

Pyruvate Kinase Isoform Expression Alters Nucleotide Synthesis to Impact Cell Proliferation

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SUMMARY

Metabolic regulation influences cell proliferation. The influence of pyruvate kinase isoforms on tumor cells has been extensively studied, but whether PKM2 is required for normal cell proliferation is unknown. We examine how PKM2 deletion affects proliferation and metabolism in nontransformed, nonimmortalized PKM2-expressing primary cells. We find that deletion of PKM2 in primary cells results in PKM1 expression and proliferation arrest. PKM1 expression, rather than PKM2 loss, is responsible for this effect, and proliferation arrest cannot be explained by cell differentiation, senescence, death, changes in gene expression, or prevention of cell growth. Instead, PKM1 expression impairs nucleotide production and the ability to synthesize DNA and progress through the cell cycle. Nucleotide biosynthesis is limiting, as proliferation arrest is characterized by severe thymidine depletion, and supplying exogenous thymine rescues both nucleotide levels and cell proliferation. Thus, PKM1 expression promotes a metabolic state that is unable to support DNA synthesis.

INTRODUCTION

Proliferating cells metabolize glucose via aerobic glycolysis (Cairns et al., 2011; Hanahan and Weinberg, 2011; Koppenol et al., 2011). Aerobic glycolysis is characterized by increased glucose uptake and lactate excretion in the presence of oxygen, and has been proposed to promote the use of glucose for biosynthetic pathways necessary for cell growth and division

(Lunt and Vander Heiden, 2011; Ward and Thompson, 2012); however, connections between aerobic glycolysis and specific pathway use are not well defined. The M2 isoform of the glycolytic enzyme pyruvate kinase (PKM2) has been associated with both aerobic glycolysis and anabolic metabolism in cancer cells (Anastasίου et al., 2012; Christofk et al., 2008a; Mazurek, 2011). PKM2 is also expressed in normal proliferating tissues (Mazurek, 2011), yet it is unclear how pyruvate kinase isoform expression influences cell metabolism to support proliferation, and whether PKM2 is required for normal cell proliferation.

Pyruvate kinase converts phosphoenolpyruvate and ADP to pyruvate and ATP in glycolysis. Four isoforms of pyruvate kinase exist in mammals, each with varying kinetic and regulatory properties adapted for different tissue types. The *PKLR* gene uses two different promoters with alternative first exons to produce either the R isoform found in red blood cells or the L isoform expressed in gluconeogenic tissues such as the liver and kidney (Domingo et al., 1992; Noguchi et al., 1987). The M1 and M2 isoforms are produced by mutually exclusive alternative mRNA splicing of the *PKM* gene. Including exon 9 in the transcript generates PKM1, while including exon 10 generates PKM2 (Noguchi et al., 1986; Yamada and Noguchi, 1999). PKM2 is found in early embryonic cells, normal proliferating cells, and tumor cells, as well as in white fat, lung, retina, and pancreatic islets (Imamura and Tanaka, 1982; Mazurek, 2011). PKM1 replaces PKM2 during development in tissues with high ATP production requirements including skeletal muscle, heart, and brain (Imamura et al., 1986; Mazurek, 2011). When cell proliferation is reactivated in nonproliferating tissues that do not express PKM2, such as during liver regeneration or carcinogenesis, PKM2 expression is observed (Hacker et al., 1998; Steinberg et al., 1999; van Veelen et al., 1977; Yamada and Noguchi, 1995), implying PKM2 may be important for proliferation.

PKM1 and PKM2 exhibit different regulatory and catalytic properties. PKM1 is not allosterically regulated and assembles into stable homotetramers with high pyruvate kinase activity

(Gui et al., 2013; Mazurek, 2011). In contrast, PKM2 can exist in an inactive nontetrameric form or active tetrameric form, and these states can be regulated by phosphotyrosine signaling, redox status, acetylation, and metabolic intermediates including FBP, amino acids, SAICAR, and fatty acids (Anastasiou et al., 2011, 2012; Chaneton et al., 2012; Christofk et al., 2008b; Keller et al., 2012; Lv et al., 2011). In addition, several nonglycolytic functions specific for PKM2 have been reported to be critical for cancer cell proliferation (Gao et al., 2012; Jiang et al., 2014; Keller et al., 2014; Luo et al., 2011; Yang et al., 2011, 2012a, 2012b), but it is unclear if any of these functions are important for proliferation of normal cells.

Here, we use nonimmortalized primary cells from PKM2-conditional mice to study the role of PKM1 and PKM2 isoform expression in cell metabolism and proliferation. Deletion of PKM2 in these cells results in PKM1 expression and proliferation arrest. Expression of PKM1 in cells that coexpress PKM2 also results in proliferation arrest, suggesting that expression of PKM1, rather than loss of PKM2, is responsible for this phenotype. Proliferation arrest is not associated with cell differentiation, senescence, changes in gene expression, or death; instead, PKM1 expression results in decreased flux to select biosynthetic pathways, with nucleotide synthesis being a critical pathway that is limiting for cell proliferation. Proliferation arrest can be rescued by exogenous pyrimidine or purine base supplementation. These data argue that PKM1 expression suppresses nucleotide biosynthesis, and that PKM2 expression supports flux into metabolic pathways to support DNA synthesis.

RESULTS

PKM1 Expression Causes Proliferation Arrest of Primary Embryonic Fibroblasts

To study the role of PKM2 in cell proliferation, we derived mouse embryonic fibroblasts (MEFs) from mice where LoxP sites flank PKM2-specific exon 10 ($PKM2^{fl/fl}$) and harbor an inducible Cre recombinase allele (*Cre-ER*) (Israelsen et al., 2013). Deletion of exon 10 disrupts the ability of cells to produce PKM2 or any truncated protein product with PKM2 activity but does not prevent production of PKM1 (Israelsen et al., 2013). When Cre recombinase remains inactive, PKM2 is expressed in $PKM2^{fl/fl}$ *Cre-ER* cells (Figure 1A), as reported for wild-type MEFs (Anastasiou et al., 2011). Addition of 4-hydroxytamoxifen (4-OHT) activates Cre recombinase, leading to loss of PKM2 protein and PKM1 expression (Figure 1A). PKM1 expression is observed 1 day after 4-OHT treatment; however, residual PKM2 can be detected for up to 4 days following 4-OHT treatment, presumably due to the half-life of PK protein being as long as 104 hr (Illg and Pette, 1979; Yee et al., 2010).

Similar to wild-type MEFs, primary $PKM2^{fl/fl}$ *Cre-ER* MEFs proliferate rapidly in culture for multiple passages (Figure 1B). When treated with 4-OHT, these MEFs exhibit a marked decrease in proliferation (Figure 1B). A similar proliferation arrest was observed in multiple lines, each independently derived from different $PKM2^{fl/fl}$ *Cre-ER* mice (see Figure S1A available online). To ensure that 4-OHT treatment itself is not affecting PK isoform expression or cell proliferation, we generated MEFs from $PKM2^{fl/fl}$ mice that do not harbor a *Cre-ER* allele. These Cre-

negative $PKM2^{fl/fl}$ MEFs express PKM2 (Figure S1B) and proliferate at a similar rate regardless of 4-OHT treatment (Figure 1C).

To determine whether expression of PKM1 or loss of PKM2 is responsible for cell proliferation arrest, we derived MEFs from $PKM2^{fl/+}$ *Cre-ER* mice. Without 4-OHT treatment, these MEFs express only PKM2 (Figure 1D) and proliferate for several passages (Figure 1E). 4-OHT treatment of $PKM2^{fl/+}$ *Cre-ER* MEFs causes deletion of the PKM2 conditional allele and results in expression of PKM1 with continued PKM2 expression from the wild-type allele (Figure 1D). As observed in $PKM2^{fl/fl}$ *Cre-ER* MEFs, 4-OHT addition caused a similar proliferation arrest in $PKM2^{fl/+}$ *Cre-ER* MEFs despite retention of PKM2 expression (Figure 1E). Cre expression alone can also slow proliferation without affecting pyruvate kinase isoform expression, but does not increase γ -H2AX levels and has less impact on cell proliferation than Cre-mediated PKM2 deletion (Figures S1C–S1E). To test whether the increase in pyruvate kinase activity associated with gain of PKM1 expression contributes to a block in proliferation, we monitored proliferation of wild-type MEFs following addition of the small molecule PKM2 activator TEPP-46 (Anastasiou et al., 2012). TEPP-46 decreases proliferation (Figure 1F), and exogenous PKM1 cDNA expression also reduces proliferation of wild-type MEFs (Figure S1F). Together, these data suggest proliferation is suppressed in $PKM2^{\Delta/\Delta}$ MEFs due to gain of PKM1 rather than loss of PKM2.

Deletion of PKM2 Does Not Induce Cell Death, Senescence, or Differentiation

Potential explanations for why PKM1 expression suppresses proliferation include an increase in cell death, cell senescence, and/or differentiation to a nonproliferating cell population. To begin to examine these possibilities in cells with and without PKM2 deletion, we quantified the percentage of viable cells by propidium iodide exclusion and the amount of cell death by lactate dehydrogenase release (Decker and Lohmann-Matthes, 1988; Nicoletti et al., 1991). No difference in cell viability or LDH release was found between $PKM2^{fl/fl}$ and $PKM2^{\Delta/\Delta}$ MEFs as late as 9 days after vehicle or 4-OHT treatment despite a large difference in cell number (Figures 2A and S2A). Consistent with these results, >90% of MEFs from both populations exclude trypan blue at this time point. These data suggest PKM1 expression following PKM2-deletion inhibits cell proliferation without increasing cell death.

