Mitochondrial Phospho-enolpyruvate Carboxykinase Regulates Metabolic Adaptation and Enables Glucose-Independent Tumor Growth

Graphical Abstract

Highlights
- Tumor cell metabolic flexibility enables glucose-independent cell proliferation
- PCK2 helps generate glycolytic intermediates under low-glucose conditions
- PCK2 expression is regulated by glucose and required for in vivo tumor growth
- PCK2 expression is elevated in non-small-cell lung carcinoma (NSCLC)

Authors
Emma E. Vincent, Alexey Sergushichev, Takla Griss, ..., Douglas J.E. Elder, Maxim N. Artyomov, Russell G. Jones

Correspondence
martyomov@pathology.wustl.edu (M.N.A.), russell.jones@mcgill.ca (R.G.J.)

In Brief
Cancer cells adapt metabolically to maintain proliferation under nutrient limitation. Vincent et al. demonstrate that cancer cells can engage the early steps of gluconeogenesis—a process regulated by mitochondrial PEPCK (PCK2)—to generate biosynthetic intermediates required for tumor cell proliferation.

Accession Numbers
GSE66556
Mitochondrial Phosphoenolpyruvate Carboxykinase Regulates Metabolic Adaptation and Enables Glucose-Independent Tumor Growth

Emma E. Vincent, 1, 2 Alexey Sergushichev, 3, 4 Takla Griss, 1, 2 Marie-Claude Gingras, 1, 5 Bozena Samborska, 1, 2 Thierry Ntimbane, 1, 6 Paula P. Coelho, 1, 2 Julianna Blagih, 1, 2 Thomas C. Raissi, 1, 2 Luc Choinière, 1,6 Gaëlle Bridon, 1, 6 Ekaterina Loginicheva, 4 Breanna R. Flynn, 1, 2 Elaine C. Thomas, 7 Jeremy M. Tavaré, 7 Daina Avizonis, 1, 6 Arnim Pause, 1, 5 Douglas J.E. Elder, 7 Maxim N. Artyomov, 4,* and Russell G. Jones 1, 2,*

1 Goodman Cancer Research Centre, McGill University, Montreal, QC H3A 1A3, Canada
2 Department of Physiology, McGill University, Montreal, QC H3G 1Y6, Canada
3 ITMO University, Saint Petersburg 197101, Russia
4 Department of Pathology and Immunology, Washington University in St. Louis, MO 63110, USA
5 Department of Biochemistry, McGill University, Montreal, QC H3G 1Y6, Canada
6 Metabolomics Core Facility, Goodman Cancer Research Centre, McGill University, Montreal, QC H3A 1A3, Canada
7 School of Biochemistry, Medical Sciences Building, University of Bristol, Bristol BS8 1TD, UK
* Correspondence: martyomov@pathology.wustl.edu (M.N.A.), russell.jones@mcgill.ca (R.G.J.)
http://dx.doi.org/10.1016/j.molcel.2015.08.013

SUMMARY

Cancer cells adapt metabolically to proliferate under nutrient limitation. Here we used combined transcriptional-metabolomic network analysis to identify metabolic pathways that support glucose-independent tumor cell proliferation. We found that glucose deprivation stimulated re-wiring of the tricarboxylic acid (TCA) cycle and early steps of gluconeogenesis to promote glucose-independent cell proliferation. Glucose limitation promoted the production of phosphoenolpyruvate (PEP) from glutamine via the activity of mitochondrial PEP-carboxykinase (PCK2). Under these conditions, glutamine-derived PEP was used to fuel biosynthetic pathways normally sustained by glucose, including serine and purine biosynthesis. PCK2 expression was required to maintain tumor cell proliferation under limited-glucose conditions in vitro and tumor growth in vivo. Elevated PCK2 expression is observed in several human tumor types and enriched in tumor tissue from non-small-cell lung cancer (NSCLC) patients. Our results define a role for PCK2 in cancer cell metabolic reprogramming that promotes glucose-independent cell growth and metabolic stress resistance in human tumors.

INTRODUCTION

One of the primary metabolic phenotypes observed in transformed cells is an increase in aerobic glycolysis (the “Warburg effect”), which is marked by increased glucose uptake and lactate production. Glucose is used to generate ATP via glycolysis or mitochondrial oxidative phosphorylation (OXPHOS) (DeBerardinis et al., 2008a), whereas glycolytic intermediates also function as key substrates for macromolecular biosynthesis, supplying carbon for the synthesis of non-essential amino acids (NEAAs), intermediates of one-carbon metabolism, nucleotides, and lipids (Locasale, 2013; Lunt and Vander Heiden, 2011). Another key nutrient for proliferating cancer cells is the NEAA glutamine. Proliferating cells maintain tricarboxylic acid (TCA) cycle function using glutamine as an anapleurotic substrate to generate mitochondrial α-ketoglutarate (α-KG) and subsequent biosynthetic intermediates (DeBerardinis et al., 2007). Glutamine enables the TCA cycle to function both as a fuel source for ATP production and as a biosynthetic hub (Ahn and Metallo, 2015; DeBerardinis et al., 2008b).

Much of our understanding of tumor cell metabolism is based on the metabolic programs engaged by cancer cells in vitro, where nutrient availability is in excess. However, glucose concentrations in tumors can be limiting—3- to 10-fold lower than in non-transformed tissues (Birsoy et al., 2014; Hirayama et al., 2009) — because of a combination of reduced tumor vascularization and high rates of glucose consumption by cancer cells. Therefore, tumor cells must enact strategies to grow and survive in metabolically unfavorable environments.

