SUPPLEMENTARY MATERIALS AND METHODS

Transgenic mouse model. In a set of experiments, the previously described immunocompromised RIP1-Tag2;Rag1-KO transgenic model of islet cell cancer was utilized (1). Anti-angiogenic treatment was started in tumor bearing animals (at 12 weeks of age) with anti-VEGFR2 blocking antibody (DC101) purified from a hybridoma culture (ATCC) at a dose of 1mg/mouse/twice a week i.p., as previously described (1). Animal housing, handling and all procedures with mice were approved by the ICO-IDIBELL institutional committee that approve and oversee research involving vertebrate animals, and all experiments were performed according to Spanish government guidelines governing animal care.

Annexin-V apoptosis assay. Cells were incubated with Annexin V-FITC in Hepes buffer containing propidium iodide (PI), using the Annexin-V Fluos Staining Kit (Roche Diagnostics, Mannheim, Germany). Labelled cells were analysed on an EPICS-XL cytofluorimeter using Expo32 sotware (Beckman Coulter, Fullerton, CA).

Glucose and lactate measurements. Glucose and lactate concentrations in the supernatants were determined by colorimetric methods on an automatic analyzer (Dimension RxL, Dade Behring, Milan, Italy). Values were normalized to cells numbers at the end of the incubation period.

Lentiviral vector-mediated transduction of shRNA in ovarian cancer cells.

The lentiviral plasmids containing AMPKα2 shRNA expression cassette or an un-relevant shRNA sequence were purchased from Sigma-Aldrich. The lentiviral vectors were produced as previously described (2). Cells expressing the shRNA were selected in puromycin-containing medium for 10-14 days prior to subsequent analysis.

Reverse Transcription PCR and Real-Time PCR assay. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA was synthesized from 0.5-1 μg of total RNA using the Superscript II reverse transcriptase (Invitrogen). Real-time PCR was performed with SYBR Green dye and Gene AMP 5700 Sequence Detection System (PE Biosystems, Foster City, CA). Five-fifty ng of cDNA were used as template; 10 μL of 2X Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) were mixed with template and primers. The total reaction volume was 20 μL. Cycling conditions were 10 min at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C. Each sample was run in duplicate. For all genes evaluated, mRNA was normalized to β_2-microglobulin (*B2M*) mRNA by subtracting the cycle threshold (Ct) value of β_2-m mRNA from the Ct value of the gene of interest (ΔCt). Fold difference ($2^{-\Delta\Delta Ct}$) was calculated by subtracting the ΔCt (treated sample)- ΔCt (reference sample), to generate a ΔΔCt. PCR efficiency was in the range 95% - 105%. The following primers were used for real-time PCR: human Hexokinase II (*HKII)* (sense, 5'-GAAGATGCTGCCCACCTTTG-3'; antisense, 5'-CACCCAAAGCACACGGAAGT-3'), human *GAPDH* (sense, 5'-GAAGGTGAAGGTCGGAGT-3'; antisense, 5'-CATGGGTGGAATCATATTGGAA-3'), human *LDH-A* (sense, 5'-GATTCAGCCCGATTCCGTTAC-3'; antisense, 5'-ACTCCATACAGGCACACTGG-3'), human *AMPKα*1 (sense, 5'-GGAGCCTTGATGTGGTAGGA-3'; antisense, 5'-

GTTTCATCCAGCCTTCCATTC-3'), human *AMPKα2* (sense, 5'-ACCAGCTTGCAGTGGCTTAT-3'; antisense, 5'- CAGTGCATCCAATGGACATC-3'), human *B2M* (sense, 5'-TGCTGTCTCCATGTTTGATGTATCT-3'; antisense 5'-TCTCTGCTCCCCACCTCTAAGT-3').

Immunoblotting. The cells exposed to the various treatments were harvested, lysed and subjected to SDS-polyacrylamide gel electrophoresis and blotting. The membrane was saturated with PBS 5% non-fat dry milk (Sigma-Aldrich, St. Louis, MO) as blocking buffer for 1 h at room temperature. Immunoreactivity levels were evaluated by hybridization using the following antibodies: polyclonal Ab anti-AMPK (1:1000, #2532,), polyclonal anti-ACC (1:750, #3662,), polyclonal anti-phosphorylated AMPK (p-AMPK) (1:1000, #2531S), polyclonal anti-phosphorylated ACC (p-ACC) (1:1000, #3661S), monoclonal Ab anti-phosphorylated (Ser473) Akt (1:1000, #4051), purchased from Cell Signaling Technology (Beverly, MA), anti-HIF-1α (1:250, Transduction Laboratories, Lexington, KY), polyclonal anti-phosporylated Akt1-2-3 (Thr308) (1:1000, SC-16646-R), polyclonal anti-Akt 1-2-3 (1:500, SC-8312), monoclonal anti-LKB1 (1:100, SC-32245) from Santa Cruz Biotechnology (Santa Cruz, CA), and mAb anti-α-tubulin (1:4000, T5168) from Sigma Aldrich. Then, the blot was hybridized with a 1:5000 diluted HRP-conjugated anti-mouse or anti-rabbit Ab (Amersham-Pharmacia, Little Chalfont, UK). Finally, the signal was detected by chemiluminescence with SuperSignal kit (Pierce, Rockford, IL).

