Loss of the tumor suppressor LKB1 promotes metabolic reprogramming of cancer cells via HIF-1α

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One of the major metabolic changes associated with cellular transformation is enhanced nutrient utilization, which supports tumor progression by fueling both energy production and providing biosynthetic intermediates for growth. The liver kinase B1 (LKB1) is a serine/threonine kinase and tumor suppressor that couples bioenergetics to cell-growth control through regulation of mammalian target of rapamycin (mTOR) activity; however, the influence of LKB1 on tumor metabolism is not well defined. Here, we show that loss of LKB1 induces a progrowth metabolic program in proliferating cells. Cells lacking LKB1 display increased glucose and glutamine uptake and utilization, which support both cellular ATP levels and increased macromolecular biosynthesis. This LKB1-dependent reprogramming of cell metabolism is dependent on the hypoxia-inducible factor-1α (HIF-1α), which accumulates under normoxia in LKB1-deficient cells and is antagonized by inhibition of mTOR complex 1 signaling. Silencing HIF-1α reverses the metabolic advantages conferred by reduced LKB1 signaling and impairs the growth and survival of LKB1-deficient tumor cells under low-nutrient conditions. Together, our data implicate the tumor suppressor LKB1 as a central regulator of tumor metabolism and growth control through the regulation of HIF-1α–dependent metabolic reprogramming.

HIF-1α | cancer metabolism | Warburg effect | Peutz-Jeghers Syndrome | PJS | glutamine metabolism

Although unchecked cell proliferation and aberrant survival are hallmark features of cancer, tumor cells must also engage pathways of cellular metabolism to generate the energy and biosynthetic intermediates required to support increased cell division (1). To meet increased energetic and biosynthetic demand, cancer cells often display fundamental changes in their cellular metabolism, including a switch to aerobic glycolysis, a phenomenon known as the “Warburg effect” (2). Increased use of glutamine (“glutaminolysis”) for mitochondrial-dependent ATP production and cellular biosynthesis is also a key feature of many tumor cells (3).

Many of the predominant driver mutations observed in cancer alter tumor-cell metabolism as part of their mode of action (4). For example, loss of the tumor suppressor PTEN can promote increased glucose uptake through elevated PI3K/Akt/mTOR signaling (5) while loss of the Von-Hippel-Lindau (VHL) tumor suppressor promotes a similar metabolic phenotype through stabilization of the hypoxia inducible factor (HIF)-1α (6). HIF-1α and HIF-2α are transcription factors whose activity is regulated by oxygen availability. HIF-1α and HIF-2α protein expression is normally stabilized only under hypoxic conditions; however, the HIFs are commonly expressed in human cancers even in the absence of hypoxia (7). Importantly, elevated expression of both HIF-1α and HIF-2α has been demonstrated in many cases of non-small cell lung cancer (NSCLC) (8), and HIF-2α has been linked to poor prognosis in lung-cancer patients (9).

The liver kinase B1 (LKB1) is a serine/threonine kinase encoded by STK11, the tumor suppressor gene responsible for Peutz-Jeghers Syndrome (PJS) (10, 11). LKB1 is a unique serine/threonine kinase, in that inactivation, rather than activation, of its kinase activity is associated with tumorigenesis. Somatic STK11 mutations are associated with a number of human cancers including lung, breast, and cervical cancer (12–15), and genetic ablation of LKB1 in mice promotes tumorigenesis in a variety of tissues (16). LKB1 is involved in a diverse array of cellular processes, including cell polarity, apoptosis, and cell growth (17, 18). All these processes play a role in cancer initiation and progression, and as such their relative contribution to LKB1-mediated tumor suppression remains unclear.

Although LKB1 is widely accepted as a regulator of cell growth control, the impact of LKB1 on tumor metabolism has remained unclear. Benign tumors haploinsufficient for LKB1 can be visualized using 18F-deoxyglucose-positron emission tomography (FDG-PET) imaging (19), suggesting that loss of LKB1 can promote increased glucose uptake by tumor cells. LKB1 may also influence ATP consumption by limiting mTORC1-dependent mRNA translation (20, 21). In this study, we have characterized the impact of LKB1 loss on cellular metabolism in both transformed and nontransformed cells. We find that silencing LKB1 in tumor cells increases glucose and glutamine consumption and promotes a metabolic switch to aerobic glycolysis. We demonstrate that HIF-1α drives the metabolic shift induced by LKB1 loss and that ablation of HIF-1α reverses the metabolic advantage of LKB1-deficient cells. Together, our data implicate LKB1 loss as a key regulator of tumor-cell metabolism and growth through regulation of HIF-1α–dependent metabolic reprogramming.

