SUPPLEMENTAL MATERIALS AND METHODS

Cell Lines and Reagents

Human CRC cell lines HCT116, HKe-3, HKh-2, DLD-1, and DKO-4 were generated as described previously (*11*). For the normoxic culture, cells were incubated in 5% CO_2-humidified atmosphere at 37°C (21% O_2). For the hypoxic culture, cells were incubated in a sealed hypoxia chamber (Billups-Rothenberg) after flushing with a mixture of 1% O_2 and 5% CO_2 balanced with N_2. U0126 was purchased from Calbiochem, LY294002 and Rapamycin were from Wako, and BKM120 was from Selleckchem.

Quantitative Reverse Transcription Polymerase Chain Reaction Analysis

Total RNA was extracted and reverse transcription was performed with oligo(dT) primer and the Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer's instructions. The resulting cDNA was quantified using StepOnePlus Real-Time PCR System (Applied Biosystems) and FastStart Universal SYBR Green Master (Roche). Primer sequences were as follows: 5′-GTCACCATCCTGGAGCTGTT-3′ and 5′-GAAGGCCGTGTTGACGATAC-3′ for *GLUT1*, 5′-CTTCTTCACGGAGCTCAA-CC-3′ and 5′-CATCTGGAGTGGACCTCACA-3′ for *HK2,* and 5′-GCAAAGACCTGTAC-GCCAAC-3′ and 5′-ACATCTGCTGGAAGGTGGAC-3′ for *ACTB*. Transcription levels for *GLUT1* and *HK2* were normalized to that for *ACTB*.

Western Blot Analysis

Cells incubated on dishes were lysed with lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 50 mM NaF, 1% NP40 and protease inhibitors). Cell lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, immunoblotted with the respective primary antibodies followed by horseradish peroxidase–conjugated secondary antibodies, and analyzed. The primary antibodies used were as follows: rabbit polyclonal anti-GLUT1 (1:400; Abcam), rabbit monoclonal anti-HK2 (1:800; Cell Signaling Technology), mouse monoclonal anti-KRAS (1:200; Santa Cruz Biotechnology), mouse monoclonal anti-HIF1α (1:500; BD Biosciences), rabbit monoclonal anti-pohospho-p44/42 kinase (Thr202/Tyr204) (1:2,000; Cell Signaling), rabbit monoclonal anti-p44/42 kinase (1:1,000; Cell Signaling), rabbit

monoclonal anti-phospho-Akt (Ser473) (1:1,000; Cell Signaling), rabbit monoclonal anti-Akt (1:1,000; Cell Signaling), rabbit polyclonal anti-phospho-p70 S6 kinase (Thr389) (1:1,000; Cell Signaling), rabbit polyclonal anti-p70 S6 kinase (1:1,000; Cell Signaling), and mouse monoclonal anti-β-actin-peroxidase (1:8,000; Sigma-Aldrich).

In Vitro ^{18}F-FDG Accumulation

Cells in Dulbecco modified Eagle medium containing 25 mM glucose were seeded at a concentration of 1.0×10^5 cells per well in triplicates in 12-well plates. After 48 h of incubation, the medium was replaced by 1 mL of glucose-free Dulbecco modified Eagle medium containing 555 kBq of ^{18}F-FDG for each well, and the cells were then incubated over times ranging from 30 min to 120 min. When ^{18}F-FDG accumulation in the tumor cells was evaluated under hypoxic conditions, the cells were seeded in the plates and incubated in normoxia for 36 h; then, these plates were incubated in 1% O_2 for 12 h. Afterward, the medium was replaced by glucose-free medium containing ^{18}F-FDG and also incubated in 1% O_2 during accumulation times. After incubation for the respective times, the medium was removed and immediately washed 3 times with ice-cold phosphate-buffered saline; the cells were then dissociated with trypsin and collected into tubes, and the ^{18}F-FDG radioactivity in the tumor cells was measured using a γ counter (Cobra II Auto-gamma; Packard). The total protein concentration was determined using the BCA assay kit (Thermo Fisher Scientific).

RNA Interference

To knock down the endogenous expression of *GLUT1, HK2, KRAS,* and *HIF-1*α*,* FlexiTube GeneSolutions (Qiagen) for *SLC2A1* (#1: SI03068436 and #2: SI03089401), *HK2* (#1: SI00004060 and #2: SI00004067), *KRAS* (#1: SI03101903, #2: SI03106824, and #3: SI02662051), and *HIF-1*α (#1: SI04249308 and #2: SI04361854), respectively, were used, and AllStars Negative Control siRNA was used for the control. Cells were transfected with 10 nM siRNA using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions.

