Four Key Steps Control Glycolytic Flux in Mammalian Cells

Graphical Abstract

Highlights
- Systematic analysis identifies four key flux-controlling steps in glycolysis
- Hexokinase and phosphofructokinase promote glycolytic flux
- Glucose and lactate transport (but not lower glycolysis) also control pathway flux
- Activation of glycolysis in cancer is achieved by induction of the four key steps

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In Brief
Here, we perform a systematic analysis of glycolytic flux control in mammalian cells. We identify four key flux-controlling steps: glucose import and phosphorylation, fructose-1,6-bisphosphate production, and lactate export. In contrast, enzyme steps in lower glycolysis do not control pathway flux. Activation of glycolysis in cancer and immune cells is associated with enhanced expression of enzymes catalyzing these four key flux-controlling steps.
Control of glycolytic rate plays an important role in mammalian physiology, contributing to circulating glucose homeostasis and providing ATP and/or biomass building blocks in contexts such as cell proliferation, immune activation, and angiogenesis (Buck et al., 2017; De Bock et al., 2013; Everts et al., 2014; Yu et al., 2017). Dysregulated glucose metabolism is a hallmark of diseases including diabetes and cancer.

Cancer cells extensively ferment glucose even in the presence of adequate oxygen (Warburg, 1956). While initially attributed by Warburg to defective mitochondria, it is now clear that most cancer cells have functional mitochondria that account for much of their ATP production (DeBerardinis and Chandel, 2016; Fan et al., 2014; Vander Heiden and DeBerardinis, 2017; Zong et al., 2016; Zu and Guppy, 2004). Accordingly, we use the term Warburg effect to refer to rapid aerobic glycolysis in cancer cells, irrespective of their utilization of oxidative phosphorylation. It has been argued that the Warburg effect promotes tumor growth by satisfying cancer cells’ high demand for both energy and central carbon metabolites for biosynthesis (Liberti and Locasale, 2016). The Warburg effect can be triggered both by oncogenic mutations (e.g., in Ras, PI3K/Akt, c-Myc) and by environmental cues (e.g., growth factors) (Saglio et al., 2011; Hay, 2016; Vander Heiden et al., 2009; Hu et al., 2016; Lunt and Vander Heiden, 2011; Shim et al., 1997; Yu et al., 2017).

Consistent with their high utilization of glycolysis, cancers and other proliferating cells exhibit increased expression of many glycolytic enzymes (Vander Heiden et al., 2009). High expression of the glucose transporters GLUT1 and GLUT3 is associated with augmented glucose uptake and oncogenic growth (Birsoy et al., 2014; Ondodera et al., 2014; Yun et al., 2009). Elevated activities of hexokinase and phosphofructokinase favor tumor initiation, immune cell activation, and angiogenesis (De Bock et al., 2013; Everts et al., 2014; Patra et al., 2013; Ros and Schulze, 2013; Webb et al., 2015; Yi et al., 2012; Ying et al., 2012; Yu et al., 2017). Aldolase A (ALDOA) has been shown to boost glycolysis upon phosphoinositide 3-kinase (PI3K)/Akt signaling (Hu et al., 2016). When upper glycolysis is activated in cancer cells, glyceroldehyde-3-phosphate dehydrogenase (GAPDH) has been reported to become a rate-limiting pathway step (Shestov et al., 2014). The importance of the final enzyme involved in pyruvate production, pyruvate kinase, to glycolytic flux control remains controversial. Earlier studies advocated for
Figure 1. Glycolytic Flux Is Controlled by Glucose Import, Fructose-1,6-Bisphosphate Production, and Lactate Export

(A) Experimental setup used to determine glycolytic flux control. Enzymes were individually overexpressed in iBMK cells and change in lactate secretion (glycolytic flux) was assessed 24 hr post transfection; $r'_l$ is the ratio between lactate secretion in enzyme-overexpressing cells versus vector-control cells.

(B) Western blot showing that the expression dynamics of different glycolytic enzymes is similar (see Figure S1 for additional enzymes).

(C) Change in lactate secretion compared with vector control upon individual overexpression of glycolytic enzymes (in DMEM supplemented with 10% dialyzed fetal bovine serum and 5 mM or 25 mM glucose as indicated) (D) and associated flux-control coefficients according to the equation in (A) (for enzymes (legend continued on next page))
the PKM2 isoform as a key driver of the Warburg effect, but recent evidence suggests that the situation is more complex (Bluemlein et al., 2011; Christofk et al., 2008; Dayton et al., 2016). Finally, lactate dehydrogenase A (LDHA) has been implicated in c-Myc-mediated transformation (Shim et al., 1997). Thus, nearly every enzyme linking glucose to lactate has been associated in some study with enhancing glycolytic flux.

Despite this extensive literature, a unified view of glycolytic flux control is lacking. In particular, the key glycolytic enzymes whose upregulation is required for flux enhancement remains unclear. This is in part likely reflects variation in flux control across cell lines and environmental conditions. It might also, however, reflect a failure to comprehensively experimentally probe the pathway. Specifically, to obtain a thorough understanding of flux control in any given biochemical pathway, it is valuable to systematically perturb each component to assess its contribution to overall pathway control. However, previous experiments have largely focused on the role of individual glycolytic enzymes in isolation, and efforts to interrogate the entire pathway have primarily been theoretical and computational (Dai et al., 2016; Shestov et al., 2014).

Here we examine, in two mouse cell lines, the degree of flux control residing in each step of glycolysis by systematically over-expressing enzymes that catalyze every glycolytic step, and by subsequently analyzing pathway flux. We find that across all enzymes tested, only four steps show significant flux control: In both cell lines, flux is controlled by the two committed steps of upper glycolysis (i.e., hexokinase and phosphofructokinase, with the complication that excessive hexokinase leads to energy stress). In one cell line but not the other, glucose import and lactate export also control flux. In both cell lines, the enzymes of lower glycolysis did not control glycolytic flux. We further show that acute optogenetic stimulation of Ras is sufficient to enhance glycolytic flux and that Ras activates the transcription of genes encoding for the same four steps revealed in our overexpression experiments. Finally, analysis of expression data from solid tumors and paired normal tissue samples reveals a transcriptional signature in which gene isoforms from each of these four steps are strongly and consistently upregulated across tumor types. While other enzymes may play additional regulatory roles in certain contexts, our findings show that glycolytic flux is controlled at a small number of pathway steps whose upregulation underlies the Warburg effect.

RESULTS

Systematic Enzyme Overexpression Can Reveal Glycolytic Flux Control

To systematically identify flux-controlling steps in glycolysis, we transiently overexpressed at least one human isozyme catalyzing every pathway step from import of extracellular glucose to secretion of lactate. Isozymes were chosen based on their high expression in glycolytic cells and human cancers (Hay, 2016). We tested multiple isoforms of glucose transporters, phosphofructokinases, and pyruvate kinases. In addition, we included fructose-1,6-bisphosphatase (FBP1), pyruvate dehydrogenase kinase 1 (PDK1), and the four isozymes of the phosphofructokinase 2 (PFK2) family, which phosphorylate fructose-6-phosphate to make fructose-2,6-bisphosphate, a key activator of the glycolytic enzyme phosphofructokinase 1 (PFK1). PFK2 isoforms, which are known as PFKFB1–4, are bifunctional and also contain phosphatase domains that can consume fructose-2,6-bisphosphate (Ros and Schulze, 2013). In total, we tested a set of 24 glycolysis-related genes. GFP was used as a negative control.

Our initial experiments were performed in immortalized baby mouse kidney cells (iBMK), which were selected for their metabolic characterization (Fan et al., 2014) and high transfection efficiency. To minimize possible metabolic alterations induced by adaptation, we measured glycolytic flux within the first 30 hr after transfection (Figures 1A, S1A, and S1B). Experiments were performed in both 25 mM glucose (common tissue culture concentration) and 5 mM glucose (typical circulating concentration in human).

Each of the tested constructs was well expressed, with substantial accumulation of the recombinant protein by 18 hr and corresponding 5-fold or greater enhancement of the enzyme’s catalytic activity as measured in lysates (Figures 1B, S1C, S1D, and S1E). Overexpression of some individual glycolytic enzymes altered both glucose consumption and lactate secretion, but none significantly changed the ratio of glucose uptake to lactate secretion: cells excreted about 82% of incoming glucose carbon as lactate (Figures 1C, S1F, and S1G). Even greater conversion of glucose to lactate was observed in cells expressing oncogenic mutant Ras (H-RasV12; Figure 1C), consistent with previous reports that oncogenic Ras inhibits pyruvate oxidation and enhances glycolytic flux (Fan et al., 2014; Gaglio et al., 2011).