Under limited-glucose conditions, cells capable of engaging OXPHOS have a greater capacity to proliferate (Birsoy et al., 2014; Chen et al., 2015). Glutamine can partially compensate for glucose to maintain TCA cycle function under conditions of metabolic stress. Acetyl-coenzyme A (CoA) can be generated from glutamine through reductive carboxylation of α-KG under conditions of hypoxia or reduced mitochondrial function (Metallo et al., 2012; Mullen et al., 2012; Wise et al., 2011). Similarly, TCA cycle function is maintained in glucose-deprived cells in part through the production of glutamine-derived pyruvate, which can be converted to acetyl-CoA and oxidized in the mitochondria (Blagih et al., 2015; Le et al., 2012; Yang et al., 2009, 2014). However, the efficiency of OXPHOS and TCA cycle maintenance...
cannot fully account for cell proliferation under glucose limitation because glycolytic intermediates are still required to generate the biosynthetic precursors essential for cell division.

Here we used metabolic and transcriptional profiling to characterize pathways of glucose-independent tumor cell growth. Using this systems-level analysis, we identified alterations in glutamine metabolism and the early steps of gluconeogenesis as key metabolic adaptations for tumor cells to proliferate under low-glucose conditions. Here we describe a role for mitochondrial phosphoenolpyruvate (PEP) carboxykinase (PEPCK-M or PCK2) in mediating tumor cell adaptation to glucose limitation, which facilitates tumor growth in vivo.

RESULTS

Network-Based Data Integration Reveals Distinct Metabolic Nodes in Tumor Cells Adapted to Glucose-Independent Proliferation

To gain insight into how cellular proliferation can be maintained under glucose-free conditions, we examined the proliferation of the non-small-cell lung cancer (NSCLC) cell lines A549 and H1299, which are capable of proliferating in medium lacking glucose but not glutamine (Figure 1A). We performed a systems-level analysis of intracellular metabolites isolated from A549 cells cultured for 48 hr in the absence of glucose. The relative metabolite abundance in glucose-starved A549 cells is shown in Figure S1. Metabolite set enrichment analysis (MSEA) of these data revealed enrichment in metabolites involved in amino acid degradation (ammonia cycling, urea cycle), the TCA cycle, glutamate metabolism, and gluconeogenesis (Figure 1B). We next integrated RNA sequencing (RNA-seq)-based transcriptional profiling with our metabolic profiling data to construct a network-based analysis of possible metabolic fluxes (Jha et al., 2015). To trace all possible metabolic transformations of the labeled atoms, we constructed a metabolic network where individual nodes are individual carbon atoms connected by edges (connecting lines) corresponding to chemical reactions (totaling about 30,000 edges). From this, we identified a subnetwork based on connectivity and changes in metabolite abundance and metabolic enzyme gene expression under conditions of glucose deprivation (Figure 1C).

From this network-based analysis, we identified five major metabolic modules regulated differentially in A549 cells undergoing glucose-independent proliferation: glycolysis, the TCA cycle, glutamate/proline metabolism, inositol metabolism, and serine metabolism (Figure 1C). Nodes for glycolysis and lactate production were, as expected, downregulated significantly in A549 cells cultured under glucose-free conditions. Conversely, genes involved in inositol and proline metabolism were upregulated selectively under glucose withdrawal, whereas the intracellular concentration of several amino acids (serine, aspartate, asparagine, tryptophan, and glycine) was also elevated. MSEA revealed enrichment in metabolites involved in glutamate metabolism and gluconeogenesis (Figure 1B). This is noteworthy because glutamine is a known gluconeogenic substrate (Stark and Kibbey, 2014). There is increased transamination of glutamate to α-KG (through ASNS and BCAT1), which is consistent with the increased glutamine dependence of A549 cells. The network also highlighted a significant upregulation in the conversion of oxaloacetate (OAA) to both aspartate (via GOT1) and PEP (via the gluconeogenic enzyme PCK2). Finally, increased levels of serine and glycine were coupled with transcriptional upregulation of phosphoglycerate dehydrogenase (PHGDH), an enzyme that directs glycolytic intermediates toward the serine biosynthesis pathway (Locasale, 2013). Taken together, these data suggest an active role for glutamine as both a carbon and nitrogen source for amino acid metabolism, which may be achieved through the malate-aspartate shuttle and early steps of gluconeogenesis under low-glucose conditions.

Glutamine Maintains TCA Cycle Metabolism and Levels of the Glycolytic Intermediate PEP under Glucose-Free Conditions

Our transcriptional-metabolic network analysis indicated increased levels of glutamine metabolism in A549 cells proliferating in the absence of glucose. To characterize how glutamine is metabolized under glucose-free conditions, A549 cells were cultured in the presence of uniformly labeled 13C-glutamine (U-[13C]-Q). Conventional metabolism of U-[13C]-glutamine in tumor cells is illustrated in Figure 2A. Similar 13C labeling patterns were observed in glutamate and TCA cycle intermediates (α-KG, succinate, fumarate, and malate) under glucose-free and glucose-replete conditions (Figure 2B), indicating that glutamine continues to be used as an anaplerotic substrate for the TCA cycle when glucose is limited. Levels of 13C-glutamine-derived glutamate, succinate, and aspartate were increased under glucose withdrawal conditions, whereas the levels of many of the TCA cycle metabolites decreased under glucose withdrawal (Figure S2A), consistent with the network analysis in Figure 1C.

