻Cys₂⁻Hcy⁺ media), then the absence of Cys₂ conferred methionine dependency to these cells (Fig. 5C). Alternatively, inhibition of xCT with sulfasalazine (SSA) (38) consistently impaired the ability of Group A cells to grow in the Met⁻Hcy⁺ media (Fig. 5D). shRNA-mediated depletion of xCT also impaired growth in Met⁻Hcy⁺ media, especially for the highly Met⁻Hcy⁺ resistant HCC70 cell line (Fig. S4B–D). The proliferation of SUM-149 cells in Met⁻Hcy⁺ media was only modestly impaired by xCT depletion (Fig. S4B–D), consistent with the observation that of the three Group A cell lines, growth of SUM149 cells in Met⁻Hcy⁺ media is least affected by Cys₂ deprivation or sulfasalazine (Fig. 5C–D). Together, these results suggest that Cys₂ uptake is required for the Group A cell lines to regulate Hcy utilization in a manner that permits proliferation in the Met⁻Hcy⁺ media.

Conversely, the methionine dependent Group C cell lines expressed lower amounts of xCT, and we hypothesized that xCT overexpression may reverse their methionine dependency. Although the rescue was not as robust as in MCF10A cells (Fig. 5A,B), xCT expression did modestly improve the survival of the Group C cells in Met⁻Hcy⁺ media, in particular for T47D cells and to a lesser extent for MCF7 cells (although this rescue was reproducible, it was small and not statistically significant) (Fig. 5E). This result suggests that while xCT may be required for the proliferation of Group A cells in Met⁻Hcy⁺ media, xCT expression was only partially sufficient in reversing the methionine dependency of two of the three Group C cells. Therefore, while xCT may partially contribute to methionine dependency in the context of these two cell lines, it is likely that other factors are also involved. Overall, these analyses in the Group A and Group C cell lines suggest that xCT is at least one of the mediators of the methionine dependency phenotype, since manipulation of its activity or expression modulated the degree to which cells can grow in Met⁻Hcy⁺ media.

Oncogenic *PIK3CA* alters methionine utilization through the transsulfuration pathway

Finally, we asked whether oncogenic *PIK3CA* alters methionine metabolism and the ability of cells to use Hcy to either regenerate Met through the methionine cycle or synthesize Cys through the transsulfuration pathway. We hypothesized that since oncogenic *PIK3CA* decreased Cys₂ uptake in MCF10A cells (Fig. 3E), *PIK3CA* mutant MCF10A cells may compensate by synthesizing Cys from Hcy through the transsulfuration pathway. Consistently, we observed increased steady-state pools of the transsulfuration pathway intermediate cystathionine in MCF10A *PIK3CA*(H1047R) cells relative to control cells (Fig. 6A). We next labeled cells with [U-¹³C₅]-methionine, which results in either (i) [M+4] cystathionine if the methionine carbon enters the transsulfuration pathway or (ii) [M+4] methionine if the methionine carbon cycles through the methionine cycle once (Fig. 6B). After one hour of labeling, a time point at which steady-state labeling had not been reached, we found greater incorporation of the ¹³C label into [M+4] cystathionine in the *PIK3CA*

mutant MCF10A cells relative to empty vector-transfected cells (Fig. 6C), which together with increased steady-state cystathionine pool sizes (Fig. 6A), suggests increased flux through the transsulfuration pathway. We then provided these cells with [U-¹³C₅]-methionine for 24 hours to reach steady-state labeling in the presence or absence of the PI3K inhibitor GDC-0941. Most of the detected cystathionine was labeled at steady-state in both the empty vector-transfected and *PIK3CA*(H1047R) cells, and the fractional contribution of [U-¹³C₅]-methionine to [M+4] cystathionine was inhibited by GDC-0941, indicating that transsulfuration pathway activity depended on PI3K signaling (Fig. 6D). Furthermore, concurrent with a decrease in [M+4] cystathionine induced by GDC-0941, PI3K inhibition also increased labeling in [M+4] methionine-containing media, suggesting that activation of PI3K directs the utilization of Met through Hcy towards the transsulfuration pathway and away from re-methylation through the methionine cycle to regenerate Met (Fig. 6E). Notably, inhibition of [M+4] cystathionine by GDC-0941 was more robust in the empty vector-transfected cells relative to the *PIK3CA*(H1047R)-expressing cells (Fig. 6D,E), which reflected the stronger inhibition of PI3K signaling by GDC-0941 in the empty vector-transfected cells under these conditions (Fig. S5). This result therefore further suggests a correlation between PI3K activity and conversion of Hcy to cystathionine. Together, these results are consistent with a model in which cells with oncogenic *PIK3CA* cannot proliferate in Met⁻Hcy⁺ media because the supplemented Hcy is used for transsulfuration and is not efficiently re-methylated to produce Met.