Primary MEFs undergo senescence upon serial passage in culture that involves entry into a state of irreversible proliferation arrest (Campisi, 2011; Kuilman et al., 2010; Odell et al., 2010). Therefore, it was possible that PKM1 expression induces early senescence. One marker of senescent cells is acid-stable β -galactosidase activity (Castro et al., 2003; Dimri et al., 1995; van der Loo et al., 1998). No difference in acid-stable β -galactosidase activity was found between early passage proliferating $PKM2^{fl/fl}$ and proliferation-arrested $PKM2^{\Delta/\Delta}$ MEFs as late as 9 days after treatment with vehicle or 4-OHT (Figure 2B, upper panels). Late-passage $PKM2^{fl/fl}$ MEFs senesce as evidenced by proliferation arrest, morphology change, and acid-stable β -galactosidase staining. Surprisingly, despite no proliferation for days, $PKM2^{\Delta/\Delta}$ MEFs stain positive for acid-stable β -galactosidase activity at the same time $PKM2^{fl/fl}$ MEFs senesce due to

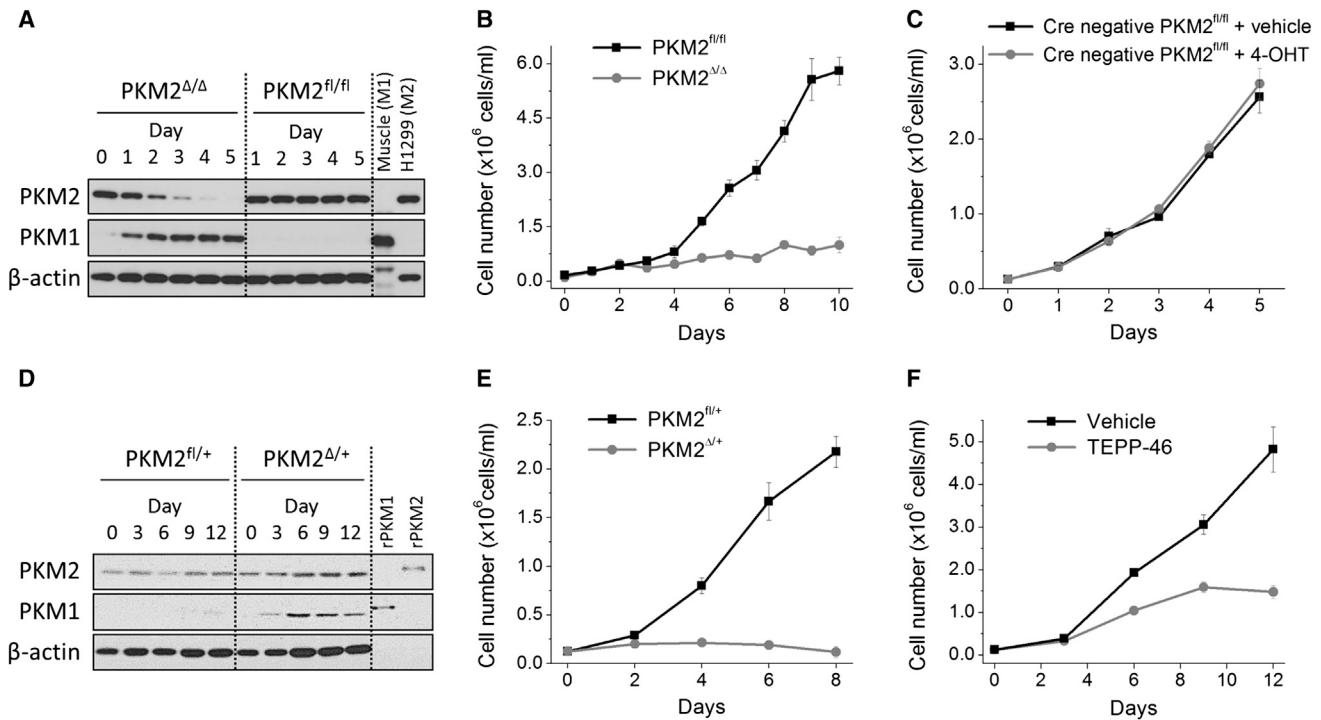


Figure 1. PKM2 Deletion with PKM1 Expression Results in Proliferation Arrest

(A) The effect of 4-OHT treatment on pyruvate kinase isoform expression in *PKM2^{fl/fl}* Cre-ER MEFs was assessed by western blot analysis. *PKM2^{fl/fl}* Cre-ER MEFs were treated with 4-OHT (*PKM2^{Δ/Δ}* Cre-ER) or vehicle (*PKM2^{fl/fl}* Cre-ER) for the indicated number of days. Muscle or H1299 cancer cell lysate was included as control samples that express only PKM1 or PKM2, respectively.

(B) Proliferation of *PKM2^{fl/fl}* Cre-ER MEFs following vehicle (*PKM2^{fl/fl}* Cre-ER) or 4-OHT (*PKM2^{Δ/Δ}* Cre-ER) treatment was assessed by counting cell numbers as shown.

(C) Proliferation of Cre negative *PKM2^{fl/fl}* MEFs treated with vehicle or 4-OHT was assessed by counting cell numbers as shown.

(D) Western blot analysis of MEFs generated from *PKM2^{fl/+}* Cre-ER mice. 4-OHT treatment results in *PKM2^{fl/Δ}* MEFs that express both PKM2 and PKM1 protein. Western blot analysis of 100 ng of recombinant PKM1 or PKM2 (rPKM1, rPKM2) is included as control.

(E) Proliferation of *PKM2^{fl/+}* Cre-ER MEFs treated with vehicle (*PKM2^{fl/+}*) or with 4-OHT (*PKM2^{Δ/+}*) was assessed by counting cell numbers as shown.

(F) Proliferation of wild-type MEFs treated with vehicle or 1 μ M of PKM2 activator TEPP-46 was assessed by counting cell numbers over time as shown.

All data are displayed as means $\pm$ SEM, $n = 3$. See also Figure S1.

high passage number (Figure 2B, lower panels). These data suggest induction of the senescence program observed with serial primary cell passage does not correlate with proliferation arrest of *PKM2^{Δ/Δ}* cells.

Primary MEFs can differentiate under specific culture conditions. For instance, MEFs can differentiate along an adipocyte-like lineage and accumulate neutral lipids (Green and Kehinde, 1974; Russell and Ho, 1976). To determine if *PKM2^{Δ/Δ}* MEFs accumulate lipids, we stained them with oil red O and found no evidence of lipid accumulation (Figure S2B). To further test whether *PKM2* deletion causes differentiation, and to determine whether proliferation arrest is observed in *PKM2^{Δ/Δ}* primary cells other than MEFs, we isolated myoblasts from *PKM2^{fl/fl}* Cre-ER mice. Myoblasts can be induced to differentiate into myotubes with characteristic morphology (Wakelam, 1985), and are a well-studied differentiation model (Abmayr and Pavlath, 2012). Similar to the phenotype observed in *PKM2^{fl/fl}* Cre-ER MEFs, treatment of *PKM2^{fl/fl}* Cre-ER myoblasts with 4-OHT results in *PKM2* deletion and PKM1 expression (Figure S2C) with proliferation arrest (Figure 2C). Similar to immortalized C2C12 myo-

blasts (Clower et al., 2010), we confirmed that primary *PKM2^{fl/fl}* Cre-ER myoblasts are capable of differentiation under conditions that favor myotube formation (Figure 2D) and express PKM1 upon differentiation (Figure S2D). However, *PKM2* deletion in myoblasts does not cause differentiation into myotubes (Figure 2D).

***PKM2^{Δ/Δ}* MEFs Do Not Incorporate EdU**

Lack of *PKM2^{Δ/Δ}* MEF proliferation implies an inability to proceed through the cell cycle. To investigate whether *PKM2^{Δ/Δ}* MEFs replicate DNA, they were incubated with EdU, a thymidine analog incorporated into DNA during S phase of the cell cycle. Consistent with lack of DNA synthesis, *PKM2^{Δ/Δ}* MEFs exhibit decreased EdU incorporation compared to *PKM2^{fl/fl}* MEFs (Figure 2E). Cells deprived of nutrients classically arrest in G1 phase of the cell cycle (Bohnsack and Hirschi, 2004). To determine whether *PKM2^{Δ/Δ}* MEFs were arrested in G1, we analyzed DNA content by flow cytometry. Both the G1 and G2/M phase DNA content peaks widened in a pattern that is most consistent with more cells in S phase (Figure 2F). Dual staining of EdU

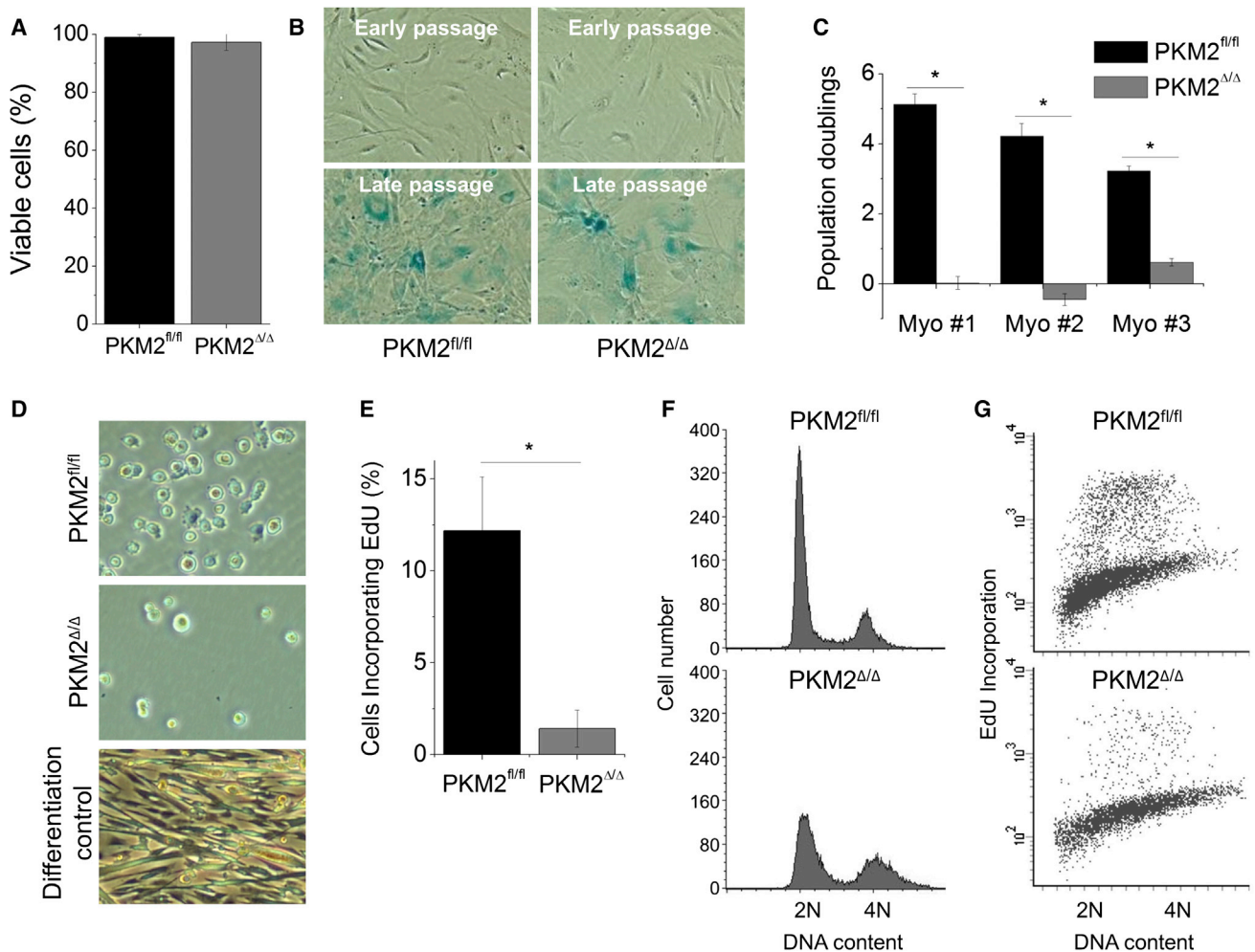


Figure 2. $PKM2^{\Delta/\Delta}$ Cells Undergo Cell Cycle Arrest

(A) Quantification of cell viability by propidium iodide staining after treatment of $PKM2^{fl/fl}$ MEFs with vehicle ($PKM2^{fl/fl}$) or 4-OHT ($PKM2^{\Delta/\Delta}$). Data are displayed as means $\pm$ SEM, $n = 3$.