Citrate production from glutamine was altered strikingly in A549 cells under glucose withdrawal. We observed a significant increase in the proportion of the citrate pool labeled from U-[13C]-glutamine, as shown by the large decrease in unlabeled citrate under glucose-free conditions (Figure 2C). The increased labeling of citrate from U-[13C]-glutamine was due to increases in both reductive carboxylation of α-KG (m+5 citrate) and the emergence of a fully labeled citrate molecule (m+6 citrate) (Figure 2C). Levels of m+6 citrate were negligible in A549 cells grown in the presence of glucose but had not yet reached steady-state levels after 6 hr of labeling with U-[13C]-glutamine under glucose-free conditions (Figure 2D).

Formation of m+6 citrate can occur when U-[13C]-glutamine is used to generate both OAA and acetyl-CoA, the latter requiring the formation of m+3 pyruvate from 13C-glutamine (Figure S2B). It has been shown previously that the decarboxylation of glutamine-derived malate (m+4) can generate pyruvate m+3 through the activity of the malic enzyme (Guay et al., 2007; Yang et al., 2014). Alternatively, PEPCK can generate PEP (m+3) from OAA, followed by conversion of PEP to pyruvate by pyruvate kinase (PK). We found that, although the overall abundance of pyruvate was lower in A549 cells growing under glucose-free conditions, the majority of pyruvate (m+3) in these cells was derived from 13C-glutamine (Figure 2E). In addition, we observed a significant increase in U-[13C]-glutamine-derived PEP specifically in A549 cells growing under glucose-free conditions (Figure 2F).
Figure 1. Network-Based Data Integration Reveals Distinct Metabolic Nodes in Tumor Cells Adapted to Glucose-Independent Proliferation

(A) Proliferation of A549 and H1299 cells over 96 hr in either nutrient-replete medium (+Glc, +Q), medium without glucose (−Glc, +Q), or medium without glutamine (+Glc, −Q). Data are represented as mean ± SEM for biological replicates (n = 5).

(B) Metabolite set enrichment analysis for differentially regulated metabolic pathways induced upon glucose deprivation. A549 cells were cultured in the presence or absence of glucose for 48 hr before metabolite abundance was determined by GC- or LC-MS (see the full metabolite profile in Figure S1).

(C) Integrated metabolic network analysis for A549 cells cultured in the presence (Glc+) or absence (Glc−) of glucose for 48 hr. The direction and magnitude of the fold change in enzyme expression or metabolite abundance between conditions is indicated on a red (enriched in Glc−) to green (enriched in Glc+) color scale. Enzymes are represented by edges (connecting lines between metabolites), with the color of the edge indicating the fold change and the thickness reflecting the significance of differential expression. Round nodes represent metabolites, with the differential abundance of each metabolite indicated by the size of the node. Major features of glucose deprivation identified by the network analysis are highlighted by bold titles and background shading.
Consistent with the network analysis (Figure 1C), lactate production was downregulated significantly in glucose-starved A549 cells (Figure S2C), and U-[13C]-glutamine was not used for its synthesis. In contrast, glutamine was used in the synthesis of both pyruvate and PEP (Figure S2D). H1299 cells displayed similar increases in m+6 citrate, m+3 pyruvate, and m+3 PEP derived from [13C]-glutamine specifically when growing under conditions of glucose withdrawal (Figure 2G). The presence of pyruvate m+3 and citrate m+6 suggests that these cells are undergoing pyruvate cycling (Yang et al., 2014), using glutamine to maintain TCA function when glucose is unavailable.

**PCK2 Is Required for the Production of Glutamine-Derived PEP**

Our RNA-seq results indicated that expression of the mitochondrial form of PEPCK (PCK2) was increased in A549 cells upon glucose withdrawal. However, no sequencing reads for PCK1, the cytosolic form of PEPCK, were detected (data not shown). Consistent with this, silencing PCK1 in A549 cells did not affect total PEPCK protein levels (Figure S3A). These data suggest that PCK2 is the sole PEPCK isoform expressed in A549 cells, in agreement with recent findings (Leithner et al., 2014).

We next investigated the role of PCK2 in glutamine metabolism by silencing PCK2 activity in A549 and H1299 cells using RNAi (Figure 3A) or 3-mercaptopicolinic acid (MPA), a specific inhibitor of PEPCK (Urbina et al., 1990). Conversion of U-[13C]-glutamine to PEP (Figure 3B) was stimulated by glucose deprivation as before but was reduced markedly in cells expressing PCK2 short hairpin RNA (shRNA) or treated with MPA (Figure 3C). Glutamine flux into PEP was also ablated in glucose-starved A549 cells expressing PCK2 shRNA (Figure S3B). This effect appeared to be specific to the formation of PEP because inhibiting PCK2 did not significantly affect...
Figure 3. PEP Production from Glutamine in Glucose-Starved Cells Is Dependent on PCK2

(A) Immunoblot for PCK2 and actin on lysates from A549 and H1299 cells expressing control or PCK2 shRNA. PCK1(1) and PCK2(2) represent two different shRNAs targeting PCK2.

(B) Schematic for proposed PCK2-mediated pyruvate cycling. Fully labeled OAA (m+4) is made from U-[13C]-Q. PCK2 activity produces fully labeled PEP (m+3) from OAA. Pyruvate can be made using PEP through the activity of pyruvate kinase, and this can re-enter the TCA cycle as acetyl-CoA.