Discussion

In the present study, we demonstrated that in breast cancer cell lines, oncogenic *PIK3CA* was associated with methionine dependency, a cancer-associated metabolic dependency in which cells cannot proliferate in Met⁻Hcy⁺ media. In mammary epithelial MCF10A cells, oncogenic *PIK3CA* was sufficient to confer methionine dependency through a PI3K-induced decrease in Cys₂ uptake mediated by both transcriptional and post-translational inhibition of the Cys₂ transporter xCT. xCT at least partially mediated the methionine dependency phenotype because manipulation of xCT activity or expression modulated the methionine dependency of breast cancer cells. Together, our data provides evidence to link oncogenic *PIK3CA* and xCT-mediated Cys₂ uptake with methionine dependency, a mechanistically poorly understood cancer-associated metabolic dependency.

The mechanism by which oncogenic *PIK3CA* decreases xCT expression remains an open question. We have previously reported that expression of an oncogenic AKT mutation in MCF10A cells also decreases xCT expression (39). The transcription factor Nrf1 has been reported to act as a repressor of xCT expression (40), and mTORC1 is an activator of Nrf1 (41). Since mTORC1 signals downstream of PI3K, one potential hypothesis is that oncogenic *PIK3CA* may decrease xCT expression through the mTORC1-mediated activation of Nrf1.

Of note, post-translational regulation of xCT downstream of PI3K resulting in phosphorylation of Ser²⁶ and inhibition of Cys₂ uptake has been previously reported (42), and as such is consistent with the results presented here. Gu *et al.* identified mTORC2 but not AKT as the kinase responsible for phosphorylation of xCT at Ser²⁶ in cells. However,

consistent with our data, they also showed that AKT can potentially phosphorylate xCT with in vitro kinase assays (42). The motif surrounding Ser²⁶ in xCT conforms only partially to both the mTOR (43, 44) and the AKT consensus motifs (45, 46), and the responsible kinase in vivo may therefore conceivably depend on distinct cellular contexts. Indeed, in our system xCT phosphorylation was robustly inhibited by the AKT inhibitors GDC-0068 and MK2206, but not the mTORC1 inhibitor rapamycin, suggesting that xCT Ser²⁶ may be phosphorylated by either AKT or a downstream kinase that is not mTORC1. However, an alternative explanation that could reconcile our results with those of Gu *et al.* is the positive feedback loop from AKT to mTORC2 (47). If this feedback loop is functional in the cells utilized in our study, then AKT inhibitors would impair activation of mTORC2 by AKT, leaving open the possibility that mTORC2 could be the responsible kinase for xCT phosphorylation, at least under these conditions. Regardless, since both AKT and mTOR catalytic inhibitors impair xCT phosphorylation, our data indicate that both mTORC2 and AKT are equally required for xCT phosphorylation.

xCT-mediated Cys₂ uptake and the transsulfuration pathway constitute two main mechanisms by which a cell can obtain Cys. Our [U-¹³C₅]-methionine tracing data suggested that concurrent with decreased Cys₂ uptake through xCT, *PIK3CA* mutant MCF10A cells utilized Met-derived Hcy to synthesize Cys through the transsulfuration pathway, as opposed to re-methylating Hcy to regenerate Met. These results are consistent with a model in which the ability of a cell to take up exogenous sources of Cys through xCT may influence whether that cell synthesizes Cys intracellularly through Hcy transsulfuration. In turn, the degree to which a cell utilizes the transsulfuration pathway for Cys may then affect the regulation of the Hcy junction by modulating how efficiently Hcy is re-methylated through the methionine cycle to produce Met. Therefore, cells with more transsulfuration pathway activity would be more methionine dependent, since less Hcy flux is devoted towards the methionine cycle to generate sufficient Met to continue proliferating in Met⁻Hcy⁺ media.

In support of this model, Group A cells, including wild-type MCF10A cells, expressed higher amounts of xCT and may therefore utilize the transsulfuration pathway less to obtain Cys. As a result, in Met⁻Hcy⁺ media, Hcy can be devoted towards Met production through the methionine cycle to restore sufficient Met levels for proliferation. Conversely, in Group C cells, including *PIK3CA* mutant MCF10A cells, Cys₂ uptake was decreased through inhibition of xCT expression and activity by oncogenic PI3K. These cells therefore utilized the transsulfuration pathway to a greater extent to obtain Cys, resulting in less Hcy to Met conversion, impaired proliferation, and cell death in Met⁻Hcy⁺ media. Of note, MDA-MB-468 cells, a Group C cell line, exhibit increased transsulfuration pathway activity in Met⁻Hcy⁺ media, which is consistent with our hypothesis (48). Finally, manipulation of xCT activity or expression may modulate the methionine dependency phenotype by altering the dependence of cells on transsulfuration-derived Cys, thereby influencing the relative diversion of Hcy into the methionine cycle compared to the transsulfuration pathway.