(B) Classical cell senescence was assayed by acid stable β -galactosidase activity. The top panels (labeled "Early passage") assess acid stable β -galactosidase activity in $PKM2^{fl/fl}$ and $PKM2^{\Delta/\Delta}$ MEFs 9 days after vehicle or 4-OHT treatment, respectively. Bottom panels (labeled "Late passage") assess acid stable β -galactosidase activity in $PKM2^{fl/fl}$ and $PKM2^{\Delta/\Delta}$ MEFs after 12 days in culture. The $PKM2^{fl/fl}$ MEFs had undergone proliferation arrest at this time point.

(C) Population doublings over 12 days for myoblasts isolated from three different $PKM2^{fl/fl}$ Cre-ER mice that were treated with vehicle ($PKM2^{fl/fl}$) or 4-OHT ($PKM2^{\Delta/\Delta}$). Data are displayed as means $\pm$ SEM, $n = 3$.

(D) Images of myoblasts generated from $PKM2^{fl/fl}$ Cre-ER mice 9 days after treatment with vehicle ($PKM2^{fl/fl}$) or 4-OHT ($PKM2^{\Delta/\Delta}$) are shown. $PKM2^{fl/fl}$ myoblasts induced to differentiate by culture in low serum for 9 days are also presented (lower panel).

(E) EdU uptake of $PKM2^{fl/fl}$ and $PKM2^{\Delta/\Delta}$ MEFs was measured in $PKM2^{fl/fl}$ Cre-ER MEFs 7 days after vehicle or 4-OHT treatment. Data are displayed as means $\pm$ SEM, $n = 3$.

(F) DNA content was assessed using propidium iodide staining and flow cytometry in $PKM2^{fl/fl}$ and $PKM2^{\Delta/\Delta}$ MEFs 7 days after $PKM2^{fl/fl}$ Cre-ER MEFs were treated with vehicle or 4-OHT.

(G) DNA content was assessed following dual staining with FxCycle Violet and EdU using flow cytometry in $PKM2^{fl/fl}$ and $PKM2^{\Delta/\Delta}$ MEFs 7 days after $PKM2^{fl/fl}$ Cre-ER MEFs were treated with vehicle or 4-OHT.

See also Figure S2.

incorporation and DNA content is also consistent with an inability to progress through the cell cycle, as less EdU incorporation is observed at all DNA contents, with much of the population having a DNA content between 2N and 4N despite no EdU uptake (Figure 2G). To compare $PKM2^{\Delta/\Delta}$ MEF arrest to cell cycle arrest induced by nutrient withdrawal, the DNA content of wild-type

MEFs was assessed following removal of the essential amino acid lysine. Lysine withdrawal also causes proliferation arrest (Figure S2E); however, unlike $PKM2^{\Delta/\Delta}$ MEFs, these cells accumulate in G1 (Figure S2F). Taken together, these results argue that lack of proliferation caused by PKM1 expression is distinct from G1 arrest caused by nutrient deprivation. Instead, these

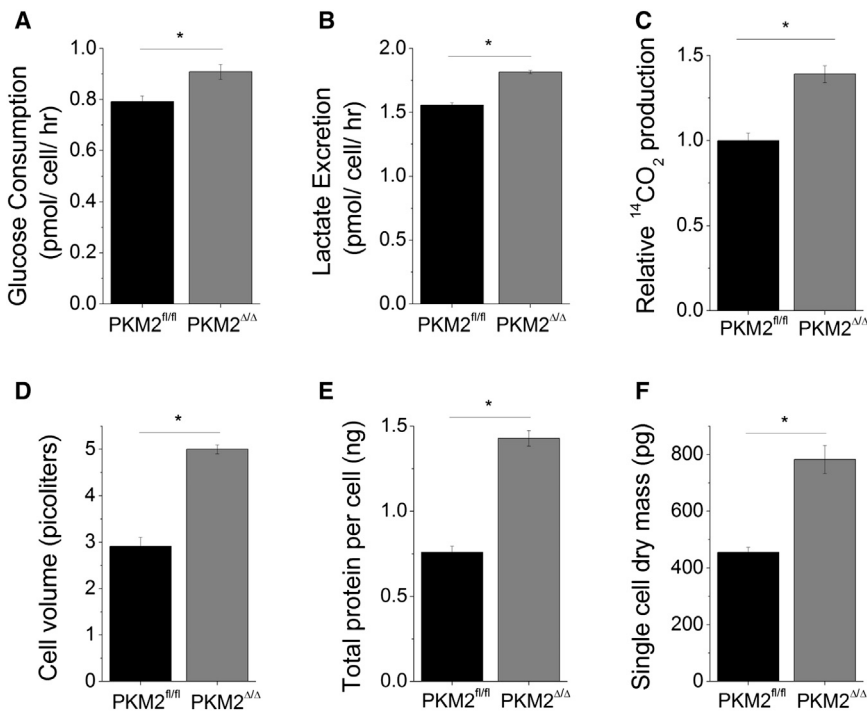


Figure 3. $PKM2^{\Delta/\Delta}$ Cells Remain Metabolically Active and Are Larger Than $PKM2^{fl/fl}$ Cells

(A) Glucose uptake rates; (B) lactate excretion rates; (C) the relative production of ^{14}C -labeled CO_2 from uniformly ^{14}C -labeled glucose; (D) average cell volumes; (E) total protein content; and (F) single cell dry mass of $PKM2^{fl/fl}$ and $PKM2^{\Delta/\Delta}$ MEFs 7 days after treatment of $PKM2^{fl/fl}$ MEFs with vehicle or 4-OHT. All data are displayed as means $\pm$ SEM, $n = 3$ ($p < 0.0001$). See also Figure S3.

data are more consistent with a failure of $PKM2^{\Delta/\Delta}$ MEFs to incorporate nucleotides into DNA.

Despite Proliferation Arrest, $PKM2^{\Delta/\Delta}$ MEFs Continue to Uptake Nutrients and Grow

Cell division can depend on cell size in eukaryotic cells (Johnston et al., 1977; Montagne et al., 1999). A possible explanation for why cells stop proliferating is that changes in metabolism lead to insufficient metabolic precursors to support cell growth, and sufficient size to complete a cell cycle is not achieved. To determine whether proliferation arrest of $PKM2^{\Delta/\Delta}$ MEFs is associated with decreased aerobic glycolysis, we measured consumption of glucose as well as lactate excretion. Surprisingly, despite proliferation arrest, $PKM2^{\Delta/\Delta}$ MEFs have higher glucose uptake and lactate excretion rates than $PKM2^{fl/fl}$ MEFs (Figures 3A and 3B). Overall glucose oxidation is also increased in $PKM2^{\Delta/\Delta}$ MEFs: measurement of $^{14}CO_2$ production from uniformly radiolabeled glucose revealed $\sim 40\%$ more $^{14}CO_2$ production from glucose over 24 hr in $PKM2^{\Delta/\Delta}$ MEFs than in $PKM2^{fl/fl}$ MEFs (Figure 3C). In addition, assessment of cell volume showed that $PKM2^{\Delta/\Delta}$ cells are ~ 1.7 times larger than proliferating $PKM2^{fl/fl}$ cells (Figure 3D). In fact, the mean volume of arrested $PKM2^{\Delta/\Delta}$ cells was larger than 85% of cycling $PKM2^{fl/fl}$ cells, suggesting $PKM2^{\Delta/\Delta}$ MEFs grow to a large size despite proliferation arrest. The cell volume increase is accompanied by increased protein amount, with total protein content of $PKM2^{\Delta/\Delta}$ MEFs ~ 1.9 times greater than total protein content in cycling $PKM2^{fl/fl}$ MEFs (Figure 3E). $PKM2^{\Delta/\Delta}$ MEFs also contain higher levels of fatty acids, with pool sizes of palmitate, oleate, and stearate >2 -fold higher in $PKM2^{\Delta/\Delta}$ MEFs compared to $PKM2^{fl/fl}$ MEFs (Figure S3). To confirm that increases in cell volume, total protein, and select fatty acids are accompanied by an increase in cell mass,

we utilized a suspended microchannel resonator (Burg et al., 2007) to determine dry mass at the single cell level (Feijó Delgado et al., 2013). The dry mass of $PKM2^{\Delta/\Delta}$ cells is greater than the dry mass of $PKM2^{fl/fl}$ cells (783 pg for $PKM2^{\Delta/\Delta}$ MEFs compared to 455 pg for $PKM2^{fl/fl}$ MEFs; Figure 3F). One million $PKM2^{\Delta/\Delta}$ MEFs also weighed $24\% \pm 4\%$ more than the same number of $PKM2^{fl/fl}$ MEFs when dried on filter paper and weighed using an analytical balance.

Together, these data suggest that despite proliferation arrest, $PKM2^{\Delta/\Delta}$ MEFs continue to metabolize glucose at high rates and grow to a larger size than proliferating $PKM2^{fl/fl}$ MEFs.