Plasmid Construction, Cotransfection of siRNA and Plasmid DNA

The coding regions of mutant-*KRAS* (G13D), *GLUT1,* and *HK2* were amplified by polymerase chain reaction from the RNA extracted from HCT116 cells. These PCR products were subcloned into pcDNA3.1+ (Invitrogen). *HIF-1α* expression plasmid vector (pcDNA3.1-HIF-1α) was kindly provided by K. Hirota (Kansai Medical University). All constructs were confirmed by sequencing. Transient cotransfection was performed with 30 pmol of siRNA and 1 μg of plasmid DNA per 6-well plate using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). In this condition, FlexiTube GeneSolutions (Qiagen) for *KRAS* (SI02662051), *SLC2A1* (SI03068436), *HK2* (SI00287329), and *HIF-1α* (SI02664431) were used. These siRNA constructs were targeting the 5′ or 3′ untranslated region of the respective genes. Cotransfected cells with AllStars Negative Control siRNA and pcDNA3.1 empty plasmid were used as the control, and they were analyzed 48 h after transfection.

Animal Tumor Model

Four-wk-old female SCID mice (CB17/Icr-*Prkdc*^{scid}/CrlCrlj) were purchased from Charles River Laboratories. Cells were resuspended in a 1:1 mix of phosphate-buffered saline and Matrigel (BD Bioscience), and a 100-μL volume containing either 1.5×10^6 cells of HCT116 or 1×10^7 cells of HKe-3 was subcutaneously injected into the flank of each mouse. At the time of assay, the average diameter of these tumors was approximately 10 mm. Tumor volumes were calculated using the following formula: $(L \times W^2) \times 0.5$, where L is length and W is width. Animal experimental studies were conducted in accordance with our institutional guidelines and approved by the Kyoto University Animal Care Committee.

Animal PET/CT Analysis

The mice were kept fasting overnight, and then 14.8 MBq of ^{18}F-FDG were injected via the tail vein and the mice were kept warm using heating pads. One hour later, a static dataset was acquired for 20 min using an energy window of 250–700 keV for each mouse. After the PET scans were finished, CT scans were obtained for anatomic reference (spatial resolution, 50 mm; 60 kV; and 310 mA). All scans were obtained on a small-animal scanner (FX3300 imager; SII NanoTechnology Inc.). Images were

reconstructed using a 3-dimensional ordered-subset expectation maximization algorithm with 20 interactions and 20 subsets, and CT images were reconstructed using a modified 3-dimensional cone-beam Feldkamp algorithm resulting in a 0.177 × 0.177 × 0.177 mm voxel size for a 512 × 512 × 512 image volume. To compare ^{18}F-FDG uptake between HCT116 and HKe-3 tumors, 3-dimensional regions of interest were drawn over the tumors and normal liver, and mean uptake values in regions of interest were measured using AMIDE software (version 1.0.4-1). For quantitative analysis, the tumor-to-liver ^{18}F-FDG uptake ratio (TLR) was calculated as follows: TLR = mean tumor uptake value/mean normal liver uptake value. Image analyses were performed using the 2-dimensional data analysis and visualization software Amira (Mercury Computer Systems, Inc.).

Immunohistochemistry

One hour after intravenous injection of pimonidazol (60 mg/kg), HCT116 and HKe-3 tumor-bearing mice (*n* = 4) were sacrificed, and xenograft tumors were immediately excised, fixed in 4% paraformaldehyde, and then paraffin-embedded. For immunohistochemical staining, these sections were stained with the respective antibodies by the avidin–biotin immunoperoxidase method. Antigen retrieval was achieved with microwave-heating in citrate buffer (pH 6.0). The primary antibodies used were as follows: rabbit polyclonal anti-GLUT1 (ab15309) (1:200; Abcam), goat polyclonal anti-GLUT1 antibody (C-20) (1:100; Santa Cruz Biotechnology), rat monoclonal anti-mouse CD34 (MEC14.7) (1:50; Abcam), rabbit polyclonal anti-HIF1α (H-206) antibody (1:200; Santa Cruz Biotechnology), rabbit monoclonal anti-HK2 (C64G5) (1:50; Cell Signaling Technology), and mouse IgG1 monoclonal antibody (MAb1, clone 4.3.11.3) (1:50; Hypoxyprobe, Inc.). Microvessel density was quantified by counting the number of CD34-positive blood vessels in 6 random fields (magnification, ×200). For primary CRC tissue, HIF-1α expression was determined by assessing the percentage of tumor cells with cytoplasmic staining and by monitoring the staining intensity, recorded as none, weak, moderate, or strong. HIF-1α expression was defined as positive when there was moderate cytoplasmic staining in more than 50% of tumor cells or strong expression in any fraction of tumor cells, as previously described (*5*).