Although xCT contributes to methionine dependency, it is unlikely to be the only functional mediator of this phenotype. In the context of the Group C breast cancer cell lines, xCT overexpression was only partially sufficient in reversing their methionine dependency.

Moreover, it is important to note that while our data provides evidence for oncogenic *PIK3CA* mutations promoting methionine dependency, the correlation between methionine dependency and alterations within various nodes of the PI3K pathway in general was not as strong. For example, in our analysis of our panel of breast cancer cell lines, although genomic mutations in both *PIK3CA* and *PTEN* clustered towards cell lines with negative Met⁻Hcy⁺ growth rates, all of the Group A cell lines are also negative for PTEN protein abundance. On one hand, this observation may reflect the idea that genetic alterations at different nodes of the PI3K pathway may not function equivalently, as has been demonstrated for other phenotypes such as drug sensitivity and organoid growth in previous studies (49–51), and may provide a rationale for testing how *PTEN* alterations contribute to methionine dependency in an isogenic system. However, this observation also points toward the likelihood that other genes may more strongly modulate the capacity to proliferate in Met⁻Hcy⁺ media. Other methods, such as focused sgRNA or cDNA overexpression screens targeting genes involved in Met and Cys metabolism, may be used to identify other factors that modify the methionine dependency phenotype.

It should also be noted that the transsulfuration pathway is restricted to certain normal tissues in an organism, such as the liver, pancreas, and kidney (52). However, this pathway is also active in various cancer cells, including breast cancer cells (48, 53–55). Moreover, the transsulfuration pathway can serve as a source of Cys for glutathione synthesis, and it is possible that the regulation of this pathway by oncogenic *PIK3CA* may support the PI3K-mediated stimulation of glutathione synthesis (39). It would also be interesting to explore whether a subset of cancer cells with transsulfuration pathway activity depend on this pathway to maintain their cellular Cys pools for growth and survival. If so, the transsulfuration pathway could be a potential therapeutic target for this subset of cancer cells.

In summary, we have identified a junction in metabolism that appears to be regulated differently in a subset of breast cancer cells: the metabolism of Hcy in either the methionine cycle to produce Met or in the transsulfuration pathway to synthesize Cys. The utilization of Hcy through these two pathways, which is in part altered by oncogenic *PIK3CA* and the degree to which cells depend on Cys₂ uptake through xCT, then contributes to the methionine dependency phenotype. This work provides another example of the role of the PI3K pathway in mediating metabolic reprogramming in various cancers (56). In addition, xCT alters the utilization of other nutrients by cancer cells. For example, xCT overexpression results in higher glutamate export, rendering cancer cells more sensitive to glucose starvation (57, 58). Increased xCT-dependent cystine uptake and glutamate release also leads to a dependence by some cancer cell lines on glutamine anaplerosis for proliferation, which results in greater sensitivity to glutaminase inhibitors (59, 60). By showing that PI3K-mediated regulation of xCT also influences methionine and cysteine metabolism in breast cancer cells, our work adds to the body of evidence describing how xCT alters nutrient utilization by cancer cells to modulate their dependency on different nutrients for survival.

Materials and Methods

Cell lines

Cell lines were obtained from the American Type Culture Collection (ATCC) and authenticated using short tandem repeat (STR) profiling. MCF10A cells with a knock-in *PIK3CA*(H1047R) mutation were obtained from Horizon Discovery. No cell lines used in this study were found in the database of commonly misidentified cell lines that is maintained by the International Cell Line Authentication Committee (ICLAC) and National Center for Biotechnology Information (NCBI) Biosample. MCF10A cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F12 (CellGro) supplemented with 5% equine serum (CellGro), 10 µg/mL insulin (Life Technologies), 500 ng/mL hydrocortisone (Sigma-Aldrich), 20 ng/mL epidermal growth factor (EGF) (R&D Systems), and 100 ng/mL cholera toxin (Sigma-Aldrich). MDA-MB-231, MDA-MB-468, and MCF7 cells were maintained in DMEM (CellGro) supplemented with 10% fetal bovine serum (FBS) (Gemini). T47D and BT549 cells were maintained in RPMI 1640 (CellGro) supplemented with 10% FBS (Gemini) and 10 µg/mL insulin (Life Technologies). HCC38, HCC70, HCC1143, HCC1806, and ZR-75-1 cells were maintained in RPMI 1640 (CellGro) supplemented with 10% FBS (Gemini). SKBR3 cells were maintained in McCoy's 5A (CellGro) supplemented with 10% FBS (Gemini). SUM149 and SUM159 cells were maintained in Ham's F12 (CellGro) supplemented with 5 µg/mL insulin (Life Technologies), 1 µg/mL hydrocortisone (Sigma-Aldrich), and 5% FBS (Gemini). Cells were passaged for no more than 6 months and routinely assayed for mycoplasma contamination.