Based on the nearly constant ratio of glucose uptake to lactate secretion across the cell lines, use of either metric leads to the same conclusions about glycolytic flux. Yet measurements of lactate secretion are more precise (Figure S1F), as the fractional drop in extracellular glucose over 6 hr is small and thus difficult to measure. Accordingly, in all subsequent analyses we used lactate secretion as a proxy for glycolytic flux. The uncertainty in the glucose uptake measurements was too large for us to determine whether the absolute flux of glucose carbon not being excreted as lactate tracks with glycolytic flux.

The extent to which a particular pathway step affects glycolytic flux can be expressed as a flux-control coefficient...
Based on the fold change in flux \( (\Delta f_{i}) \) induced by a given fractional change in enzyme catalytic activity \( (\Delta E) \) (Fell, 1998; Heinrich and Rapoport, 1974; Kacser and Burns, 1973),

\[
C^E_J = \frac{(\Delta f_{i} / f_{i}^0)}{(\Delta E / E)} \quad \text{(Equation 1)}
\]

Experimentally, measurement of \( C^E_J \) based on Equation 1 is difficult. It requires quantitating small changes in both fluxes and enzyme activities. Fortunately, \( C^E_J \) can be approximated based on the fold change in flux \( (\Delta f_{i} / f_{i}^0) \) (measured as the flux ratio between cells with enhanced versus basal enzyme activity) that occurs upon massively increasing the activity of a given enzymatic step (Small and Kacser, 1993a) (see STAR Methods),

\[
C^E_J = (f_{i}^0 - 1) / f_{i}^0 \quad \text{(Equation 2)}
\]

Note that dramatically increasing the activity of a given step does not correspondingly increase pathway flux, unless that is the sole rate-limiting pathway step, in which case \( C^E_J = 1 \). For steps that are not rate-controlling, the pathway may not respond, and \( C^E_J = 0 \).

Flux Is Controlled by Glucose Import, Phosphofructokinase, and Lactate Export

Of the 24 individually overexpressed genes, only seven significantly increased glycolytic flux in iBMK cells cultured in both high- and low-glucose media (Figures 1D and 1E): GLUT1, GLUT3, and GLUT5 (glucose transporters); MCT4 (lactate transporter); PFKP (phosphofructokinase; PFK1); and PFKFB1 and PFKFB3 (phosphofructo-2-kinase/fructose-2,6-bisphosphatases; PFK2). The flux-control coefficient for PFKP was comparable with previous literature values determined in other mammalian cells (Small and Kacser, 1993a; Torres et al., 1986). Our results are in agreement with literature pointing to the importance of glucose uptake and FBP production in controlling glycolytic flux (Birsoy et al., 2014; Onodera et al., 2014; Ros and Schulze, 2013; Webb et al., 2015; Yi et al., 2012; Ying et al., 2012; Yun et al., 2009). In contrast, no enzymes in lower glycolysis significantly affected flux, including enzymes suggested in prior literature as inducers of the Warburg effect: ALDOA (aldolase), GAPDH, PKG (phosphoglycerate kinase), PKM1 and PKM2 (pyruvate kinases), and LDHA (lactate dehydrogenase). Nevertheless, we did observe significant flux control in the lactate export step, as overexpression of MCT4 led to a substantial increase in glycolytic flux (Figures 1C–1E).

Certain genes, such as PKM2 and LDHA, have been classically associated with the Warburg effect but did not show any impact on glycolytic metabolism here. To rule out that these genes promote Warburg metabolism by suppressing oxidative phosphorylation or impairing glucose entry into the tricarboxylic acid (TCA) cycle, we also examined oxygen consumption rate and contribution of glucose carbon to TCA intermediates. Although there was a trend toward higher oxygen consumption with GLUT3 overexpression, the oxygen consumption rate did not vary significantly in response to overexpression of GLUT3, PFKP, GAPDH, PKM2, LDHA, or MCT4 (Figure S1H). Glucose contribution to TCA was slightly but significantly decreased by PFKP overexpression but not affected by any of the other genes (Figure S1I). Thus, the GLUT, PFK, and MCT steps promote glycolytic flux, while most other steps neither drive glycolytic flux nor modulate glucose oxidation.

The summation theorem of metabolic control analysis states that the sum of the flux-control coefficients in a linear pathway must equal 1 (Kacser and Burns, 1973). This theorem refers to the sum of flux control across the pathway steps, not across iso-enzymes. In our case, we observe flux control of about 30% for glucose uptake, 30% for FBP production (either via expression of phosphofructokinase or its activators PFKFB1 or PFKFB3), and 40% for lactate export, together summing to approximately 100% (Figure 1F). Thus, our data are roughly consistent with the summation theorem.

We then tested the potential to further activate glycolytic flux in iBMK cells grown in 25 mM glucose by coexpressing GLUT3, PFKP, and MCT4 (Figure 1G). The ATP synthase inhibitor oligomycin was used as a positive control (Huijing and Slater, 1961; Lardy et al., 1958). Combined overexpression of GLUT3, PFKP, and MCT4 triggered a 2-fold increase in glycolytic flux, which is comparable with oligomycin and significantly greater than any individual gene on its own (Figure 1G). Similar increases in glycolytic flux were obtained with coexpression of either transporter with PFKP, but not the two transporters together. The roles for glucose import, phosphofructokinase, and lactate export in controlling iBMK cell glycolysis are highlighted in Figure 1H.

PFN2 Isoforms Exhibit Divergent Effects on Glycolytic Flux

Overexpression of 5 of the 24 tested genes significantly decreased glycolytic flux in both glucose conditions (Figure 1D). The reduction in glycolytic flux observed upon overexpression of fructose-1,6-bisphosphate phosphatase (FBP1) is consistent with its role in gluconeogenesis and inhibition of tumor progression (Li et al., 2014; Tejwani, 1983). We also captured the well-documented anti-glycolytic function of the TP53-induced glycolysis and apoptosis regulator (TIGAR) (Bensad et al., 2006). In contrast, we were surprised to find that hexokinase 2 (HK2) overexpression decreased flux, an observation that results from energy stress based on its excessive overexpression, as described below.

Another anti-glycolytic gene was the phosphofructokinase-2 isozyme PFKFB2. This contrasted with PFKFB1 and PFKFB3, which as expected increased glycolysis, and PFKFB4, which had no discernible effect (Figure 1D). While PFK2 isoforms are generally considered pro-glycolytic, their impact on glycolysis depends on their balance of kinase and phosphatase activity. Recent in vitro biochemical studies have assigned the highest kinase-to-phosphatase ratio to the PFKFB3 isoform (>700:1) (Ros and Schulze, 2013), which we found most strongly induced glycolytic flux. Existing biochemical data on the other three PFK2 isoforms, however, does not explain their divergent glycolytic flux phenotypes, as their in vitro kinase and phosphatase activities are similar. Our results highlight the functional diversity of PFK2 isoforms in cells, with the PFKFB2 isozyme acting to slow glycolysis.

Intracellular FBP Concentration Mirrors Glycolytic Flux

To explore potential connections between enzyme induction, flux, and metabolite levels, we measured changes in glycolytic intermediate levels by liquid chromatography-mass spectrometry.
Figure 2. Intracellular FBP Levels Mirror Glycolytic Flux

(A) Heatmap summarizing changes in intracellular metabolite levels upon overexpression of glycolytic enzymes. Red and blue indicate increased and decreased intracellular metabolites, respectively. Light-yellow-colored boxes highlight the identified flux-controlling steps from Figure 1. Glycolytic enzymes with positive glycolytic flux control are colored red.

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These metabolomics data confirmed the functional activities of the overexpressed enzymes (Figure 2A). For example, overexpression of PFKP led to a decrease in fructose-6-phosphate (F6P) and to a concomitant increase in FBP, whereas the opposite was observed for fructose bisphosphatase (FBP1). Increased ALDOA activity resulted in lower FBP but elevated dihydroxyacetone phosphate (DHAP). We observed that 2,3-bisphosphoglycerate (2,3BPG) accumulated in BPGM-overexpressing cells, which likely explains their decreased glycolytic flux (Figures 1D and 2A). Our analysis also confirmed recently reported side products of GAPDH and pyruvate kinase (Collard et al., 2016): we found that GAPDH overexpression induced erythronate, and PKM1 and especially PKM2 generated 2-phospholactate (Figures S2A and S2B). TIGAR-overexpressing cells were characterized by a marked reduction of lower glycolytic intermediates, specifically 2,3BPG and phosphoenolpyruvate (PEP), but not FBP, and we did not detect any increase in hexose-phosphate species. These observations are consistent with recent evidence showing greater TIGAR phosphatase activity toward 2,3BPG and PEP than fructose-2,6-bisphosphate (Gerin et al., 2014). Collectively, these metabolomics data support the successful induction of the desired enzymatic activities via protein overexpression, including for enzymes that did not significantly impact glycolytic flux.