Patients, Clinicopathologic Data, and PET Imaging

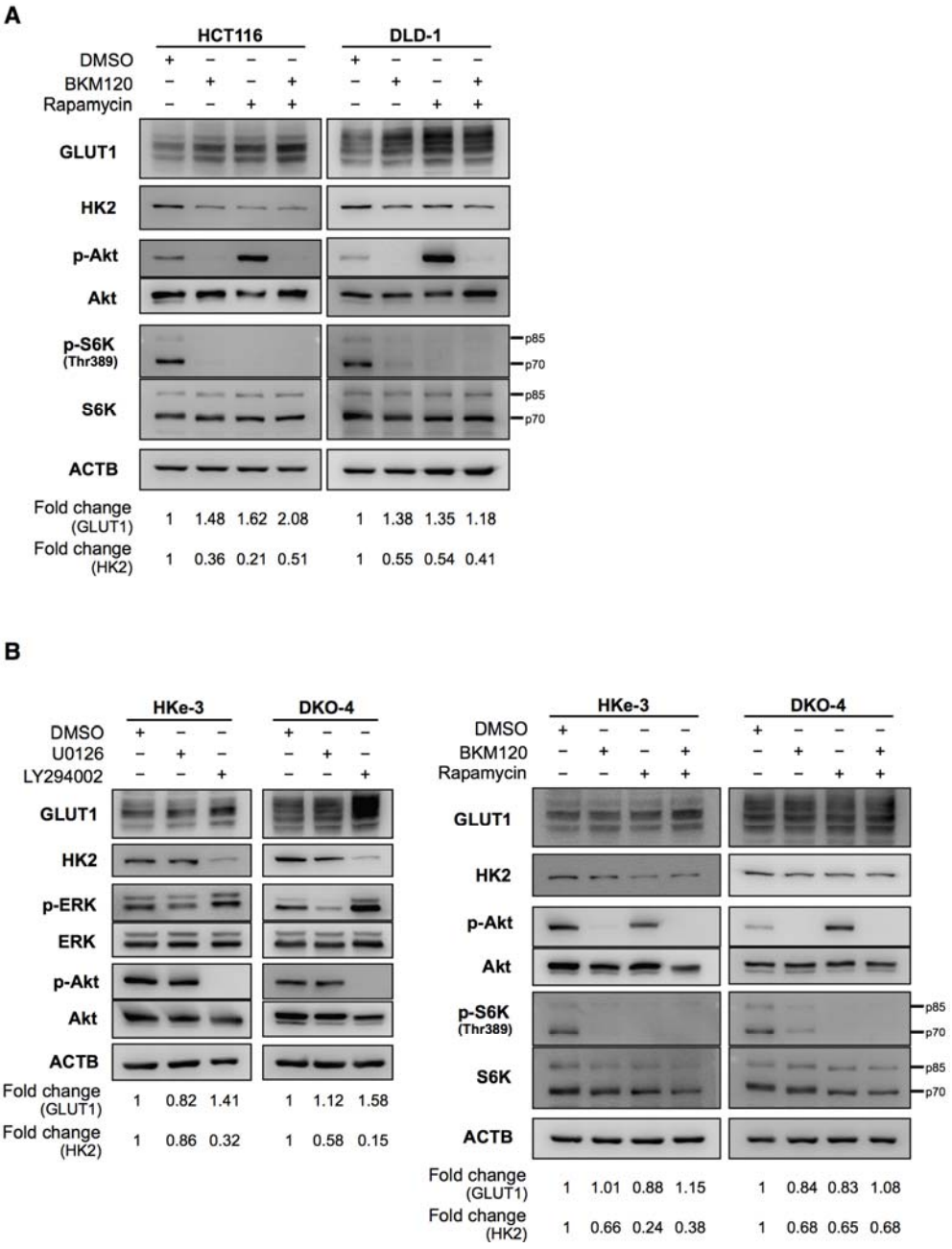
The clinicopathologic data of 51 patients with CRC who underwent ^{18}F-FDG PET before tumor resection at Kyoto University Hospital between April 2009 and September 2010 were previously described (*10*). The methods for PET imaging and quantitative analysis were previously described (*10*). This study protocol was approved by the institutional review board of Kyoto University, and patients provided their written consent for data handling.