Liver kinase B1 (LKB1) is a serine/threonine kinase often inactivated in human cancer. We demonstrate here that loss of LKB1 expression in cancer cells promotes a progrowth metabolic profile that enables increased cell growth and proliferation. Loss of LKB1 promotes increased tumor cell metabolism through mammalian target of rapamycin complex 1- and reactive oxygen species–dependent increases in hypoxia-inducible factor-1α (HIF-1α). LKB1-null cells are dependent on HIF-1α to maintain cellular ATP and viability under poor nutrient conditions, raising the possibility of targeting HIF-1α for synthetic lethality in LKB1-deficient tumors. Together, our data reveal that regulation of cellular metabolism is a key function of LKB1 that may contribute to its tumor-suppressor function in human cancer.


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Results

Loss of LKB1 Promotes Enhanced Glucose and Glutamine Metabolism.

To examine the metabolic consequences of LKB1 loss, we manipulated LKB1 expression in mouse embryonic fibroblasts (MEFs) harboring a conditional mutation in the stki gene (LKB1fl/fl). We used Cre recombinase to generate isogenic MEFs expressing (Cre−) or lacking (Cre+) LKB1 expression (Fig. 1A) and then examined the effect of LKB1 loss on nutrient uptake. LKB1-deficient MEFs displayed increased glucose (Fig. 1B) and glutamine consumption (Fig. 1C) relative to control cells expressing LKB1. We next measured the basal extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) for control or LKB1-deficient MEFs using a flux analyzer (22). Cells lacking LKB1 displayed a twofold increase in ECAR (Fig. 1D), with no significant change in oxygen consumption (Fig. 1E). This change in ECAR displayed by LKB1-deficient MEFs correlated with increased lactate production by these cells (Fig. 1F).

We next cultured control or LKB1-null MEFs with uniformly labeled (U-13C) glucose or glutamine, and examined the total 13C contribution to intracellular metabolite pools. Cells lacking LKB1 (Fig. 1G, filled bar) displayed a slight increase in intracellular lactate levels derived from glucose (Fig. 1H). The strong increase in ECAR (Fig. 1D) and extracellular lactate (Fig. 1F) associated with LKB1 loss suggests that intracellular lactate is rapidly exported once generated. Using 13C-glutamine, we observed increased glutamine conversion to glutamate and α-ketoglutarate (Fig. 1H and J), suggesting an increase in glutaminolysis in LKB1-deficient MEFs.

LKB1-Deficient Tumor Cells Display Enhanced Glycolytic and TCA Cycle Flux. To investigate the role of LKB1 loss on tumor-cell metabolism, we examined the metabolic activity of A549 NSCLC cells, which naturally lack LKB1 expression (Fig. 2A and SI4). Reexpression of LKB1 in A549 cells (A549/LKB1) promoted an ~20% decrease in ECAR relative to control cells lacking LKB1 expression (A549/Vec) whereas OCR was unaffected (Fig. 2B). A549 cells reexpressing LKB1 also displayed reduced production of glutamine-derived glutamate compared with control cells, consistent with a reduction in glutaminolysis in these cells (Fig. 2C). We observed similar effects of LKB1 expression on glycolysis using an independent LKB1-deficient NSCLC cell line (A427) (23). A427/Vec cells lacking LKB1 displayed an ~twofold higher ECAR relative to A427 cells reexpressing LKB1 (Fig. 2D). Moreover, the proportion of these metabolites containing 13C label was also elevated in LKB1-null A549 cells. A549 cells displayed enrichment of 13C-glucose carbon in
lactate (Fig. 2D, black bars), consistent with the increased glycolytic activity of these cells. However, the relative proportion of 13C-glucose–derived carbon in TCA metabolites downstream of citrate was low for both cell lines, despite the total metabolite levels remaining higher in A549 cells lacking LKB1. The decreased labeling from glucose in TCA cycle metabolites was compensated for by an increase in glutamine-derived carbons entering the TCA cycle at α-ketoglutarate, metabolites was compensated for by an increase in glutamine-derived carbons entering the TCA cycle at α-ketoglutarate, providing the majority of carbon pools for the TCA cycle (Fig. 2D, gray bars). Again, LKB1-deficient A549 cells displayed increased labeling from 13C-glutamine in alpha-ketoglutarate, fumarate, malate, and citrate (Fig. 2D, gray bars).

