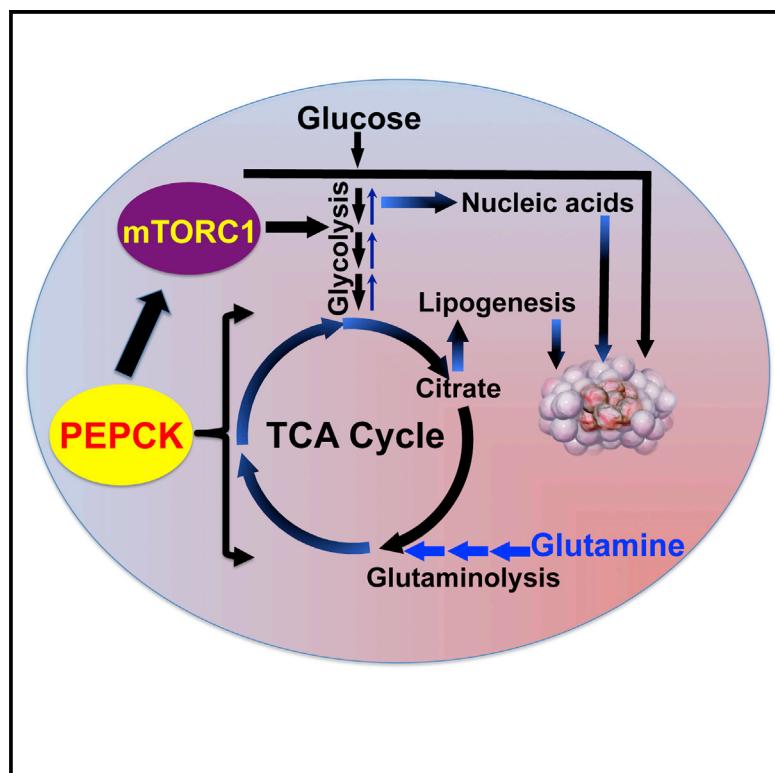


Molecular Cell

PEPCK Coordinates the Regulation of Central Carbon Metabolism to Promote Cancer Cell Growth

Graphical Abstract



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In Brief

Montal et al. report that PEPCK increases tumor growth by promoting metabolic flexibility. PEPCK increases the utilization of both glucose and glutamine toward anabolic metabolism. In addition, the ability of PEPCK to increase glucose metabolism and cell proliferation was mediated in part via activation of mTORC1.

Highlights

- PEPCK promotes tumor growth by increasing glucose and glutamine metabolism
- PEPCK increases anabolic metabolism, including ribose synthesis from glutamine
- PEPCK promotes mTORC1 activity
- PEPCK increases glycolysis and proliferation via mTORC1 activation



PEPCK Coordinates the Regulation of Central Carbon Metabolism to Promote Cancer Cell Growth

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SUMMARY

Phosphoenolpyruvate carboxykinase (PEPCK) is well known for its role in gluconeogenesis. However, PEPCK is also a key regulator of TCA cycle flux. The TCA cycle integrates glucose, amino acid, and lipid metabolism depending on cellular needs. In addition, biosynthetic pathways crucial to tumor growth require the TCA cycle for the processing of glucose and glutamine derived carbons. We show here an unexpected role for PEPCK in promoting cancer cell proliferation *in vitro* and *in vivo* by increasing glucose and glutamine utilization toward anabolic metabolism. Unexpectedly, PEPCK also increased the synthesis of ribose from non-carbohydrate sources, such as glutamine, a phenomenon not previously described. Finally, we show that the effects of PEPCK on glucose metabolism and cell proliferation are in part mediated via activation of mTORC1. Taken together, these data demonstrate a role for PEPCK that links metabolic flux and anabolic pathways to cancer cell proliferation.

INTRODUCTION

Reprogramming of cellular metabolism in cancer cells is crucial for maintaining biosynthetic demands, proliferation, energy, and reducing equivalents for macromolecular synthesis. Many studies have focused on addiction to either glucose or glutamine

as a basis for cancer therapy (DeBerardinis et al., 2008a, 2008b; Gatenby and Gillies, 2004). However, studies are beginning to suggest that these are not universal features in cancer and that cancer cells display metabolic flexibility (Lim et al., 2014; Marin-Valencia and DeBerardinis, 2011). For example, when glutamine utilization is inhibited, cells adapt by switching to glucose as a nutrient source. Conversely, when glucose utilization is blocked, cells increase their utilization of glutamine or other nutrient sources (Choo et al., 2010; Le et al., 2012; Lim et al., 2014). This enables cancer cells to adapt metabolically to proliferate and survive stress associated with reduced nutrient availability to satisfy bioenergetic and anabolic demands. Therefore, targeting the ability of cancer cells to utilize glucose and glutamine would provide a significant therapeutic advantage.

Phosphoenolpyruvate carboxykinase (PEPCK) is the rate-limiting enzyme of gluconeogenesis in the liver and kidney. Following the conversion of amino acids and other non-carbohydrate sources to oxaloacetate (OAA) in the TCA cycle, PEPCK catalyzes the conversion of OAA into phosphoenolpyruvate (PEP). PEP is then converted to glucose via a series of enzymes of glycolysis and several unique enzymes. Despite this well-known role of PEPCK, tracer analysis demonstrates an even more important role for PEPCK in regulating TCA cycle flux (Burgess et al., 2007). For example, reducing PEPCK more than 90% leads to a similar reduction in TCA cycle flux; gluconeogenesis is reduced by only 40%. In addition, it remains unclear whether PEPCK promotes gluconeogenesis in intestinal epithelium (Previs et al., 2009).

The TCA cycle is a central hub of carbon metabolism coordinating the metabolism of glucose, glutamine, other amino acids, respiration, and biosynthetic pathways such as lipogenesis and

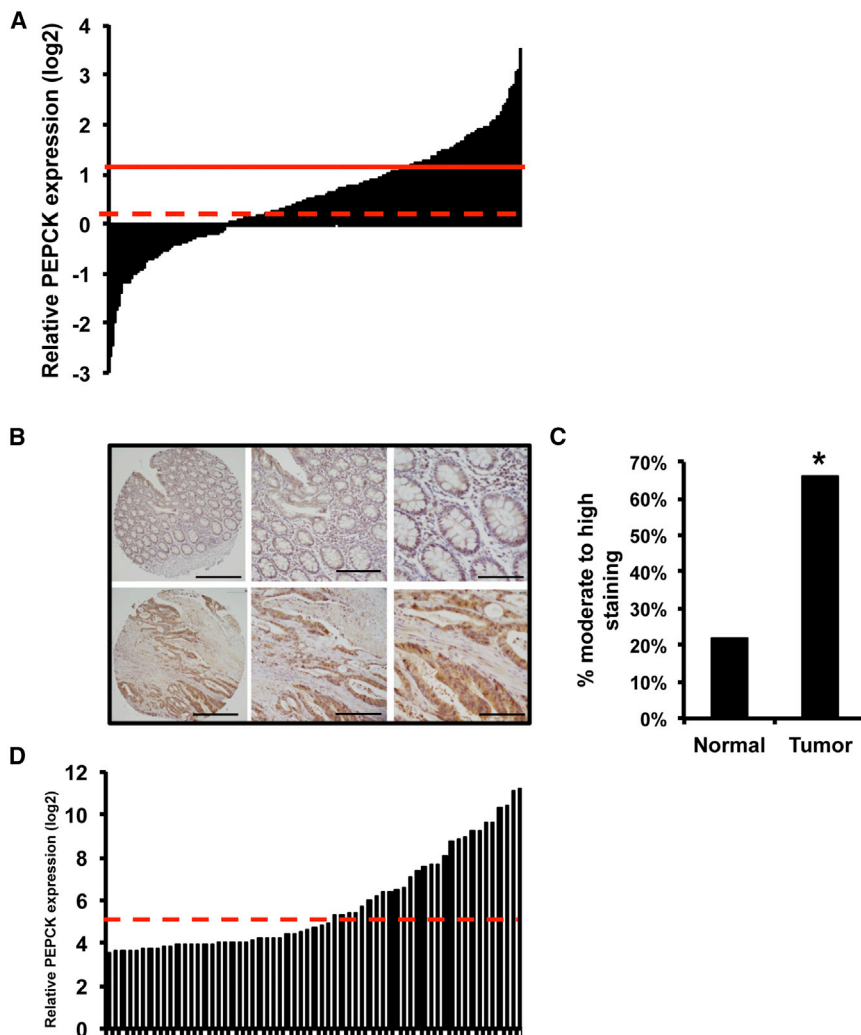


Figure 1. PEPCK Expression Is Increased in Colon-Derived Tumors and Cell Lines

(A) CBioPortal database analysis for *PEPCK* expression in 198 patient samples. Solid line indicates $z = 2$, dashed line indicates mean.

(B and C) Representative IHC of PEPCK in normal and tumor tissue from colon TMA (B) and the percent of moderate to high PEPCK staining in normal and tumor tissue (C).