Measurement of Metabolites

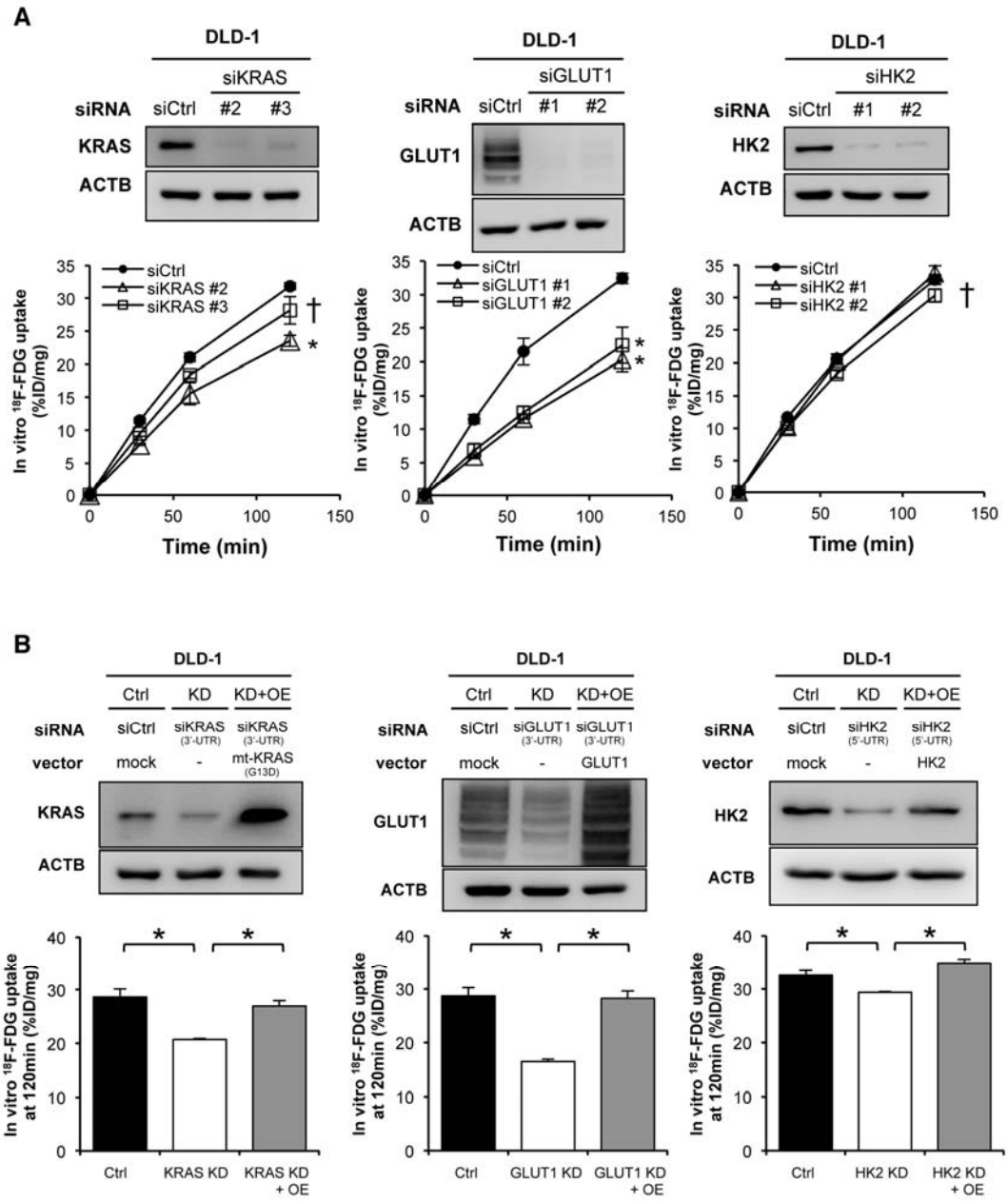
A dish of cultured cells (10^6 cells/sample) was used for the extraction of intracellular metabolites. The medium was aspirated from the dish, and the cells were washed twice by 5% mannitol solution. The cells were then treated with 800 μL of methanol and left at rest for 30 s to inactivate enzymes. Next, the cell extract was treated with 550 μL of water containing internal standards (H3304-1002; Human Metabolome Technologies, Inc.) and left at rest for another 30 s. The extract was obtained and centrifuged at 2,300*g* and 4°C for 5 min, and then 800 μL of the upper aqueous layer were centrifugally filtered through a Millipore 5-kDa-cutoff filter at 9,100*g* and 4°C for 240 min. The filtrate was centrifugally concentrated and resuspended in 50 μL of water for capillary electrophoresis mass spectrometry analysis. Metabolome measurements were performed through a facility service at Human Metabolome Technology Inc.