Although overall lactate and TCA cycle metabolite abundance, as well as flux into these pathways from glucose or glutamine, was elevated in LKB1-null cells, the relative distribution of mass isotopomers in the metabolite pools did not vary dramatically in A549 cells regardless of LKB1 status. One consistent difference was a small elevation in glutamine-dependent reductive carboxylation in A549 cells lacking LKB1, which was apparent by the slight increase in m+5 citrate production from 13C-glutamine (Fig. S2A). Similar trends in metabolite abundance and labeling patterns from glucose and glutamine were observed in LKB1-deficient MEFs relative to controls (Fig. S1B). Thus, under normal growth conditions, loss of LKB1 in tumor cells appears to enhance glucose and glutamine flux but does not dramatically alter the metabolic fate of these carbon sources.

LKB1-Null Cells Display Enhanced Growth and Biosynthetic Capacity. Given the observation of increased carbon flow of glucose and glutamine in LKB1-null cells, we examined the impact of LKB1 loss on proliferation and de novo lipid biosynthesis. LKB1-deficient MEFs displayed increased rates of proliferation (Fig. 3A) and displayed a 15% increase in cell size (Fig. 3F) relative to control cells. Given the enhanced carbon flow from glucose and glutamine observed in LKB1-null cells, we measured levels of glucose- and glutamine-derived lipid synthesis in these cells. MEFs were pulsed with radioactively labeled (14C) glucose or glutamine, and 14C-labeling in lipids was measured. LKB1-null cells displayed a twofold increase in glucose-dependent lipid biosynthesis relative to control cells (Fig. 3C) whereas no appreciable difference in 13C-glutamine incorporation to fatty acids was observed between cell lines (Fig. 3D). We next used GC-MS to measure the total abundance of fatty acids in LKB1-deficient cells. The overall abundance of several fatty acid species was increased when LKB1 was absent in A549 cells (Fig. 3E). Cumulatively, these data indicate that loss of LKB1 enhances biosynthetic pathways that support cell growth and proliferation.

LKB1 Deletion Promotes HIF-1α Protein Expression in Cancer Cells Under Normoxia. It has previously been shown that LKB1-deficient MEFs display enhanced HIF-1α protein levels under normoxia (19). Acute deletion of LKB1 in MEFs also resulted in increased HIF-1α protein levels under normoxic conditions (Fig. 4A). HIF-1α mRNA levels were also elevated fivefold in LKB1-deficient MEFs relative to isogenic controls (Fig. 4B). Similar to LKB1-null MEFs, A549 cells lacking LKB1 displayed elevated levels of HIF-1α protein expression under normoxia, which was reduced by reexpression of LKB1 (Fig. 4C). Similarly, HIF-1α protein was detectable in A278 cells under normoxic conditions and was reduced upon ectopic expression of LKB1 in these cells (Fig. S3A). Furthermore, reducing LKB1 expression by siRNA treatment promoted an increase in HIF-1α protein levels in U20S cells (Fig. S2B) and HCT116 cells (Fig. S3C). We next examined the expression of several HIF-1α target genes involved in metabolic control, assessing both mRNA (Fig. 4D) and protein (Fig. 4E) levels. The expression levels of Aldolase A, pyruvate dehydrogenase kinase 1 (PDHK1), and lactate dehydrogenase A (LDHA) were all specifically elevated in LKB1-null MEFs (Fig. 4E).

LKB1-Dependent HIF1α Expression Is Regulated by mTORC1 and ROS. Aberrant mTOR signaling has been linked to deregulated HIF-1α protein expression under normoxic conditions (19, 24). Consistent with previous reports (21), acute deletion of LKB1 in MEFs promoted heightened activation of mTORC1 signaling marked with previous reports (21), acute deletion of LKB1 in MEFs promoted heightened activation of mTORC1 signaling marked by increased rS6 phosphorylation and hyperphosphorylation of 4E-BP1 (Fig. 5A). A549 cells lacking LKB1 also displayed increased...
rS6 phosphorylation (Fig. 5B). To test whether elevated HIF-1α protein levels in LKB1-null cells were supported by mTOR activity, we treated cells with the mTORC1 inhibitor rapamycin and measured HIF-1α protein levels in cell lysates. Rapamycin treatment reduced HIF-1α protein expression in LKB1-deficient A549 cells under normoxic conditions (Fig. 5B). Moreover, HIF-1α mRNA levels in LKB1-null cells were reduced in response to rapamycin treatment (Fig. 5C). Finally, we examined HIF-1α protein expression in LKB1-deficient MEFs with specific ablation of mTORC1 signaling by reducing expression of the mTORC1 complex component Raptor using RNAi. Similar to rapamycin, knockdown of Raptor ablated normoxic HIF-1α protein expression in LKB1-null MEFs (Fig. 5D).