(C) Relative abundance of U-[13C]-Q incorporation into PEP in A549 and H1299 cells expressing control or PCK2 shRNAs or upon treatment with the PEPCK inhibitor MPA. Cells were cultured in the presence or absence of glucose for 12 hr, with U-[13C]-Q added for the last 6 hr of incubation. Far right: A549 cells were treated with either DMSO (vehicle) or MPA throughout the 12-hr incubation. Con, control.

(D) Relative abundance of U-[13C]-Q incorporation into pyruvate in A549 and H1299 cells expressing control or PCK2 shRNA or upon treatment with the PCK2 inhibitor MPA. Cells were incubated as in (C).

(C and D) Data are represented as mean ± SEM of three independent cultures. See also Figure S3.
TCA cycle anaplerosis from glutamine in A549 cells regardless of glucose availability (Figure S3C).

To address whether PCK2 contributed to pyruvate cycling in A549 cells growing under glucose limitation (Figure 3B), we measured the formation of m+3 pyruvate (Figure 3D) and m+6 citrate (Figure S3D) in cells expressing PCK2 shRNA. A549 cells expressing PCK2 shRNA displayed reduced labeling of pyruvate (m+3, Figure 3D) and citrate (m+6, Figure S3D) from U-[13C]-glutamine specifically under conditions of glucose withdrawal. Similarly, MPA treatment reduced the production of m+3 pyruvate from U-[13C]-glutamine in A549 cells growing in the absence of glucose (Figure 3D).

**Glutamine-Derived PEP Is Used as a Biosynthetic Intermediate under Low-Glucose Conditions**

The reduced labeling of m+3 pyruvate and m+6 citrate upon PCK2 inhibition (Figure 3D; Figure S3D) was modest compared with the large reduction in m+3 PEP observed in NSCLC cells expressing PCK2 shRNA (Figure 3C), suggesting that glutamine-derived PEP may be formed for a purpose other than pyruvate cycling. Our network analysis indicated a specific increase in the serine biosynthesis pathway in glucose-deprived A549 cells (Figure 1C). Therefore, we assessed 13C enrichment in serine and glycine in A549 cells cultured with U-[13C]-glutamine under glucose-free conditions. 13C-glutamine-derived carbon was detected in serine (Figure 4A; Figure S4A) and glycine (Figure 4B; Figure S4A) from A549 cells undergoing glucose-independent proliferation. H1299 cells demonstrated a similar shift toward glutamine-dependent serine and glycine biosynthesis under glucose-free conditions, indicating that glutamine can substitute for glucose as a carbon source for serine biosynthesis (Figures 4C and 4D; Figure S4B). The contribution of 13C-glutamine-derived carbon to the serine and glycine pools of glucose-starved A549 cells increased exponentially over time and did not reach steady-state labeling over the 6-hr labeling period (Figures 4A and 4B). Indeed, the contribution of 13C-glutamine-derived carbon to cellular serine and glycine pools reached 40% and 20%, respectively, when glucose-starved A549 cells were labeled continuously with 13C-glutamine for 48 hr (Figures S4C and S4D).

Next we addressed whether PCK2 could regulate the production of 13C-glutamine-derived serine and glycine in tumor cells. Silencing PCK2 in A549 cells ablated the ability of glucose-starved cells to produce serine (Figure 4E) and glycine (Figure 4F) from U-[13C]-glutamine. 13C-glutamine flux into the serine biosynthesis pathway was ablated completely in glucose-starved cells expressing PCK2 shRNA (Figures S4E and S4F). Treatment of A549 cells with MPA also blocked glutamine-dependent production of serine (Figure 4G).

We hypothesized that PEP generated from OAA is exported from the mitochondria (Passarella et al., 2003; Satrustegui et al., 2007), converted by enolase to 3-phosphoglycerate (3-PG), and then directed into the serine biosynthesis pathway by the activity of PHGDH (Figure 4H). To test this, we measured 13C enrichment in serine (from the U-[13C]-glutamine tracer) in A549 cells with enolase or PHGDH expression silenced using small interfering RNAs (siRNAs) (Figure S4G). Although levels of 13C-labeled serine from U-[13C]-glutamine increased significantly upon glucose deprivation in control cells, cells transfected with siRNAs targeting ENO1 or PHGDH displayed a marked reduction in glutamine-dependent serine biosynthesis (Figure S4H).

Glucose-derived serine and glycine contribute to the synthesis of purine nucleotides required for cell proliferation (Lunt and Vander Heiden, 2011). Therefore, we investigated whether glutamine could substitute for glucose in supplying carbon for purine biosynthesis by measuring 13C enrichment from U-[13C]-glutamine in the purine nucleotide ATP. We detected a significant increase in the abundance of glutamine-derived ATP in cells grown under glucose-free conditions, which was blocked in cells treated with MPA (Figure 4I). An increased proportion of ATP (~12%) was labeled from U-[13C]-glutamine, with the majority of the labeling at m+3 (Figure 4J).

**PCK2 Is Required for Glucose-Independent Cancer Cell Proliferation and Tumor Growth In Vivo**

Silencing PCK2 by siRNA (Figure 5A) or shRNA (Figure S5B) blocked the ability of A549 and H1299 cells to proliferate in the absence of glucose. Consistent with the requirement of enolase and PHGDH to mediate glutamine–dependent serine biosynthesis (Figure S4H), knockdown of these enzymes also reduced the proliferation of A549 cells under glucose-free conditions (Figure 5B). Cell viability was unaffected by PCK2, ENO1, or PHGDH knockdown regardless of glucose availability (Figure S5C).

