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*$^{fl/fl}$) were generated by timed mating and immortalized with SV40 Large T Antigen as previously described (1). A549 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). *LKB1*$^{fl/fl}$ mouse embryonic fibroblasts (MEFs) were transduced with either MiCD8t for control virus or MiCD8t-Cre to delete *stk11*-floxed alleles. *LKB1*$^{fl/fl}$ MEFs and A549 non-small cell lung cancer (NSCLC) cells were also transduced with pKD-HIF-1αhp 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) dialyzed 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 FACSCalibur (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 $MgCl_2$, 1 mM EGTA, 0.5 mM CHAPS, 10% glycerol, 5 mM NaF) supplemented with the following protease additives: protease and phosphatase tablets (Roche), DTT (1 μg/mL), and benzamidine (1 μg/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*, *aldoA, 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 5×10^5 per well in 625 μL of nonbuffered DMEM containing 25 mM glucose and 2 mM glutamine. Cells were incubated in a CO_2-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 (3 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 Biomedical). Glucose-derived lipid biosynthesis was determined by culturing cells in medium containing ^{14}C-glucose or ^{14}C-glutamine (Perkin-Elmer) for 3 d, and extracting lipids using a 1:1:1 Water/Methanol/Chloroform extraction procedure (7). Following extraction, the organic layer was isolated, dried via N_2 stream, and resuspended in methanol, and incorporated radioactivity was measured using a MicroBeta Liquid Scintillation Counter (Perkin-Elmer).

GC-MS Analysis of ^{13}C Metabolites or Free Fatty Acids. For GC-MS analysis, protocols have been outlined previously (6). Briefly, cells ($2–5 \times 10^6$ 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-^{13}C-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 N_2 stream, and saponified in sodium hydroxide overnight at 60 °C. The free fatty acids were reextracted and dried, derivatized as tert-butyldimethylsilyl (TBDMS) 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% O_2, 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: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

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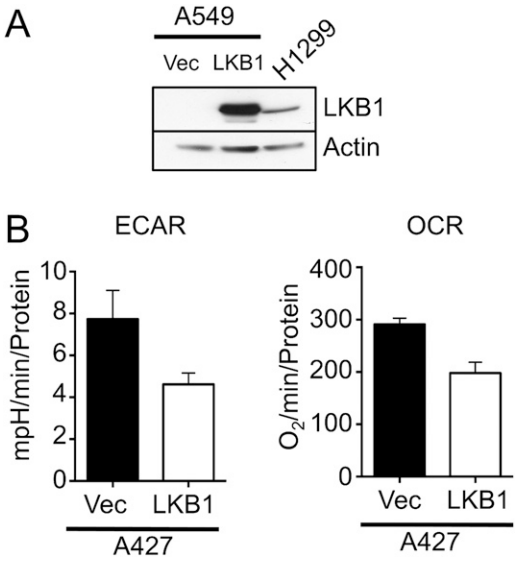

Fig. S1. Expression of LKB1 and metabolism of NSCLC cell lines. (*A*) LKB1 immunoblot on lysates from A549 cells transduced with empty vector (Vec) or LKB1 cDNA (LKB1), and H1299 cells. (*B*) Basal extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) for A427 cells expressing empty vector (Vec) or LKB1 cDNA (LKB1).