$PKM2$ Supports Specific Aspects of Anabolic Metabolism

Despite growing to a larger size, a specific metabolite(s) or metabolic flux limitations could account for the inability of $PKM2^{\Delta/\Delta}$ cells to proliferate. To explore this hypothesis, we used isotope-labeled nutrients and mass spectrometry-based metabolite analysis to probe metabolism of $PKM2^{\Delta/\Delta}$ MEFs in comparison to $PKM2^{fl/fl}$ MEFs. Analysis of intracellular metabolites after culture in the presence of uniformly labeled ^{13}C -glucose ($[U-^{13}C_6]$ glucose) and glutamine ($[U-^{13}C_5]$ glutamine) reveals that $PKM2^{\Delta/\Delta}$ MEFs have decreased labeling from glucose and glutamine in some biosynthetic precursors including the nucleotide uridine monophosphate (UMP) and the fatty acid palmitate (Figures 4 and S4). Moreover, ^{13}C -glucose incorporation into serine and glycine was also decreased in $PKM2^{\Delta/\Delta}$ MEFs. In contrast, a higher percentage of TCA cycle intermediates are labeled from $[U-^{13}C_6]$ glucose in $PKM2^{\Delta/\Delta}$ MEFs (Figure 4A), suggesting increased glucose contribution and/or decreased glutamine contribution to the TCA cycle. Labeling studies using $[U-^{13}C_5]$ glutamine show that $PKM2^{\Delta/\Delta}$ MEFs have lower glutamine contribution to TCA cycle intermediates (Figure 4B), implying that proliferation-arrested $PKM2^{\Delta/\Delta}$ MEFs rely more on glucose than glutamine to support the TCA cycle. These results confirm that PKM1 expression leads to increased glucose catabolism, and support a model in which PKM1 expression curtails the use of glucose and glutamine for production of some biosynthetic precursors.

Glucose is the major precursor for de novo palmitate synthesis in both $PKM2^{\Delta/\Delta}$ and $PKM2^{fl/fl}$ MEFs, despite less palmitate

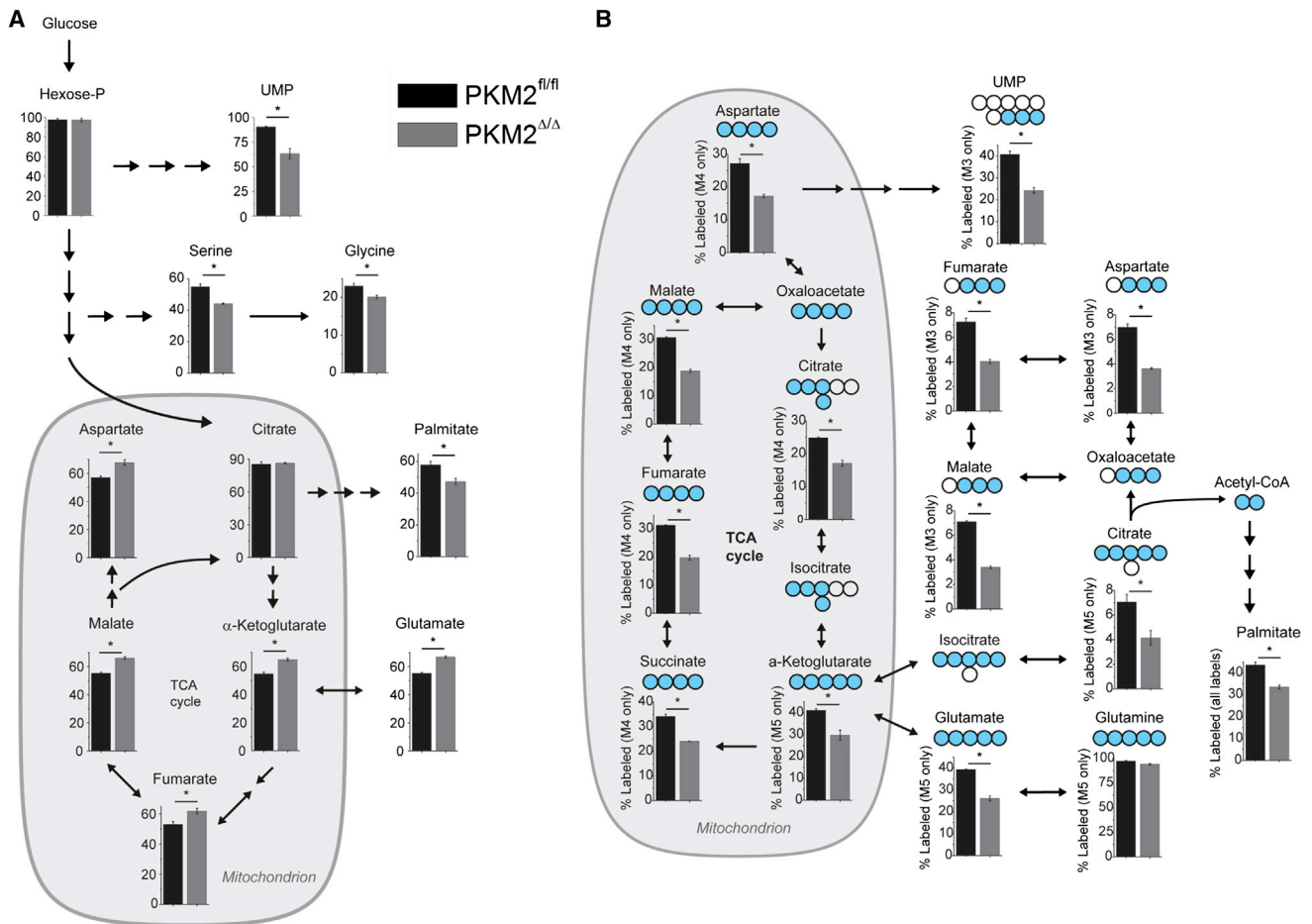


Figure 4. Pyruvate Kinase Isoform Expression Affects the Use of Specific Metabolic Pathways

(A) Labeling of intracellular metabolites with [U-¹³C]glucose or (B) [U-¹³C]glutamine was measured using mass spectrometry in *PKM2^{fl/fl}* and *PKM2^{Δ/Δ}* MEFs 7 days after *PKM2^{fl/fl} Cre-ER* MEFs were treated with vehicle or 4-OHT. Complete isotopomer distributions used to calculate these values are included in Table S1. The y axis for all graphs in (A) is the calculated percent total glucose contribution (Fendt et al., 2013a).

See also Figure S4.

synthesized from both glucose and glutamine in *PKM2^{Δ/Δ}* MEFs relative to *PKM2^{fl/fl}* MEFs (Figure S4). The rate of total fatty acid synthesis decreased by 22% with PKM2 deletion in these cells, consistent with decreased lipid synthesis observed previously upon PKM1 expression (Anastasiou et al., 2012). A fraction of palmitate is produced from glutamine in *PKM2^{fl/fl}* and *PKM2^{Δ/Δ}* MEFs, with a labeling pattern consistent with synthesis by reductive metabolism in both cases (Fendt et al., 2013b; Metallo et al., 2012). Although *PKM2^{Δ/Δ}* MEFs exhibit decreased fatty acid synthesis, they contain higher levels of intracellular fatty acids compared to their *PKM2^{fl/fl}* counterparts (Figure S3). To accumulate high levels of fatty acids despite a decrease in biosynthesis, it is likely that *PKM2^{Δ/Δ}* MEFs import exogenous fatty acids. Lipid scavenging from the media has been observed previously in cancer cells (Kamphorst et al., 2013).

Proliferation of *PKM2^{Δ/Δ}* MEFs Is Limited by Nucleotides

Pool sizes of intracellular metabolites were measured to assess whether specific metabolites are limiting for cell proliferation in

PKM2^{Δ/Δ} MEFs. While levels of most metabolites in *PKM2^{Δ/Δ}* cells were unchanged or increased relative to *PKM2^{fl/fl}* cells (Figure S5A), nucleotide levels were lower, and the most-depleted intracellular metabolite measured in *PKM2^{Δ/Δ}* MEFs is thymidine monophosphate (dTMP; Figure 5A). This is consistent with decreased glucose and glutamine contribution to UMP observed in *PKM2^{Δ/Δ}* MEFs (Figure 4), as UMP is a biosynthetic precursor of dTMP.

To explore the possibility that de novo nucleotide synthesis is decreased in *PKM2^{Δ/Δ}* MEFs, we performed dynamic labeling experiments and flux modeling. Dynamic labeling with uniformly labeled ¹³C-glucose and ¹³C-glutamine confirms decreased glucose and glutamine flux to UMP in *PKM2^{Δ/Δ}* MEFs (Figure 5B). The same labeling studies also suggest flux of glucose carbon into IMP and AMP is decreased slightly in *PKM2^{Δ/Δ}* MEFs, while glutamine labeling is not informative, as this amino acid does not contribute carbons to de novo purine synthesis. Analysis of metabolic fluxes from steady-state labeling data (Antoniewicz et al., 2007; Young et al., 2008) supports the notion that de

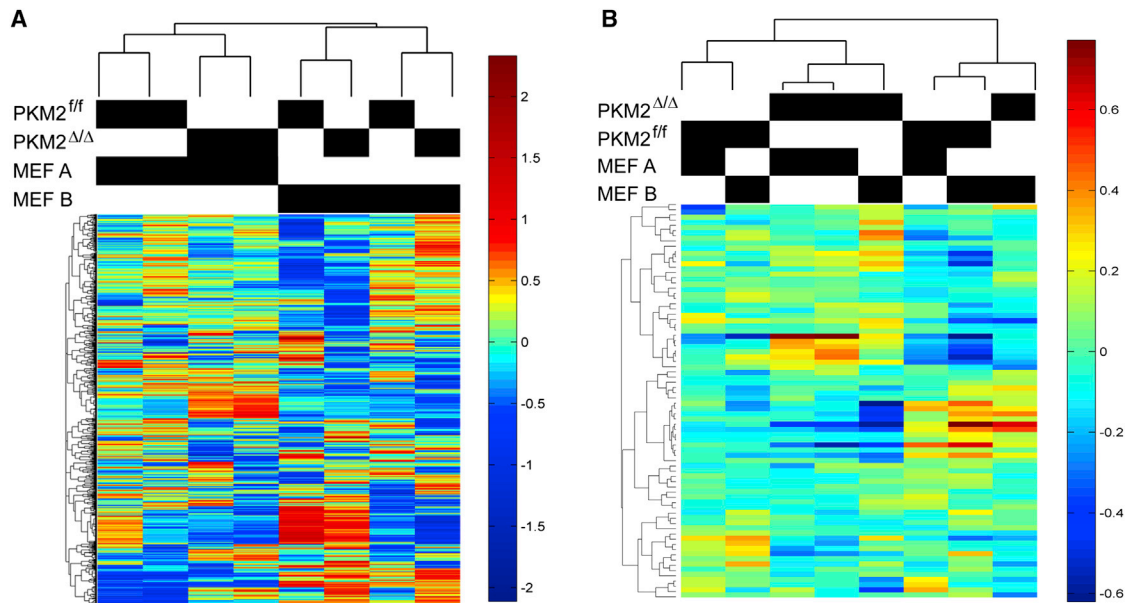


Figure 6. Genome-Scale Gene Expression Analysis Reveals No Correlation between Gene Expression and Proliferation Arrest of *PKM2*^{Δ/Δ} Cells

(A) Unsupervised hierarchical clustering of *PKM2*^{fl/fl} and *PKM2*^{Δ/Δ} MEF expression profiles. RNA was isolated from *PKM2*^{fl/fl} and *PKM2*^{Δ/Δ} MEFs 7 days after *PKM2*^{fl/fl} Cre-ER MEFs were treated with vehicle or 4-OHT. MEFs were generated from two independent *PKM2*^{fl/fl} Cre-ER MEFs. RNA was analyzed using an Affymetrix GeneChip Mouse Gene 1.0 ST array.