Elevated levels of mitochondrial reactive oxygen species (ROS) have been shown to promote increased HIF-1α activity (25–28). Consistent with recent results in LKB1-null tumor cells (23), LKB1-deficient MEFs displayed an ~twofold increase in ROS levels that could be reduced via addition of the ROS scavenger N-acetyl-cysteine (NAC) (Fig. 5E). The addition of NAC also reduced HIF-1α protein levels in LKB1-null MEFs back to levels observed in control MEFs (Fig. 5F). Together, these data suggest that both mTOR signaling and cellular ROS levels contribute to increased HIF-1α protein expression in cells lacking LKB1.

**HIF-1α Drives the Metabolic Phenotype Induced by LKB1 Loss.** HIF-1α has well-established roles in redirecting metabolism in response to stress (29). To assess the contribution of HIF-1α to the metabolic phenotypes induced by LKB1 loss, we used siRNA to knock down HIF-1α protein expression in LKB1-null MEFs (Fig. 6A). Knockdown of HIF-1α had no effect on the level of lactate production by control cells but specifically reduced the level of lactate produced by MEFs lacking LKB1 (Fig. 6B). Reductions in lactate production were also observed in LKB1-null MEFs (Fig. S4A) and A549 cells (Fig. S4B) treated with rapamycin. Reducing HIF-1α expression decreased the size of LKB1-deficient MEFs, restoring cell size to control levels (Fig. 6C). Next we reduced HIF-1α expression in A549 cells via stable expression of shRNA specific for HIF-1α (Fig. 6D). Knockdown of HIF-1α in A549 cells lacking LKB1 promoted an ~30% decrease in glutamine consumption by these cells (Fig. 6E). Glutaminolysis, as measured by 13C-glutamate conversion to 13C-glutamate, was similarly reduced in A549 cells when HIF-1α signaling was ablated (Fig. 6F).

**HIF-1α Promotes the Growth and Survival of LKB1-Deficient Cells Under Conditions of Nutrient Limitation.** Data presented in Figs. 1–3 indicate that loss of LKB1 promotes increased nutrient acquisition and processing, and ultimately increased cell growth. Given the importance of HIF-1α in directing metabolism and bioenergetics in the absence of LKB1, we next assessed the requirement of HIF-1α in regulating the growth and survival of LKB1-deficient tumor cells. A549 cells expressing control or HIF-1α shRNAs were grown under full (25 mM) or low (0.04 mM) glucose conditions, and cell counts were measured over 72 h. A549 cells lacking HIF-1α displayed a slight reduction in proliferative
rate compared with control cells under full-glucose conditions (Fig. 7A, Left). However, under low-glucose conditions, the proliferative capacity of A549 cells expressing HIF-1α shRNA was significantly impaired (Fig. 7A, Right).

To further assess the dependence of A549 cells on HIF-1α for cell growth, we measured the proliferation of A549/Vec or A549/ shHIF1-α cells under low-glucose conditions along with serum starvation and/or hypoxia (1% O2). A549 cells cultured under low glucose displayed considerable blocks in cell growth when serum or oxygen was limiting (Fig. S5A). Interestingly, A549 cells lacking HIF-1α displayed increased sensitivity to combined glucose and serum starvation, but not glucose starvation combined with hypoxia (Fig. S5A). In addition, A549 cells expressing HIF-1α shRNA displayed reduced viability under glucose and glutamine withdrawal relative to A549 cells expressing control shRNA (Fig. 7B). A549 cells lacking HIF-1α displayed increased caspase-3 activation at low-glucose concentrations (Fig. 7C), indicating the induction of apoptosis in these cells.

To investigate whether the increased apoptosis of A549 cells lacking HIF-1α was due to defects in cellular bioenergetics, we characterized the bioenergetic profile of LKB1-deficient tumor cells lacking HIF-1α. A549 cells with reduced HIF-1α displayed a modest increase in oxygen consumption under low-glucose conditions (Fig. S5B). However, these cells also displayed a 50% reduction in spare respiratory capacity (SRC) (Fig. S5C), suggesting reduced mitochondrial fitness in these cells (30). We also measured the ATP content of A549 cells with or without HIF-1α expression (Fig. 7D). Under basal growth conditions (25 mM glucose, 4 mM glutamine), silencing HIF-1α had little effect on cellular ATP levels. However, following overnight glucose withdrawal, A549 cells expressing HIF-1α shRNA displayed a significant drop in cellular ATP levels relative to control tumor cells. Although glutamine starvation also stimulated a decrease in cellular ATP levels, this drop appeared to be HIF-1α-independent (Fig. 7D). Together, these data suggest that LKB1-null tumor cells require HIF-1α to maintain mitochondrial respiratory capacity, ATP levels, and cell viability in response to nutrient limitation.