In addition, our metabolite concentration data support a picture in which upper glycolytic enzymes control both glycolytic flux and metabolite levels. Overexpression of upper glycolytic enzymes caused strong metabolite changes across the entire pathway. In contrast, lower glycolytic enzymes tended to marginally increase lower glycolytic intermediates (with strong effects only on 2,3BPG) and to weakly deplete upper glycolytic intermediates (Figure 2A). Although the overexpressed glucose and lactate transporters generally had only weak effects on metabolite concentrations, they did elevate FBP and to a lesser extent DHAP, which is linked to FBP via the reversible aldolase reaction.

Consistent with prior literature in both microbes and mammalian cells (Kochanowski et al., 2013; Kotte et al., 2010; Peeters et al., 2017; Zhang et al., 2017), among the glycolytic intermediates FBP and DHAP concentrations correlated most strongly with glycolytic flux (Figure 2B). AMP, ADP, ATP, and citrate are textbook regulators of glycolysis (Berg et al., 2002; Garland et al., 1963; Llorente et al., 1970; Newsholme et al., 1977), but none showed a comparable correlation with glycolytic flux (Figure S2C). With the exception of cells overexpressing toxic levels of hexokinase (see below), flux across the cell lines was generally well explained by a Michaelis-Menten function of FBP concentration (p < 10⁻⁴) (Figure 2C). The fitted K_m value of 1.5 mM (p = 0.002) is within the range of the basal FBP level in iBMK cells (1.8 mM), and the V_max value of 0.3 μmol hr⁻¹ μL cell⁻¹ (p < 10⁻⁷) is 2-fold higher than the basal glycolytic flux (0.15 μmol hr⁻¹ μL cell⁻¹) and equal to the maximum glycolytic flux obtained upon oligomycin treatment or combined GLUT3, PFKP, and MCT4 overexpression. Thus, glycolytic flux appears to be a saturable function of FBP levels.

Hexokinase Is Pro-glycolytic but Toxic in Excess

Hexokinase catalyzes an effectively irreversible step of glycolysis that has previously been shown to have significant flux control (Marín-Hernández et al., 2006). HK2 overexpression resulted in accumulation of most glycolytic intermediates, including FBP (Figure 2A). Accordingly, we were perplexed by the negative effect of HK2 overexpression on glycolytic flux (Figure 1D). To address this discrepancy, we measured the temporal dynamics of glycolytic flux in HK2-overexpressing cells as well as in cells overexpressing GLUT1, GLUT3, PFKP, PFKFB3, and PKM2 (Figures 2D and S2D). This analysis revealed that HK2 initially accelerates glycolytic flux but suppresses it at later time points. No other enzyme showed a similar biphasic response. Thus, HK2 exerts positive glycolytic flux control, but over time also impairs glycolysis.

To obtain a better understanding of the underlying mechanism, we measured the temporal evolution of metabolite concentrations in HK2-overexpressing cells (Figures 2E and S2E). We found that glycolytic intermediates accumulated monotonically. AMP and, to a lesser extent, ADP also built up, suggestive of energy stress. ATP levels and adenylate energy charge dropped at the same time as glycolytic flux decreased (Figures 2D and 2F). A lower energy charge also manifested in depletion of creatine phosphate (Figure S2F). These metabolic alterations likely result from excessive HK2 enzyme activity detrimentally consuming ATP through overactive hexose phosphorylation, with downstream glycolysis unable to maintain pace. Cell growth and viability were, however, largely maintained over this time interval (Figure S2G). This HK2-induced energy stress is evocative of other instances where excessive ATP-driven phosphorylation leads to energy stress, e.g., glucose-induced ATP depletion in yeast lacking the enzyme trehalose-6-phosphate synthase (TPS1), which acts in part as a brake on hexokinase (Van Aelst et al., 1993; Ernandes et al., 1998; González et al., 1992) and fructose-induced drainage of ATP in hepatocytes via ketohexokinase (Abdelmalek et al., 2012; Ishimoto et al., 2012; Johnson et al., 2013).
To prove that HK2 is fundamentally pro-glycolytic but that excessive HK2 activity causes energy stress, we transfected iBMK cells with serial dilutions of plasmid DNA encoding HK2 or, for comparison, PFKP (Figure 2G). Both low and high copy numbers of PFKP had similar effects, increasing glycolytic flux by \( \sim 40\% \). In contrast, at the 30-hr time point used in Figure 1, while concentrated HK2 decreased glycolytic flux, dilute HK2 increased glycolytic flux by \( \sim 40\% \). Glycolytic flux began to decrease as the introduced hexokinase activity exceeded endogenous activity by more than 5-fold (Figure S2H). Thus, hexokinase exerts positive flux control in iBMK cells, but this was masked in our initial experiments involving massive overexpression because excessive hexokinase activity causes ATP depletion.

**Hexokinase and Phosphofructokinase Also Control Flux in NIH3T3 Fibroblasts**

To examine glycolytic flux control in another mouse cell line, we conducted single enzyme overexpression experiments in NIH3T3 mouse fibroblasts (3T3). Parental 3T3 cells were transduced by lentiviruses carrying a glycolytic gene of interest and lactate secretion was measured after 48 hr. Overall, the results closely mirrored those observed in iBMK cells (Figures 3A and S3A). Of the eight glycolytic reactions that did not substantially control flux in iBMK cells, none had a significant effect in 3T3 cells. Similar to iBMK cells, the hexokinase and phosphofructokinase steps exerted substantial flux control, with expression of the PFK1-activator PFKFB3 yielding an effect similar to that of PFKP itself. In contrast to iBMK cells, we did not observe increased flux in response to single overexpression of glucose or lactate transporters. Overall, our results suggest that hexokinase and phosphofructokinase are common flux-controlling enzymes in glycolysis, with other core glycolytic enzymes exerting limited flux control.

**Acute Ras Stimulation Transcriptionally Activates All Four Flux-Controlling Steps**

The Ras/Erk pathway is a well-studied growth-promoting signal transduction pathway whose constituents are among the most frequently mutated genes in human cancers (Prior et al., 2012). Oncogenic mutations in the Ras/Erk pathway have been shown to enhance both uptake and utilization of glucose (Flier et al., 1987; Prior et al., 2012; Racker et al., 1985; Ying et al., 2012; Yun et al., 2009). We have previously observed that acute optogenetic stimulation of wild-type Ras is sufficient to drive 3T3 cell transcription and proliferation (Toettcher et al., 2013; Wilson et al., 2017). We wondered whether this acute Ras-driven proliferation was associated with glycolytic enhancement, and, if so, whether the enhanced flux is driven by the flux-controlling reactions identified by our overexpression analysis.

To address these questions, we employed the optogenetic system (Opto-SOS) to activate Ras signaling in 3T3 cells (Gaglio et al., 2011). Serum-starved 3T3 cells expressing the light-activatable Opto-SOS system were stimulated with red light, and glycolytic flux was measured at 6 hr and cell growth after 24 hr (Figure 3B). As expected, cell growth was increased by acute Ras signaling, and even at the 6-hr time point glycolytic flux was already enhanced (Figures 3C, 3D, and S3B).

To obtain a more thorough understanding of early metabolic perturbations upon Ras signaling, we measured glycolytic intermediates after 4 hr and 24 hr of activating light (Figure 3E). We observed a general increase in glycolytic intermediates and adenosine nucleotide levels at 4 hr, similar to changes observed upon HK2 overexpression in iBMK cells (Figures 2A and 3E). FBP and DHAP were the only metabolites that remained elevated after 24 hr of Ras activation. The 2-fold elevation of FBP at 4 hr was associated with a 1.5-fold increase in glycolytic flux, consistent with the relationship between FBP and glycolytic flux in iBMK cells (Figures 2B, 2C, 3C, and 3E).