Met⁺Hcy⁻ and Met⁻Hcy⁺ media

DMEM lacking, glutamine, pyruvate, methionine, and cystine was obtained from Gibco and was supplemented as necessary with 2 mM glutamine (Gibco), 0.2 mM L-methionine (Sigma), 0.2 mM L-cystine (Sigma), 0.1 mM L-homocystine (Sigma), and 10% dialyzed FBS (Cellgro). For MCF10A cells, media was also supplemented with 5% dialyzed FBS (CellGro), 10 µg/mL insulin (Life Technologies), 500 ng/mL hydrocortisone (Sigma-Aldrich), 20 ng/mL EGF (R&D Systems), and 100 ng/mL cholera toxin (Sigma-Aldrich).

Growth Factors and Inhibitors

For insulin stimulation, cells were serum-starved for 20–24 h and stimulated with 100 nM insulin (Life Technologies). Inhibitors were used as follows: GDC-0941 (LC Laboratories), 1 µM; GDC-0068 (Active Biochem), 1 µM; MK2206 (Selleck Chemicals), 1 µM; rapamycin (Cayman Chemicals), 100 nM; Torin1 (Tocris), 1 µM; sulfasalazine (SSA) (Sigma), 1 mM.

Antibodies

All antibodies were purchased from Cell Signaling Technology: p-AKT Ser⁴⁷³ (#4060, 1:1000), p-AKT Thr³⁰⁸ (#2965, 1:1000), AKT (#4691, 1:1000), p-PRAS40 Thr²⁴⁶ (#2997, 1:1000), PRAS40 (#2691, 1:1000), PARP (#9542, 1:1000), p-S6 Ser^{240/244} (#2215, 1:1000), p-RxxS/T (#9614, 1:1000); HA-tag (#2367, 1:1000); xCT/SLC7A11 (#12691, 1:1000); β-actin (#4970, 1:5000); conformation specific mouse anti-rabbit IgG HRP conjugate (#5127, 1:3000).

Plasmids

pJP1520-HA-GFP, pJP1520-HA-*PIK3CA* WT, pJP1520-HA-*PIK3CA*(E545K), and pJP1520-HA-*PIK3CA*(H1047R) were generous gifts from Dr. Albert S. Baldwin (61). pENTR223-*SLC7A11* was obtained from the Dana-Farber/Harvard Cancer Center DNA Resource Core, and *SLC7A11* was sub-cloned into the pHAGE-C-HA-FLAG lentiviral expression vector (W. Harper, Harvard Medical School). The *SLC7A11* S26A mutation was generated by site-directed mutagenesis (Qiagen). pLKO.1 vector was used as empty vector control (EV).

Immunoblotting and Immunoprecipitation

Cells were washed with phosphate buffered saline (PBS) at 4°C and lysed in radioimmunoprecipitation assay buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.5), proteinase inhibitor cocktail, 50 nmol/L calyculin, 1 mmol/L sodium pyrophosphate, and 20 mmol/L sodium fluoride) for 15 minutes at 4°C. Cell extracts were pre-cleared by centrifugation at 14,000 rpm for 5–10 minutes at 4°C, and protein concentration was measured with the Bio-Rad DC protein assay. For HA-tag immunoprecipitations, lysates were incubated with 10 µl of HA beads (ThermoFisher Scientific) for 3 h. Beads were then washed four times with NETN buffer (20 mmol/L Tris-HCl, pH 8, 100 mmol/L NaCl, 0.5% NP40, and 1 mM EDTA) and one time with PBS prior to elution with SDS sample buffer. For endogenous xCT immunoprecipitations, lysates were collected as described above and incubated with anti-xCT primary antibody for 2 h, followed by 2 more hours with 20 µl of Protein A beads. Lysates were then resolved on acrylamide gels by SDS-PAGE and transferred electrophoretically to nitrocellulose membrane (Bio-Rad) at 100 V for 90 minutes. The blots were blocked in Tris-buffered saline (TBST) buffer (10 mmol/L Tris-HCl, pH 8, 150 mmol/L NaCl, and 0.2% Tween 20) containing 5% (w/v) nonfat dry milk for 1 hour, and then incubated with the specific primary antibody diluted in blocking buffer at 4°C overnight. Membranes were washed three times in TBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hour at room temperature. For endogenous xCT immunoprecipitations, a conformation specific secondary antibody conjugated to HRP (mouse anti-rabbit-HRP, CST #5127) was used to facilitate the detection of endogenous xCT phosphorylation. Membranes were washed three times and developed using enhanced chemiluminescence substrate (EMD Millipore).