**Discussion**

In this study, we provide evidence that the tumor suppressor LKB1 promotes a metabolic checkpoint that regulates carbon utilization in proliferating cells. To date, the main link between LKB1 and tumor metabolism has been the observation of increased FDG-PET signal in vivo in benign LKB1-<sup>−/−</sup> tumors (19). Here, we show that silencing LKB1 is sufficient to promote both aerobic glycolysis and glucoinolysis (Figs. 1 and 2) and that this increase in glucose and glutamine metabolism fuels cell growth and lipid biosynthesis in cells lacking LKB1 (Fig. 3). The progrowth metabolic program induced by LKB1 loss is mediated by the transcription factor HIF-1α, which displays increased protein stabilization under normoxia when LKB1 is deleted (Fig. 4). We find that the metabolic and biosynthetic phenotypes of LKB1-null cells are dependent upon HIF-1α (Fig. 6) and that targeting HIF-1α impairs the growth and survival of LKB1-deficient tumor cells (Fig. 7). This work highlights the existence of a metabolic circuit regulated by HIF-1α that coordinates cellular bioenergetics when LKB1 activity is suppressed.

Our data suggest that LKB1 loss disrupts normal metabolic homeostasis in cells, which paradoxically has a net positive effect on cell growth and proliferation. Glucose-derived pyruvate is a key intermediate in lipid biosynthesis (31). Flux of glucose-derived pyruvate into citrate is enhanced by LKB1 loss, as is glucose-dependent lipid biosynthesis and overall lipid content. This change occurs despite HIF-1α-dependent elevation of PDK1, which has been shown to negatively regulate pyruvate entry into the mitochondrion under hypoxic conditions (32, 33). Interestingly, although glucoinolysis is increased in LKB1-deficient cells, LKB1 loss did not appear to promote increased glutamine-dependent lipid biosynthesis or the differential use of glutamine in pathways such as reductive carboxylation of alpha-ketoglutarate (34–36). LKB1-null cells appear to use glutamine as an anaplerotic substrate to support mitochondrial metabolic processes.

Our work here identifies HIF-1α as a key mediator of the metabolic transformation triggered by LKB1 loss. Using multiple cell systems, we demonstrate that acute down-regulation of LKB1 is sufficient to increase HIF-1α protein levels under normoxic conditions. Reducing HIF-1α levels reverses the metabolic effects triggered by LKB1 loss in cells (Fig. 6). We show here that targeting the mTORC1 complex, either by using rapamycin or through Raptor knockdown, reduces HIF-1α protein expression in LKB1-null cells, suggesting that deregulated mTORC1 activity links LKB1 loss to elevated HIF-1α activity. However, we also demonstrate that elevated ROS levels may contribute to HIF-1α protein expression in LKB1-null cells. LKB1 loss has previously been reported to promote enhanced levels of intracellular ROS in A549 cells (23). Here, we observe a similar trend in MEFs lacking LKB1. Reducing ROS levels with NAC abrogated the increase in HIF-1α levels in LKB1-null cells. It is unclear whether these two systems (mTORC1 and ROS) work separately or in concert to affect HIF-1α protein expression. One possibility is that increased metabolic activity of LKB1-deficient cells is driven by mTORC1 and that mitochondrial ROS generated as a consequence of this increased metabolic activity promotes HIF-1α expression, thus reinforcing the progrowth metabolic program induced by LKB1 deletion.