(D) PEPCK expression of cell lines from a variety of tissues analyzed using the CBioPortal database. $p < 0.0002$. Solid line indicates $z = 2$, dashed line indicates mean. See also Figure S1.

nucleic acid synthesis. The TCA cycle occurs in the mitochondria and although the Warburg effect was thought to be at odds with oxidative metabolism, mitochondrial function is actually required for transformation and tumor growth. Therefore, the TCA cycle represents a nexus point of cancer cell metabolism. The important role that PEPCK plays in regulating the TCA cycle coupled with the requirement of tumor cells to coordinate the use of glucose and glutamine prompted us to determine the role of PEPCK in colorectal cancer.

RESULTS

PEPCK Expression Is Elevated in Colorectal Cancer

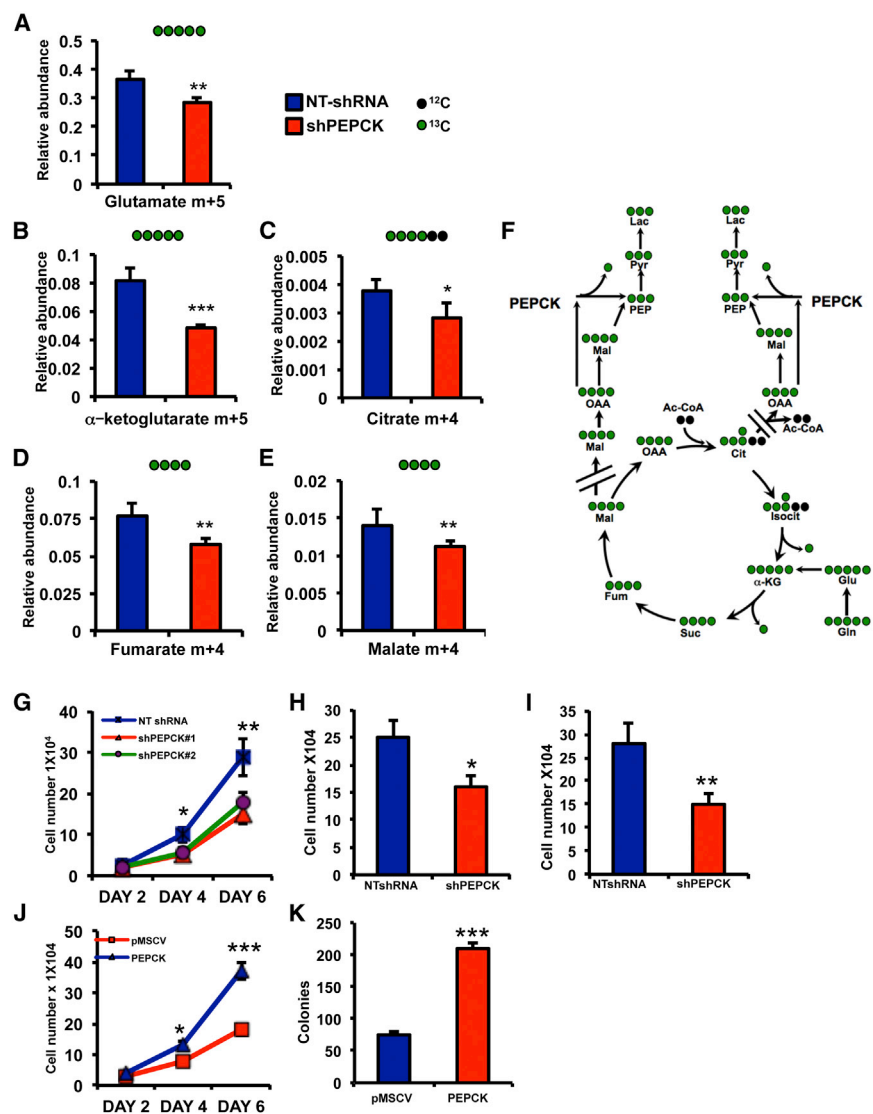
In an effort to determine the role of PEPCK in cancer, we examined two different databases for the expression of PEPCK in cancer cell lines derived from different tissues. There was variability between different tissues, and colorectal cancer cell lines consistently appeared to have higher expression of PEPCK compared to other cancer types (Figures S1A and S1B). Next, we examined the CBioPortal for expression of PEPCK in colon cancer samples. Figure 1A shows that PEPCK was amplified

or overexpressed in $\sim 17\%$ of colon derived tumors (Figure 1A). We also used a tissue microarray composed of normal and colon derived tumor tissue to determine the expression of PEPCK in colon cancer. Almost 50% of tumors had moderate to high PEPCK expression, compared to $\sim 20\%$ of non-tumor tissue (Figures 1B and 1C). Furthermore, when including tissues that express PEPCK at low, medium, or high expression, over 80% of tumors and 78% of non-tumor tissue expressed PEPCK (not statistically significant) (Figure S1C). There did not appear to be a relationship between tumor grade and expression of PEPCK. There are two isoforms of PEPCK, cytosolic PEPCK (PEPCK1, PCK1, which we refer to as PEPCK) and a mitochondrial isoform of PEPCK (PEPCK2 or PCK2). Recent studies show that PEPCK2 also promotes cell proliferation (Leithner et al., 2015; Mendez-Lucas et al., 2014).

Therefore, we also examined the CBioPortal for the expression of PEPCK2. Less than 3% of colon cancers had increased expression of PEPCK2, and there were no amplifications (Figure S1D). Given these results and previous studies showing that the cytosolic form of PEPCK regulates TCA cycle flux, we focused on the cytosolic form of PEPCK.

PEPCK Regulates the TCA Cycle and Cell Proliferation

Next, we sought to identify colon cancer-derived cell lines expressing PEPCK expression. For initial studies, we used the Colo205 cell line, which expresses abundant levels of PEPCK (Figures 1D and S1E). We knocked down PEPCK in the Colo205 cells using different shRNAs (Figure S2A) and observed increased OAA and decreased in PEP confirming functional knockdown of PEPCK (Figures S2B and S2C). Glutamine is the main non-carbohydrate nutrient source for cancer cells (DeBerardinis and Cheng, 2010). Furthermore, the utilization of glutamine for energy, lactate, and macromolecule biosynthesis requires metabolism of glutamine-derived carbons through the TCA cycle. Next, we used uniformly labeled $[U5]-^{13}C$ glutamine to determine whether PEPCK knockdown alters the relative



abundance of TCA cycle intermediates derived from glutamine. The abundance of m+5-labeled glutamate, the first product of glutaminolysis from [U5]- ^{13}C glutamine, was reduced in the PEPCK knockdown cells (Figure 2A). The relative abundance of m+5 α -ketoglutarate (α KG), the first TCA cycle intermediate generated from glutaminolysis, was decreased more than 40% in the PEPCK knockdown cells (Figure 2B). In general there was a decrease in the relative abundance of α KG isotopomers, suggesting that PEPCK was reducing flux as previously shown (Burgess et al., 2007) (Figure S2D). Similarly, there was a decrease in m+4 labeling of citrate, fumarate, and malate. We also observed decreases in the relative abundance of isotopomers of these TCA cycle intermediates further supporting an effect of PEPCK on TCA cycle flux (Figures S2D–S2G). Taken together, these data demonstrate that PEPCK is regulating glutamine utilization and flux through the TCA cycle as previously described (Burgess et al., 2007). Interestingly, despite the role of PEPCK in gluconeogenesis, we did not observe conversion of

Figure 2. PEPCK Regulates Flux through the TCA Cycle and Tumor Cell Proliferation

(A–E) NT-shRNA and shPEPCK Colo205 cells were incubated with ^{13}C glutamine and GC/MS performed for m+5 labeled glutamate (A), or m+4 labeled α -ketoglutarate (B), citrate (C), fumarate (D), and malate (E).

(F) Schematic for conversion of [U5] ^{13}C glutamine into various metabolites.

(G) Proliferation of NT-shRNA and shPEPCK stable knockdown Colo205 cells over 6 days.

(H) Proliferation of NTshRNA and shPEPCK Colo205 cells after 6 days.

(I) Proliferation of Moser human CRC cells with stable knockdown of PEPCK after 6 days.

(J and K) Proliferation (J) and clonogenic survival (K) of control and PEPCK overexpressing HT29 cells. $n = 3 \pm \text{SD}$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. See also Figure S2.