To explore potential mechanisms by which acute Ras activation might enhance glycolysis, we analyzed published RNA-sequencing data collected from Opto-SOS 3T3 cells stimulated with activating red light over a 2-hr time course (Wilson et al., 2017). Most gene expression changes were, as expected, in signaling pathways (Figure S3C). Among metabolic pathways, nucleotide biosynthesis was substantially increased, likely to support cell proliferation. In central carbon metabolism, four glycolytic genes were upregulated more than 2-fold at some time point. In contrast, such upregulation was observed for only a single gene in the pentose phosphate pathway (ribose-5-phosphate isomerase A [RPIA]) and none in the TCA cycle (Figure S3D). The more than 2-fold upregulated glycolytic genes were GLUT1, HK2, PFKFB3, and MCT1, corresponding to the four flux-controlling steps identified in the overexpression studies. PFKP was up to a lesser extent (Figure 3F). Induction of GLUT1 and HK2 was particularly strong and rapid. These acute changes differ from those resulting from chronic Ras activation, which also induces multiple lower glycolytic enzymes (Gaglio et al., 2011; Ying et al., 2012). In addition, we observed Ras-dependent decreases in expression of some glycolytic enzymes (BPGM and PFKFB2), which were “anti-glycolytic” in the single enzyme overexpression experiments (Figure 1D). We did not observe altered expression of any glycolytic genes with low flux-control coefficients. Taken together, our results suggest that Ras controls a coordinated expression program to both up- and downregulate glycolytic enzyme targets in a manner that increases glycolytic flux. Figure 3G summarizes the flux-controlling steps identified in iBMK and 3T3 cells together with the transcriptional targets induced by acute Ras signaling.

**Solid Tumors Consistently Overexpress Genes for Glucose Transport, FBP Production, and Lactate Export**

Enhanced glycolysis was the first molecular phenotype assigned to cancer, and occurs in tumors originating from many different tissues and harboring distinct oncogenes and genomic alterations (Dang, 2012; Vander Heiden et al., 2009; Warburg, 1956). We wondered whether the same four glycolytic flux-controlling steps identified in our cell culture data would also emerge in gene expression data across a variety of human tumors. To this end, we analyzed expression of glycolytic genes in 666 paired solid tumor tissue samples from The Cancer Genome Atlas (TCGA) (http://cancergenome.nih.gov/). Based on KEGG database annotations, we curated a set of 40 glycolytic genes, covering all isozymes of glycolytic enzymes, including PFK2 isoforms and glucose and lactate transporters (Kanehisa and Goto, 2000; Kanehisa et al., 2016, 2017). Gene expression of tumor...
Figure 3. Glycolytic Flux Increase upon Ras Activation Is Controlled by Glucose Import, Glucose-6-Phosphate Production, and Lactate Export

(A) Change in glycolytic flux in 3T3 cells upon individual overexpression of enzymes catalyzing every step in glycolysis. Means and 95% confidence intervals (n = 3 biological replicates) are plotted.

(B) Experimental setup to investigate acute metabolic perturbation in 3T3 cells upon optogenetic Ras activation.

(C–D) Changes after red light exposure in (C) lactate secretion at 6 hr (n = 12 biological replicates from two independent experiments; p < 10^{-4}) and (D) cell growth at 24 hr (n = 3 biological replicates; p < 0.02). Means and 95% confidence intervals are plotted.

(E) Changes in intracellular metabolites 4 hr and 24 hr after activating red light exposure. The 4-hr red light exposed time point was normalized to the mean of the 0-hr time point; the stimulated (Ras ON) 24-hr samples were normalized to the mean of the 24-hr non-stimulated (Ras OFF) samples.

(F) Gene expression dynamics of glycolytic enzymes (from RNA-sequencing data) upon optogenetic Ras activation (data from a recent study [Wilson et al., 2017]).

(G) Overview of results in iBMK and 3T3 cells. For the iBMK and 3T3 columns, red boxes indicate significant flux control based on enzyme overexpression experiments. For the Ras column, red boxes indicate significant transcriptional upregulation upon acute optogenetic Ras activation. See also Figure S3.
samples was normalized to the corresponding benign adjacent tissue. Unsupervised clustering of solely this limited set of glycolytic genes was sufficient to group tumor samples based on their tissue of origin (Figures 4A and 4B).

At least one isoform of every glycolytic step was generally upregulated in each patient tumor (Figure 4A). In most cancer types, strong increases were seen in at least one isoform of GLUT, of PFK, and of MCT, representing three of the four key flux-controlling nodes (Figures 4C and S4A). While no hexokinase isozyme was particularly strongly expressed in lung, liver, or breast cancer, HK2 was the most strongly upregulated glycolytic enzyme in kidney renal clear cell carcinoma and kidney renal papillary cell carcinoma tumors, and HK3 in thyroid tumors. The less consistent high upregulation of HK expression may reflect the toxicity of excess HK activity.

To more systematically evaluate overexpression at different glycolytic steps, we ranked the glycolytic gene isoforms in each individual tumor specimen based on their extent of transcriptional upregulation relative to adjacent benign tissue. Figure 4D shows the frequency with which gene isoforms catalyzing different glycolytic steps were among the four topmost upregulated glycolytic genes in a given patient. Consistent with our findings regarding flux control in cultured cells, we find that the most overexpressed glycolytic genes in human cancer encode for glucose transport (GLUT1 and GLUT5), glucose phosphorylation (HK2 and HK3), FBP production (PFKFB4), PEP production (ENO2), and lactate export (MCT1 and MCT4). Except for enolase, whose catalytic cysteine can be oxidized, may exert flux control in contexts where it is substantially inactivated by binding to the actin cytoskeleton (Hu et al., 2016). GAPDH, whose catalytic cysteine can be oxidized, may exert flux control in the context of oxidative stress (Liberti et al., 2017; Raiser et al., 2007, 2009; Shesov et al., 2014; Yun et al., 2015). More generally, any enzyme of glycolysis can gain flux control if sufficiently inhibited, e.g., by genetic or pharmacological manipulation. For this reason, while studies examining the effects of enzyme depletion are valuable to understand gene essentiality, they do not shed light on intrinsic flux control (Small and Kacser, 1993a). In contrast, for the specific cell line in which they are conducted, overexpression studies yield quantitative estimates of flux-control coefficients.

Systematic assessment of pathway regulation by metabolic control analysis, however, has revealed that rate-limiting steps are an oversimplification (Fell, 1992, 1998; Kacser and Burns, 1973). Indeed, as much as every pathway step must be thermodynamically forward driven and thus consume some of the available pathway free energy, every step also exerts at least some modicum of flux control (Fell, 1998; Noor et al., 2014; Park et al., 2016). Therefore, a pragmatic understanding of metabolic control requires figuring out which steps substantially control flux.

Here we assessed flux control in mammalian glycolysis using three approaches: glycolytic enzyme overexpression, optogenetic activation of Ras, and analysis of gene expression data from human tumors. The first of these approaches is particularly informative, as it directly probes the relationship between enzyme levels and flux, with our data covering each step of glycolysis. All three approaches consistently point to flux through glycolysis being controlled by glucose uptake and phosphorylation, FBP production, and lactate export, and not by lower glycolytic enzymes including pyruvate kinase.

Our measurements of flux control apply to the context in which the enzyme overexpression was carried out (i.e., iBMK and 3T3 cells). This reflects flux-control coefficients depending on relative enzyme concentrations. As the level or activity of an enzyme goes down (compared with pathway flux), its flux control goes up. A relatively high basal capacity for glucose transport and lactate secretion, relative to glucose phosphorylation and FBP production, likely explains why GLUT and MCT do not exert flux control in 3T3 cells. Moreover, while we do not find evidence for substantial flux control in any of the lower glycolytic enzymes, these enzymes may exert flux control in cellular contexts where their relative activities are lower. For example, aldolase A may gain flux control in contexts where it is substantially inactivated by binding to the actin cytoskeleton (Hu et al., 2016). GAPDH, whose catalytic cysteine can be oxidized, may exert flux control in the context of oxidative stress (Liberti et al., 2017; Raiser et al., 2007, 2009; Shesov et al., 2014; Yun et al., 2015). More generally, any enzyme of glycolysis can gain flux control if sufficiently inhibited, e.g., by genetic or pharmacological manipulation. For this reason, while studies examining the effects of enzyme depletion are valuable to understand gene essentiality, they do not shed light on intrinsic flux control (Small and Kacser, 1993a). In contrast, for the specific cell line in which they are conducted, overexpression studies yield quantitative estimates of flux-control coefficients.