In vitro Kinase Assay

HEK293T expressing EV, pHAGE-C-HA-FLAG xCT, or pHAGE-C-HA-FLAG xCT(S26A) were serum starved for 20 h and then treated with 1 µM MK2206 for 30 min prior to collection of lysates and HA-xCT immunoprecipitation, as described above. Immunoprecipitated samples were incubated at 30°C for 1 h in the presence of Kinase Assay reaction buffer (Cell Signaling Technology #9802), adenosine triphosphate (ATP) (200 µM), and dithiothreitol (DTT) (2mM), with or without 0.5 µg of active GST-AKT1 (Sigma-Aldrich #SRP5001). The reaction was stopped by adding 12 µL of 6x SDS loading buffer. Samples were resolved by Western blot, as described above.

Proliferation Assays

Cells were seeded into 24-well or 96-well plates (Corning) at the following densities- MCF10A: 25000 cells/well (24-well), 3000–8000 cells/well (96-well); MDA-MB-468: 75000 cells/well (24-well); MCF7: 75000 cells/well (24-well); T47D: 50000 cells/well (24-well); HCC38: 30000 cells/well (24-well); HCC70: 75000 cells/well (24-well); SUM149: 50000 cells/well (24-well). After 24 h, the media was changed to either Met⁺Hcy⁻ Met⁻Hcy⁺ + Cys₂⁻Hcy⁺ or Met⁻Cys₂⁻Hcy⁺ media every two days. 200 µl of media were used per well on a 96-well plate, and 1 ml of media was used per well on a 24-well plate. Cell number was measured at the indicated time points using sulforhodamine B staining, as previously described (62).

Propidium Iodide Viability Assay

Cell viability was assayed with a propidium iodide-based plate reader assay, as previously described (63). Briefly, cells in 96-well plates were treated with a final concentration of 30 µM propidium iodide for 60 min at 37°C. The initial fluorescence intensity was measured in a SpectraMax M5 (Molecular Devices) at 530 nm excitation/620 nm emission. Digitonin was then added to each well at a final concentration of 600 µM. After incubating for 30 min at 37°C, the final fluorescence intensity was measured. The fraction of dead cells was calculated by dividing the background-corrected initial fluorescence intensity by the final fluorescence intensity. Viability was calculated by (1 – fraction of dead cells).

Growth Rate and Gene Expression Correlation Analysis

The proliferation data for each cell line in Met⁺Hcy⁻ or Met⁻Hcy⁺ media were fit to an exponential curve to calculate growth rates and doubling times. For the correlation of Met⁻Hcy⁺ growth rates to the expression of Met and Cys metabolism genes, RNA expression data was obtained from (36) for the following genes: *GOT2*, *MAT2A*, *ODC1*, *AHCYL1*, *AHCY*, *AMD1*, *MARS*, *SRM*, *DNMT1*, *SMS*, *MTRR*, *SAT1*, *MPST*, *MTR*, *CDO1*, *MTAP*, *MAT1A*, *CTH*, *BHMT*, *TRDMT1*, *MTHFR*, *GOT1*, *SHMT1*, *GNMT*, *AHCYL2*, *CBS*, *SHMT2*, *IL4I1*, *ADI1*, *ENOPH1*, *MAT2B*, *DNMT3A*, *APIP*, *MSRB2*, *MSRA*, *BHMT2*, *DNMT3L*, *DNMT3B*, and *SLC7A11*. A Spearman rank correlation was then conducted between the Met⁻Hcy⁺ growth rates of the panel of cell lines with the expression of the listed genes.

Quantitative Real-Time PCR

Total RNA was isolated with the NucleoSpin RNA Plus (Macherey-Nagel) according to the manufacturer's protocol. Reverse transcription was performed using the TaqMan Reverse Transcription Kit (ThermoFisher Scientific). Quantitative real-time PCR was performed using an ABI Prism 7700 sequence detector. *SLC7A11* primer: sense, 5'-TGCTGGGCTGATTATCTTCG-3', antisense, 5'-GAAAGGGCAACCATGAAGAGG-3'; *MAT1A* primer: sense, 5'-ATGCTACCGACGAGACAGAG-3', antisense, 5'-GATGACGATGGTGTGGATGC-3'; *18S* primer: sense, 5'-GTAACCCGTTGAACCCCAT-3', antisense, 5'-CCATCCAATCGGTAGTAGCG-3'. PCR reactions were carried out in triplicate. Quantification of mRNA expression was calculated by the CT method with *18S* rRNA as the reference gene.