Disruption of the downstream LKB1 effectors AMPK (37) or TSC2 (24) promotes elevated mTORC1 activity and increased ATP levels. The loss of mTORC1 activity in LKB1-deficient cells is consistent with the decrease in cellular ATP levels measured in LKB1-null cells. The decrease in ATP levels is unlikely to be due to reduced cellular ATP production, at least in part, due to the increase in glucose uptake and utilization by LKB1-null cells. This decrease in ATP levels is more likely to be due to increased ATP consumption, which is consistent with the increase in cell growth and proliferation observed in LKB1-null cells. Therefore, these results suggest that the decrease in ATP levels measured in LKB1-null cells is not due to reduced cellular ATP production, but due to increased ATP consumption.
HIF-1α protein levels under normoxia. We have recently demonstrated that loss of AMPK activity is sufficient to promote the Warburg effect in tumor cells (37), suggesting that LKB1 may be linked to metabolic control through its upstream regulation of AMPK (16). However, LKB1 and AMPK appear to influence HIF-1α protein expression through different mechanisms. Silencing LKB1 promotes both increased transcription and translation of HIF-1α, events which are sensitive to mTORC1 inhibition. In contrast, loss of AMPK results in increased HIF-1α protein levels with no discernible changes in mRNA levels (37). Moreover, mTORC1 inhibition has little effect on HIF-1α protein levels when AMPK is silenced (37). These data suggest the existence of both AMPK-dependent and -independent mechanisms linking LKB1 to the HIF-1α and metabolic reprogramming.

Our observation that LKB1 loss promotes a progrowth metabolic profile in tumor cells raises the prospect that there may be selective pressure for tumors to lose or silence LKB1-AMPK signaling (38), as suggested by the frequent inactivation of LKB1 in NSCLC (39). We speculate that the metabolic effects of LKB1 inactivating mutations may also synergize with other genetic lesions, ultimately favoring the selection of tumor cells with distinct metabolic advantage. For example, oncogenic K-ras mutations (G12D) in pancreatic ductal carcinoma have been shown to redirect glucose metabolism from the TCA cycle to fuel increased pentose phosphate shunt activity and ribose biosynthesis (39). Interestingly, comutation of LKB1 and K-ras is frequently observed in NSCLC (40). We speculate that LKB1 inactivating mutations synergize with oncogenic K-ras to accelerate tumorigenesis in mouse models of lung cancer (41). Thus, LKB1 loss may augment the metabolic activities of other driver mutations in cancer by enhancing their ability to promote nutrient acquisition and utilization by tumor cells. However, although loss of LKB1 reprograms cancer-cell metabolism, it also confers a dependence on HIF-1α, rendering LKB1-null tumor cells more susceptible to apoptosis under poor nutrient conditions. This observation raises the possibility of targeting HIF-1α for synthetic lethality in LKB1-deficient cancers. Given that mTORC1 inhibition affects both aberrant mTORC1 signaling and HIF-1α expression in LKB1-deficient cancer cells, mTORC1-targeting compounds may be particularly effective for treating tumors with somatic LKB1 mutations or cancers associated with PJS.

Materials and Methods

Full methods are available as SI Materials and Methods. Primary mouse embryonic fibroblasts (MEFs) conditional for stk11 (LKB1Δ<sup>−−</sup>) were generated by targeting and immortalized with SV40 Large T Antigen as previously described (42). A549 and A427 NSCLC cell lines expressing LKB1 have been previously described (23). OCR and ECAR were measured using an XF24 Extracellular Flux Analyzer (Seahorse Bioscience), and GC/MS analysis of metabolites was conducted using established protocols (37). Statistics were determined using paired Student’s t test, ANOVA, or Log-rank (Mantel–Cox) test using Prism software (GraphPad). Statistical significance is represented in figures as follows: * < 0.05; ** < 0.01; *** < 0.001.

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Supporting Information

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SI Materials and Methods

Cell Lines, DNA Constructs, and Cell Culture. Primary mouse embryonic fibroblasts (MEFs) conditional for stk11 (LKB1-Δβ) were generated by timed mating and immortalized with SV40 Large T Antigen as previously described (1). AS49 and A427 cells have been previously described (2). DNA plasmids MigCD8t, pKD-HIF-1α hp, and LMP-based shRNAs against mouse and human liver kinase B1 (LKB1) have been described previously (1, 3, 4). Transduction of cell lines with high-titer retrovirus was conducted as previously described (1). Retrovirus-infected cells were cultured in 2 μg/mL puromycin and/or sorted 7 d postinfection by flow cytometry (for GFP or CD8t-expressing cells). LKB1Δβ mouse embryonic fibroblasts (MEFs) were transduced with either MigCD8t for control virus or MCD8t-Cre to delete stk11-foxed alleles. LKB1Δβ MEFs and AS49 non-small cell lung cancer (NSCLC) cells were also transduced with pKD-HIF-1α hp or control retrovirus. For siRNA transfections, cells were subjected to two rounds of reverse transfection with pooled siRNAs against hypoxia-inducible factor-1α (HIF-1α) (5).