In considering how overexpression of a single enzyme increases glycolysis, it is important to keep in mind that increased pathway flux requires enhancement of flux through every enzymatic step of the pathway (Hackett et al., 2016). A straightforward way to achieve this is increased expression of each pathway enzyme. Overexpression of a single enzyme may, however, drive flux through upstream and downstream pathway steps by changing metabolite levels. This appears to be the case for hexokinase and phosphofructokinase. The former increases all glycolytic intermediates. The latter depletes hexose phosphates (thereby relieving product inhibition of glucose import and phosphorylation) and increases FBP and all downstream metabolites (increasing substrate to drive these reactions). It remains unclear how GLUT overexpression drives.
downstream glycolytic reactions (or MCT upstream ones), as neither transporter markedly alters glycolytic intermediate concentrations.

The lag between GLUT expression and flux response raises the possibility of adaptation being involved in inducing the flux increase. Consistent with this, once we include the positive flux-control coefficient for hexokinase that was masked by energy stress in our initial measurements, we observe a surplus of flux control in iBMK cells (i.e., sum of flux-control coefficients greater than 1), suggesting that overexpression of certain enzymes may activate other enzymes beyond simply providing substrate or consuming product. Notably, near maximal glycolytic rate in iBMK cells is achieved by simultaneous upregulation of both FBP production and a transport step (but not concomitant increase of both flux-controlling transport steps). The reasons for these non-linear interactions between different steps in glycolysis are not evident from the individual flux-control coefficients and are an important topic for future research.

As glycolytic rate is enhanced by overexpression of any particular flux-controlling enzyme, flux control will tend to transfer to other pathway steps. This may explain why HIF induces a broad spectrum of glycolytic enzymes including also phosphoglycerate kinase and LDH, why tumors overexpress many glycolytic enzymes including also aldolase and enolase (Graham et al., 2017; Hu et al., 2013; Nilsson et al., 2014), and why tumor types associated with particularly high glycolytic rates due to mitochondrial defects (i.e., renal and thyroid cancer) overexpress hexokinase, even though excessive hexokinase activity can be toxic (Abu-Amero et al., 2005; Davis et al., 2014; Su et al., 2016).

While our study does not address the extent of mitochondrial respiration in cancer cells, our finding that overexpression of certain glycolytic enzymes is sufficient to drive glycolysis without altering glucose entry into the TCA cycle is consistent with emerging evidence that control of glycolytic flux and TCA cycle flux are substantially independent (Faubert et al., 2017; Hui et al., 2017). This reinforces the appropriateness of defining the Warburg effect as aerobic glycolysis in cancer, a robust clinical phenotype proved by fluorodeoxyglucose positron emission tomography imaging.

In summary, we provide a thorough experimental assessment of glycolytic flux control in two mammalian cell lines. We find that flux control is partially distributed, with 4 of the 13 steps collectively dominant. The four flux-controlling steps reside at the top (glucose import), at the two committed phosphorylation steps (hexokinase and phosphofructokinase) and at the bottom (lactate export) of glycolysis. In contrast, at least in these cells, lower glycolytic enzymes do not substantially control glycolytic flux. The identified flux-controlling enzymes stand out for being transcriptionally induced by acute Ras signaling and for being strongly upregulated in human tumors, immune cell activation, and angiogenesis, arguing for the generality of their biological importance. By providing a systematic assessment of glycolytic flux control in mammalian cells, we hope that this work helps both to contextualize research on individual glycolytic enzymes and to motivate comprehensive studies of flux control in additional pathways.

**STAR METHODS**

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

- **KEY RESOURCES TABLE**
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- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
- **METHOD DETAILS**
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  - Transient Overexpression of Glycolytic Genes in iBMK Cells
  - Western Blotting
  - Glycolytic Flux Measurements
  - Measurement of Oxygen Consumption Rates
  - Calculation of Flux Control Coefficients
  - Oligomycin Treatment
  - Metabolomic Analysis Using Mass Spectrometry
  - Enzyme Assays for HK, GAPDH, PKM and LDH Activities
  - Overexpression of Glycolytic Genes in 3T3 Cells by Lentivirus Transduction
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  - RNaseq Analysis of 3T3 Opto-SOS Cells
  - Gene Expression Analysis of TCGA Data
- **QUANTIFICATION AND STATISTICAL ANALYSIS**

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**Figure 4. Increased Glucose Uptake, FBP Production, and Lactate Export Is a General Hallmark of Tumor Metabolism**

(A) Expression levels of glycolytic enzymes in solid tumor tissue samples compared with paired healthy tissue samples analyzed from TCGA data. (B) Clustering of tumor types based on glycolytic gene expression data only: bladder urothelial carcinoma (BLCA), breast invasive carcinoma (BRCA), cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC), colon adenocarcinoma (COAD), esophageal carcinoma (ESCA), head and neck squamous cell carcinoma (HNSC), kidney chromophobe (KICH), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), pancreatic adenocarcinoma (PAAD), prostate adenocarcinoma (PrAD), rectum adenocarcinoma (READ), sarcoma (SARC), stomach adenocarcinoma (STAD), thyroid carcinoma (THCA), and uterine corpus endometrial carcinoma (UCEC). Kidney (CP) refers to clear cell and papillary cell tumors. Kidney (CH) refers to chromophobe tumors. (C) Rank order of glycolytic genes from the most to the least expressed relative to benign adjacent tissue, on average, for different tumor types. Red represents highly ranked gene isoforms catalyzing glucose transport, FBP production, and lactate export for each tumor type. Black indicates the highest expressed hexokinase isoform. (D) Relative frequencies with which gene isoforms across glycolysis are among the four most upregulated glycolytic genes in an individual patient tumor. The expected frequency for each gene due to chance alone (78 occurrences across 666 tumors) is defined as a relative frequency of 1. Statistically significant enrichment of a given gene isoform in the top four most upregulated genes glycolytic genes was calculated using a Fisher’s exact test with Bonferroni correction for multiple hypothesis testing. Significant enrichment was found for GLUT1, GLUT5, HK2, HK3, PKFB4, ENO2, MCT1, and MCT4. See also Figure S4; Tables S2 and S3.
SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and three tables and can be found with this article online at https://doi.org/10.1016/j.cels.2018.06.003.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS


DECLARATION OF INTERESTS

The authors declare no competing interests.

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REFERENCES


# STAR METHODS

## KEY RESOURCES TABLE

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### Experimental Models: Organisms/Strains

| Organism/Strain                                                  | Source                        | Identifier       |
|                                                               |                               |                  |
| HEK293T LX                                                      | Clontech Laboratories         | Cat# 632180      |
| Immortalized baby mouse kidney cell (iBMK D3)                  | Eileen White Lab, Rutgers University | N/A             |
| Immortalized baby mouse kidney cell H-RasV12G (iBMK Ras)        | Eileen White Lab, Rutgers University | N/A             |
| Immortalized baby mouse kidney cell myr-Akt (iBMK Akt)          | Eileen White Lab, Rutgers University | N/A             |
| NIH3T3 cells                                                   | ATCC                          | Cat# CRL-1658    |
| NIH3T3 OptoSOS (NIH 3T3 + Phy-mCherry-CAAX + YFP-SOS2cat)       | Jared Toettcher Lab, Princeton University | N/A            |

### Oligonucleotides

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<td>copGFP: PB-Cuo-MCS-ires-GFP-EF1a-CymR-Puro Inducible cDNA Cloning and Expression Vector</td>
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CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Joshua D. Rabinowitz (joshr@princeton.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

All cell lines used in this study are listed in the Key Resources Table and were cultured in standard Dulbecco’s Modified Eagle Medium (DMEM; with 4.5 g/L glucose and 0.584 g/L L-glutamine, but without sodium pyruvate) supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO2. For passaging, cells were washed once with 1x phosphate buffer saline (PBS) and detached by trypsin/EDTA (0.05%). For glycolytic flux measurements and metabolomics analysis, DMEM was supplemented with 10% dialyzed FBS.

METHOD DETAILS

All reagents and resources used in this study are listed in the Key Resources Table.