^{13}C glutamine into glucose in either the non-target control or PEPCK knockdown cells (data not shown). This is in line with previous studies showing that PEPCK plays a greater role in regulating the TCA cycle than gluconeogenesis and that the intestines are not a source of gluconeogenesis under normal conditions (Previs et al., 2009; Yang et al., 2009). In line with these observations, G6Pase, which converts glucose-6-phosphate (G6P) to glucose, was not detectable by RT-PCR or western blotting (data not shown). Although we did not observe ^{13}C -labeled glucose (i.e., gluconeogenesis), we did observe ^{13}C -labeled G6P. There was a trend toward reduced ^{13}C -labeled G6P in PEPCK knockdown cells, but it was not statistically significant (Figure S2H). However, the relative abundance of ^{13}C -labeled glycolytic intermediates, phosphoenolpyruvate, and pyruvate were significantly reduced in the PEPCK knockdown cells (Figures S2I and S2J)

phenolpyruvate, and pyruvate were significantly reduced in the PEPCK knockdown cells (Figures S2I and S2J)

Next we examined the effect of altering PEPCK levels affects cell proliferation. Loss of PEPCK led to an approximately 40% decrease in cell number using distinct shRNAs in the Colo205 cell line (Figures 2G, 2H, and S2K). We also knocked down PEPCK in the Moser human colon cancer cell line, which expresses PEPCK, albeit lower levels than Colo205s, using a distinct shRNA (Figures S1E and S2L, inset). Knocking down PEPCK significantly reduced proliferation and clonogenic survival in the Moser cells despite already its lower PEPCK expression (Figures 2I and S2L). This suggests that inhibiting PEPCK can reduce growth even in cancer cells without abundant PEPCK. We examined whether the effect of PEPCK on cell proliferation is specific for colon cancer-derived cells. We used the Fao rat hepatoma cell line, which expresses abundant levels of PEPCK (Scott et al., 1998). Knocking down PEPCK in the Fao cell line (using a rat-specific shRNA) reduced cell proliferation

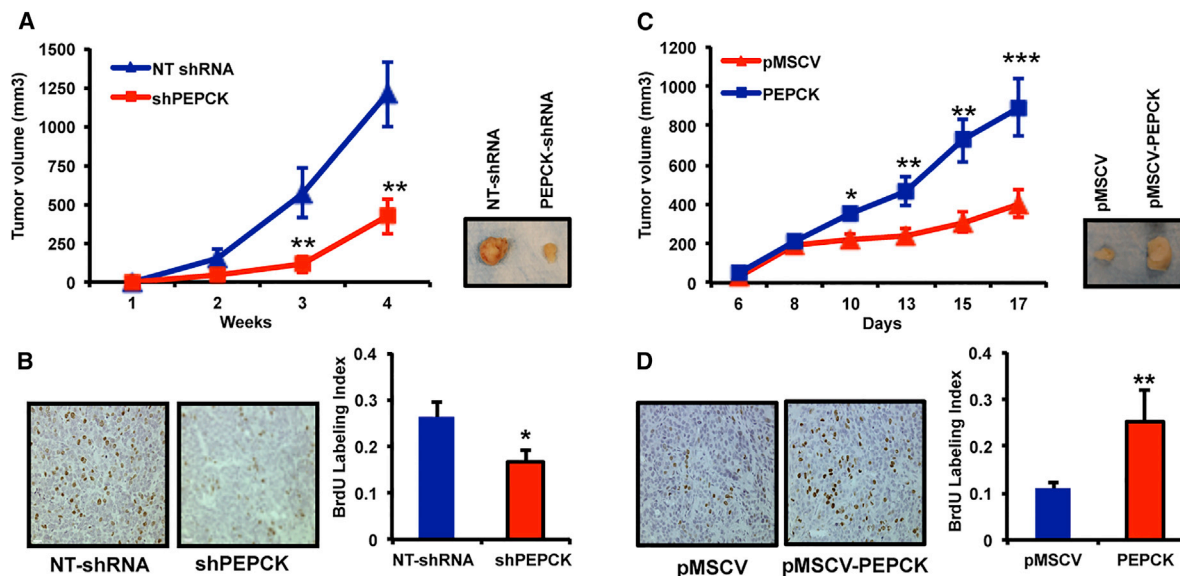


Figure 3. PEPCK Promotes Tumor Growth In Vivo

(A) Tumor growth of Colo205 NT-shRNA and Colo205 PEPCK-shRNA xenografts.

(B) BrdU positive nuclei per field in xenografts from non-target and PEPCK knockdown cell lines.

(C) Tumor growth of HT29 pMSCV and HT29-PEPCK xenografts.

(D) BrdU positive nuclei per field in xenografts from pMSCV control and PEPCK overexpressing HT29 cell lines. $n = 8 \pm \text{SD}$. * $p < 0.05$, ** $p < 0.01$.

as evidenced by decreased clonogenic growth (Figure S2M). We also sought to determine whether PEPCK was sufficient to promote cell proliferation. We used HT29 cells, which do not express PEPCK and generated stable cell lines expressing PEPCK (Figures S1E and S2N). Ectopic expression of PEPCK significantly increased cell proliferation (Figure 2J). Likewise, clonogenic growth was increased almost 3-fold (Figure 2K).

Next, we examined the effect of PEPCK on tumor growth in vivo. Knocking down PEPCK reduced the growth of tumors by almost 70% (Figure 3A) compared to control NTshRNA cells. This was associated with a decrease in proliferation as determined by a reduction in BrdU-positive nuclei (Figure 3B). In contrast, tumors overexpressing PEPCK grew over three times as large as the vector control HT29 cells (Figure 3C) and showed increased in BrdU incorporation (Figure 3D).

PEPCK Promotes Glutamine Utilization

The ability of PEPCK to promote anaplerosis of glutamine into TCA cycle intermediates described above, prompted us to determine how PEPCK affects glutamine beyond the TCA cycle using [U5]- ^{13}C glutamine. The generation of lactate is an important metabolite of cancer cells (DeBerardinis et al., 2008a; Gatenby and Gillies, 2004). For many years, it was believed that lactate was derived from glucose. It is now appreciated that tumor cells generate a fraction of their lactate pools from glutamine via flux through the TCA cycle (DeBerardinis et al., 2007). A small percent of lactate was derived from glutamine (<5%, Figure S3A). However, loss of PEPCK led to a decrease in the relative abundance of m+3-labeled lactate as well as the percent of labeled lactate (Figures 4A and S3A). Conversely, overexpression of PEPCK led to an increase in the relative abundance of ^{13}C -labeled lactate

and a small but significant increase in the fractional labeling of lactate from glutamine (Figures 4A and S3B). Although lactate is usually considered a gluconeogenic substrate, these studies show that PEPCK can actually promote lactate production from glutamine. Hence, in addition to the malate shuttle, PEPCK provides another route for lactate production from glutamine. Importantly, the increase in lactate derived from glutamine further demonstrates that PEPCK is increasing TCA cycle flux.

PEPCK Promotes Anabolic Metabolism

Our initial studies show that PEPCK knockdown reduced the relative abundance of labeled glutamine-derived citrate. Citrate is a precursor for de novo lipid synthesis, another common feature of many tumors (Menendez and Lupu, 2007). Glutamine is incorporated into fatty acid pools in proliferating cells after being processed through the TCA cycle (DeBerardinis et al., 2008a, 2008b) (Figures S3C and S3D). This prompted us to examine the effect of PEPCK on fatty acid synthesis in the Colo205 cells. Almost 30% of the palmitate, and its chain-shortened product, myristate, were ^{13}C labeled, indicating significant amounts of basal anabolic use of glutamine by Colo205 cells for lipid synthesis (Figure 4B). Knocking down PEPCK significantly decreased ^{13}C incorporation into palmitate and myristate. To confirm that PEPCK is sufficient to promote lipogenesis, we examined the incorporation of ^{13}C from glutamine into palmitate and myristate in cells overexpressing PEPCK. Basal incorporation of glutamine into palmitate and myristate were ~7% and 6%, respectively, with the remainder coming from non-labeled sources. However, ^{13}C -labeled palmitate and myristate were increased ~30% and 60%, respectively, in cells overexpressing PEPCK (Figure 4C).