¹⁴C-Cystine Uptake Assay

Fresh media was added to the cells 2 h before the experiment. [¹⁴C]-Cystine (L-[3,3'-¹⁴C]-Cystine, 110 mCi/mmol; PerkinElmer) was then added to the media at 0.9 μ M for 5 min at 37°C. Uptake was terminated by rinsing cells two times with ice-cold PBS. Cells were then lysed with 400 μ l of 0.2 M NaOH containing 1% SDS, and lysates were centrifuged at 13,000 rpm for 5 min at room temperature. 250 μ l of the lysate was mixed with 5 ml of scintillation cocktail (Ultima Gold, PerkinElmer), and radioactivity was measured using a scintillation counter. The remaining lysate was used for protein determination by the Bio-Rad DC protein assay, and the scintillation counts for each sample was normalized by protein concentration.

Glutamate Release Rate

MCF10A cells were seeded at 250,000 cells/well on a 6-well plate. On the following day, cells were washed twice with PBS, and 2 mL of Met⁺Hcy⁻ media were placed in each well. 1 mL of media was immediately removed, centrifuged at 3000 rpm, and the supernatant was frozen for analysis. Cell number on the plate was also determined using the SRB assay. One day later, media was harvested and cell number was determined in the same manner. Glutamate concentrations in the media were measured by gas chromatography-mass spectrometry (GC-MS). 10 μ l of media was added to 10 μ l of isotopically labeled internal standards of amino acids (Cambridge Isotope Laboratory, MSK-A2-1.2), and this mixture was extracted in 300 μ l of ice cold HPLC-grade methanol (Sigma-Aldrich), vortexed for 10 min, and centrifuged at 15,000 rpm for 10 min. 280 μ l of the supernatant was dried under nitrogen gas, and the remaining pellet was analyzed by GC-MS following methoxamine (MOX)-tert-butyldimethylsilyl (TBDMS) derivatization, as previously described (59). To calculate the glutamate release rate (pmol/cell/day), changes in glutamate concentration were normalized to the integrated growth curve of each culture (number of cell-days during which glutamate was released), which was calculated by using the cell numbers at the initial day and day 1 counts to fit an exponential growth equation.

LC-MS/MS Metabolomic Profiling

Cells were maintained in the indicated growth media, and fresh media was added 2 h before the experiment. For metabolite extraction, media was aspirated, and 80% (v/v) methanol at dry ice temperatures was added. Cells and the metabolite-containing supernatants were collected, and the insoluble material in lysates was centrifuged at 4,000xg for 5 min. The resulting supernatant was evaporated using a refrigerated SpeedVac. Samples were re-suspended using 20 μ l HPLC grade water for mass spectrometry. Ten microliters were injected and analyzed using a 5500 QTRAP hybrid triple quadrupole mass spectrometer (AB/SCIEX) coupled to a Prominence UFLC HPLC system (Shimadzu) via selected reaction monitoring (SRM) of a total of 254 endogenous water soluble metabolites for steady-state analyses of samples. Some metabolites were targeted in both positive and negative ion mode for a total of 285 SRM transitions using positive/negative switching. ESI voltage was +4900 V in positive ion mode and -4500 V in negative ion mode. The dwell time was 4 ms per SRM transition and the total cycle time was 1.89 s. Approximately 9–12 data points were acquired per detected metabolite. Samples were delivered to the MS via

normal phase chromatography using a 4.6 mm i.d × 10 cm Amide Xbridge HILIC column (Waters Corp.) at 300 µl/min. Gradients were run starting from 85% buffer B (HPLC grade acetonitrile) to 42% B from 0–5 min; 42% B to 0% B from 5–16 min; 0% B was held from 16–24 min; 0% B to 85% B from 24–25 min; 85% B was held for 7 min to re-equilibrate the column. Buffer A was comprised of 20 mM ammonium hydroxide/20 mM ammonium acetate (pH = 9.0) in water:acetonitrile (95:5). Peak areas from the total ion current for each metabolite SRM transition were integrated using MultiQuant v2.0 software (AB/SCIEX). For ¹³C-labeled experiments, SRMs were created for expected ¹³C incorporation in various forms for targeted LC-MS/MS. Data analysis was performed in MatLab. Metabolite total ion counts represent the normalized (by cellular protein content), integrated total ion current from a single SRM transition.