Reagent or Resource Source Identifier
LDHA: MGC Human LDHA Sequence-verified cDNA (CloneId:4096518) Dharmacon Cat# MHS6278-202840043
Lenti-Envelope: pMD2.G Addgene Cat# 12259
Lenti-Packing: pCMVdR8.91 Trono lab, École Polytechnique Fédérale de Lausanne, Switzerland N/A
Lenti-Vector: pHR SFFVp iRES-CIBN-BFP-CAAX Jared Toettcher lab, Princeton University N/A
MCT4: MGC Human SLC16A3 Sequence-verified cDNA (CloneId:8327739) Dharmacon Cat# MHS6278-211688899
PDK1: PDK1 (NM_002610) Human cDNA Clone Origene Cat# SC321678
PFKFB1: MGC Human PFKFB1 Sequence-verified cDNA (CloneId:40000713) Dharmacon Cat# MHS6278-211689324
PFKFB2: MGC Human PFKFB2 Sequence-verified cDNA (CloneId:30915381) Dharmacon Cat# MHS6278-202856883
PFKFB3: PFKFB3 (NM_004566) Human cDNA Clone Origene Cat# SC117238
PFKFB4: MGC Human PFKFB4 Sequence-verified cDNA (CloneId:2900872) Dharmacon Cat# MHS6278-202755424
PFKP: MGC Human PFKP Sequence-verified cDNA (CloneId:5180268) Dharmacon Cat# MHS6278-202800861
PGAM1: pReceiver M11 DNA plasmid expressing FLAG-PGAM1 Tom Muir, Princeton University N/A
PGK1: MGC Human PGK1 Sequence-verified cDNA (CloneId:3917989) Dharmacon Cat# MHS6278-202757198
PKM1: Homo sapiens pyruvate kinase, muscle (PKM), transcript variant 2, mRNA. Genecopoeia Cat# Z5842
PKM2: Homo sapiens pyruvate kinase, muscle (PKM), transcript variant 1, mRNA. Genecopoeia Cat# Z7438
TIGAR: TIGAR (NM_020375) Human cDNA Clone Origene Cat# SC320794
TPI1: Triosephosphate isomerase (TPI1) (NM_000365) Human cDNA Clone Origene Cat# SC111041
Other
Cell culture plates and dishes Falcon/Cell Treat N/A
LB Agar plates with 100 µg/mL Ampicillin Teknova Cat# L1004
PCV Packed Cell Volume Tube TPP Cat# 87005
Seahorse XF24 Cell Culture Microplates Agilent Cat# 100777-004

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Cloning of Transient Overexpression and Lentivirus Constructs

All purchased human cDNAs of the 24 glycolytic enzymes are listed above and corresponding primers are listed in Table S1. For cloning into the pCMV-6-A-puro mammalian expression vector, the vector was digested by HindIII (5’-end) and NotI (3’-end) restriction enzymes for 4.5 hr at 37°C. The digested vector was run on a 1% agarose gel (with ethidium bromide) and the cut band was gel purified using the Wizard® SV Gel and PCR Clean-Up System. The open reading frame (ORF) of a given gene was amplified by polymerase chain reaction (PCR) using corresponding primers and the 2x PluUltra ll Hotstart PCR Master Mix according to manufacturer’s instructions. PCR products were cleaned up with the Wizard® SV Gel and PCR Clean-Up System before Gibson Assembly. For all Gibson Assembly reactions, a molar ratio of 1:3 (vector:insert) was used and the reaction was performed at 50°C for 1 hr as per manufacturer’s instructions. Assembled constructs were transformed into NEB 5-alpha competent E. coli cells and grown on agar plates (100 μg/mL ampicillin) at 37°C overnight. Single colonies were inoculated in Luria broth (100 μg/mL ampicillin) overnight and plasmid DNA was extracted using the QIAprep Spin Miniprep Kit. Constructs were sequence verified by Sanger sequencing (Genewiz, South Plainfield, NJ, USA) and stocks for all experiments were prepared using the QIAGEN Maxi Kit.

For lentivirus transductions, ORFs were cloned into a pHR™ SFFVp lentiviral vector, upstream of IRES CIBN-BFP-CAAX, using the In-Fusion™ HD Cloning Kit according to the manufacturer’s instructions. Briefly, the vector was linearized by backbone PCR and the ORFs were amplified by PCR as described above. The linearized vector and PCR products were run on a 1% agarose gel (with ethidium bromide) and the cut bands were cleaned up with the Wizard® SV Gel and PCR Clean-Up System. Using a molar ratio of 1:3 (vector:insert), all In-Fusion reactions were performed at 50°C for 15 min as per manufacturer’s instructions. Assembled constructs were transformed into Clontech Stellar competent E. coli cells and grown on agar plates (50 μg/mL carbenicillin) at 37°C overnight. Single colonies were inoculated in Luria broth (50 μg/mL carbenicillin) overnight. Plasmid DNA was prepared by the QIAprp Spin Miniprep Kit and constructs were sequence verified by Sanger sequencing (Genewiz, South Plainfield, NJ, USA).

Transient Overexpression of Glycolytic Genes in iBMK Cells

iBMK cells were transfected using Lipofectamine 2000 as described in the manufacturer’s protocol. 24 hr before transfection, 0.5 μl packed cell volume (PCV) iBMK cells were plated per well in a 6-well plate in DMEM (10% dFBS). For each well, 2.5 μg of plasmid DNA, 10 μl of transfection reagent and 200 μl of OptiMEM were used. The DNA to lipofectamine ratio was kept equal for all experiments. For coexpression experiments, the total amount of plasmid DNA was 2.5 μg and the amount of the different constructs was kept equal. The single expression controls were adjusted to the lowest concentration and mixed with vector plasmid DNA to keep the total amount of plasmid DNA at 2.5 μg. Correspondingly, serial dilutions of a given construct were supplemented with vector plasmid DNA to keep the 2.5 μg total plasmid DNA amount. iBMK cells were incubated with the plasmid DNA-lipofectamine complexes in 2 ml DMEM (10% dFBS) for 6 hr before switching to fresh DMEM (10% dFBS). Metabolomics experiments were performed in 6 cm dishes. For transfection in 6 cm dishes, 1 μl PCV iBMK cells were plated and grown overnight before transfection with 8 μg of plasmid DNA; 20 μl of lipofectamine as well as 500 μl of OptiMEM incubated in 5 ml of DMEM (10% dFBS) for 6 hr. Number of viable cells was counted at the indicated time points. Cells were transfected in 6-well plates as described above and viable cells were counted using Trypan blue exclusion assays and a Countess™ II FL Automated Cell Counter (ThermoFisher, MA).

Western Blotting

iBMK cells were collected at the indicated time points after transfection. Cells were washed once with ice cold 1x PBS and scraped into 200 μl RIPA buffer supplemented with phosphatase and protease inhibitors. Collected iBMK cells were vortexed, incubated for 10 min on ice and centrifuged at 17,000 g at 4°C. Supernatant was transferred to a fresh tube and protein content was determined by Pierce™ BCA Protein Assay Kit. 10 μg of protein per sample was analyzed by SDS-PAGE and western blotting. Primary and secondary antibodies (horse-radish peroxidase conjugated) were used at 1:1000 and 1:10,000 respectively. The SuperSignal™ ELISA Pico Chemiluminescent Substrate was used for protein detection.

Glycolytic Flux Measurements

Glucose and lactate measurements were performed in 6-well plates between 24 hr and 30 hr post transfection unless stated otherwise. Cells were washed twice with 2 ml of 1x PBS before 1 ml of fresh DMEM (10% dFBS) was added. After 6 hr of incubation, 300 μl of supernatant was collected and analyzed by a YSI 2900D Biochemistry Analyzer (YSI, Yellow Springs, OH, USA). Measured glucose consumption and lactate secretion were normalized to the average PCV during the 6 hr measurement interval as determined based on the terminal PCV measurement at 30 hr and the known growth rate (0.028 h⁻¹) of GFP expressing cells (Figure S1A):

\[
PCV_{Average} = \frac{1}{0.028h^{-1}} \left( \frac{PCV_{End} - PCV_{End} \times e^{0.028h^{-1} \times 6h}}{6h} \right)
\]

Measurement of the secreted lactate to consumed glucose ratio was done in 5 mM glucose conditions. To make growth medium with 5 mM glucose, DMEM powder without glucose, glutamine, or phenol red was dissolved in deionized water and supplemented with L-glutamine (0.584 g/l), phenol red (0.015 g/l), sodium bicarbonate (3.7 g/l), glucose (0.9 g/l) and 10% dFBS. Data was presented either as absolute values or the percentage change normalized to the vector control as indicated in the corresponding figure panels.
Measurement of Oxygen Consumption Rates
Oxygen consumption rates were measured using a Seahorse XF24 extracellular flux analyzer (Agilent, Santa Clara, CA). Briefly, 100 μl of a 0.5 μl cells ml⁻¹ solution were plated per well in a XF24 cell culture microplate. The next day, cells were transfected with Vector, GFP, GLUT3, PFKP, GAPDH, PKM2, LDHA and MCT4 constructs as described above. 24 hr post transfection, cells were equilibrated for 1 hr in DMEM supplemented with 25 mM glucose and L-glutamine (0.584 g/l) in a non-CO₂ incubator. Oxygen consumption rates were monitored at baseline (5 measurements) and were normalized to cell number as determined by the CyQUANT Cell Proliferation Assay Kit. Experiments were performed with four replicates per overexpression condition and the average of three independent experiments from different days were reported with standard errors.