To assess the impact of PCK2 on tumor growth in vivo, A549 and H1299 cells expressing either control or PCK2-specific shRNAs (Figures S5D and S5F) were injected into the flanks of nude mice, and tumor growth was assessed over 65 days (Figures 5C and 5E). The majority of tumors expressing control shRNAs grew steadily over time. However, tumors expressing PCK2 shRNA failed to establish and grow substantially in vivo (Figures 5C and 5E). This was reflected in the reduced weight of PCK2 shRNA-expressing tumors at the end of the experiment (Figures 5D and 5F). PCK2 protein expression was readily detected in end-stage control tumors, whereas suppression of PCK2 protein levels was maintained in tumors derived from cells expressing PCK2 shRNA (Figures S5E and S5G).

**The Hypoxia-Inducible Factors HIF-1α and EPAS1 Regulate PCK2 Expression under Glucose Limitation**

We next examined the mechanisms of PCK2 regulation by glucose availability. Expression of both PCK2 mRNA (Figure 6A) and protein (Figure 6B) was induced in NSCLC cells under conditions of glucose limitation. The activity of PCK2 is also dependent on the production of mitochondrial guanosine triphosphate (mtGTP). mtGTP is generated by the SUCLG2 form of succinyl-CoA synthetase (SCS) in the TCA cycle (Stark et al., 2009). We found that the expression of the SUCLG2 transcript (Figure 6C) and its metabolite product succinate (Figure S2A) was elevated in glucose-starved A549 cells. Notably, the expression of SUCL2A, the ATP-generating form of SCS, was not affected by glucose availability (Figure 6C). Consistent with the requirement of SUCLG2 expression for PCK2 activity, silencing of SUCLG2 (Figure S6A) led to a reduction in the glucose-independent proliferation of A549 cells (Figure 6D), similar to what was observed when PCK2 was silenced.
Figure 4. Serine, Glycine, and ATP Are Made from Glutamine upon Glucose Withdrawal in a PCK2-Dependent Manner

(A–D) U-[13C]-Q incorporation into serine and glycine in glucose-starved cells. A549 (A and B) or H1299 (C and D) cells were cultured for 12 hr in the presence or absence of glucose, followed by culture with U-[13C]-Q for the last 6 hr of the 12-hr incubation. Shown is the proportion of the serine (A) or glycine (B) pools labeled by U-[13C]-Q in A549 cells over time. Levels of U-[13C]-Q-derived serine (C) and glycine (D) in glucose-starved H1299 cells is also shown.

(E–G) Relative abundance of U-[13C]-Q incorporation into serine (E and G) and glycine (F) in A549 cells expressing control and PCK2 shRNA (E and F) or upon treatment with MPA (G). Cells were cultured in the presence or absence of glucose for 12 hr and then cultured with U-[13C]-Q for the last 6 hr of the 12-hr incubation.

(H) Schematic of PCK2-mediated labeling of serine from OAA. Fully labeled OAA (m+4) is made from U-[13C]-Q. PCK2 activity produces fully labeled PEP (m+3) from OAA. Serine and glycine can be made using PEP through the activities of enolase and PHGDH. Eno, enolase.

(I) Abundance of 13C-ATP in A549 cells cultured with U-[13C]-Q for 48 hr in the presence or absence of glucose. Cells were treated with either DMSO (vehicle) or MPA throughout the 48-hr incubation.

(J) Mass isotopologues of the ATP pool in A549 cells cultured as in (I). Data are represented as mean ± SEM of three independent cultures.

See also Figure S4.

(Figure 5A). Silencing SNUCLA2 did not affect the glucose-independent proliferation of A549 cells (Figure 6D; Figure S6A).

The hypoxia-inducible transcription factors HIF-1α and EPAS1/EPAS1 prevent PCK2 expression in K-ras mutant tumor cells (Chun et al., 2010). To test the role of HIF-1α and EPAS1 in glucose-dependent control of PCK2 expression in our system, these genes were silenced in A549 cells (Figure S6B). Glucose-dependent stimulation of PCK2 mRNA was not altered in A549 cells expressing HIF-1α siRNA and only partially reduced upon EPAS1 knockdown (Figure S6C). However, silencing both HIF-1α and EPAS1 led to a ~25% reduction in PCK2 mRNA expression. Similarly, silencing both HIF-1α and EPAS1 prevented the full induction of PCK2 protein by glucose withdrawal (Figure 6E).

Finally, we assessed the impact of HIF expression during glucose-independent proliferation. Knockdown of HIF-1α or EPAS1 alone had minimal effects on cell proliferation, whereas silencing both impaired the ability of A549 cells to proliferate under glucose-free conditions (Figure 6F).
PCK2 Expression Is Deregulated in Human Cancer

We next investigated PCK2 expression in human tumors through analysis of The Cancer Genome Atlas (TCGA) expression datasets for a variety of tumor types. PCK2 mRNA expression was increased in a variety of tumor types (Figure 7A). The highest PCK2 expression was found in thyroid, bladder, breast, kidney, and non-small-cell lung cancer tissue (Figure 7A). We next asked whether elevated PCK2 expression was found in thyroid, bladder, breast, kidney, and non-small-cell lung cancer tissue (Figure 7A). The highest PCK2 sets for a variety of tumor types.