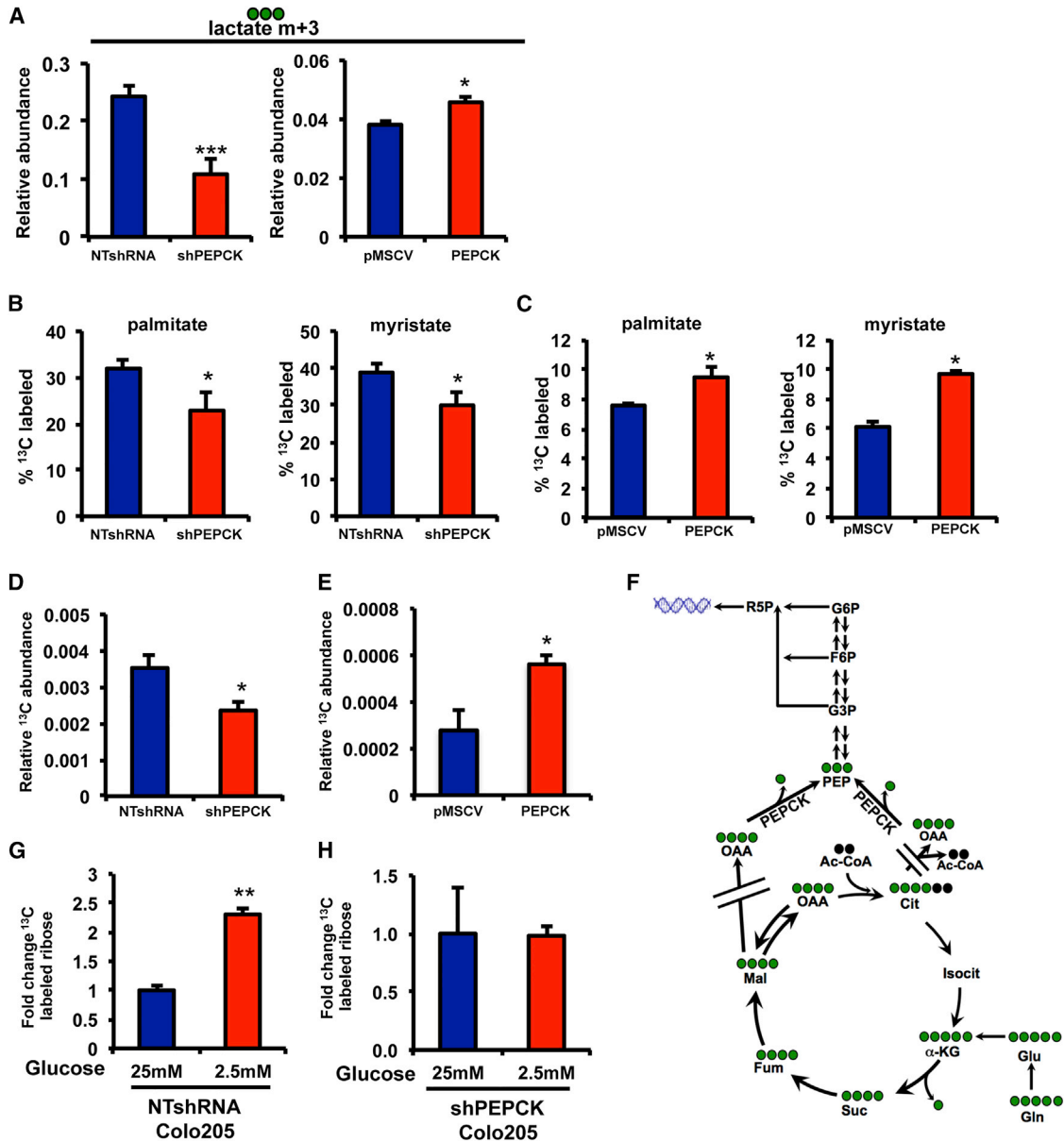


Figure 4. PEPCK Promotes Glutamine Utilization toward Lipogenesis and Ribose Synthesis

(A) ¹³C lactate levels in media (m+3) in NT and shPEPCK Colo205 cells and pMSCV and PEPCK overexpressing HT29 cells cultured with ¹³C glutamine.

(B and C) Percent of ¹³C labeled palmitate and myristate from NT or PEPCK knockdown Colo205 cells (B) or pMSCV and PEPCK overexpressing cells (C).

(D) Relative abundance of ¹³C labeled ribose derived from ¹³C glutamine in NT and shPEPCK Colo205 cells.

(E) Relative abundance of ¹³C labeled ribose derived from glutamine from HT29 pMSCV or HT29 PEPCK cells.

(F) Pathway for ribose synthesis from glutamine.

(G and H) Fold change in ¹³C labeled ribose from ¹³C glutamine was determined in non-target (G) or PEPCK knockdown (H) Colo205 after culturing cells in media with reduced glucose levels.

n = 3 ± SD. *p < 0.05, **p < 0.01, ***p < 0.001. See also Figure S3.

The presence of glutamine-derived G6P and other glycolytic intermediates promoted us to address the fate of these metabolites. Another branch point in glycolysis/gluconeogenesis is the pentose phosphate pathway (PPP), which plays a critical role in cancer proliferation (Tong et al., 2009). The PPP generates ribose for nucleic acid synthesis and NADPH for

biosynthetic and antioxidant pathways. It is well established that glutamine provides the amine groups for purine and pyrimidines (Salzman et al., 1958). However, its role in ribose synthesis has not been previously described. In non-target shRNA Colo205 cells, ~2.5% of the ribose was derived from glutamine (Figure S3E). The remaining unlabeled ribose most likely reflects

glucose-derived ribose and existing pools. Knocking down PEPCK led to a 30% decrease in the relative abundance of ^{13}C ribose and fractional labeling derived from glutamine (Figures 4E and S3E). We also examined the effect of PEPCK on ribose production in two additional knockdown cell lines using ^{14}C glutamine. Incorporation of glutamine-derived carbons into the ribose/RNA fraction was decreased in PEPCK knockdown cells. (Figures S3F and S3G). Conversely, overexpression of PEPCK increased the relative abundance and fractional labeling of ^{13}C ribose derived from glutamine (Figures 4E and S3H). This was a surprising finding since the incorporation of non-carbohydrate sources such as glutamine into ribose and especially promotion of this process by PEPCK has not been previously described (Figure 4F).

The levels of glutamine-derived ribose do not make up a significant fraction of the ribose pool under the cell culture conditions we used (<5%, Figures S3E and S3H). The majority of ribose is derived from glucose. However, physiological glucose levels are ~5 mM and tumor cells are often under conditions of hypoglycemia due to increased glucose utilization coupled with disorganized microvasculature (Gatenby and Gillies, 2004). As a result, glucose levels in tumors can be reduced almost 90% compared to surrounding normal tissues (Hirayama et al., 2009). This would require cancer cells to synthesize ribose via alternative sources. Glutamine concentrations in tumors are similar to normal surrounding tissue despite the dependence of cancer cells on glutamine (Hirayama et al., 2009). Therefore, we examined the incorporation of ^{13}C glutamine into ribose under reduced glucose conditions. In the NT cells expressing PEPCK, reducing glucose caused ^{13}C glutamine-derived ^{13}C ribose to increase almost 3-fold (Figure 4G). In contrast, relative levels of labeled ribose derived from glutamine carbons did not change in the PEPCK knockdown cells (Figure 4H). These data demonstrate that PEPCK enables cells to utilize the carbons from glutamine as an additional source of ribose for nucleic acid synthesis, especially when glucose levels are limiting. It also suggests a coordinated role for glutamine in nucleic acid synthesis, whereby glutamine contributes the amine groups of bases and also the carbon backbone of the ribose group.

We also established xenografts in mice with tumor cells expressing PEPCK or with PEPCK knockdown to determine the role of PEPCK on glutamine metabolism in vivo. Similar to our in vitro studies, there was reduced abundance of ^{13}C citrate (i.e., derived from [U5]- ^{13}C glutamine. Furthermore, we observed reduced enrichment of ^{13}C labeled into palmitate. Likewise, the relative abundance of labeled ribose, which actually made up a greater fraction of labeled ribose than in vitro, was also reduced (Figures S3G–S3I and data not shown). Thus, despite the well-known gluconeogenic roles of PEPCK, these data demonstrate that PEPCK promotes anabolic metabolism with increased lipid and nucleic acid synthesis from glutamine.

PEPCK Protects Cells against Inhibition of Glutaminolysis

Although glutamine is not considered an essential amino acid, it becomes conditionally essential during periods of increased

proliferation and growth (Lacey and Wilmore, 1990). This prompted us to determine whether inhibiting glutaminolysis blocks the growth promoting effects of PEPCK. We inhibited glutaminolysis with aminooxyacetate (AOA) or BPTES (DeBerardinis et al., 2007; Robinson et al., 2007). Surprisingly, inhibiting glutaminolysis in NT Colo205 cells expressing endogenous PEPCK had only a modest effect on cell proliferation (Figures 5A and 5B). In contrast, the PEPCK knockdown cells appeared more sensitive to inhibition of glutaminolysis with cell numbers reduced ~25%–40% compared to NT Colo205 cells. Similarly, BPTES and AOA had a much greater effect on cell number in HT29-pMSCV, which lack PEPCK, compared to the HT29 expressing PEPCK cells (Figures 5C and 5D). Therefore, while PEPCK promotes glutamine utilization, the presence of PEPCK appears to protect cells against reduced glutaminolysis. This suggests that PEPCK can promote the utilization of additional/alternative nutrients. This might be especially important in the tumor micro-environment where nutrients supplies are often limited.

PEPCK Promotes Glucose Utilization

Glucose is an important nutrient in tumor metabolism and one of the main carbon sources for nucleic acid and fatty acid synthesis (DeBerardinis et al., 2008a, 2008b; Hatzivassiliou et al., 2005). In addition, a recent study demonstrated a role for PEPCK in promoting glucose metabolism during the metastases of melanoma cells (Li et al., 2015). Therefore, we examined ^{14}C 2-deoxyglucose uptake into cells with gain and loss of PEPCK. Despite the well-known role of PEPCK in glucose production, we observe an increase in ^{14}C 2-deoxyglucose uptake into cells expressing higher levels of PEPCK (Figure 5E). Next, we wanted to determine the fate of the increased glucose uptake and whether PEPCK was affecting glycolysis.