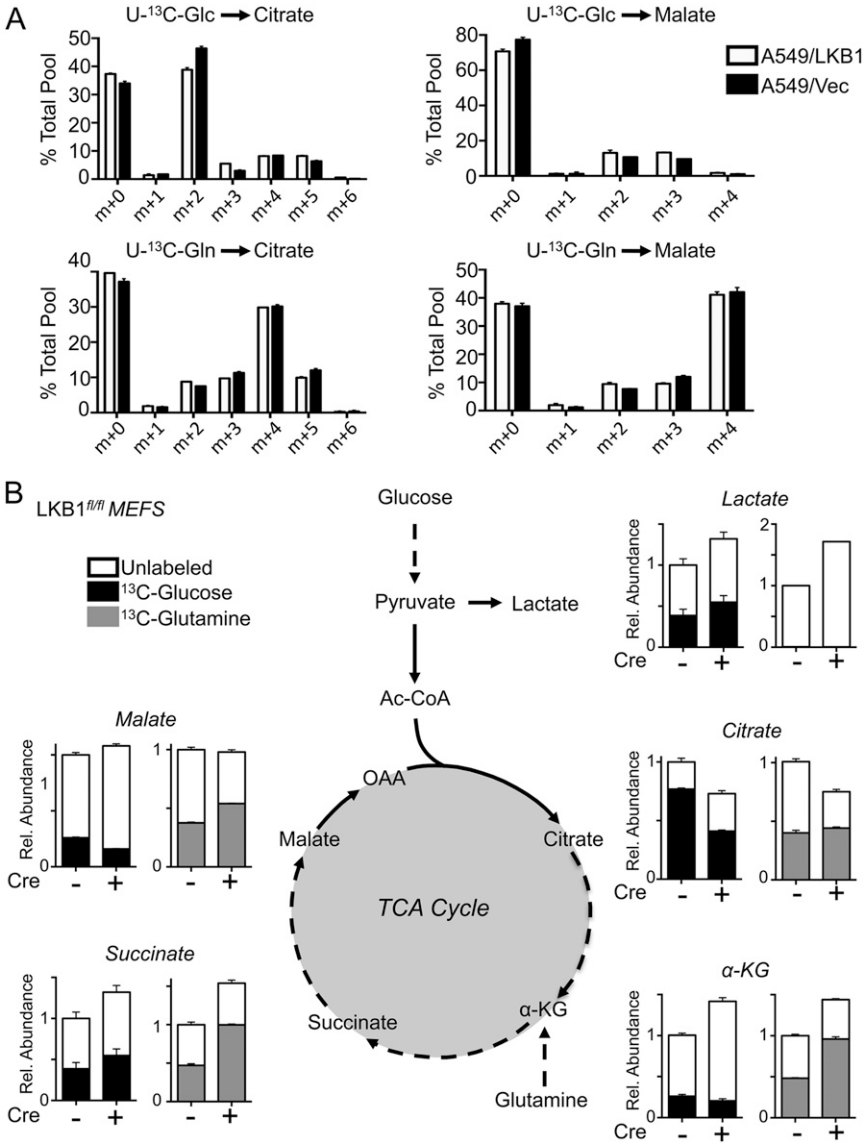

Fig. S2. LKB1-deficient cells display enhanced glycolytic and TCA cycle flux. (*A*) Mass isotopomer profiles of A549 tumor cells with LKB1 reexpression (open bar) or lacking LKB1 (filled bar). Cells were pulsed with ^{13}C-labeled glucose (Glc) or glutamine (Gln) for 1 h before metabolite extraction. Mass isotopes for citrate and malate are indicated. (*B*) MEFs expressing LKB1 (Cre−) or deficient for LKB1 (Cre+) were pulsed with ^{13}C-glucose or ^{13}C-glutamine for 1 h, and ^{13}C incorporation into lactate and TCA cycle metabolites was determined by GC-MS as in Fig. 2. The relative incorporation of ^{13}C into total metabolite pools (lactate, citrate, α-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.

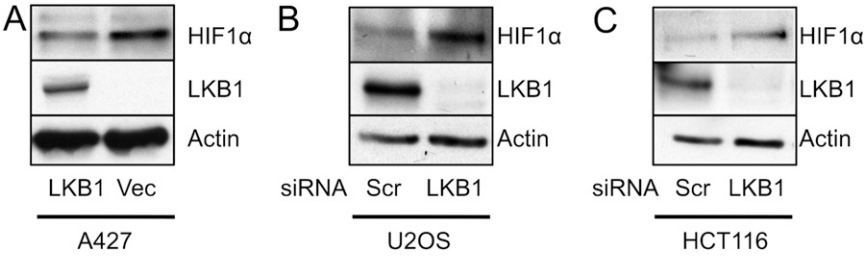

Fig. S3. Expression of HIF-1α protein levels in cell models of LKB1 deficiency. (*A*) Immunoblot for HIF-1α in A427 cells expressing empty vector (Vec) or LKB1 cDNA (LKB1). (*B*) Immunoblot for HIF-1α in U20S cells transfected with 25 nM scrambled (Scr) siRNA or siRNA targeting LKB1 (LKB1). A representative immunoblot is shown. (*C*) Immunoblot for HIF-1α in HCT116 cells transfected with control (Scr) or LKB1-targeting (LKB1) siRNA.