Isotope Labeling

For labeling in MCF10A cells, DMEM lacking glutamine, pyruvate, methionine, and cystine was obtained from Gibco and was supplemented with 2 mM glutamine (Gibco), 0.2 mM L-cystine (Sigma), 0.2 mM [U-¹³C₅]-L-methionine (Cambridge Isotope Labs), 5% dialyzed FBS (Cellgro), 10 µg/mL insulin (Life Technologies), 500 ng/mL hydrocortisone (Sigma-Aldrich), 20 ng/mL EGF (R&D Systems), and 100 ng/mL cholera toxin (Sigma-Aldrich). Fresh unlabeled media with or without 1 µM GDC-0941 was added to the cells 2 h prior to labeling. Labeled media with or without 1 µM GDC-0941 was then added to the cells, and cellular metabolites were extracted as described above at 1 and 24 h.

Statistics and Reproducibility

Sample sizes and reproducibility for each figure are denoted in the figure legends. Unless otherwise noted, all western blots are representative of at least three biologically independent experiments. Statistical significance between conditions was assessed by two-tailed Student t-tests. All error bars represent s.e.m., and significance between conditions is denoted as **P* < 0.05; ***P* < 0.01; and ****P* < 0.001.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Proliferation of breast cancer cell lines in Met⁻Hcy⁺ media

(A) Schematic of the methionine cycle and transsulfuration pathway. Met, methionine; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; Hcy, homocysteine; Ser, serine; Cys, cysteine; α KB, alpha-ketobutyrate; MAT, methionine adenosyltransferase; AHCY, adenosylhomocysteinase; MTR, 5-methyltetrahydrofolate-homocysteine methyltransferase; CBS, cystathionine-beta-synthase; CTH, cystathionine gamma-lyase. (B) Cell lines were screened for their growth in Met⁻Hcy⁺ media for four days, and the proliferation of cells was determined using the sulforhodamine B (SRB) assay ($n = 3$ independent replicates). (C) Proliferation data from (B) were fit to an exponential curve to calculate the growth rate of each cell line in Met⁻Hcy⁺ media. (D) Pearson correlation of the growth rates of the cell lines in Met⁻Hcy⁺ media with their doubling time in Met⁺Hcy⁻ media. All error bars represent s.e.m.

Figure 2. Oncogenic *PIK3CA* confers methionine dependency to MCF10A cells

(A) Cells were grown in Met⁻Hcy⁺ media for four days, and the proliferation of cells was determined using the sulforhodamine B (SRB) assay (n = 3 biologically independent replicates). (B) Cells were grown in Met⁺Hcy⁻ or Met⁻Hcy⁺ media for two days, and cell death was measured using a propidium iodide-based plate reader assay (n = 3 biologically independent replicates). (C) Cells were grown in Met⁺Hcy⁻ or Met⁻Hcy⁺ media for two days and were then immunoblotted for the indicated proteins (data is representative of three independent experiments). All error bars represent s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001 by a two-sided Student's t-test.

Figure 3. Decreased xCT expression and cystine uptake is associated with increased methionine dependency

(A) Spearman correlation of the growth rates of the panel of breast cancer cell lines in Met⁻Hcy⁺ media from Fig. 1C with their expression of a selected set of methionine and cysteine metabolism genes (see Materials and Methods for full list). Values above the red dashed line represents $p < 0.05$. (B) *SLC7A11* and *MAT1A* mRNA abundances were measured by qRT-PCR and are expressed as fold changes relative to MCF10A empty vector-transfected (EV) cells ($n = 3$ biologically independent replicates). (C) *SLC7A11* mRNA abundance was measured by qRT-PCR ($n = 3$ biologically independent replicates). (D) Cells were grown in Met⁺Hcy⁻ media and immunoblotted for the indicated proteins (data is representative of two independent experiments). (E) [¹⁴C]-cystine uptake was measured over 5 min and is expressed as fold changes relative to MCF10A EV cells ($n = 5$ biologically independent replicates). All error bars represent s.e.m. ** $P < 0.01$ by a two-sided Student's t -test.