We also assessed PCK2 protein levels in tumor samples isolated from human NSCLC patients. Examples of PCK2 protein expression in normal and tumor tissue from the lungs of seven NSCLC patients are shown in Figure 7B, whereas Figure S7 presents PCK2 protein levels in normal and tumor tissue from each of the 29 patients analyzed. Quantification of PCK2 protein expression in these primary human lung cancer samples revealed a significant increase in PCK2 protein levels in 15 of 29 (52%) lung tumors (at p < 0.05, Kruskal-Wallis test) compared to patient-matched normal tissue (Figure 7C) and compared to a decrease in PCK2 expression in only 10% of tumors (3 of 29, p < 0.05). Together, these data indicate that PCK2 expression is enriched in a number of tumor types, including NSCLC.

DISCUSSION

Glucose is an important carbon source for proliferating cells and is used to generate both ATP and precursors for macromolecular synthesis (Lunt and Vander Heiden, 2011). However, reduced glucose availability combined with the high demand for nutrients by cancer cells can lead to metabolic stress in tumors (Cantor and Sabatini, 2012; Jones and Thompson, 2009). Mounting evidence indicates that cancer cells and some non-malignant cells can engage glucose-independent metabolism to maintain cell proliferation and viability when glucose is limited (Birsoy et al., 2014; Blagih et al., 2012; Jones and Thompson, 2009). Mounting evidence indicates that cancer cells and some non-malignant cells can engage glucose-independent metabolism to maintain cell proliferation and viability when glucose is limited (Birsoy et al., 2014; Blagih et al., 2015; Glick et al., 2014; Le et al., 2012; Pasto et al., 2014). Tumor cells capable of using alternate substrates for biosynthetic and bioenergetic needs would gain a survival and proliferative advantage in microenvironments with limited resources. Using a systems-level analysis combining both transcriptional and metabolic profiling data, we identified the gluconeogenic enzyme PCK2 as a key regulator of tumor cell metabolic flexibility. We demonstrate here that PCK2 mediates a metabolic shunt in response to glucose deprivation that supplies carbon from glutamine, rather than glucose, to generate glycolytic pathway intermediates required for biosynthesis and cell proliferation. Our data implicate PCK2-dependent metabolic reprogramming as a mechanism for glucose-independent tumor cell growth.

TCA cycle metabolism and OXPHOS are essential for maintaining energetic homeostasis in glucose-starved cells. This is achieved in part by glutamine-dependent pyruvate cycling through malic enzyme activity (Guay et al., 2007). Our data indicate that pyruvate cycling can also be mediated by PCK2 in glucose-starved tumor cells, similar to that observed in pancreatic β cells (Stark et al., 2009). However, silencing PCK2 in glucose-starved A549 cells only modestly affected pyruvate cycling, suggesting alternate fates for PEP in tumor cells.

Figure 5. Cancer Cells Require PCK2 to Maintain Glucose-Independent Proliferation and Tumor Growth In Vivo

(A) Proliferation of A549 and H1299 cells in glucose-free media over 96 hr following transfection with control or PCK2 siRNA.

(B) Proliferation of A549 cells in glucose-free media over 96 hr following transfection with the indicated siRNAs.

(A and B) Each data point in (A) and (B) represents the average cell number of five wells in a 384-well plate.

(C–F) A549 and H1299 cells were injected into the flanks of nude mice. Growth of tumors derived from A549 cells expressing either control (n = 6) or shRNA targeting PCK2 (n = 7) (C) and from H1299 cells expressing either control (n = 7) or shRNA targeting PCK2 (n = 7) (D) is shown over 65 days. Tumor weights at the end of the experiment are also shown for A549 (D) and H1299 (F) cells. Data are represented as mean ± SEM. **p < 0.01, ***p < 0.001.

See also Figure S5.
found that PCK2-dependent production of PEP could also provide tumor cells growing in a low-glucose environment with a supply of glycolytic intermediates needed to maintain cell proliferation.

Serine biosynthesis is a key metabolic pathway for cell proliferation, contributing carbon to many anabolic processes, such as protein, glutathione, nucleotide, and phospholipid biosynthesis (Locasale, 2013; Vander Heiden et al., 2011). Under standard growth conditions, either exogenous serine or serine generated de novo from the glycolytic intermediate 3-PG can be used for anabolic growth (Chaneton et al., 2012; Labuschagne et al., 2014; Locasale et al., 2011). Here we demonstrate that glutamine can substitute for glucose in serine and glycine biosynthesis in tumor cells with elevated PCK2 expression. Interestingly, despite the availability of exogenous serine and glycine, PHGDH was still required for tumor cell proliferation under low-glucose conditions, suggesting that flux through this pathway contributes to cell growth even when the end products of the pathway are plentiful. PHGDH amplification is observed in some cancers (Locasale et al., 2011; Possemato et al., 2011), which may provide metabolic flexibility to tumors by fueling serine biosynthesis from multiple carbon sources.

Our results also indicate that glutamine can be used as a carbon source for nucleotide biosynthesis under conditions of glucose deprivation. Two carbons in purine nucleotides are derived from glycine, and two one-carbon units are provided by N-14-formyl-tetrahydrofolate, which acquires most of its carbon units from serine (Lunt and Vander Heiden, 2011). Therefore, de novo serine biosynthesis could account for the 15C-glutamine labeling patterns we observed in ATP. Another possibility is that 15C-glutamine-derived glyceraldehyde 3-phosphate enters the non-oxidative arm of the pentose phosphate pathway to generate 5-phosphoribosyl-α-pyrophosphate (PRPP) for nucleotide biosynthesis. It is unclear whether glutamine is sufficient to supply the glycolytic intermediates to generate nucleotide sugars or whether these are sourced from other nutrients in glucose-starved cells.