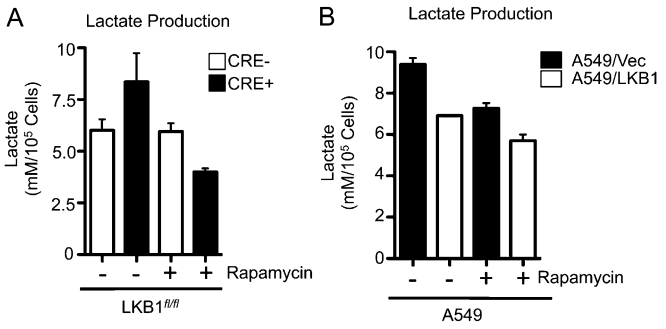

Fig. S4. Rapamycin treatment of LKB1-null cells reduces lactate production. MEFs expressing (Cre−) or lacking (Cre+) LKB1 expression (*A*) or A549 cells with (LKB1) or without (Vec) LKB1 reexpression (*B*) were treated for 24 h with 25 nM rapamycin, and lactate in the extracellular medium was measured via enzymatic assay. Lactate levels are expressed as the mean ± SD for triplicate samples.

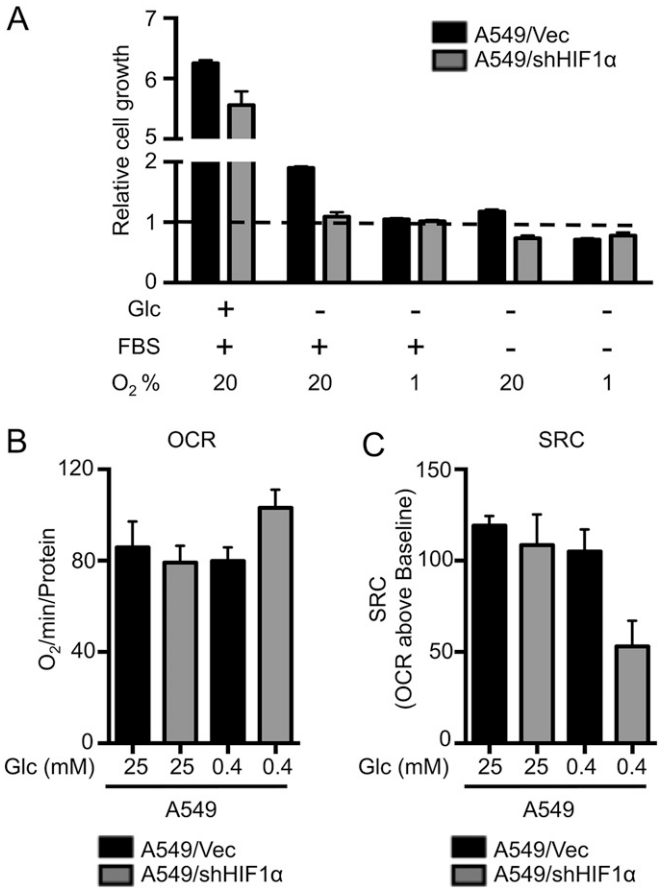

Fig. S5. A549 cells lacking HIF-1α expression display enhanced sensitivity to glucose and serum withdrawal. (*A*) Growth curves of A549 cells expressing control (black bar) or HIF-1α (gray bar) shRNAs, and grown under full (+, 25 mM) or no glucose (−, 0 mM) conditions. Cells were additionally cultured in the presence or absence of serum (FBS) or under normoxic (20% O_2) or hypoxic (1% O_2) 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*.