Determination of Cell Proliferation, Apoptosis Assays, and Cell Size. Growth curves for all cell lines were determined by cell counting using trypan blue exclusion and a TC10 Automated Cell Counter (BioRad). Apoptosis assays were performed by washing cells twice with PBS and incubating in glucose- or glutamine-free media, containing 10% (vol/vol) diazylated FCS. Cells were incubated for times indicated, and apoptosis measurements were performed using propidium iodide (PI) staining and analyzed on FACS. Size of viable cells was measured by flow cytometry and quantified as the mean fluorescence intensity for forward scatter (FSC). Measurement of reactive oxygen species (ROS) was performed by incubating cells for 30 min with 2′,7′-dichlorofluorescein diacetate (DCF-DA), followed by quantification using flow cytometry. All flow cytometry was conducted using BD FACScan (BD Biosciences) or Gallios (Beckman Coulter) flow cytometers and analyzed with FlowJo software (Tree Star).

Western Blots. Cells were lysed in modified CHAPS buffer (10 mM Tris-HCl, 1 mM MgCl2, 1 mM EGTA, 0.5 mM CHAPS, 10% glycerol, 5 mM NaF) supplemented with the following protease additives: protase and phosphatase tablets (Roche), DTT (1 μM/mL), and benzamidine (1 μM/mL). Cleared lysates were resolved by SDS/PAGE, transferred to nitrocellulose, and incubated with primary antibodies. Primary antibodies to LKB1 (total), p70 S6-kinase (pT389-specific and total), S6 ribosomal protein (pS235/236-specific and total), 4E-BP1 (pT37/46-specific and total), LDHA, PDK1, Aldolase, and Actin, as well as HRP-conjugated anti-rabbit and anti-mouse secondary antibodies were obtained from Cell Signaling Technology. Anti-HIF-1α antibodies were from Cayman Chemical. LKB1 antibodies were from Santa Cruz Biotechnologies.

Quantitative Real-Time PCR. Total mRNA was isolated from cells using TRIzol (Invitrogen), and cDNA was synthesized from total RNA using the SuperScript VILO cDNA Synthesis Kit (Invitrogen). Quantitative PCR was performed using SYBR Green qPCR SuperMix (Invitrogen) and an MX3005 qPCR machine (Agilent) using primers against hif1a, aldA, ldha, pdk1, and actin. All samples were normalized to β-actin mRNA levels. Primer sequences have been previously described (6).

Seahorse XF24 Respirometry. Respirometry [oxygen consumption rate (OCR)] and the extracellular acidification rate (ECAR) of cells were measured using an XF24 Extracellular Flux Analyzer (Seahorse Bioscience) as previously described (6). In brief, cells were plated at 5 × 104 per well in 625 μL of nonbuffered DMEM containing 25 mM glucose and 2 mM glutamine. Cells were incubated in a CO2-free incubator at 37 °C for 1 h to allow for temperature and pH equilibration before loading into the XF24 apparatus. XF assays consisted of sequential mix (5 min), pause (3 min), and measurement (5 min) cycles, allowing for determination of OCR/ECAR every 10 min.

Metabolic Assays. Glucose, lactate, and glutamine levels in culture medium were measured using a Flex Bioanalyzer (NOVA Biological). Glucose-derived lipid biosynthesis was determined by culturing cells in medium containing 14C-glucose or 13C-glutamine (Perkin-Elmer) for 3 d, and extracting lipids using a 1:1 Water/Methanol/Chloroform extraction procedure (7). Following extraction, the organic layer was isolated, dried via N2 stream, and resuspended in methanol, and incorporated radioactivity was measured using a MicroBeta Liquid Scintillation Counter (Perkin-Elmer).

GC-MS Analysis of 13C Metabolites or Free Fatty Acids. For GC-MS analysis, protocols have been outlined previously (6). Briefly, cells (2–5 × 106 per 10-cm dish) were cultured for 3 d and were lysed using ice-cold 80% methanol followed by sonication. For isotopomer-labeling experiments, cells were treated with U-13C-glucose or -glutamine (Cambridge Isotopes) (8), and metabolites from tissue culture cells were extracted as described previously (9).

For free fatty acid profiles, cells were grown for 72 h under standard growth conditions. Triglycerides and other lipids were extracted using a modified Folch method (7) substituting methylene chloride for chloroform. Following extraction, the organic layer was isolated, dried in a warm N2 stream, and saponified in sodium hydroxide overnight at 60 °C. The free fatty acids were reextracted and dried, derivatized as tert-butylimidemethylsil (TBDMSS) esters, and analyzed on GC-MS.