Immunofluorescence analysis. For immunofluorescence analysis of LKB1 expression, cells were grown on Lab-Tek chambers (Nunc, Rochester, NY) for 20 h

under glucose starvation and hypoxic conditions (0.5% pO_2), fixed, washed with PBS and then incubated for 1 h at room temperature with a saturating solution consisting of 5% goat serum, 1% BSA, 0.1% Triton-X in PBS. After saturation, slides were incubated with anti-LKB1 mAb (1:50, SC-32245), washed and incubated with Alexa488-labelled secondary antibody (Invitrogen). Nuclei were stained with TO-PRO-3 iodide (Invitrogen). Confocal laser scanning microscopy was carried out with a Zeiss LSM 510 microscope (Zeiss, Jena, Germany) using Argon (488 nm) and Helium-Neon (543-633 nm) laser sources. Images were collected at a total magnification of 400 X.

Bioluminescence imaging. Reaction solutions containing specific enzymes linking the metabolite of interest to the luciferase of *Photobacterium fischeri* or *Photinus pyralis*, respectively, were applied to the cryosections. Light emission was induced in a temperature-stabilized reaction chamber, which was placed under a microscope (Axiophot, Zeiss, Oberkochen, Germany) connected to a 16bit CCD camera with an imaging photon counting system (iXonEM+ DU-888, Andor Technology PLC, Belfast, Northern Ireland). The resulting images of the spatial distribution of light intensities were calibrated using appropriate standards. Metabolite content was calculated in µmol/g tumor tissue. Images were displayed in colors coding for tissue concentration of metabolites in units of µmol/g. Computerized image analysis allowed for separate data assessment in selected histological areas of xenotransplanted tumors, e. g., vital tumor regions. Four tumors of OC316 controls, seven tumors of A4.6.1 treated OC316, and ten tumors of IGROV-1 controls or A4.6.1 treated, respectively, were analyzed using generally three sections from each tumor. All metabolite concentrations shown

here were acquired exclusively from vital tumor regions. Box plots display the following values: minimum, 25th percentile, median, mean, 75th percentile, and the maximum.

***In vitro* MRS Analysis.** Aqueous extracts (from 20 x 10^6 cells/sample) were prepared in EtOH 70% according to an established protocol (3). Briefly, samples were ultra-sonicated at 20 kHz by a MSE ultrasonic disintegrator Mk2 (Crawley, Sussex, United Kingdom) and centrifuged at 14000 x g for 30 min. Supernatants were lyophilized twice in a RVT 4104 Savant lyophilizer (Mildford, ME), and the residue resuspended in 0.7 ml D_2O (Sigma-Aldrich, St. Louis, MO, USA) containing 0.1 mM 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (TSP) as internal standard. High-resolution NMR experiments (25°C) were performed at 9.4T and 16.4 T (Bruker AVANCE spectrometers, Karlsruhe, Germany). ^{1}H-NMR spectra of cell extracts were acquired using 90° flip angle, 30 s repetition time, 32K time domain data points and 128 transients (3).

MRI/MRS Analysis. Animals were anaesthetized with isofluran 2.5-1.5% in O_2, 1 L/min. ^{1}H MRI/MRS of tumors was carried out weekly. Coronal multislice spin echo images were acquired from the s.c. implants with the following parameters: 4 transients, 15 slices, FOV starting from 12 x 12 mm^2 and increasing according to tumor growth, matrix 256 x 128, thickness = 0.6 mm (corresponding to voxel dimensions up to 47 x 93 x 600 μm^3) and TR/TE = 3000/70, 500/16 and 3000/16 ms for T2W, T1W and PD images, respectively. Diffusion weighted imaging (DWI, TR/TE = 2500/50 ms, 2 transients, 11 slices, FOV = 20 x 20 mm^2, matrix 128 x 64, thickness = 1 mm, b-values =123, 491, 1105 s/mm^2), which

allowed quantitative determination of water ADC in tumors was also performed.

Localized spectroscopy was acquired by using a PRESS sequence in vital and necrotic regions between 10 and 35 days post injection (dpi). The repetition time of 4 s was chosen in order to make negligible the effects due to the T1 relaxation time of water and metabolites (4). A short echo time (23 ms) was adopted for tumor metabolic characterization. Spectra at longer echo times (136 and 272 ms) were also acquired for lactate editing (5): in fact, since the two groups of protons present in the lactate molecule are weakly coupled, the signal at 1.3 ppm is inverted in spectra acquired by using TE=136 ms and comes back as a positive signal at 272 ms, albeit reduced up to about 40% of the original signal (6). Water suppression was achieved by using the VAPOR presequence (7). Finally, T2 measurements have been performed to water signals.