(B) Unsupervised hierarchical clustering of *PKM2*^{fl/fl} and *PKM2*^{Δ/Δ} MEF expression profiles for genes involved in nucleotide metabolism.

See also Figure S6.

in clustering by the embryo from which the MEFs were generated rather than by pyruvate kinase isoform expression. As an additional approach, we used fold change analysis to compare gene expression differences between *PKM2*^{Δ/Δ} and *PKM2*^{fl/fl} MEFs. This analysis revealed a small number of genes that show differences according to pyruvate kinase isoform expression, and these results were confirmed by RT-PCR (Figure S6A); however, none of the genes are directly related to metabolism or cell cycle control.

Since metabolic analysis of *PKM2*^{Δ/Δ} and *PKM2*^{fl/fl} MEFs revealed deficiencies in nucleotide metabolism, we reanalyzed the gene expression data, examining only nucleotide metabolism genes (Figure 6B). Again, unsupervised hierarchical clustering does not show a correlation between expression changes and pyruvate kinase isoform expression, and most genes show minimal change in expression levels. To confirm these results, we examined relative protein and gene expression levels of several key nucleotide metabolism genes by western blotting and RT-PCR (Figures S6B and S6C). None showed differences in expression between *PKM2*^{Δ/Δ} and *PKM2*^{fl/fl} MEFs. These results argue that changes in gene expression do not account for either impaired nucleotide synthesis or proliferation arrest of *PKM2*^{Δ/Δ} MEFs.

***PKM2*^{Δ/Δ} MEFs Cannot Complete DNA Synthesis**

To determine whether de novo pyrimidine production is limiting for proliferation of *PKM2*^{Δ/Δ} MEFs, we attempted to prevent proliferation arrest by supplementing the media with thymine at the time of 4-OHT addition. Thymine supplementation prevented

proliferation arrest of *PKM2*^{Δ/Δ} MEFs (Figure 7A). Thymine supplementation also prevents depletion of intracellular dTMP and dTTP levels, as well as levels of other nucleotides (Figure 5A) and some other depleted metabolites (Figure S5A) but has no effect on proliferation of *PKM2*^{fl/fl} MEFs or Cre expressing MEFs (Figures S7A and S1C). The ability of thymine supplementation to sustain proliferation of *PKM2*^{Δ/Δ} MEFs is maintained for up to 10 days and does not increase *PKM2*^{Δ/Δ} MEF cell number by inducing a burst of proliferation following addition of thymine. The pyrimidines cytosine and uracil and purines inosine, guanine, and adenine also partially prevent proliferation arrest of *PKM2*^{Δ/Δ} MEFs, but to a lesser extent (Figure 7A). To confirm that changes in serine are not limiting for cell proliferation, we supplemented *PKM2*^{Δ/Δ} MEFs with serine; however, neither serine nor other substrates used in nucleotide synthesis rescued proliferation (Figure 7A).

To determine whether thymine supplementation can restore proliferation of arrested *PKM2*^{Δ/Δ} MEFs, we added thymine to the media 5 days after 4-OHT addition, a time point when *PKM2*-deleted cells have stopped proliferating (Figure 1B). Thymine addition restores proliferation that is sustained for several days; however, the cells senesce around day 10 and thus undergo fewer population doublings (Figure S7B). Even when media is changed every 3 days without replating, no spontaneous recovery has been observed in *PKM2*^{Δ/Δ} MEFs despite following cultures for up to 18 days after proliferation arrest.

To determine whether DNA synthesis might be limited by nucleotide pools in *PKM2*^{Δ/Δ} MEFs, we performed DNA labeling

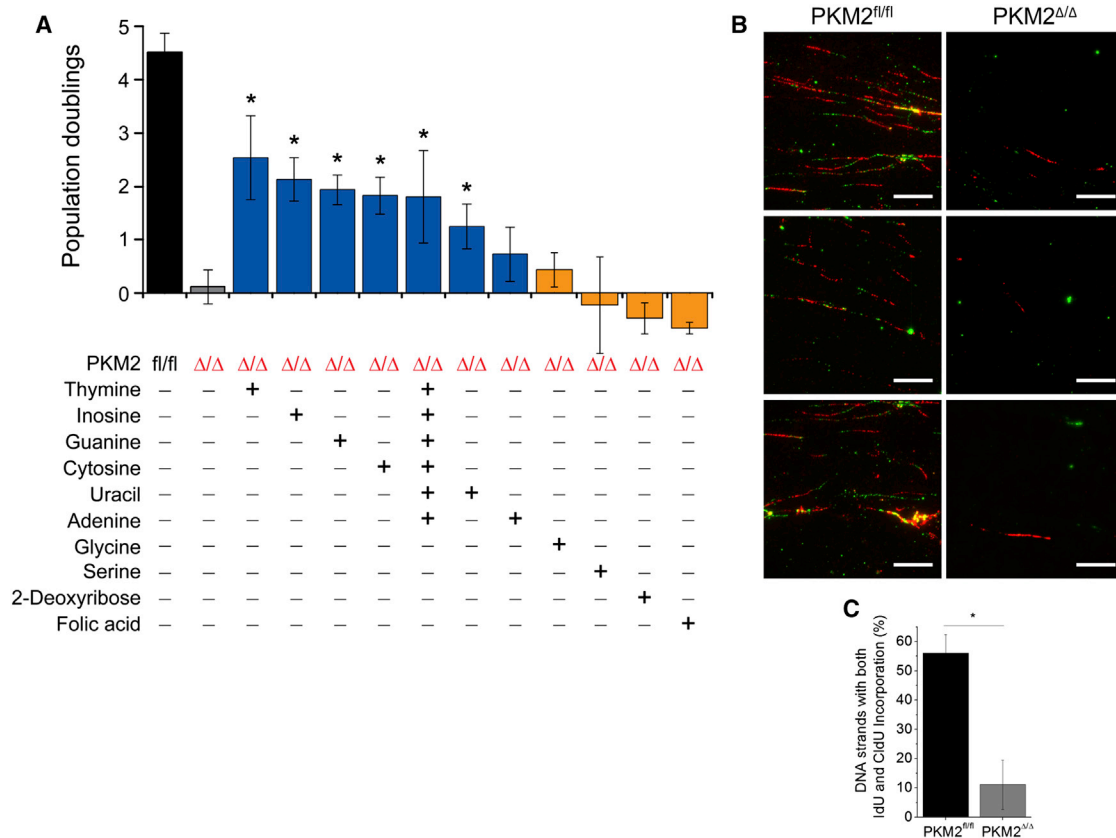


Figure 7. Thymine Supplementation Restores Proliferation of *PKM2*^{Δ/Δ} MEFs

(A) Population doublings over 10 days of *PKM2*^{fl/fl} and *PKM2*^{Δ/Δ} MEFs were measured with or without the indicated metabolites supplemented in the media at a concentration of 250 μ M. Data are displayed as means $\pm$ SEM, $n = 3$. *Population doubling is statistically significant ($p < 0.05$) when compared to the population doubling of *PKM2*^{Δ/Δ} MEFs without any supplements.

(B) Three representative images of DNA strands incorporating CldU (red) and IdU (green) from *PKM2*^{fl/fl} and *PKM2*^{Δ/Δ} MEFs 5 days after *PKM2*^{fl/fl} Cre-ER MEFs were treated with vehicle or 4-OHT are shown; scale bar, 15 μ m.

(C) Quantitation of the percentage of DNA strands incorporating both CldU and IdU is shown. For this analysis 237 strands from *PKM2*^{fl/fl} MEFs and 54 strands from *PKM2*^{Δ/Δ} MEFs prepared as described in (B) were evaluated.

See also Figure S7.

studies to directly visualize nucleotide incorporation into DNA over time (Figure 7B). While *PKM2*^{fl/fl} MEFs incorporate both 5-chloro-2'-deoxyuridine (CldU) and 5-iodo-2'-deoxyuridine (IdU) to produce long DNA strands over a 20 min period, *PKM2*^{Δ/Δ} MEFs are only able to incorporate CldU and IdU for short bursts, and strands with both CldU and IdU are rarely observed (Figures 7B and 7C). These data suggest at least some *PKM2*^{Δ/Δ} MEFs are in S phase because limited CldU and IdU incorporation into DNA is observed. The data also argue that *PKM2*^{Δ/Δ} MEFs are unable to sustain DNA synthesis, and are consistent with nucleotide depletion causing proliferation arrest. Together, our experiments argue PKM1 expression leads to low nucleotide levels and an inability to replicate DNA.

DISCUSSION

While most proliferating cells express PKM2, the exact role of PKM2 in cell proliferation remains controversial. Unlike constitutively active PKM1, PKM2 can be regulated to assume either

a low or high activity state, and the inactive state of PKM2 is favored in proliferating tumor cells (Israelsen et al., 2013). It has been proposed that less-active PKM2 allows buildup of upstream glycolytic metabolites that can feed into biosynthetic pathways, with the subsequent ability to use these metabolites for anabolic metabolism (Christofk et al., 2008b; Eigenbrodt and Glossmann, 1980). Consistent with this idea, our data show that expression of constitutively active PKM1 results in decreased de novo nucleotide biosynthesis. However, contrary to this hypothesis, PKM1 expression did not deplete upstream glycolytic intermediates or impair all anabolic metabolism. Instead, pools of upstream glycolytic intermediates and most intracellular metabolites were unchanged, and the cells continued to accumulate protein and lipids to grow to a larger size. This suggests protein and nucleotide synthesis can be uncoupled in these cells. This is in contrast to findings in some cancer cells where coordinate regulation of protein synthesis and nucleotide synthesis is observed (Cunningham et al., 2014).