Statistical Analysis

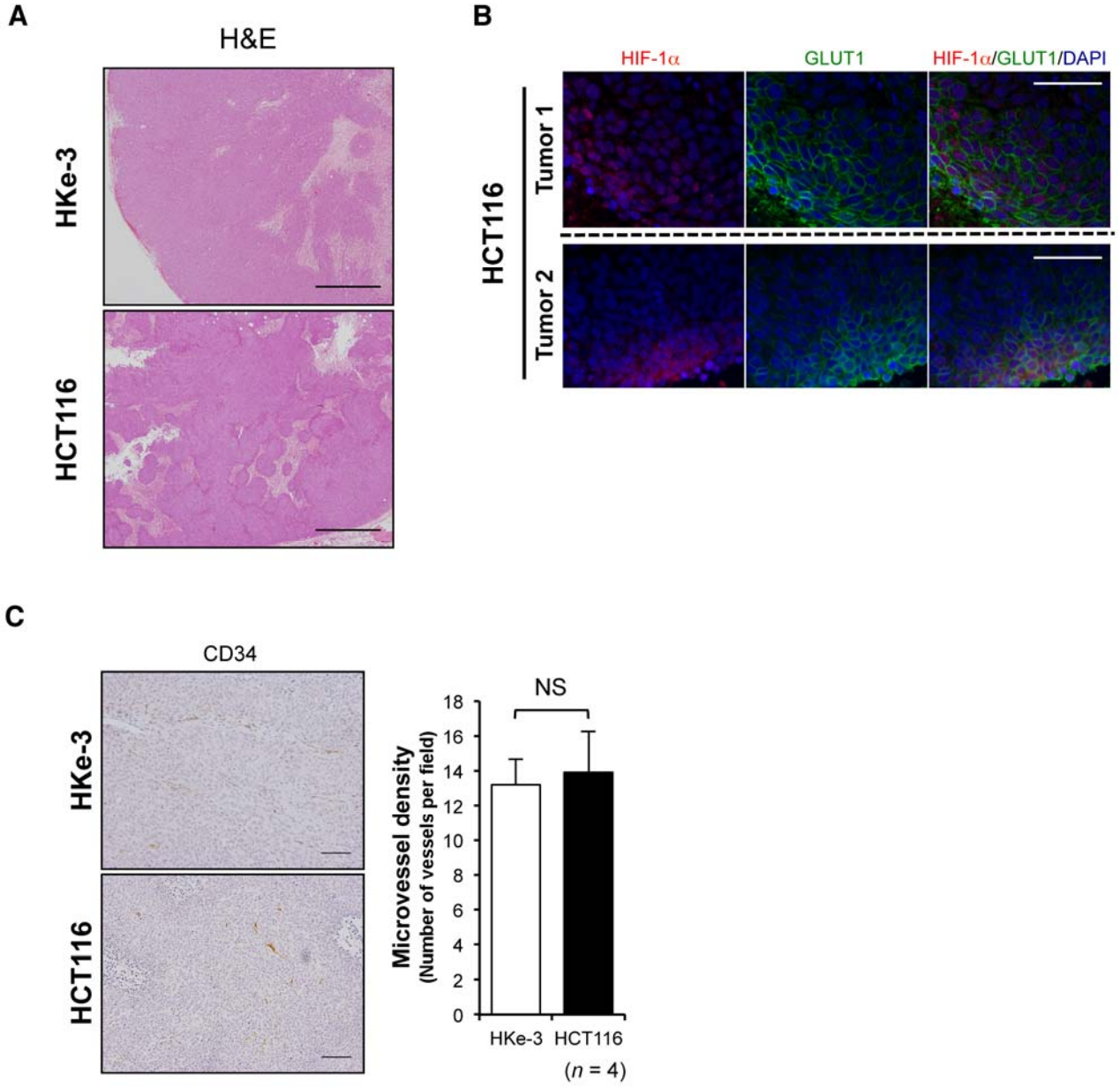
All values were expressed as mean ± SD. The statistical significance of differences was determined by the Student *t* test, χ^2 test, or Mann–Whitney *U* test. All analyses were 2-sided, and differences with a *P* value of less than 0.05 were considered significant. Statistical analyses were conducted with JMP software (version 9; SAS Institute, Inc.).



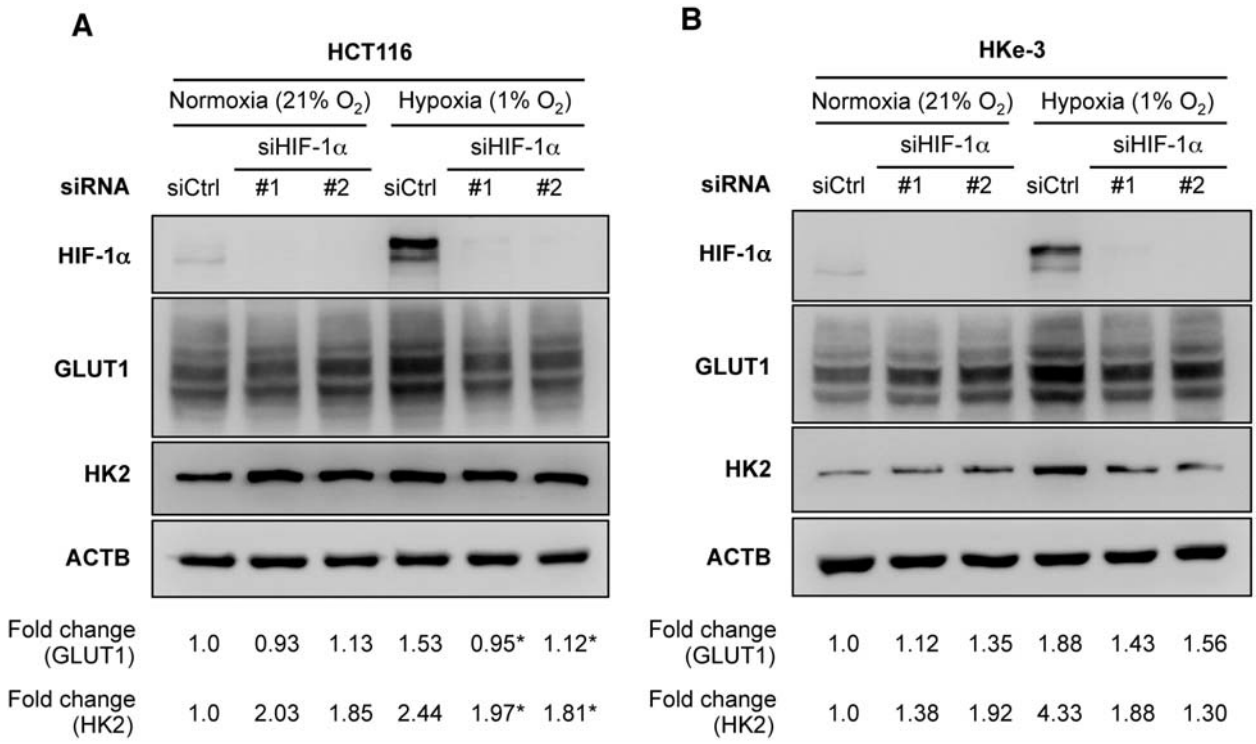
SUPPLEMENTAL FIGURE 1. GLUT1 and HK2 expression in CRC cells treated with signal inhibitors. Western blot analyses of HCT116 and DLD-1 cells treated with dimethyl sulfoxide (DMSO), 1 μM BKM120 (PI3K inhibitor), and 20 nM rapamycin (mammalian-target-of-rapamycin inhibitor) for 48 h (A); HKe-3 and DKO-4 cells treated with DMSO, 20 μM U0126 (MEK inhibitor), and 50 μM LY294002 (PI3K inhibitor) for 48 h (B, left); and HKe-3 and DKO-4 cells treated with DMSO, 1 μM BKM120 (PI3K inhibitor), and 20 nM rapamycin (mTOR inhibitor) for 48 h (B, right). Protein levels were normalized to β actin, and densitometry values are expressed as fold change compared with DMSO-treated cells.