Spectra were analyzed using LCModel (8) which calculates the best fit to the experimental spectrum as a linear combination of model spectra (spectra of metabolites' solutions). Eleven metabolites (resulting from in vitro analyses of tissue extracts of these tumors) were included in the basis set: alanine (Ala), creatine (Cr), phosphocreatine (PCr), glucose (Glc), glutamate (Glu), glutamine (Gln), glycerophosphorylcholine (GPC), phosphorylcholine (PCho), *myo*-inositol (Ins), lactate (Lac), and taurine (Tau). Spectra of lipids and macromolecules were also included in the basis set.

Only those metabolites which were estimated to have Cramer-Rao lower bounds (CRLB) less than 20%, corresponding to an estimated concentration error <0.2 μmol/g (6), were included into the quantitative analysis.

Reporter gene assay. IGROV-1 cells were transiently co-transfected using lipofectamine 2000 (Invitrogen) with a HIF-1α-responsive luciferase reporter

construct kindly provided by Dr. Celeste M. Simon (Abramson Cancer Research Institute, Philadelphia, PA) - carrying the luciferase reporter gene under the control of a promoter containing HRE sequences - and a plasmid encoding β-galactosidase under the control of the human CMV promoter, which was used to normalize transfection efficiency. Cell lysates were harvested 24 h post-transfection and luciferase and β-galactosidase assays were carried out using BriteLite Plus (Perkin-Elmer, Waltham, Massachusetts) and Tropix® Galacto-Light™ (Applied Biosystems, Foster City, CA), respectively, on a plate luminometer (Perkin-Elmer).

Cell proliferation assay. IGROV-1 cells and their derivatives were plated in triplicate in 96 well plates at 2.5 x 10^3 cells/well and subsequently cultivated for 72 hours. ATP levels were determined by the ViaLight HS Kit (Lonza, Basel, Switzerland) at different time points according to manufacturer's instructions, and used as surrogate indicator of cell proliferation.

Analysis of LKB1 gene mutations. Genomic DNA (100ng) was used for gene amplification. PCR of genomic DNA for exons 1-9 was carried out using AmpliTaq Gold (Applied Biosystems). PCR conditions of exons 1, 2, 6, 7, and 8 were: one cycle of 95°C for 10 min, 35 cycles of 95°C for 30s, 62°C for 30s, 72°C for 40s, and one cycle of 72°C for 10 min; exons 3, 4-5, 9 one cycle of 95°C for 10 min, 35 cycles of 95°C for 30s, 60°C for 30s, 72°C for 50s, and one cycle of 72°C for 10 min. PCR products were sequenced using Big Die Terminators Kit v1.1 (Applied Biosystems). Sequencing reaction products were separated electrophoretically on ABI PRISM 3130 apparatus (Applied Biosystems) and the forward and reverse

sequences obtained were analyzed by manual review. PCR primers were previously published (9).

REFERENCES

1. Casanovas O, Hicklin DJ, Bergers G, Hanahan D. Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors. Cancer Cell 2005;8:299-309.

2. Indraccolo S, Tisato V, Tosello V, Habeler W, Esposito G, Moserle L, et al. Interferon-alpha gene therapy by lentiviral vectors contrasts ovarian cancer growth through angiogenesis inhibition. Hum Gene Ther 2005;16:957-70.

3. Iorio E, Mezzanzanica D, Alberti P, Spadaro F, Ramoni C, D'Ascenzo S, et al. Alterations of choline phospholipid metabolism in ovarian tumor progression. Cancer Res 2005;65:9369-76.

4. Mlynarik V, Gruber S, Moser E. Proton T (1) and T (2) relaxation times of human brain metabolites at 3 Tesla. NMR Biomed 2001;14:325-31.

5. Matsumura A, Isobe T, Takano S, Kawamura H, Anno I. Non-invasive quantification of lactate by proton MR spectroscopy and its clinical applications. Clinical Neurol Neurosurg 2005;107:379-84.

6. Ernst T, Hennig J. Coupling effects in volume selective 1H spectroscopy of major brain metabolites. Magn Reson Med 1991;21:82-96.

7. Tkac I, Andersen P, Adriany G, Merkle H, Ugurbil K, Gruetter R. In vivo 1H NMR spectroscopy of the human brain at 7 T. Magn Reson Med 2001;46:451-6.

8. Provencher SW. Estimation of metabolite concentrations from localized in vivo proton NMR spectra. Magn Reson Med 1993;30:672-9.

9. Onozato R, Kosaka T, Achiwa H, Kuwano H, Takahashi T, Yatabe Y, Mitsudomi T. LKB1 gene mutations in Japanese lung cancer patients. Cancer Sci 2007;98:1747-51.