PKM1 expression led to severe thymidine depletion and proliferation arrest that is rescued by thymine supplementation. Although all nucleotides were depleted, depletion of pyrimidines may be most severe because they depend on both glucose and glutamine as carbon sources, while purines only depend on glucose. Thymine supplementation was most effective; however, supplementation of other nucleotide bases could also partially prevent proliferation arrest. Nucleotide levels in cells are tightly regulated, with complex allosteric control of synthesis maintaining balanced pools in cells such that changes in one nucleotide pool can impact the availability of other nucleotides (Cohen et al., 1983; Reichard, 1988). Although pyrimidines and purines cannot be interconverted, changes in pyrimidine production impact both purine and pyrimidine levels (Reaves et al., 2013). Methotrexate impairs thymidine synthesis and depletes both purines and pyrimidines in tumor cells (Hryniuk, 1975; Hryniuk et al., 1975; Tattersall et al., 1974). Thymidine supplementation alone rescues methotrexate toxicity in some cancer cells, while others require both purine and pyrimidine supplementation (Harrap et al., 1977).

Thymidine depletion is the basis for antiproliferative therapies that range from antibiotics (Hawser et al., 2006; Kwon et al., 2010) to cancer chemotherapy (Gangjee et al., 2007; Wither spoon et al., 2013). The success of drugs that target thymidine pools highlights the importance of generating this nucleotide in proliferating cells. Nucleotide depletion can trigger cell cycle arrest in human neuroblastoma cell lines (Messina et al., 2004) and T lymphocytes (Laliberté et al., 1998). Similar to our findings, proliferation arrest induced by nucleotide depletion in T lymphocytes could be reversed with nucleotide supplementation (Turka et al., 1991). Accumulation of cells in S phase and a slow DNA replication rate has also been observed in cells treated with hydroxyurea, a ribonucleotide reductase inhibitor that lowers deoxynucleotide pools (Anglana et al., 2003). Additional studies have shown low nucleotides can impair cancer progression (Cunningham et al., 2014) or cause oncogene-induced senescence (Aird et al., 2013), suggesting nucleotide levels can limit proliferation in other contexts.

Some mammalian cells can continue DNA synthesis with imbalanced deoxyribonucleoside pools (Meuth, 1989); however, nucleotide insufficiency can also result in DNA polymerase stalling, collapsed replication forks, and activation of the DNA damage response (Bester et al., 2011). Activation of the DNA damage response is one mechanism that could account for S phase cell cycle arrest in *PKM2^{Δ/Δ}* cells, although increased γ -H2AX staining was not observed. The lack of sustained DNA replication in *PKM2^{Δ/Δ}* cells suggests they enter S phase with insufficient nucleotides to complete this phase of the cell cycle. It has been suggested that one function of the G1-S cell size checkpoint is to ensure sufficient nutrients are available to complete S phase (Foster et al., 2010), while others have argued that mammalian cells only have sufficient precursor stores to sustain DNA replication for a few minutes and therefore must continuously replenish deoxynucleotide pools to replicate the genome (Reichard, 1988). These data argue that regulation of pyruvate kinase isoform expression can impact the ability of cells to generate nucleotides and progress through S phase.

To avoid a high mutation rate, cells may decrease oxidative metabolism during S phase to protect the genome from DNA damage (Tu et al., 2005). PKM2 expression can favor the use of aerobic glycolysis (Christofk et al., 2008a); however, apart from protecting the genome by limiting oxidative phosphorylation, these data imply that aerobic glycolysis may also be important for supporting nucleotide synthesis to complete S phase. Supporting nucleotide production may be another mechanism by which aerobic glycolysis keeps the mutation rate low by preventing collapsed replication forks caused by insufficient nucleotides.

Serine activates PKM2 and decreases new serine synthesis (Chaneton et al., 2012). Increased pyruvate kinase activity from PKM1 expression decreases 3PG to serine flux; however, serine levels are not affected in these cells despite decreased serine production. In fact, serine levels are maintained despite increased protein and cell mass in *PKM2^{Δ/Δ}* relative to *PKM2^{fl/fl}* MEFs.

Nonmetabolic functions of PKM2 have also been reported (Gao et al., 2012; Keller et al., 2014; Luo et al., 2011; Yang et al., 2011, 2012a, 2012b), including a role for PKM2 in regulating mitosis (Jiang et al., 2014). However, the failure of most *PKM2^{Δ/Δ}* MEFs to arrest in G2/M, and the ability to rescue proliferation with thymine supplementation both argue that defects in chromosome segregation are not a major cause of proliferation arrest in these cells. Because most nonmetabolic PKM2 functions are proposed to alter gene expression, the observation that loss of PKM2 causes minimal gene expression changes suggests that these PKM2 activities are less active in primary MEFs.

The inability of *PKM2^{Δ/Δ}* MEFs to sustain DNA synthesis is consistent with a model where a switch from PKM2 to PKM1 expression leads to low deoxynucleotide levels, an inability to replicate DNA, and decreased proliferation without affecting cell mass accumulation. How pyruvate kinase activity regulates nucleotide biosynthesis remains unknown. PKM2 can influence ribose production via the oxidative pentose phosphate pathway in cancer cells (Anastasiou et al., 2011), but altered ribose production was not observed in *PKM2^{Δ/Δ}* MEFs. Regardless of how pyruvate kinase influences nucleotide synthesis, these findings support the notion that independent of nutrient uptake, how nutrients are metabolized determines whether cells engage in proliferation.

EXPERIMENTAL PROCEDURES

All mouse studies were performed in accordance with institutional guidelines and approved by the MIT Committee on Animal Care.

Isolation and Culture of Primary Mouse Embryonic Fibroblasts

Primary MEFs were isolated and cultured using published methods (Springer et al., 2001). Embryos were isolated at E11.5–E14.5 of gestation and each embryo processed separately. MEFs were cultured in MEF medium (Dulbecco's modified Eagle's medium with 10% heat-inactivated FBS, 1% streptomycin/penicillin, 1 mM glutamine, and 0.1 mM 2-mercaptoethanol) and plated at a density of 5×10^6 cells per 10 cm plate. Cells were split before reaching confluence and replated at 750,000 cells per 10 cm plate. A total of 1 μ M 4-hydroxytamoxifen in ethanol was used to induce recombination of *PKM2^{fl}* alleles. Cells starved of lysine were incubated in lysine-free DMEM and doubling time analyzed over 10 days.

Cell Proliferation Analysis

A total of 750,000 cells were seeded in triplicate in 10 cm plates, and cell counts were obtained using a Cellometer Auto T4 Cell Counter (Nexcelom). Cells were replated at 70% confluence, counted, and reseeded at a density of 750,000 per 10 cm plate. Population doubling (PD) was calculated using the following formula:

$$PD = \frac{\log(\frac{\text{number of cells counted}}{\text{number of cells seeded}})}{\log(2)} + \text{previous PD}.$$

When used, all metabolite supplements were added to MEF medium at a concentration of 250 μ M.

Metabolite Uptake and Excretion

Conditioned medium was sampled after 72 hr. Levels of glucose and lactate were measured using YSI 7100 Select Biochemistry Analyzer. The following equation was used to calculate metabolite consumption/excretion per 10^6 cells per hour (α):

$$\alpha = \frac{[\text{metabolite}]_{\text{initial}} - [\text{metabolite}]_{\text{final}}}{\frac{X_{\text{initial}}}{\mu} (e^{\mu \Delta t} - 1)}$$

where

$$\mu = \text{specific growth rate} = \frac{1}{t} \ln \left(\frac{X_{\text{final}}}{X_{\text{initial}}} \right)$$

and X is cell number and t is time in hours. For μ of 0 (specific growth rate of 0), α was calculated using the following equation:

$$\alpha = \frac{[\text{metabolite}]_{\text{initial}} - [\text{metabolite}]_{\text{final}}}{X \cdot \Delta t}.$$

Metabolite Extraction and Analysis

For metabolite analysis using gas chromatography/mass spectrometry, cells were cultured for ~ 72 hr in glucose- and glutamine-free DMEM-based MEF media with dialyzed FBS and the appropriate tracer added. [$U\text{-}^{13}\text{C}_6$] glucose, [$U\text{-}^{13}\text{C}_5$] glutamine, and [$5\text{-}^{13}\text{C}$] glutamine were all from Cambridge Isotopes Laboratories, Inc.

Cell Cycle Analysis

Cells were fixed in ice cold ethanol at 4°C overnight, washed twice in PBS with 1% BSA, treated with RNase, and resuspended in propidium iodide buffer to a final concentration of $50 \mu\text{g} \cdot \text{mL}^{-1}$. Cells were analyzed on a Becton Dickinson FACScan flow cytometer. To assay for new DNA synthesis, the Click-iT EdU Alexa Fluor 488 kit was used with 3 hr exposure of cells to $10 \mu\text{M}$ EdU (Invitrogen C35002) and imaged on a Nikon Eclipse TE2000-U microscope with a 4 s exposure time and analyzed using ImageJ software. Cells were dual stained for DNA content and new DNA synthesis with the Click-iT EdU Alexa Fluor 488 Kit and FxCycle Violet Stain (Life Technologies F-10347), respectively, and analyzed on a BD FACSCanto II using FACS Diva software. Viability was assessed by propidium iodide dye exclusion using a BD FACScan flow cytometer.

Measurement of Cell Mass and Volume

Dry mass of single cells was measured as previously described (Feijó Delgado et al., 2013). Briefly, cells were resuspended in $1 \times$ PBS and loaded into a $15 \times 20 \mu\text{m}$ cross-section suspended microchannel resonator. The buoyant mass of each cell was measured first in $1 \times$ PBS and then in $1 \times$ D_2O -PBS. Each experiment was conducted for ~ 1.5 hr at room temperature. For measurement of cell dry weight from batch culture, 10^6 cells were captured onto membrane filters, dried at 37°C for several days, and weighed using an analytical balance. Weights of control membrane filters loaded only with PBS before drying were subtracted to obtain the weight of 10^6 cells. Cell volumes were measured using a Coulter counter.