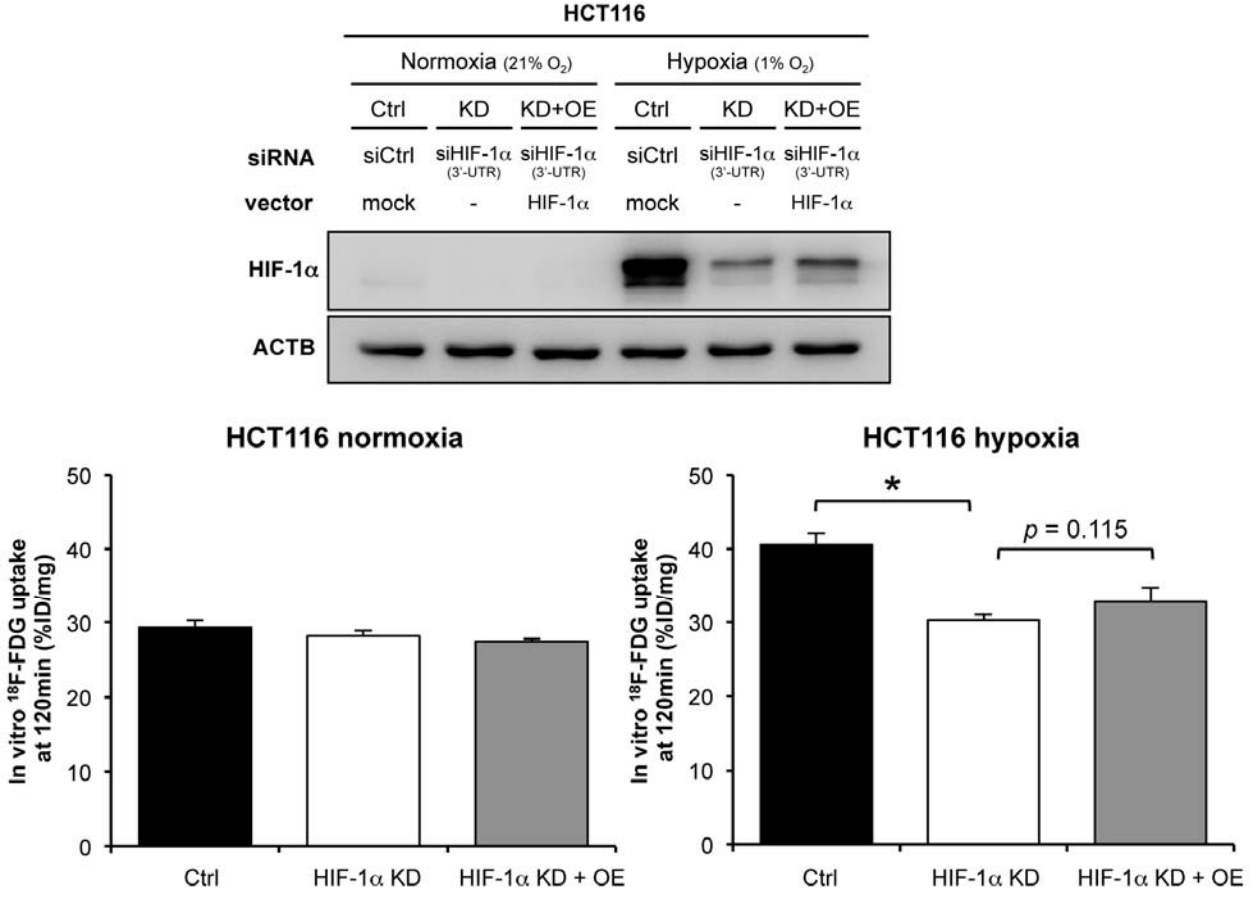
SUPPLEMENTAL FIGURE 2. In vitro ^{18}F-FDG accumulation in DLD-1 cells. (A) DLD-1 cells were treated with 2 independent siRNA constructs (#1 or #2 and #3) targeting *KRAS, GLUT1,* and *HK2,* respectively, and negative control (Ctrl) siRNA. (B) Mutant-*KRAS, GLUT1,* and *HK2* expression plasmid vectors were transiently cotransfected with siRNAs targeting 3′ or 5′ untranslated region of respective genes into DLD-1 cells. Forty-eight hours after transfection, expression levels of each protein were confirmed with Western blotting (A and B, upper panels). In vitro ^{18}F-FDG accumulations are presented as mean ± SD of triplicate measurements (A and B, lower panels). **P* < 0.01. †*P* < 0.05. Ctrl = control; KD = knockdown; OE = overexpression.