Calculation of Flux Control Coefficients
Flux control coefficients $C_{E}$ for every step in glycolysis were calculated using the relationship described by Small and Kacser accounting for large changes in enzymatic activity (Small and Kacser, 1993a):

$$f_{E}^{f} = \frac{1}{1 - \frac{r_{E} - 1}{r_{E}} C_{E}^{f}}$$  \hspace{1cm} (Equation 1.1)

where $f_{E}^{f}$ is the fractional glycolytic flux $J$ (for enzyme overexpression compared to control) as a function of fractional enzyme activity $r_{E}$. The derivation of this equation assumes an unbranched pathway with linear reaction kinetics, neither of which applies to glycolysis. The equation is, nevertheless, a valid approximation, as long as flux is measured through the branch in which the enzyme variation occurred (Small and Kacser, 1993a, 1993b). This is true in our case as we are modulating glycolytic enzymes and measuring glycolytic flux. Moreover, based on numerical simulations, the approximation is robust to enzymes that follow Michaelis-Menten as opposed to linear reaction kinetics, with typical deviations < 10% (Small and Kacser, 1993a, 1993b). Thus, we utilized this equation to approximate flux control coefficients.

Solving Equation 1.1 for $C_{E}^{f}$ results in the following equation:

$$C_{E}^{f} = \left( \frac{r_{E} - 1}{r_{E}} \right)^{-1} \left( \frac{r_{E}}{1 - \frac{r_{E} - 1}{r_{E}} C_{E}^{f}} \right)$$  \hspace{1cm} (Equation 1.2)

With the assumption that changes in protein levels of a given enzyme $E$ are directly proportional to changes in enzyme activities $r_{E}$, and since ectopic overexpression generally results in large changes in protein levels, the term $r_{E} / (r_{E} - 1)$ approximates 1 and Equation 1.2 can be simplified as Equation 2:

$$C_{E}^{f} = \left( \frac{r_{E} - 1}{r_{E}} \right)^{-1}$$  \hspace{1cm} (Equation 2)

Equation 2 is shown in Figure 1A. $C_{E}^{f}$ for a given overexpressed enzyme $E$ were calculated using Equation 2 based on the determined fold-change in lactate secretion $f_{E}^{l} = J_{E}/J_{\text{Vector}}$ from experiments performed in 25 mM glucose conditions. Results were presented as the mean with 95% confidence intervals from $n \geq 6$ biological replicates of at least two independent experiments. For the calculation of the sum of determined $C_{E}^{f}$ in iBMK cells, only constructs exhibiting positive flux control in both 5 mM and 25 mM glucose conditions were considered:

$$\sum C_{E}^{f} = C_{GLUT}^{f} + C_{PFK}^{f} + C_{MCT}^{f}$$ \hspace{1cm} (Equation 4.1)

whereby $C_{E}^{f}$ for different gene isoforms acting on the same glycolytic step were averaged:

$$C_{GLUT}^{f} = \frac{C_{GLUT1}^{f} + C_{GLUT3}^{f} + C_{GLUT5}^{f}}{3}$$ \hspace{1cm} (Equation 4.2)

$$C_{PFK}^{f} = \frac{C_{PFKP}^{f} + C_{PFKFB}^{f} + C_{PFKFB}^{f}}{3}$$ \hspace{1cm} (Equation 4.3)

$$C_{MCT}^{f} = C_{MCT4}^{f}$$ \hspace{1cm} (Equation 4.4)

Oligomycin Treatment
Analogous to the transient overexpression experiments, 0.5 μl PCV iBMK cells were plated per well of a 6-well plate in DMEM (10% dFBS) the day before the experiment. The next day, iBMK cells were washed twice with 1x PBS and incubated with 1 ml of fresh DMEM (10% dFBS) supplemented with 1 μM oligomycin. Supernatant was analyzed after 6 hr of incubation and measured lactate was normalized to PCV. Data was presented as the percentage change in lactate secretion compared to vector control.
Metabolomic Analysis Using Mass Spectrometry
Metabolomics experiments were performed in 6 cm dishes. 24 hr post transfection, iBMK cells were switched to fresh DMEM (10% dFBS) and incubated for another 2 hr before extraction. Supernatant was subsequently aspirated and metabolism was directly quenched on dry ice using -20 °C methanol:water (80:20 v/v %). Quenched cells were left on dry ice for at least 10 min before scraping and collecting them into fresh Eppendorf tubes. Samples were vortexed and centrifuged at 17,000 g at 4 °C for 10 min. Supernant was transferred to a fresh Eppendorf tube and dried under a stream of nitrogen. Freshly extracted samples were subjected to liquid chromatography mass spectrometry (LC-MS) analysis on the same day.

For labeling experiments using U-13C-glucose, cells were washed twice with 1x PBS and switched into the glucose labeled cell culture medium 24 hr post transfection. The cells were kept in the labeled medium for 6 hr before quenching. To make U-13C-glucose-containing cell growth medium, DMEM powder was dissolved in deionized water and supplemented with L-glutamine (0.584g/l), phenol red (0.015g/l), sodium bicarbonate (3.7g/l), U-13C-glucose (4.5 g/l) and 10% dFBS.

For LC-MS, dried samples were reconstituted in HPLC grade water at 40 µl per 1 µl PCV cells. At least 50 µl of sample were transferred to a fresh MS vial and 10 µl of sample were injected. Negatively charged metabolites were analyzed by reverse-phase ion-pairing chromatography coupled to an Exactive Orbitrap mass spectrometer (ThermoFisher Scientific, San Jose, CA, USA). For LC separation, a previously described method was modified (Lu et al., 2010) and an Atlantis T3 column (150 mm × 2.1 mm, 3 µm particle size, Waters, Milford, MA) was used. Solvent A consisted of 97:3 water:methanol (v/v %) with 10mM tributylamine and 15mM acetic acid, and solvent B was 100% methanol. The LC gradient was run as following: 0 min, 0% B, 200 µl/min; 2 min, 0% B, 200 µl/min; 4 min, 20% B, 200 µl/min; 13 min, 80% B, 200 µl/min; 17 min, 100% B, 200 µl/min; 17.5 min, 100% B, 300 µl/min; 20 min, 100% B, 300 µl/min; 20.5 min, 0% B, 300µl/min; 24 min, 0% B, 300 µl/min; 25 min, 0% B, 200 µl/min. The column and autosampler temperatures were kept at 25 °C and 5 °C, respectively. The mass spectrometer was operated in negative ion mode with resolving power of 100,000 at m/z 200 and scanning range of m/z 75-1000.

Data were analyzed using the open source software MAVEN (Melamud et al., 2010) and presented as log2 fold-changes compared to vector control unless stated otherwise. Previously determined absolute metabolite concentrations from iBMK cells (Park et al., 2016) were used to convert the measured metabolite intensities into absolute concentrations (assuming any effect of the empty vector expression on absolute metabolite concentrations is small). Adenylate energy charge was calculated using the following equation:

$$\text{Adenylate Energy Charge (EC)} = \frac{[\text{ATP}] + 0.5[\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]}$$

(Equation 5)

Enzyme Assays for HK, GAPDH, PKM and LDH Activities
Cell culture for enzyme activity assays was performed in 6-well plates and iBMK cells were collected 24 hr post transfection. Briefly, cells were washed once with ice-cold 1x PBS and directly scraped in 200 µl ice-cold assay buffer supplemented with phosphatase and protease inhibitors. Cells were lysed by sonication and whole cell lysates were centrifuged at 17,000 g at 4 °C for 10 min. The supernatant was transferred to a fresh Eppendorf tube and 10 µl of whole cell lysate were used for enzyme assays. For GAPDH, PKM and LDH activity assays, cell lysates were diluted 50-fold. Colorometric assay kits from Sigma (hk), Sciencell (GAPDH) and Abcam (PKM and LDH) were used to measure enzyme activities according to manufacturer’s instructions. Enzyme activities were normalized to the protein content as determined by the Pierce™ BCA Protein Assay Kit.