Lactate production from glucose is one of the hallmarks of cancer cell metabolism. We initially examined the total concentration of lactate in the media of NT and shPEPCK cells using a NOVA biochemical analyzer. Lactate levels were decreased ~20% in the PEPCK knockdown cells (Figure 5F). This coincided with an increase in pH due to the decrease in lactate (Figure S4A). Next, we incubated Colo205 NTshRNA and shPEPCK cells with uniformly labeled ^{13}C glucose to determine whether PEPCK affected the levels of glycolytic intermediates specifically from glucose. The relative abundance of m+3 lactate in the media, the main lactate species derived from [U6]- ^{13}C glucose via glycolysis, was reduced 40% in the shPEPCK cell line (Figure 5G). Similarly, m+3 lactate abundance was increased about 30% in cells overexpressing PEPCK (Figure 5G). Several other glycolytic intermediates, PEP, and pyruvate were also reduced in the PEPCK knockdown cells, reinforcing the ability of PEPCK to promote glycolysis (Figures 5H and 5I). We also used the Seahorse Bioanalyzer to determine whether PEPCK was affecting glycolysis, using ECAR as a measure of glycolysis. Basal ECAR was significantly lower in the PEPCK knockdown cells (Figure 5J). Next, we examined whether PEPCK had an effect on the flux of glucose-derived carbons into the TCA cycle. The relative abundance of citrate, malate, and αKG were reduced in the PEPCK knockdown cells (Figures 5K–5M). We observed a similar effect of PEPCK knockdown on the incorporation of ^{13}C carbon from glucose into lactate, pyruvate, and citrate in

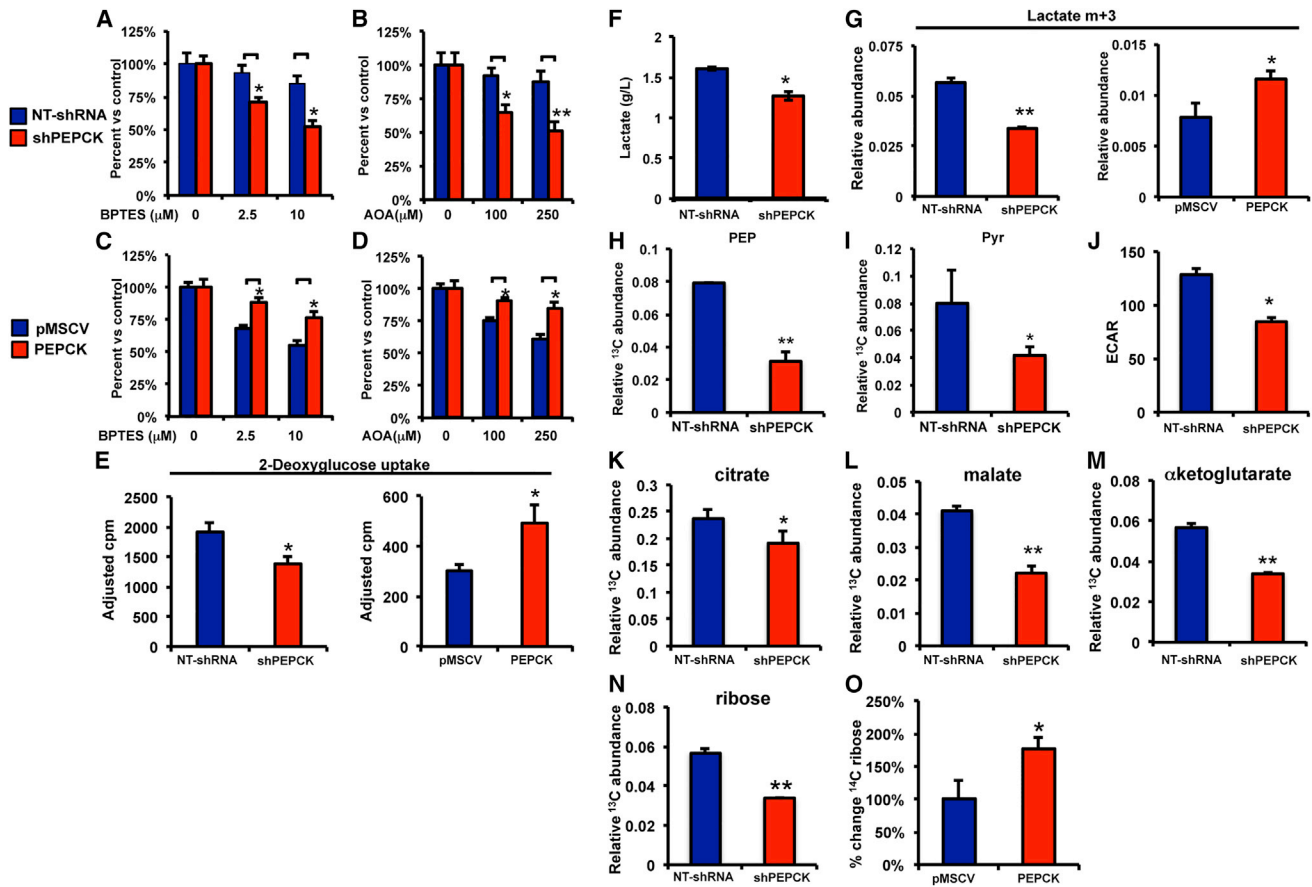


Figure 5. PEPCK Promotes Glucose Utilization

(A–D) Cell proliferation in NTshRNA and shPEPCK Colo205 (A and B) or pMSCV or PEPCCK-expressing HT29 cells (C and D) following treatment with BPTES (A and C) or AOA (B and D) for 48 hr.

(E) ¹⁴C 2-deoxyglucose uptake in PEPCCK knockdown (Colo205) and PEPCCK overexpressing (HT29) cell lines.

(F) Media lactate from NTshRNA and shPEPCK cells determined using a Nova Bioanalyzer.

(G) Relative abundance of ¹³C m+3-labeled lactate from NTshRNA and shPEPCK Colo205 cells and pMSCV or PEPCCK-expressing HT29 cells.

(H and I) Relative abundance of ¹³C-labeled phosphoenolpyruvate (H) or pyruvate (I) from NTshRNA and shPEPCK Colo205 cells.

(J) Glycolysis as determined by ECAR using a Seahorse Bioanalyzer. n = 10 ± SD.

(K–M) Relative abundance of ¹³C-labeled citrate (K), malate (L), or αketoglutarate (M) from NTshRNA and shPEPCK Colo205 cells.

(N and O) Relative abundance of ¹³C-labeled ribose derived from ¹³C glucose in NTshRNA and shPEPCK Colo205 cells (N) or ¹⁴C-labeled RNA/ribose from ¹⁴C glucose in pMSCV or PEPCCK-expressing HT29 cells (O). n = 3 ± SD, *p < 0.05. See also Figure S5.

the Fao cells after PEPCCK knockdown (Figures S4B–S4D). This further supports the ability of PEPCCK to promote both glycolysis and increased flux of glucose into the TCA cycle.

Glucose is one of the most important substrates for ribose production in cells. This prompted us to examine how PEPCCK affects ribose synthesis. Almost 50% of the ribose was derived from the ¹³C glucose tracer in NT Colo205 cells, with the remainder coming from existing pools or the salvage pathway (Figure S4E). However, in PEPCCK knockdown cells there was a significant reduction in the relative abundance of ¹³C-labeled ribose and fractional labeling (Figures 5N and S4E). We observed a similar reduction in ¹³C ribose abundance in the Fao PEPCCK knockdown cell line (Figure S4F). We also incubated HT29 cells overexpressing PEPCCK with ¹⁴C glucose to determine whether PEPCCK was sufficient to promote RNA/ribose synthesis. Indeed,

overexpression of PEPCCK increased ribose synthesis from ¹⁴C glucose (Figure 5O). Next, we wanted to determine whether PEPCCK could promote glucose metabolism in vivo. Following establishment of PEPCCK-expressing and PEPCCK knockdown xenografts, mice were administered a bolus of ¹³C glucose. There was a significant reduction in the relative abundance of lactate and citrate in PEPCCK knockdown tumors compared to non-target PEPCCK-expressing tumors (Figures S4G and S4H).

Our data show that PEPCCK-expressing cells are able to survive better in the presence of glutaminolysis inhibition. Our data suggest that this is due to increased glucose utilization. Therefore, we wanted to determine whether PEPCCK promotes glucose utilization to a greater degree when glutamine utilization is inhibited. We treated cells with BPTES in the presence of [U-¹³C] glucose and examined changes in glucose utilization.