DNA Strand Labeling

Cells were incubated in medium containing $100 \mu\text{M}$ CldU (Sigma Aldrich C6891) for 20 min, washed with PBS, and then incubated in medium contain-

ing $250 \mu\text{M}$ IdU (Sigma Aldrich I7125) for 20 min. Following trypsinization, labeled cells were resuspended in PBS with 10-fold excess of unlabeled cells, and $2.5 \mu\text{L}$ were transferred to a glass slide with $7.5 \mu\text{L}$ of spreading buffer (200 mM Tris-HCl [pH 7.5], 50 mM EDTA, 0.5% SDS). Slides were dried and fixed in cold 3:1 methanol:acetic acid for 2 min. Following a 30 min treatment with 2.5 N HCl to denature the DNA, slides were washed with PBS and blocked at 37°C with PBST + 2% BSA. The samples were then incubated at 37°C for 1 hr in PBST + 1% BSA containing primary antibodies to CldU (AbD Serotec OB0030) and IdU (BD 347580), washed with PBST, and incubated at 37°C for 30 min in PBST + 1% BSA containing secondary antibodies conjugated to Cy3 (Jackson Immunochemicals 712-165-153) and Alexa Fluor 488 (Jackson Immunochemicals 715-545-151) with minimal crossreactivity to mouse and rat IgG, respectively. Samples were imaged using the DeltaVision Elite imaging system (Applied Precision) and microscope (model IX-71; Olympus) with a $100\times$ objective lens, and the CoolSNAP HQ2 camera (Photometrics).

ACCESSION NUMBERS

Data are deposited in the Gene Expression Omnibus under accession number GSE60499.

SUPPLEMENTAL INFORMATION

Supplemental information includes seven figures, two tables, Supplemental Experimental Procedures, and the description of the flux model with the complete flux data provided as a separate Excel spreadsheet and can be found with this article online at <http://dx.doi.org/10.1016/j.molcel.2014.10.027>.

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REFERENCES

- Abmayr, S.M., and Pavlath, G.K. (2012). Myoblast fusion: lessons from flies and mice. *Development* 139, 641–656.
- Aird, K.M., Zhang, G., Li, H., Tu, Z., Bitler, B.G., Garipov, A., Wu, H., Wei, Z., Wagner, S.N., Herlyn, M., and Zhang, R. (2013). Suppression of nucleotide metabolism underlies the establishment and maintenance of oncogene-induced senescence. *Cell Rep.* 3, 1252–1265.
- Anastasiou, D., Poulgiannis, G., Asara, J.M., Boxer, M.B., Jiang, J.K., Shen, M., Bellinger, G., Sasaki, A.T., Locasale, J.W., Auld, D.S., et al. (2011). Inhibition of pyruvate kinase M2 by reactive oxygen species contributes to cellular antioxidant responses. *Science* 334, 1278–1283.

- Anastasiou, D., Yu, Y., Israelsen, W.J., Jiang, J.-K., Boxer, M.B., Hong, B.S., Tempel, W., Dimov, S., Shen, M., Jha, A., et al. (2012). Pyruvate kinase M2 activators promote tetramer formation and suppress tumorigenesis. *Nat. Chem. Biol.* 8, 839–847.
- Anglana, M., Apiou, F., Bensimon, A., and Debatisse, M. (2003). Dynamics of DNA replication in mammalian somatic cells: nucleotide pool modulates origin choice and interorigin spacing. *Cell* 114, 385–394.
- Antoniewicz, M.R., Kelleher, J.K., and Stephanopoulos, G. (2007). Elementary metabolite units (EMU): a novel framework for modeling isotopic distributions. *Metab. Eng.* 9, 68–86.
- Bester, A.C., Roniger, M., Oren, Y.S., Im, M.M., Sarni, D., Chaoat, M., Bensimon, A., Zamir, G., Shewach, D.S., and Kerem, B. (2011). Nucleotide deficiency promotes genomic instability in early stages of cancer development. *Cell* 145, 435–446.
- Bohnsack, B.L., and Hirschi, K.K. (2004). Nutrient regulation of cell cycle progression. *Annu. Rev. Nutr.* 24, 433–453.
- Burg, T.P., Godin, M., Knudsen, S.M., Shen, W., Carlson, G., Foster, J.S., Babcock, K., and Manalis, S.R. (2007). Weighing of biomolecules, single cells and single nanoparticles in fluid. *Nature* 446, 1066–1069.
- Cairns, R.A., Harris, I.S., and Mak, T.W. (2011). Regulation of cancer cell metabolism. *Nat. Rev. Cancer* 11, 85–95.
- Campisi, J. (2011). Cellular senescence: putting the paradoxes in perspective. *Curr. Opin. Genet. Dev.* 21, 107–112.
- Castro, P., Giri, D., Lamb, D., and Iltmann, M. (2003). Cellular senescence in the pathogenesis of benign prostatic hyperplasia. *Prostate* 55, 30–38.
- Chaneton, B., Hillmann, P., Zheng, L., Martin, A.C.L., Maddocks, O.D.K., Chokkathukalam, A., Coyle, J.E., Jankevics, A., Holding, F.P., Vousden, K.H., et al. (2012). Serine is a natural ligand and allosteric activator of pyruvate kinase M2. *Nature* 491, 458–462.
- Christoff, H.R., Vander Heiden, M.G., Harris, M.H., Ramanathan, A., Gerszten, R.E., Wei, R., Fleming, M.D., Schreiber, S.L., and Cantley, L.C. (2008a). The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. *Nature* 452, 230–233.
- Christoff, H.R., Vander Heiden, M.G., Wu, N., Asara, J.M., and Cantley, L.C. (2008b). Pyruvate kinase M2 is a phosphotyrosine-binding protein. *Nature* 452, 181–186.
- Clower, C.V., Chatterjee, D., Wang, Z., Cantley, L.C., Vander Heiden, M.G., and Krainer, A.R. (2010). The alternative splicing repressors hnRNP A1/A2 and PTB influence pyruvate kinase isoform expression and cell metabolism. *Proc. Natl. Acad. Sci. USA* 107, 1894–1899.
- Cohen, A., Barankiewicz, J., Lederman, H.M., and Gelfand, E.W. (1983). Purine and pyrimidine metabolism in human T lymphocytes. Regulation of deoxyribonucleotide metabolism. *J. Biol. Chem.* 258, 12334–12340.
- Cunningham, J.T., Moreno, M.V., Lodi, A., Ronen, S.M., and Ruggero, D. (2014). Protein and nucleotide biosynthesis are coupled by a single rate-limiting enzyme, PRPS2, to drive cancer. *Cell* 157, 1088–1103.
- Decker, T., and Lohmann-Matthes, M.-L. (1988). A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *J. Immunol. Methods* 115, 61–69.
- Dimri, G.P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E.E., Linskens, M., Rubelj, I., Pereira-Smith, O., et al. (1995). A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc. Natl. Acad. Sci. USA* 92, 9363–9367.
- Domingo, M., Einig, C., Eigenbrodt, E., and Reinacher, M. (1992). Immunohistological demonstration of pyruvate kinase isoenzyme type L in rat with monoclonal antibodies. *J. Histochem. Cytochem.* 40, 665–673.
- Eigenbrodt, E., and Glossmann, H. (1980). Glycolysis one of the keys to cancer? *Trends Pharmacol. Sci.* 1, 240–245.
- Feijó Delgado, F., Cermak, N., Hecht, V.C., Son, S., Li, Y., Knudsen, S.M., Olcum, S., Higgins, J.M., Chen, J., Grover, W.H., and Manalis, S.R. (2013). Intracellular water exchange for measuring the dry mass, water mass and changes in chemical composition of living cells. *PLoS ONE* 8, e67590.
- Fendt, S.-M., Bell, E.L., Keibler, M.A., Davidson, S.M., Wirth, G.J., Fiske, B., Mayers, J.R., Schwab, M., Bellinger, G., Csibi, A., et al. (2013a). Metformin decreases glucose oxidation and increases the dependency of prostate cancer cells on reductive glutamine metabolism. *Cancer Res.* 73, 4429–4438.
- Fendt, S.-M., Bell, E.L., Keibler, M.A., Olenchok, B.A., Mayers, J.R., Wasylenko, T.M., Vokes, N.I., Guarente, L., Heiden, M.G.V., and Stephanopoulos, G. (2013b). Reductive glutamine metabolism is a function of the α -ketoglutarate to citrate ratio in cells. *Nat. Commun.* 4, 2236.
- Foster, D.A., Yellen, P., Xu, L., and Saqena, M. (2010). Regulation of G1 cell cycle progression distinguishing the restriction point from a nutrient-sensing cell growth checkpoint(s). *Genes Cancer* 1, 1124–1131.
- Gangjee, A., Jain, H.D., and Kurup, S. (2007). Recent advances in classical and non-classical antifolates as antitumor and antiopportunistic infection agents: Part I. *Anticancer Agents Med. Chem.* 7, 524–542.
- Gao, X., Wang, H., Yang, J.J., Liu, X., and Liu, Z.R. (2012). Pyruvate kinase M2 regulates gene transcription by acting as a protein kinase. *Mol. Cell* 45, 598–609.
- Green, H., and Kehinde, O. (1974). Sublines of mouse 3T3 cells that accumulate lipid. *Cell* 1, 113–116.
- Gui, D.Y., Lewis, C.A., and Vander Heiden, M.G. (2013). Allosteric regulation of PKM2 allows cellular adaptation to different physiological states. *Sci. Signal.* 6, pe7.
- Hacker, H.J., Steinberg, P., and Bannasch, P. (1998). Pyruvate kinase isoenzyme shift from L-type to M2-type is a late event in hepatocarcinogenesis induced in rats by a choline-deficient/DL-ethionine-supplemented diet. *Carcinogenesis* 19, 99–107.
- Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of cancer: the next generation. *Cell* 144, 646–674.
- Harrap, K.R., Taylor, G.A., and Browman, G.P. (1977). Enhancement of the therapeutic effectiveness of methotrexate and protection of normal proliferating tissues with purines and pyrimidines. *Chem. Biol. Interact.* 18, 119–128.
- Hawser, S., Lociuio, S., and Islam, K. (2006). Dihydrofolate reductase inhibitors as antibacterial agents. *Biochem. Pharmacol.* 71, 941–948.
- Hryniuk, W.M. (1975). The mechanism of action of methotrexate in cultured L5178Y leukemia cells. *Cancer Res.* 35, 1085–1092.
- Hryniuk, W.M., Brox, L.W., Henderson, J.F., and Tamaoki, T. (1975). Consequences of methotrexate inhibition of purine biosynthesis in L5178Y cells. *Cancer Res.* 35, 1427–1432.
- Illg, D., and Pette, D. (1979). Turnover rates of hexokinase I, phosphofructokinase, pyruvate kinase and creatine kinase in slow-twitch soleus muscle and heart of the rabbit. *Eur. J. Biochem.* 97, 267–273.
- Imamura, K., and Tanaka, T. (1982). Pyruvate kinase isozymes from rat. In *Methods in Enzymology*, A.W. Willis, ed. (Waltham, MA: Academic Press), pp. 150–165.
- Imamura, K., Noguchi, T., and Tanaka, T. (1986). Markers of Human Neuroectodermal Tumors, G.E.J. Staal and C.W.M. van Veelen, eds. (Boca Raton, FL: CRC Press), pp. 191–222.
- Israelsen, W.J., Dayton, T.L., Davidson, S.M., Fiske, B.P., Hosios, A.M., Bellinger, G., Li, J., Yu, Y., Sasaki, M., Horner, J.W., et al. (2013). PKM2 isoform-specific deletion reveals a differential requirement for pyruvate kinase in tumor cells. *Cell* 155, 397–409.
- Jiang, Y., Li, X., Yang, W., Hawke, D.H., Zheng, Y., Xia, Y., Aldape, K., Wei, C., Guo, F., Chen, Y., and Lu, Z. (2014). PKM2 regulates chromosome segregation and mitosis progression of tumor cells. *Mol. Cell* 53, 75–87.
- Johnston, G.C., Pringle, J.R., and Hartwell, L.H. (1977). Coordination of growth with cell division in the yeast *Saccharomyces cerevisiae*. *Exp. Cell Res.* 105, 79–98.
- Kamphorst, J.J., Cross, J.R., Fan, J., de Stanchina, E., Mathew, R., White, E.P., Thompson, C.B., and Rabinowitz, J.D. (2013). Hypoxic and Ras-transformed cells support growth by scavenging unsaturated fatty acids from lysophospholipids. *Proc. Natl. Acad. Sci. USA* 110, 8882–8887.