Increased glutamine/glutamate metabolism forms only one node of the metabolic adaptation network mediated by PCK2, suggesting that other substrates may feed into the network to help supply biosynthetic intermediates. Recent work suggests that PCK2 can mediate the conversion of lactate to PEP in lung cancer cells (Leithner et al., 2014), although this would require a supply of lactate from the tumor microenvironment because very little lactate is found in tumor cells experiencing glucose deprivation. Other anapleurotic substrates capable of generating OAA could function as substrates for PCK2-dependent PEP production under low-glucose conditions, such as...
branched-chain amino acids (BCAAs), which can enter the TCA cycle through metabolism of propionyl-CoA (Stark and Kibbey, 2014). Fructose-1,6-bisphosphatase, another enzyme involved in gluconeogenesis, can promote the viability of metastatic breast cancer cells by increasing glutamine and BCAA metabolism (Chen et al., 2015). Indeed, branched-chain amino acid transaminase 1 (BCAT1) is upregulated significantly in our metabolic network, as are the levels of BCAAs (leucine, valine, and isoleucine).

β-oxidation of lipids may provide another carbon source for biosynthetic growth under glucose limitation (Vacanti et al., 2014).

PCK2 appears to be a glucose-regulated gene because both mRNA and protein levels are induced significantly upon glucose withdrawal in tumor cells. PCK2 activity also depends on mtGTP, which is generated by the SUCLG2 form of SCS in the mitochondrial matrix (Stark et al., 2009). Expression of both SUCLG2 and PCK2 mRNA is stimulated by glucose withdrawal, suggesting a coordinated effort to enhance PCK2 activity under low-glucose conditions. Our data indicate that HIF-1α and EPAS1/HIF-2α act synergistically to induce PCK2 expression and facilitate proliferation under glucose-free conditions. Although HIF-1α has been implicated in gluconeogenesis and regulation of PCK1 expression in the liver (Choi et al., 2005; Tajima et al., 2009), PCK1 was not abundantly expressed in NSCLC cells, nor was HIF-1α alone required for PCK2 expression, suggesting a more prominent role for EPAS1/HIF-2α in the glucose-dependent control of PCK2. Therefore, although HIFs play prominent roles in metabolic regulation during hypoxia (Mucaj et al., 2012), HIF-dependent re-wiring of the TCA cycle through PCK2 helps provide tumor cells with additional metabolic flexibility for glucose-independent cell growth.

To date, there has been little focus on PEPCK in the context of cancer. Here we present evidence for PCK2-mediated metabolic adaptation in tumor cells and elevated PCK2 expression in human tumor subsets, including NSCLC. We hypothesize that PCK2 could provide a selective growth advantage to tumor cells growing in nutrient-poor environments, enabling them to use PEP, supplied by glutamine or other anaplerotic substrates, to support TCA cycle metabolism and glycolytic intermediates for biosynthesis. In this light, targeting PCK2-mediated metabolic adaptation may be an effective strategy for certain cancer subtypes, particularly NSCLC.
**EXPERIMENTAL PROCEDURES**

**Cell Culture**
The A549 and H1299 cell lines were obtained from the ATCC. Cells were cultured in growth medium (DMEM [A549 cells] or RPMI medium [H1299 cells]) supplemented with 10% fetal bovine serum (FBS), penicillin, streptomycin, glutamine, and non-essential amino acids (for H1299 cells). For glucose limitation experiments, cells were cultured in glucose- and glutamine-free DMEM supplemented with 10% dialysed FBS (Wisent) and glutamine (2 mM) and glucose (25 mM) added as required. PCK2 knockdown was achieved using lentiviral shRNA vectors from The RNAi Consortium (TRC) shRNA collection (ID numbers are listed in the Supplemental Experimental Procedures). Lentiviral supernatants were generated as described previously (Huang et al., 2012). Transient knockdown of PCK2, ENO1, PHGDH, HIF1A, EPAS1, SUCLG2, and SUCLA2 was achieved using SMARTpool ON-TARGETplus siRNA reagent (composed of four individual siRNAs for each target) from GE Dharmacon. The PEPCX inhibitor MPA was obtained from Santa Cruz Biotechnology.

**Cell Proliferation and Viability Assays**
Cells were seeded in 384-well plates (500 cells/well) in growth medium as described previously (Vincent et al., 2015). After 24 hr, the growth medium was replaced with fresh medium containing 25 or 0 mM glucose. Cells were fixed with 4% formaldehyde, stained with Hoechst DNA stain, and then the cell number was determined by nucleus counting. Images were taken using an Operetta high content imaging system and analyzed using Harmony high content imaging and analysis software (PerkinElmer). Cell viability was determined by viability dye exclusion by flow cytometry using propidium iodide (PI). Cells were analyzed using a Galaxios flow cytometer (Beckman Coulter), and data were analyzed using FlowJo software (TreeStar).