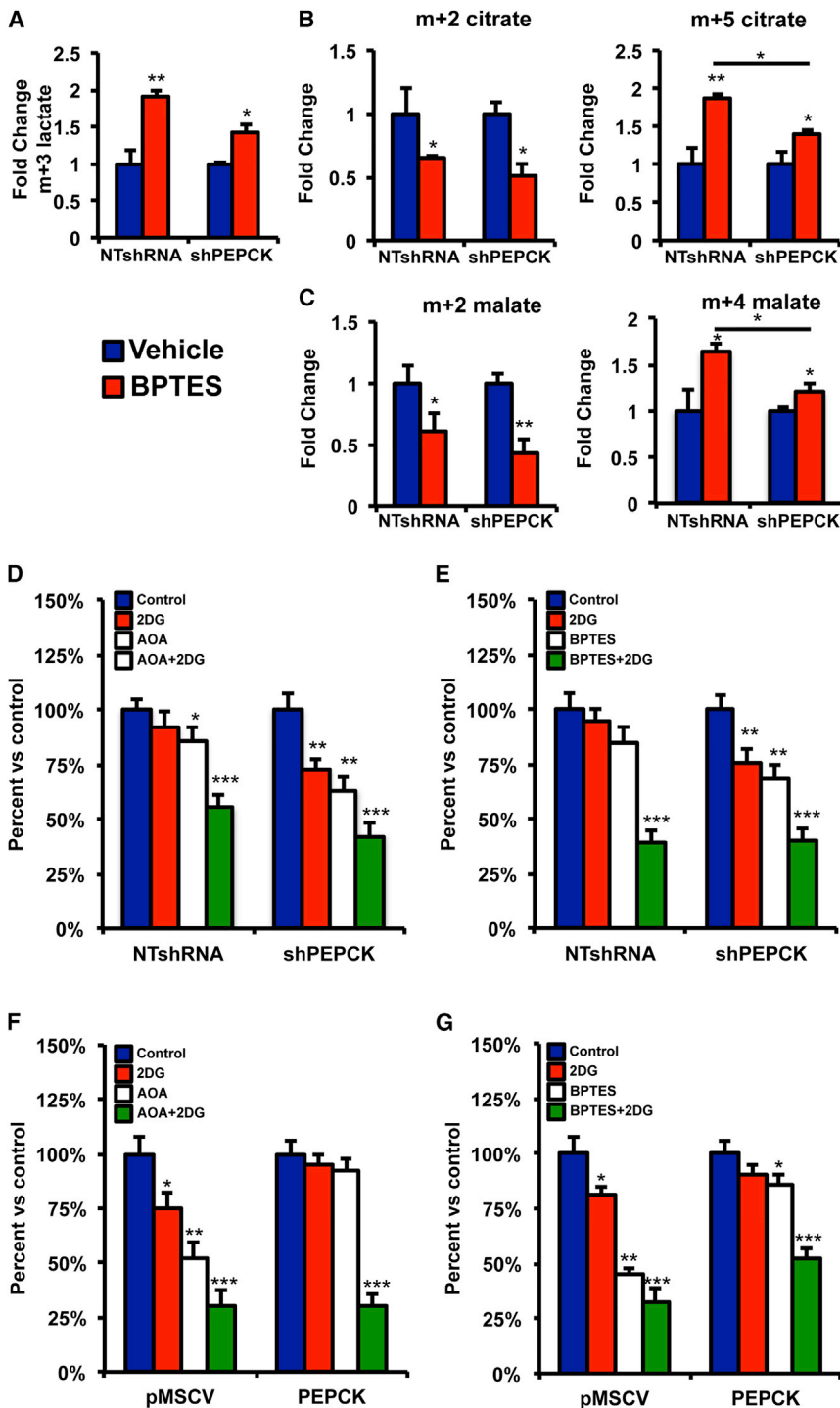


Figure 6. PEPCK Promotes Glucose Utilization when Glutamine Utilization Is Inhibited

(A) Relative abundance of m+3 media lactate from NTshRNA and shPEPCK Colo205 cells following incubation with ^{13}C glucose in the absence and presence of $10\ \mu\text{M}$ BPTES.

(B) Relative abundance of m+2 and m+5 citrate from NTshRNA and shPEPCK Colo205 cells following incubation with ^{13}C glucose in the absence and presence of $10\ \mu\text{M}$ BPTES.

(C) Relative abundance of m+2 and m+4 malate from NTshRNA and shPEPCK Colo205 cells following incubation with ^{13}C glucose in the absence and presence of $10\ \mu\text{M}$ BPTES.

(D–G) Colo205 NT-shRNA and Colo205 PEPCK-shRNA (D and E) or HT29-pMSCV and HT29-PEPCK (F and G) cells were treated with 2.5 mM 2DG alone or in combination with $200\ \mu\text{M}$ AOA (D and F) or BPTES (E and G) for 2 days and cell number determined. $n = 3 \pm \text{SD}$. * $p < 0.05$, ** $p < 0.01$.

m+2-labeled citrate was reduced in the presence of BPTES (Figure 6B). This is most likely due to the fact that OAA (four unlabeled carbons) derived from amino acids such as glutamine is reduced due to inhibition of glutaminolysis. Therefore, cells are unable to use citrate synthase to condense ^{13}C -labeled AcCoA (m+2) with unlabeled OAA (m+0) to generate m+2 citrate. We then examined the levels of m+5 citrate, which would result from ^{13}C -labeled AcCoA (m+2) condensing with M+3 OAA. Treatment of cells with BPTES in the presence of ^{13}C glucose led to a >2-fold increase in m+5 labeled citrate, which was blunted in the PEPCK knockdown cells. The labeling of malate reinforces these data. Labeling of malate via acetyl Co would lead to m+2 malate. However, m+4 malate would be derived from m+5 citrate. Indeed, we see more m+4 malate after treatment with BPTES in the PEPCK-expressing cells, whereas it was not induced in the knockdown cells (Figure 6C). There are two main routes by which m+3-labeled OAA could be generated. Pyruvate carboxylase can convert mitochondrial pyruvate to OAA, or PEPCK can convert PEP to OAA. Since both reactions produce the same ^{13}C -labeling

pattern from $[\text{U}6]\text{-}^{13}\text{C}$ glucose, it is not possible to determine which reaction is responsible. However, we do not see a difference in PC expression, and despite the normal view of PEPCK converting OAA to PEP, the ability of PEPCK to convert PEP to OAA has been shown (Wang et al., 2010).

As shown in Figure 6A, in the presence of BPTES, media lactate increased about 2-fold in the NTshRNA PEPCK-expressing cells most likely due to increased glucose utilization. In contrast, media lactate only increased ~50% in the PEPCK knockdown cells.

We did not see a significant change in total labeled citrate following treatment with BPTES in the NTshRNA cells and a small decrease in the shPEPCK cells (data not shown). However,

Our data shows that PEPCK promotes both glutamine and glucose utilization, especially when one of them is limiting as

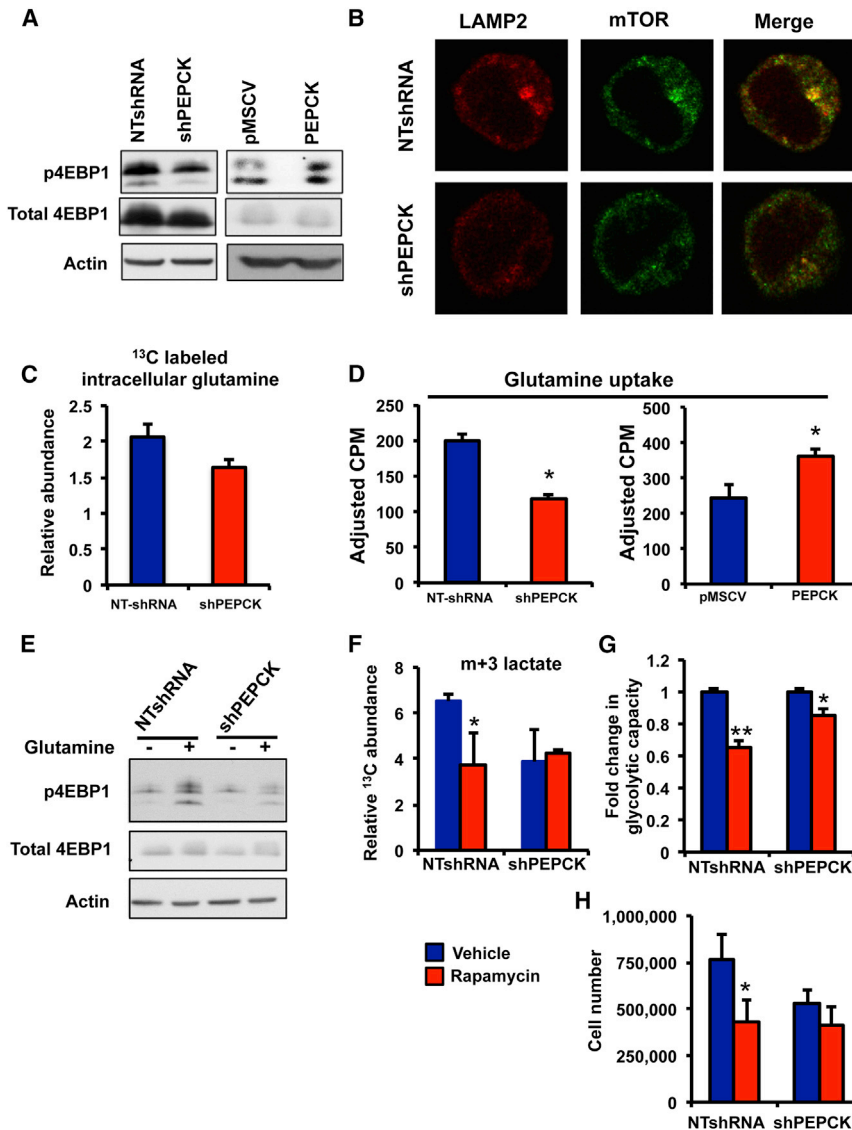


Figure 7. PEPCK Promotes mTORC1 Activation

(A) PEPCK increases phosphorylation of 4EBP1. Immunoblotting for total and phospho4EBP1 was performed for protein of cells following knockdown of PEPCK (shPEPCK) or cells with increased PEPCK expression. Actin was used to control for loading.