Growth Assays. For analysis of adherent cell growth, cells were seeded (8,000 cells per well) in a 96-well plate in DMEM containing 10% (vol/vol) FBS and penicillin/streptomycin. After 24 h, medium was replaced with fresh DMEM containing 25 mM or 0.04 mM glucose. Cells were fixed with 100 μL of 4% paraformaldehyde at 0, 24, 48, and 72 h. Plates were incubated at 4 °C for 20 min. Cells were washed 2 × 5 min in 200 μL of PBS, and 100 μL of crystal violet solution [0.05% (wt/vol) crystal violet and 20% (vol/vol) 95% ethanol] was added to each well. Plates were incubated at room temperature for 30 min. Cells were washed 3 × 5 min in 200 μL of PBS and solubilized on a plate shaker for 1 h in 100 μL of 1% SDS in PBS. The plates were analyzed at 595 nm on a Molecular Devices Spectramax plate reader. Hypoxia experiments were conducted by incubating cells at 1% O2 in a Hera Cell 150 incubator (Mandel).

Statistical Analysis. Statistics were determined using paired Student t test, ANOVA, or Log-rank (Mantel–Cox) test using Prism software (GraphPad). Data are calculated as the mean ± SEM unless otherwise indicated. Statistical significance is represented in figures as follows: *P < 0.05; **P < 0.01; ***P < 0.001.

**Fig. S1.** Expression of LKB1 and metabolism of NSCLC cell lines. (A) LKB1 immunoblot on lysates from A549 cells transduced with empty vector (Vec) or LKB1 cDNA (LKB1), and H1299 cells. (B) Basal extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) for A427 cells expressing empty vector (Vec) or LKB1 cDNA (LKB1).
**Fig. S2.** LKB1-deficient cells display enhanced glycolytic and TCA cycle flux. (A) Mass isotopomer profiles of A549 tumor cells with LKB1 reexpression (open bar) or lacking LKB1 (filled bar). Cells were pulsed with \(^{13}\text{C}\)-labeled glucose (Glc) or glutamine (Gln) for 1 h before metabolite extraction. Mass isotopes for citrate and malate are indicated. (B) MEFs expressing LKB1 (Cre−) or deficient for LKB1 (Cre+) were pulsed with \(^{13}\text{C}\)-glucose or \(^{13}\text{C}\)-glutamine for 1 h, and \(^{13}\text{C}\) incorporation into lactate and TCA cycle metabolites was determined by GC-MS as in Fig. 2. The relative incorporation of \(^{13}\text{C}\) into total metabolite pools (lactate, citrate, \(\alpha\)-ketoglutarate, succinate, and malate) is indicated by shaded bars for glucose (black) and glutamine (gray). Metabolite abundance is expressed as the mean ± SD for triplicate samples and expressed relative to basal levels in control (Cre-negative) cells.

**Fig. S3.** Expression of HIF-1\(\alpha\) protein levels in cell models of LKB1 deficiency. (A) Immunoblot for HIF-1\(\alpha\) in A427 cells expressing empty vector (Vec) or LKB1 cDNA (LKB1). (B) Immunoblot for HIF-1\(\alpha\) in U2OS cells transfected with 25 nM scrambled (Scr) siRNA or siRNA targeting LKB1 (LKB1). A representative immunoblot is shown. (C) Immunoblot for HIF-1\(\alpha\) in HCT116 cells transfected with control (Scr) or LKB1-targeting (LKB1) siRNA.
Fig. S4. Rapamycin treatment of LKB1-null cells reduces lactate production. MEFs expressing (Cre−) or lacking (Cre+) LKB1 expression (A) or A549 cells with (LKB1) or without (Vec) LKB1 reexpression (B) were treated for 24 h with 25 nM rapamycin, and lactate in the extracellular medium was measured via enzymatic assay. Lactate levels are expressed as the mean ± SD for triplicate samples.

Fig. S5. A549 cells lacking HIF-1α expression display enhanced sensitivity to glucose and serum withdrawal. (A) Growth curves of A549 cells expressing control (black bar) or HIF-1α (gray bar) shRNAs, and grown under full (+, 25 mM) or no glucose (−, 0 mM) conditions. Cells were additionally cultured in the presence or absence of serum (FBS) or under normoxic (20% O₂) or hypoxic (1% O₂) conditions. (B) Oxygen consumption rates of A549 cells expressing empty vector (Vec) or HIF1α shRNA (shHIF1α). Cells were grown under full glucose (25 mM) or low glucose (0.4 mM) conditions. (C) Spare respiratory capacity (SRC) of cells treated as in B.