**Metabolite Profiling by GC-MS and LC-MS**
Cellular metabolites were extracted and analyzed either by gas chromatography-mass spectrometry (GC-MS) or liquid chromatography (LC)-MS using protocols described previously (Dupuy et al., 2013; Faubert et al., 2014; McGuirk et al., 2013). For GC-MS, metabolite extracts were derived using N-[tert-butyldimethylsilyl]-N-methyltrifluoroacetamide (MTBSTFA) as described previously (Faubert et al., 2013). D-myristic acid (750 ng/sample) was added as an internal standard to metabolite extracts, and metabolite abundance was expressed relative to the internal standard and normalized to cell number. Liquid chromatography was performed using a 1290 Infinity ultraperformance LC system (Agilent Technologies) equipped with a Scherzo 3-µm, 3.0 x 150 mm SM-C18 column (Imtakt). LC-MS analysis was performed on an Agilent 6540 ultra-high definition (UHD) Accurate-Mass Q-TOF mass spectrometer (Agilent). For stable isotope tracer analysis (SITA) experiments, cells were cultured with U-[13C]glutamine (Cambridge Isotopes) for the indicated times. Mass isotopomer distribution was determined using a custom algorithm developed at McGill University (McGuirk et al., 2013).

**RNA-Seq Analysis and Network-Based Data Integration**
For RNA-seq experiments, A549 cells were cultured in the presence or absence of glucose (25 mM) for 48 hr prior to RNA extraction. cDNA synthesis and library construction were conducted as described previously (Jha et al., 2015). Libraries were sequenced at the Centre for Applied Genomics (SickKids, Toronto) using a HiSeq 2500 (Illumina) using 50 bp single-end sequencing.

To construct the integrated metabolic network illustrated in Figure 1, A549 cells were cultured in medium containing 25 or 0 mM glucose for 48 hr prior to either RNA or metabolite extraction and analysis. Integration of metabolite and RNA expression datasets was conducted as described previously (Jha et al., 2015). Additional details are provided in the Supplemental Experimental Procedures.

**Immunoblotting and Quantitative Real-Time PCR**
Lysates of NSCLC cell lines or tissue were subjected to SDS-PAGE and western blotting as described previously (Vincent et al., 2011). Primary antibodies to PCK2 and actin as well as HRP-conjugated anti-rabbit and anti-mouse secondary antibodies were obtained from Cell Signaling Technology. All human NSCLC tissue lysate immunoblots were incubated with fluorescently labeled anti-rabbit or anti-mouse secondary antibodies and analyzed and quantified using an Odyssey 5a infrared imaging system (LI-COR Biosciences). qPCR was performed as described previously (Faubert et al., 2013). Primer sequences have been described previously (Faubert et al., 2013) or are listed in Table S1.

**Tumor Xenograft Assays**
Animals were maintained under specific pathogen-free conditions at McGill University. A549 and H1299 cells expressing either control or shRNA targeting PCK2 were counted (2 million cells/injection), resuspended in 50% Matrigel/50% PBS (200 µl/injection), and injected subcutaneously into the flanks of nude mice (Charles River Laboratories). Tumor length (l) and width (w) were measured every 3–4 days with calipers, and the tumor volume (V) was calculated ($V = \frac{1}{2}lw^2$). After 65 days, the mice were sacrificed, and the tumors were dissected out and weighed.

**TCGA Data Analysis**
TCGA data for 35 tumor studies that contained mRNA expression data were accessed using the R-interface cgdss to the cbio Cancer Genomics Portal (Cerami et al., 2012; Gao et al., 2013). For each dataset, PCK2 expression relative to ACTB was determined for each patient sample. Nine studies with different tissues from the whole spectrum of relative PCK2 expression were analyzed.

**NSCLC Patients and Tissue Samples**
Collection of samples, details of the cohort, and sample extraction have been described previously (Vincent et al., 2011, 2014). Briefly, three distinct samples of lung tumor tissue and adjacent normal lung from the resection margin were taken, flash-frozen in liquid nitrogen, and stored at −80°C until further analysis. Only NSCLC tumor samples comprising at least 90% of tumor tissue were analyzed. Tissues were homogenized in 1% NP40 lysis buffer using a Polytron homogenizer to generate tissue lysates for SDS-PAGE analysis.

**Statistical Analysis**
Statistics were determined using paired Student’s t test using Prism software (GraphPad) unless stated otherwise. Data were calculated as the mean ± SEM for biological triplicates unless stated otherwise. Statistical significance is represented in figures as follows: *p < 0.05,* **p < 0.01,** ***p < 0.001,** ****p < 0.0001.

**ACCESSION NUMBERS**
The accession number for the raw and processed sequencing data reported in this paper is GEO: GSE66556.

**SUPPLEMENTAL INFORMATION**
Supplemental Information includes seven figures, one table, and Supplemental Experimental Procedures and can be found with this article at dx.doi.org/10.1016/j.molcel.2015.08.013.

**ACKNOWLEDGMENTS**
We acknowledge M. Hetzel, J. Pawade, M. Sohail, and L. Phillips for the collection of NSCLC tissue. W. Reintsch provided technical assistance, and S. Huang provided access to the shRNAs. This work was supported by grants from the McGill Integrated Cancer Research Training Program (to E.E.V., P.P.C., and B.R.F.); the Government of the Russian Federation (to A.S., V.L.S., and V.I.G.); the Fonds de recherche Sante´ Que´ be´ c (to T.G.); the Wellcome Trust Seeding Drug Discovery Award (to J.M.T); and CIHR (MOP-93799), the Terry Fox Foundation, and the Cancer Research Society (to R.G.J.).

Received: February 23, 2015
Revised: June 23, 2015
Accepted: August 17, 2015
Published: October 15, 2015