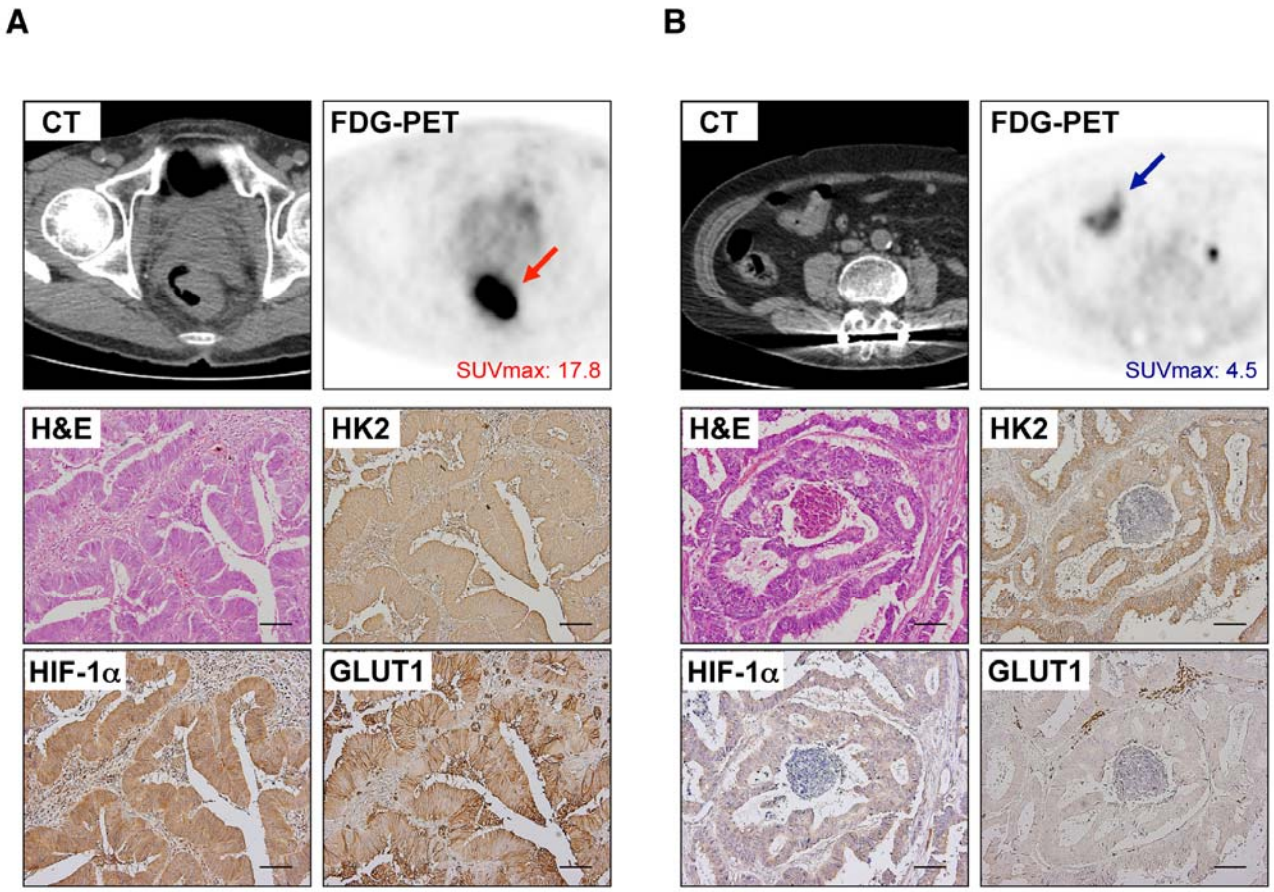
SUPPLEMENTAL FIGURE 3. Histologic examination of xenograft tumors. (A) Hematoxylin and eosin (H&E) staining of HKe-3 and HCT116 tumor sections. (B) Double immunofluorescence staining for HIF-1α and GLUT1 of HCT116 tumors. Scale bar = 50 μm. Immunohistochemistry for CD34 is shown in left panels, and quantification of microvessel density in xenograft tumors is shown in right panels. Microvessel densities are presented as mean ± SD of counting number in 6 random fields (magnification, ×200). Scale bar = 100 μm.