Figure 4. xCT Ser²⁶ is phosphorylated upon PI3K pathway activation

(A) Comparison of the consensus AKT substrate motif with the amino acid sequence surrounding xCT Ser²⁶. (B) MCF10A *PIK3CA*(+/+) and *PIK3CA*(H1047R/+) knock-in cells expressing EV, xCT-HA-FLAG, or xCT(S26A)-HA-FLAG were serum-starved. HA immunoprecipitates from cell lysates were immunoblotted for the indicated proteins (data is representative of three independent experiments). (C) Serum-starved MCF10A cells expressing EV or xCT-HA-FLAG were treated with 100 nM insulin for the indicated times. HA immunoprecipitates from cell lysates were immunoblotted for the indicated proteins (data is representative of three independent experiments). (D) Serum-starved MCF10A *PIK3CA*(+/+) and *PIK3CA*(H1047R/+) knock-in cells were treated with vehicle, 1 μ M GDC-0941, 1 μ M GDC-0068, 1 μ M MK2206, 1 μ M Torin1, or 100 nM rapamycin for 15 minutes. HA immunoprecipitates from cell lysates were immunoblotted for the indicated proteins (data is representative of three independent experiments). (E) xCT-HA-FLAG or xCT(S26A)-HA-FLAG were immunoprecipitated from HEK293T cells treated with 1 μ M MK2206 and incubated with active GST-AKT1 for an in vitro kinase assay. Lysates were immunoblotted for the indicated proteins (data is representative of three independent experiments). (F) Release of glutamate into media by the indicated cell lines was measured over 24 h (n = 3 biologically independent replicates). IP, immunoprecipitation; IB, immunoblot; WCL, whole cell lysate; p, phospho. All error bars represent s.e.m. *P < 0.05 by a two-sided Student's t-test.

Figure 5. xCT and cystine uptake functionally contribute to the methionine dependency phenotype

(A) Cells were grown in Met^+Hcy^+ media for four days, and the proliferation of cells was determined using the sulforhodamine B (SRB) assay ($n = 3$ biologically independent replicates). (B) Cells were grown in Met^+Hcy^+ or Met^-Hcy^+ media for two days, and cell death was measured using a propidium iodide-based plate reader assay ($n = 3$ biologically independent replicates). (C, D) Cells were grown in (C) Met^+Hcy^+ $Cys_2^-Hcy^+$ or $Met^-Cys_2^-Hcy^+$ media or (D) Met^+Hcy^+ media with or without 1 mM SSA for four days, and the proliferation of cells was determined using the sulforhodamine B (SRB) assay ($n = 3-4$ biologically independent replicates). (E) Cells were grown in Met^+Hcy^+ media for six days,

and the proliferation of cells was determined using the sulforhodamine B (SRB) assay ($n = 3$ biologically independent replicates). All error bars represent s.e.m. * $P < 0.05$ by a two-sided Student's t-test. # $P < 0.05$ by a paired t-test.

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Figure 6. Oncogenic *PIK3CA* alters methionine utilization through the transsulfuration pathway (A) Steady-state total pool sizes of cystathionine measured by LC-MS/MS in MCF10A cells growing in standard culture media (n = 3 biologically independent replicates). (B) Schematic of [U-¹³C₅]-methionine labeling into the methionine cycle ([M+4] methionine) or the transsulfuration pathway ([M+4] cystathionine), which are detected by LC-MS/MS. (C) Total ion counts of [M+4] cystathionine in MCF10A cells labeled with [U-¹³C₅]-methionine for 1 h (n = 3 biologically independent replicates). (D) Fractional labeling of [M+4] cystathionine in MCF10A cells labeled with [U-¹³C₅]-methionine for 24 h and treated with or without 1 μM GDC-0941 (n = 3 biologically independent replicates). (E) Total ion counts of [M+4] cystathionine and [M+4] methionine in MCF10A cells labeled with [U-¹³C₅]-methionine for 24 h and treated with or without 1 μM GDC-0941 (n = 3 biologically independent replicates). All error bars represent s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001 by a two-sided Student's t-test.

Table 1**PI K3CA**and *PTEN* mutational status of breast cancer cell lines studied

Group	Cell Line	<i>PIK3CA</i> mutation status ^a	<i>PTEN</i> mutation status ^a	<i>PTEN</i> protein abundance ^b
A	HCC70	WT	267delT (F90fs*9)	Negative
	HCC38	WT	WT	Negative
	SUM-149	WT	WT	Negative
B	MDA-MB-231	WT	WT	Positive
	HCC1806	WT	WT	Positive
	HCC1143	WT	WT	N.D.
	SKBR3	WT	WT	Positive
	BT-549	WT	821delG (V275fs*1)	Negative
	ZR-75-1 ^b	WT	323T>G (L108R)	Positive
	SUM-159 ^c	3140A>T (H1047L)	WT	Positive
C	T47D	3140A>G (H1047R)	WT	Positive
	MCF7	1633G>A (E545K)	WT	Positive
	MDA-MB-468	WT	253+1G>T (A72fs*5)	Negative

^aReported in COSMIC database (<http://cancer.sanger.ac.uk>, (64))^bReported in (50) and (65)^cReported in (66)

N.D., not determined