(B) Immunofluorescence for LAMP2, mTOR, or the colocalization (merge, yellow) in Colo205 cells expressing PEPCK (NTshRNA) or with PEPCK knocked down (shPEPCK).

(C) PEPCK increases intracellular glutamine levels. Colo205 Non-target and PEPCK knockdown cells were cultured in the presence of [U-¹³C] glutamine and intracellular glutamine levels determined using GC/MS. See also Figure S6.

(D) PEPCK increases glutamine uptake. Colo205 Non-target and PEPCK knockdown cells or HT29 cells with empty vector or PEPCK overexpression were cultured in ¹⁴C glutamine and intracellular glutamine uptake determined.

(E) Glutamine is responsible for the PEPCK-mediated activation of mTORC1. Cells were cultured Colo205 non-target and PEPCK knockdown cells were cultured in media containing glutamine or without glutamine overnight. Proteins were isolated and immunoblotting performed for total and phospho4EBP1.

(F–H) mTORC1 mediates the effects of PEPCK on glycolysis and proliferation.

(F) Colo205 Non-target and PEPCK knockdown cells were cultured in the presence of ¹³C glucose and the presence or absence of 10 nM rapamycin. The relative abundance of lactate m+3 was monitored by GC/MS.

(G) Colo205 Non-target and PEPCK knockdown cells were seeded in Seahorse Flux plates and treated with 10 nM rapamycin and glycolytic capacity determined. n = 10 ± SD.

(H) Colo205 non-target and PEPCK knockdown cells were seeded and then treated with rapamycin for 48 hr and cell number determined using an automated cell counter. n = 3 ± SD unless otherwise noted. *p < 0.05, **p < 0.001.

might be found in the tumor microenvironment. Therefore, we inhibited glutaminolysis, using AOA or BPTES, or glycolysis using 2DG, alone or in combination. PEPCK-expressing cells were less sensitive to growth inhibition by BPTES or AOA alone (Figures 6D–6G). Interestingly, PEPCK-expressing cells also appeared resistant to growth inhibition by 2DG. However, inhibition of both glycolysis and glutaminolysis in PEPCK-expressing cells had a synergistic effect on growth inhibition. The combination of 2DG with BPTES or AOA in PEPCK knockdown cells or cells without PEPCK did not have a similar synergistic effect. This supports a role for PEPCK in enabling cells to utilize both glucose and glutamine, especially when one of these nutrients is limiting.

PEPCK Promotes the Activation of mTORC1

Given the ability of PEPCK to promote glycolysis, we examined the expression of several glycolytic genes known to promote

tumor cell growth. Overall, we did not observe a difference in expression of any of these genes (Figures S5A and S5B). This raised the question as to how PEPCK regulates glycolysis. mTOR is well known for its role in nutrient sensing and metabolism. Despite the role of mTORC1 and mTORC2 in regulating metabolism, mTORC1 is more responsive to amino acid changes than mTORC2 (Sancak et al., 2008). Amino acids are essential for mTORC1 activation, with leucine and glutamine playing important roles in mTORC1 activation (Csibi et al., 2013; Durán et al., 2012; Jewell et al., 2015). Loss of PEPCK increased mTORC1 activity as judged by phosphorylation of eukaryotic translation initiation factor 4E binding protein 1 (4EBP1), S6kinase (S6K) and ribosomal S6 (S6) (Figures 7A and S5C). Conversely, overexpression of PEPCK led to the activation of mTORC1 as determined by increased phosphorylation of 4EBP1. We did not see any changes to mTORC2 activity as judged by phosphorylation of serine 473 on AKT (Figure S5D).

The lysosome plays a major role in mediating the full activation of mTORC1 since localization of mTOR with the lysosome is required for full mTORC1 activation (Sancak et al., 2010). Therefore, we investigated whether PEPCK altered the localization of mTOR with the lysosome. In PEPCK-expressing cells, we observed mTORC1 activation as evidenced by greater mTOR colocalization (yellow) with the lysosomal protein lysosome-associated membrane protein 2 (LAMP2), than in PEPCK knockdown cells (Figure 7B).

We also examined the metabolism of ^{13}C leucine in cells with altered PEPCK. However, we do not see a difference in leucine metabolism into the TCA cycle in cells expressing PEPCK compared to knockdown cells (Figure S5E). Next, we wanted to determine whether inhibiting glutamine utilization affected mTORC1 activation. Surprisingly, treatment of cells with BPTES or another inhibitor (Compound 968) had no effect on mTOR activation (Figure S5F; data not shown). Therefore, glutaminolysis does not appear responsible for mTORC1 activation. A more recent study showed that intracellular glutamine could activate mTORC1 (Jewell et al., 2015). Therefore, we examined glutamine levels in cells following incubation with [U5]- ^{13}C glutamine. The abundance of intracellular ^{13}C -labeled glutamine was greater in cells expressing PEPCK compared to PEPCK knockdown cells (Figure 7C). Similarly, ^{14}C glutamine uptake was reduced in PEPCK knockdown cells, whereas glutamine uptake was increased in cells overexpressing PEPCK (Figure 7D).

In order to determine whether glutamine is responsible for PEPCK-mediated mTORC1 activation, cells were cultured in the presence and absence of glutamine. The absence of glutamine decreased mTORC1 activation in general. However, mTORC1 activation was blunted to a much greater extent in PEPCK-expressing cells compared to PEPCK knockdown cells in the absence of glutamine (Figures 7E and S5G). Therefore, these data suggest that PEPCK-induced glutamine utilization does not affect mTORC1 activity; rather, its ability to increase cellular glutamine levels appears responsible for mTORC1 activation.

Next, we wanted to determine whether the activation of mTORC1 in PEPCK-expressing cells was mediating the observed effects on metabolism and proliferation. Initially, we examine the effect of rapamycin on glutamine metabolism. Despite previous studies showing that inhibition of mTOR decreases glutaminolysis, in our cells, we did not see an effect as determined by changes in the relative abundance of ^{13}C αKG and glutamate (Figures S5H and S5I). Next, we examined the effect of mTOR inhibition on PEPCK-induced glucose utilization. Unlike glutamine utilization, rapamycin treatment appeared to decrease glucose metabolism. The relative abundance of ^{13}C -labeled lactate in the media in PEPCK-expressing cells was decreased after treatment with rapamycin (Figure 7F). By contrast, we did not see a further reduction in relative abundance of ^{13}C -labeled lactate in the PEPCK knockdown cells treated with rapamycin. This effect was blunted in the PEPCK knockdown cells. To further corroborate these studies, we examined the effect of rapamycin on glycolytic capacity using the Seahorse Bioanalyzer. Rapamycin decreased glycolytic capacity in the NTshRNA Colo205 cells about 40% (Figure 7G). In contrast, in the PEPCK knockdown cells, rapamycin decreased glycolytic capacity was reduced only ~20%.

Finally, we wanted to determine whether the effect of PEPCK on cell proliferation involved mTORC1. NTshRNA and shPEPCK cells were incubated in the absence and presence of rapamycin for 48 hr and cell number was determined. Cell number was reduced almost 50% in NTshRNA cells treated with rapamycin compared to vehicle-treated NTshRNA cells (Figure 7H). In contrast, there was a small but insignificant decrease in cell number in PEPCK knockdown cells after treatment with rapamycin. Therefore, mTORC1 activation by PEPCK appears to increase glucose metabolism and proliferation but does not appear to alter glutamine metabolism.

DISCUSSION

PEPCK is most well-known for its role in GNG. However, its presence in non-GNG tissues suggests alternative roles for PEPCK. Indeed, the role of PEPCK in the intestinal epithelium is controversial (Previs et al., 2009). The intestinal epithelium appears to utilize glutamine for energy rather than GNG by oxidizing glutamine into the TCA cycle, a process that would be promoted by PEPCK (Yang et al., 2009). These studies highlight a role for PEPCK in driving cell growth by promoting anabolic metabolism and metabolic flexibility. PEPCK coordinates the ability of cells to take up and metabolize both glucose (glycolysis) and glutamine (glutaminolysis) for energy and anabolic purposes, features of tumor cells that require the TCA cycle.

Anabolic metabolism is recognized as crucial for tumor cell proliferation (Deberardinis et al., 2008b). We show that PEPCK promotes the utilization of glucose and glutamine for anabolic metabolism, including fatty acid and nucleic acids. Two of the main anabolic pathways driving tumor cell proliferation are lipogenesis and ribose synthesis. In order for cells to convert glucose or glutamine to fatty acids, they must enter the TCA cycle and be converted to citrate. Citrate then leaves the mitochondria via the citric acid transporter and is cleaved to OAA and acetyl CoA by ATP citrate lyase. Acetyl CoA is then used in the synthesis of a variety of lipids. De novo lipogenesis from glucose and glutamine play crucial roles in cancer supporting not only generation of biomass, but also bioactive lipids and lipid molecules driving signal transduction (Louie et al., 2013; Menendez and Lupu, 2007). Lipids can also serve as energy source allowing cancer cells to survive when under nutrient-deprived conditions (Zaugg et al., 2011). Therefore, the ability of PEPCK to promote lipid synthesis helps to coordinate a key feature of cancer metabolism.