Overexpression of Glycolytic Genes in 3T3 Cells by Lentivirus Transduction
Lentivirus production was performed in 6-well plates, with one plate (or 6 wells) used per individual construct. 30% to 40% confluent HEK293T LX cells were transfected with 1.33 µg of pCMVdR8.91 (packaging), 0.17 µg pMD2.G (envelope) and 1.5 µg of construct DNA using 9 µl of FuGENE. Supernatant was collected between 48 hr and 52 hr after transfection and filtered using a 0.45 µm filter. Concentrated virus was diluted 100-fold and used to transduce 3T3 cells on the same day.

0.3 µl PCV of 3T3 cells were plated per well of a 6-well plate in DMEM (10% dFBS) the day prior to transduction. Cell growth medium was aspirated and 700 µl of freshly-produced virus supplemented with polysine (5µg/ml) and HEPES (10mM) was added directly to each well. Cells were incubated for 4 hr to 6 hr before adding 2 ml of fresh DMEM (10% dFBS; 5µg/ml polysine) to the inoculum. Lactate secretion was measured between 42 hr and 48 hr after transduction as described above. Transduction efficiency was assessed using confocal imaging of blue fluorescent protein (BFP), which was expressed on the same lentiviral vector downstream of the target glycolytic gene using an IRES, and GFP. Briefly, transduced 3T3 cells were harvested via trypsinization, seeded on black glass-bottom 96 well plates (Cellvis, Mountain View, CA, USA), and allowed to adhere for at least 6 hr in normal growth medium. Subsequently, live-cell imaging was carried out with a Nikon Eclipse Ti Spinning Disk Confocal Microscope (Nikon Instruments, Melville, NY, USA), using a 20x objective and 405 nm (BFP) and 488 nm (GFP) laser lines.

Optogenetic Stimulation of Ras Signaling in 3T3 Opto-SOS Cells
Optogenetics experiments were performed as previously described (Goglia et al., 2017). Briefly, clonal 3T3 cells expressing the light-activatable Opto-SOS system were plated in DMEM (10% dFBS) and grown overnight. The next day, 4 hr to 6 hr prior to stimulation, the cells were washed twice with 1xPBS and serum starved in fresh DMEM (without FBS) supplemented with 10 µM phycocyanobilin.
(PCB). For all steps following PCB addition, the cells were carefully protected from ambient light (e.g., experiments performed in low-light conditions and plates wrapped in aluminum foil) to prevent activation of the Opto-SOS system. Subsequently, the cells were either exposed to 650 nm red light (stimulated) or were left in the dark (unstimulated) until the end of the experiment. Glycolytic flux measurements were performed in 6-well plates and change in lactate secretion was assessed after 6 hr of stimulation as described above. For metabolomics experiments, cells were grown in 6 cm dishes and intracellular metabolites were extracted and analyzed after 4 hr and 24 hr of stimulation as described earlier. Cell growth was determined by measuring change in PCV at 24 hr post-stimulation.

Kinetic glycolytic flux profiling of stimulated and unstimulated cells using U-\(^{13}\)C-glucose was performed in 6 cm dishes. Serum starved, PCB-treated cells were either pre-exposed to activating red light or left in the dark for 30 min before washing twice with 1xPBS and switching to 2 ml of U-\(^{13}\)C-glucose-containing DMEM. Cells were subsequently collected at the indicated time points and subjected to LC-MS analysis as described above.

**RNAseq Analysis of 3T3 Opto-SOS Cells**

Changes in gene expression of 3T3 Opto-SOS cells upon Ras activation were reanalyzed from a previously published dataset (GEO accession # GSE100816) (Wilson et al., 2017). The reads for the light activated samples (0, 30, 60, and 120 min) were analyzed using the Galaxy Workflow system (Afgan et al., 2016) and custom scripts (https://bitbucket.org/princeton_genomics/tanner_glycolysis_flux_maseq/) implemented in R (R Core Team, 2017). Briefly, adapters were removed and reads were quality trimmed using Trim Galore! V0.4.3.1 with default parameters (Krueger, 2017). The trimmed reads were mapped to the GRCm38/mm10 genome using TopHat 2.1.1 (Kim et al., 2013). The GTF option was used with the genes.gtf file from Illumina’s iGenomes (https://support.illumina.com/sequencing/sequencing_software/igenome.html) and the no-novel-juncs option was specified to ensure all samples used the same set of splice junctions. FeatureCounts 1.4.6.p5 (Liao et al., 2014) was used to determine the read counts for each gene specified in the iGenomes gtf file. Finally, DESeq2 1.16.1 (Love et al., 2014) was used to calculate normalization factors for each sample. Technical replicates were combined and genes with counts lower than 200 were excluded from further analysis. Differential expression was calculated at each time point compared to 0 min and the data was presented as log2-fold-ratios. Gene set enrichment analysis (GSEA) was performed using the GSEA software (Mootha et al., 2003; Subramanian et al., 2005). Briefly, only genes exhibiting a two-fold change on at least one time point were chosen and enrichment for hallmark gene sets and KEGG annotated gene sets was calculated.

**Gene Expression Analysis of TCGA Data**

RNAseq data from human solid tumor tissue samples were downloaded from The Cancer Genome Atlas (TCGA) Research Network (http://cancergenome.nih.gov/) using the open-source software TCGA-assembler (Lu et al., 2010) executed in R (R Core Team, 2017). Only primary tumor samples (TCGA barcode identifier: 01) with paired adjacent normal tissue samples (TCGA barcode identifier: 11) were considered for further analysis. This resulted in a final set of 666 paired human solid tumor tissue samples from 19 different tumor types (Table S2).

A list of 40 glycolytic enzymes and transporters was manually curated based on annotation in KEGG pathways (Kanehisa and Goto, 2000; Kanehisa et al., 2016, 2017). Gene expression of primary tumor tissue samples was normalized to the corresponding adjacent healthy tissue samples and the normalized values were presented as a log2 fold ratio. Gene and samples were clustered by pearson correlation distances (centered) with average-linkage implemented in Cluster3.0 (de Hoon et al., 2004). Calculated dendrograms and heat maps were subsequently visualized using Java TreeView (Saldanha, 2004). The clustered dendrogram heat map was generated using aheatmap command in the R package NMF (Gaujoux and Seoighe, 2010).

For rank order analysis of the 40 glycolytic genes, the average fold-expression change relative to benign adjacent tissue of different glycolytic genes across all samples of a given tumor type was ranked from highest to lowest. The topmost up-regulated GLUT, PFK and MCT isoforms were highlighted until at least one gene isoform of each of these three glycolytic steps was covered.

To determine the four most up-regulated glycolytic steps in each tumor sample, gene isoforms were ranked based on their extent of up-regulation relative to adjacent benign tissue. Subsequently, the frequency with which gene isoforms were among the four topmost up-regulated glycolytic genes was tabulated. Gluconeogenic and anti-glycolytic (FBP1, PFKFB2 &TIGAR), poorly annotated (HKDC1) and pyruvate dehydrogenase (PDHA1 & PDHB) gene isoforms were excluded from this analysis (Table S3). A Fisher’s exact test (with Bonferroni correction for multiple hypothesis testing) was performed to probe the statistical significance of whether a given isoform is more likely to rank in the four top most up-regulated genes than expected.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

All statistical analyses were done in R (R Core Team, 2017). The following packages were additionally used for general data preparation: dplyr (Wickham and Francois, 2016), plotrix (Lemon, 2006), reshape/reshape2 (Wickham, 2007), stringr (Wickham, 2017a) and tidyr (Wickham, 2017b). For all experiments, mean and 95% confidence intervals of at least n=3 biological replicates were plotted unless stated otherwise. Statistically significant differences (p<0.05) were calculated by two-tailed unpaired Student’s t test and multiple hypothesis correction (q<0.05) was performed using the qvalue package (Storey, 2015). Michaelis-Menten curves were fitted.
using the `nls` function and $R^2$ for fits were calculated by the residual sum of squares (RSS) and total sum of squares (TSS) using the following equation:

$$R^2 = 1 - \frac{RSS}{TSS} \quad \text{(Equation 6)}$$

95% confidence intervals of the Michaelis-Menten fit were calculated using the `drc` package (Ritz et al., 2015). P values for Michaelis-Menten fits were calculated by linear regression of the predicted versus measured values. Metabolomics heat maps were generated with the `pheatmap` package (Kolde, 2015). All other plots were generated using `ggplot2` (Wickham, 2009) and all the figures were formatted in Illustrator (Adobe, San Jose, CA, USA).