- Keller, K.E., Tan, I.S., and Lee, Y.-S. (2012). SAICAR stimulates pyruvate kinase isoform M2 and promotes cancer cell survival in glucose-limited conditions. *Science* 338, 1069–1072.
- Keller, K.E., Doctor, Z.M., Dwyer, Z.W., and Lee, Y.S. (2014). SAICAR induces protein kinase activity of PKM2 that is necessary for sustained proliferative signaling of cancer cells. *Mol. Cell* 53, 700–709.
- Koppenol, W.H., Bounds, P.L., and Dang, C.V. (2011). Otto Warburg's contributions to current concepts of cancer metabolism. *Nat. Rev. Cancer* 11, 325–337.
- Kuilman, T., Michaloglou, C., Mooi, W.J., and Peeper, D.S. (2010). The essence of senescence. *Genes Dev.* 24, 2463–2479.
- Kwon, Y.K., Higgins, M.B., and Rabinowitz, J.D. (2010). Antifolate-induced depletion of intracellular glycine and purines inhibits thymineless death in *E. coli*. *ACS Chem. Biol.* 5, 787–795.
- Laliberté, J., Yee, A., Xiong, Y., and Mitchell, B.S. (1998). Effects of guanine nucleotide depletion on cell cycle progression in human T lymphocytes. *Blood* 91, 2896–2904.
- Lunt, S.Y., and Vander Heiden, M.G. (2011). Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. *Annu. Rev. Cell Dev. Biol.* 27, 441–464.
- Luo, W., Hu, H., Chang, R., Zhong, J., Knabel, M., O'Meally, R., Cole, R.N., Pandey, A., and Semenza, G.L. (2011). Pyruvate kinase M2 is a PHD3-stimulated coactivator for hypoxia-inducible factor 1. *Cell* 145, 732–744.
- Lv, L., Li, D., Zhao, D., Lin, R., Chu, Y., Zhang, H., Zha, Z., Liu, Y., Li, Z., Xu, Y., et al. (2011). Acetylation targets the M2 isoform of pyruvate kinase for degradation through chaperone-mediated autophagy and promotes tumor growth. *Mol. Cell* 42, 719–730.
- Mazurek, S. (2011). Pyruvate kinase type M2: a key regulator of the metabolic budget system in tumor cells. *Int. J. Biochem. Cell Biol.* 43, 969–980.
- Messina, E., Gazzaniga, P., Micheli, V., Guaglianone, M.R., Barbato, S., Morrone, S., Frati, L., Aglianò, A.M., and Giacomello, A. (2004). Guanine nucleotide depletion triggers cell cycle arrest and apoptosis in human neuroblastoma cell lines. *Int. J. Cancer* 108, 812–817.
- Metallo, C.M., Gameiro, P.A., Bell, E.L., Mattaini, K.R., Yang, J., Hiller, K., Jewell, C.M., Johnson, Z.R., Irvine, D.J., Guarente, L., et al. (2012). Reductive glutamine metabolism by IDH1 mediates lipogenesis under hypoxia. *Nature* 481, 380–384.
- Meuth, M. (1989). The molecular basis of mutations induced by deoxyribonucleoside triphosphate pool imbalances in mammalian cells. *Exp. Cell Res.* 181, 305–316.
- Montagne, J., Stewart, M.J., Stocker, H., Hafen, E., Kozma, S.C., and Thomas, G. (1999). *Drosophila* S6 kinase: a regulator of cell size. *Science* 285, 2126–2129.
- Nicoletti, I., Migliorati, G., Pagliacci, M.C., Grignani, F., and Riccardi, C. (1991). A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J. Immunol. Methods* 139, 271–279.
- Noguchi, T., Inoue, H., and Tanaka, T. (1986). The M1- and M2-type isozymes of rat pyruvate kinase are produced from the same gene by alternative RNA splicing. *J. Biol. Chem.* 261, 13807–13812.
- Noguchi, T., Yamada, K., Inoue, H., Matsuda, T., and Tanaka, T. (1987). The L- and R-type isozymes of rat pyruvate kinase are produced from a single gene by use of different promoters. *J. Biol. Chem.* 262, 14366–14371.
- Odell, A., Askham, J., Whibley, C., and Hollstein, M. (2010). How to become immortal: let MEFs count the ways. *Aging* 2, 160–165.
- Reaves, M.L., Young, B.D., Hosios, A.M., Xu, Y.-F., and Rabinowitz, J.D. (2013). Pyrimidine homeostasis is accomplished by directed overflow metabolism. *Nature* 500, 237–241.
- Reichard, P. (1988). Interactions between deoxyribonucleotide and DNA synthesis. *Annu. Rev. Biochem.* 57, 349–374.
- Russell, T.R., and Ho, R. (1976). Conversion of 3T3 fibroblasts into adipose cells: triggering of differentiation by prostaglandin F₂α and 1-methyl-3-isobutyl xanthine. *Proc. Natl. Acad. Sci. USA* 73, 4516–4520.
- Springer, M.L., Rando, T.A., and Blau, H.M. (2001). Gene delivery to muscle. In *Current Protocols in Human Genetics* (New York: John Wiley & Sons, Inc.).
- Steinberg, P., Klingelhöffer, A., Schäfer, A., Wüst, G., Weisse, G., Oesch, F., and Eigenbrodt, E. (1999). Expression of pyruvate kinase M2 in preneoplastic hepatic foci of N-nitrosomorpholine-treated rats. *Virchows Arch.* 434, 213–220.
- Tattersall, M.H.N., Jackson, R.C., Jackson, S.T.M., and Harrap, K.R. (1974). Factors determining cell sensitivity to methotrexate: studies of folate and deoxyribonucleoside triphosphate pools in five mammalian cell lines. *Eur. J. Cancer* 10, 819–826.
- Tu, B.P., Kudlicki, A., Rowicka, M., and McKnight, S.L. (2005). Logic of the yeast metabolic cycle: temporal compartmentalization of cellular processes. *Science* 310, 1152–1158.
- Turka, L.A., Dayton, J., Sinclair, G., Thompson, C.B., and Mitchell, B.S. (1991). Guanine ribonucleotide depletion inhibits T cell activation. Mechanism of action of the immunosuppressive drug mizoribine. *J. Clin. Invest.* 87, 940–948.
- van der Loo, B., Fenton, M.J., and Erusalimsky, J.D. (1998). Cytochemical detection of a senescence-associated β-galactosidase in endothelial and smooth muscle cells from human and rabbit blood vessels. *Exp. Cell Res.* 241, 309–315.
- van Veelen, C.W.M., Staal, G.E., Verbiest, H., and Vlug, A.M.C. (1977). Alanine inhibition of pyruvate kinase in gliomas and meningiomas. A diagnostic tool in surgery for gliomas? *Lancet* 2, 384–385.
- Wakelam, M.J. (1985). The fusion of myoblasts. *Biochem. J.* 228, 1–12.
- Ward, P.S., and Thompson, C.B. (2012). Metabolic reprogramming: a cancer hallmark even warburg did not anticipate. *Cancer Cell* 21, 297–308.
- Witherspoon, M., Chen, Q., Kopelovich, L., Gross, S.S., and Lipkin, S.M. (2013). Unbiased metabolite profiling indicates that a diminished thymidine pool is the underlying mechanism of colon cancer chemoprevention by alpha-difluoromethylornithine. *Cancer Discov.* 3, 1072–1081.
- Yamada, K., and Noguchi, T. (1995). [Alteration of isozyme gene expression during cell differentiation and oncogenesis]. *Nippon Rinsho* 53, 1112–1118.
- Yamada, K., and Noguchi, T. (1999). Regulation of pyruvate kinase M gene expression. *Biochem. Biophys. Res. Commun.* 256, 257–262.
- Yang, W., Xia, Y., Ji, H., Zheng, Y., Liang, J., Huang, W., Gao, X., Aldape, K., and Lu, Z. (2011). Nuclear PKM2 regulates β-catenin transactivation upon EGFR activation. *Nature* 480, 118–122.
- Yang, W., Xia, Y., Hawke, D., Li, X., Liang, J., Xing, D., Aldape, K., Hunter, T., Alfred Yung, W.K., and Lu, Z. (2012a). PKM2 phosphorylates histone H3 and promotes gene transcription and tumorigenesis. *Cell* 150, 685–696.
- Yang, W., Zheng, Y., Xia, Y., Ji, H., Chen, X., Guo, F., Lyssiotis, C.A., Aldape, K., Cantley, L.C., and Lu, Z. (2012b). ERK1/2-dependent phosphorylation and nuclear translocation of PKM2 promotes the Warburg effect. *Nat. Cell Biol.* 14, 1295–1304.
- Yee, J.C., Jacob, N.M., Jayapal, K.P., Kok, Y.-J., Philp, R., Griffin, T.J., and Hu, W.-S. (2010). Global assessment of protein turnover in recombinant antibody producing myeloma cells. *J. Biotechnol.* 148, 182–193.
- Young, J.D., Walther, J.L., Antoniewicz, M.R., Yoo, H., and Stephanopoulos, G. (2008). An elementary metabolite unit (EMU) based method of isotopically nonstationary flux analysis. *Biotechnol. Bioeng.* 99, 686–699.