The pentose phosphate pathway is responsible for ribose synthesis. Ribose synthesis serves two important roles. The oxidative branch of the PPP generates ribose and NADPH. NADPH is used as a biosynthetic precursor and antioxidant, protecting cancer cells from excessive ROS. The non-oxidative branch of the PPP generates ribose. These two sources of ribose can then be incorporated into nucleic acid for RNA and DNA synthesis. Glucose is the primary substrate for ribose synthesis. Surprisingly, we observed that loss of PEPCK reduced the incorporation of glucose into ribose. Even more surprisingly, we observed a role for PEPCK in promoting ribose synthesis from glutamine. Although the synthesis of ribose from glutamine and its regulation by PEPCK was an unexpected finding, the ability

of PEPCK to promote ribose synthesis from glutamine is not a far divergence from its classic metabolic role. PEPCK promotes the entry of carbons from non-carbohydrate sources into the glycolytic pathway via their conversion to PEP from OAA. Once PEP is generated, it is reasonable that carbons derived from PEP can enter the pentose phosphate pathway (Figure 4G). A role for glutamine as the amine donor in purine synthesis and carbon donor in pyrimidine synthesis is well established (Salzman et al., 1958). Therefore the ability to synthesize ribose from glutamine or even other glucogenic amino acids would provide additional sources for RNA and DNA synthesis.

Recent studies show that tumor cells display a high degree of metabolic flexibility. Therefore, a fascinating observation in our studies was the ability of PEPCK to promote the use of multiple carbon sources. This becomes especially important when the reduced nutrient availability of the tumor microenvironment is taken into account. This effect became evident when we inhibited glutaminolysis. PEPCK-expressing cells showed increased survival by increasing glucose utilization to meet their metabolic needs. Conversely, when glucose utilization was inhibited, cells expressing PEPCK were able to survive due to increased glutamine utilization. This is particularly relevant to the tumor microenvironment since glucose levels in tumors are approximately one-tenth of the level found in serum or normal tissue (Hirayama et al., 2009). Indeed, we observed greater incorporation of glutamine into ribose when glucose levels were reduced in PEPCK-expressing cells. Therefore, PEPCK can promote metabolic flexibility by promoting the use of glutamine and perhaps other non-carbohydrate sources for energy and anabolic metabolism, especially when nutrients are limiting, as is often seen in the tumor microenvironment.

We found PEPCK overexpressed or amplified in almost 20% of colorectal cancers from the CBioPortal. While the mechanism for overexpression is not clear, PEPCK is located on chromosome 20q13, which is frequently amplified in several epithelial derived cancers (Hidaka et al., 2000; Iwabuchi et al., 1995; Tanner et al., 1994). The significance of PEPCK amplification in colon cancer is underscored by our observation that increased 20q13 copy number drives higher expression of PEPCK in colon cancer, similarly to that observed with ZNF217, a known oncogenic driver gene of this region (Quinlan et al., 2007) (Figures S1F and S1G). This suggests that PEPCK might also be contributing the oncogenic role of 20q13 amplification. Although previous studies showed that PEPCK levels were reduced in colon-derived cancers, only a few cell lines or tumors were examined (Blouin et al., 2010). In contrast, we examined almost 200 colon tumors from the CBioPortal and about 100 colon tumors by IHC in the TMA.

There are two isoforms of PEPCK, cytosolic PEPCK and a mitochondrial isoform of PEPCK, which appear to have a comparable function (PEPCK2 or PCK2). We focused on the cytosolic isoform PEPCK since it is inducible, and as we show here, overexpressed in a significant number of colon cancers. Recent studies do show that PEPCK2 affects cell proliferation (Leithner et al., 2015; Mendez-Lucas et al., 2014). These effects were primarily seen under conditions of nutrient deprivation or stress pathway activation. Furthermore, the significance of those

studies is also unclear since less than 3% of colon cancers have increased expression of PEPCK2, and there were no amplifications. It remains to be seen whether PEPCK and PEPCK2 have similar effects on metabolism.

One of our unexpected findings was the ability of PEPCK to increase glycolysis. Our data suggest two potential related mechanisms. Cells must maintain the TCA cycle in order to survive. PEPCK is one of the main cataplerotic enzymes of the TCA cycle and will promote the net efflux of TCA cycle intermediates. In order for the TCA cycle to function and maintain cell survival, anaplerotic reactions are required to compensate for the cataplerotic action of PEPCK. The siphoning off of intermediates and their incorporation into anabolic molecules (such as lipids and ribose) further reduces carbon availability to replenish the TCA cycle. Therefore, cells will increase the oxidative and anaplerotic entry of glucose into the TCA cycle. Support for the glycolytic flux is supported by our data showing that when glutamine anaplerosis is blocked, PEPCK-expressing cells increase glycolysis and glucose utilization into the TCA cycle.

While mass action may explain in part the increased glucose flux, another surprising finding was the ability of PEPCK to regulate mTORC1 activity. Although glutaminolysis can activate mTORC1, inhibiting glutamine metabolism did not affect mTORC1 activity nor blunt the PEPCK-mediated increase in mTORC1 activity. Our data suggest that regulation of mTORC1 activity by PEPCK is at the level of intracellular glutamine levels. PEPCK increased intracellular glutamine levels and removal of glutamine from the media blunted the effect of PEPCK on mTORC1 activation. This is in line with a recent study showing that glutamine itself can activate mTORC1 (Jewell et al., 2015). Currently, we are investigating how PEPCK increases intracellular glutamine levels, leading to mTORC1 activation.

Tumor cells are under significant nutrient stress. The metabolic flexibility of tumor cells to utilize different nutrients provides them with a strong advantage in the face of changing nutrient environment to meet bioenergetic, anabolic, and signaling needs. The TCA cycle represents a key nodal point in metabolism linking cellular carbohydrate, amino acid, and bioenergetics, with anabolic and catabolic pathways. This enables cancer cells to promote metabolic flexibility and sustain tumor growth. PEPCK is a key enzyme regulating TCA cycle flux. In summary, our studies show that PEPCK promotes anabolic metabolism from glucose and glutamine to support tumor growth. In addition, we demonstrate a role for PEPCK in activating mTORC1 in part via its ability to regulate amino acid metabolism.

EXPERIMENTAL PROCEDURES

Cell Culture

All cancer cell lines were obtained from ATCC except the Moser colon cancer cell line, which were a gift from Dr. Bruce Spiegelman. Additional information can be found in the [Supplemental Experimental Procedures](#).

Analysis of PEPCK Expression in Human Tumors

Expression profiling array data from a published study was used to analyze the PEPCK expression in multiple tumors. Immunohistochemistry was performed on a commercial TMA by the Stony Brook Histopathology Core. Additional information can be found in the [Supplemental Experimental Procedures](#).

Growth Assay

Cells were seeded at 10,000–25,000 cells/well in 6-well plates, and allowed to grow at 37°C, 5% CO₂. For clonogenic assay, cells were seeded at 2,000–5,000 cells/well in 6-well plates and allowed to grow at 37°C, 5% CO₂. Cells were fixed and stained using crystal violet and colony counting performed using ImageJ. Additional information can be found in the [Supplemental Experimental Procedures](#).

Animal Work

Animal experiments were performed according to procedures approved by the University of Maryland, Baltimore and Stony Brook University IACUC. Additional information can be found in the [Supplemental Experimental Procedures](#).

¹³C Tracer Studies

Cells were seeded in triplicate into 100 mm or 60 mm dishes and allowed to grow overnight. [U-¹³C₅] glutamine or [U6-¹³C₆] glucose (Cambridge Isotope Labs) were used as tracers since they provide excellent analysis of overall central carbon metabolism and in particular the TCA cycle. Additional information can be found in the [Supplemental Experimental Procedures](#).

Gas Chromatography/Mass Spectrometry

Mass spectral data were obtained on the HP5973 mass selective detector connected to an HP6890 gas chromatograph. Additional information can be found in the [Supplemental Experimental Procedures](#).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at <http://dx.doi.org/10.1016/j.molcel.2015.09.025>.

AUTHOR CONTRIBUTIONS

E.D.M., R.D., and K.B., Conducted and helped design experiments and were involved with the writing of the manuscript. O.U., B.J.H., A.E.R., C.G., W.-J.L., and J.S., Conducted experiments. L.G.B., Analysis of metabolic data and manuscript preparation. W.T., Pathological analysis. K.R.S., Pathological analysis, tumor scoring, and involvement with manuscript preparation. R.S.P., S.D., and R.D., Assisted with analysis and interpretation of gene expression data. R.J.D., Helped design metabolic studies and was involved in manuscript preparation. J.M.R. and M.B.B., Helped with the drug design studies and other aspects of medicinal chemistry. K.-K.W., Helped with interpretation of data and guidance on studies. G.D.G., Oversaw direction of project, conducted experiments, interpretation of data, and wrote manuscript.

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