SUPPLEMENTAL FIGURE 4. Expression of HIF-1α, GLUT1, and HK2 protein under normoxic and hypoxic conditions. Western blot analyses show protein expression of HIF-1α, GLUT1, and HK2 in HCT116 (A) and HKe-3 (B) that were treated with 2 independent siRNA constructs targeting *HIF-1α* (si*HIF-1α* #1 and si*HIF-1α* #2) and negative control (Ctrl) siRNA under normoxic and hypoxic cultures. GLUT1 and HK2 protein levels were normalized to β actin, and densitometry values are expressed as fold change compared with siCtrl in normoxia. **P* < 0.01 vs. siCtrl in hypoxia.



SUPPLEMENTAL FIGURE 5. In vitro ^{18}F-FDG accumulation in HCT116 cells after knockdown or overexpression of *HIF-1α*. *HIF-1α* expression plasmid vector was transiently cotransfected with siRNAs targeting 3′ untranslated region of *HIF-1α* into HCT116 cells. Forty-eight hours after transfection, expression levels of HIF-1α protein under normoxic and hypoxic cultures (for 12 h) were confirmed with Western blotting (upper panels). In vitro ^{18}F-FDG accumulation at 120 min are presented as mean ± SD of triplicate measurements (lower panels, **P* < 0.01). %ID = percentage injected dose; Ctrl = control; KD = knockdown; OE = overexpression.



SUPPLEMENTAL FIGURE 6. Representative cases of human CRC samples. Images of CT and ^{18}F-FDG PET scans of stage I rectal cancer with mutated *KRAS* (A) and stage II transverse colon cancer with wild-type *KRAS* (B) are shown. Hematoxylin and eosin (H&E) and immunohistochemical staining for HIF-1α, GLUT1, and HK2 of primary tumors of respective patients are also shown. Scale bar = 100 μm.

SUPPLEMENTAL TABLE 1: Metabolomic Analysis of CRC Cell Lines With or Without *KRAS* Mutation

Compound name	Pathway label	Concentration (pmol/10^6 cells)			
		***KRAS* MT**	***KRAS* WT**	***KRAS* MT**	***KRAS* WT**
		HCT116	HKe-3	DLD-1	DKO-4
Glucose 6-phosphate	G6P	212	161	291	294
Fructose 6-phosphate	F6P	41	24	72	77
Fructose 1,6-diphosphate	F1,6P	2,370	864	5,677	7,242
Fructose 1-phosphate	D-F1P	74	N.D.	220	261
Dihydroxyacetone phosphate	DHAP	899	535	1,134	2,027
Glyceraldehyde 3-phosphate	G3P	347	262	614	246
2,3-diphosphoglyceric acid	DPG	65	35	56	62
3-phosphoglyceric acid	3-PG	145	117	156	93
Phosphoenolpyruvic acid	PEP	36	16	21	2.6
Pyruvic acid	Pyruvic acid	203	209	263	234
Lactic acid	Lactic acid	11,566	13,296	14,120	9,730
6-phosphogluconic acid	6-PG	58	98	114	106
Ribulose 5-phosphate	Ru5P	50	37	68	75
Ribose 5-phosphate	R5P	43	24	79	85
Xylulose 5-phosphate	X5P	63	N.D.	191	123
Sedoheptulose 7-phosphate	S7P	N.D.	N.D.	N.D.	N.D.
Erythrose 4-phosphate	E4P	N.D.	N.D.	N.D.	N.D.
Citric acid	Citric acid	697	1,460	1,082	1,582
cis-aconitic acid	*cis*-aconitic acid	4.7	12	11	18
Isocitric acid	Isocitric acid	N.D.	N.D.	17	29
2-oxoglutaric acid	2-OG	578	416	680	598
Succinic acid	Succinic acid	314	319	297	445
Fumaric acid	Fumaric acid	367	593	749	431
Malic acid	Malic acid	1,917	2,610	2,749	2,366
Acetyl CoA	AcCoA	6.5	2.7	10	4.8

MT = mutated; WT = wild-type.