Supplemental Information

Metformin targets central carbon metabolism and reveals mitochondrial requirements in human cancers

Xiaojing Liu, Iris L. Romero, Lacey M. Litchfield, Ernst Lengyel, and Jason W. Locasale

Supplemental Figures

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Patient#	Treatment	Age	BMI	FIGO Stage	Recurrence	Decease	Other treatment	Diabetes
1	Metformin	62	35	FIGO IIIC	No	No	Atorvastatin (Caduet, Lipitor)	Yes
2	Metformin	61	33	FIGO IIIC	Yes	No	None	Yes
3	Metformin	64	29	FIGO IIIC	Yes	No	None	Yes
4	Metformin	60	25	FIGO IIIC	Yes	Yes	Lovastatin (Mevacor, Altocor, Altoprev, Advicor)	Yes
5	Metformin	54	38	FIGO IV	Yes	No	Atorvastatin (Caduet, Lipitor)	Yes
6	Metformin	43	37	FIGO IIIC	Yes	No	Atorvastatin (Caduet, Lipitor)	Yes
7	Metformin	71	49	FIGO IIIC	No	No	None	Yes
8	Metformin	72	41	FIGO IIIC	Yes	Yes	None	Yes
9	Metformin	67	24	FIGO IV	Yes	Yes	Pravastatin (Pravachol, Pravigard, Selektine)	Yes
10	Metformin	64	34	FIGO IIIC	Yes	Yes	Rosuvastatin (Crestor)	Yes
11	No	65	20	FIGO IIIC	Yes	Yes	None	No
12	No	66	24	FIGO IIIC	Yes	No	Simvastatin (Vytorin, Simcor, Zocor, Juvisync)	No
13	No	66	20	FIGO IIIC	No	No	None	No
14	No	54	28	FIGO IIIC	Yes	No	Simvastatin (Vytorin, Simcor, Zocor, Juvisync)	No
15	No	66	32	FIGO IIIC	Yes	Yes	None	No
16	No	68	28	FIGO IIIC	Yes	No	None	No
17	No	69	20		Yes	No	None	No
18	No	62	24	FIGO IIIC	Yes	Yes	None	No
19	No	66	25	FIGO IV	Yes	Yes	None	No
20	No	66	25		Yes	Yes	Pravastatin (Pravachol, Pravigard, Selektine)	No

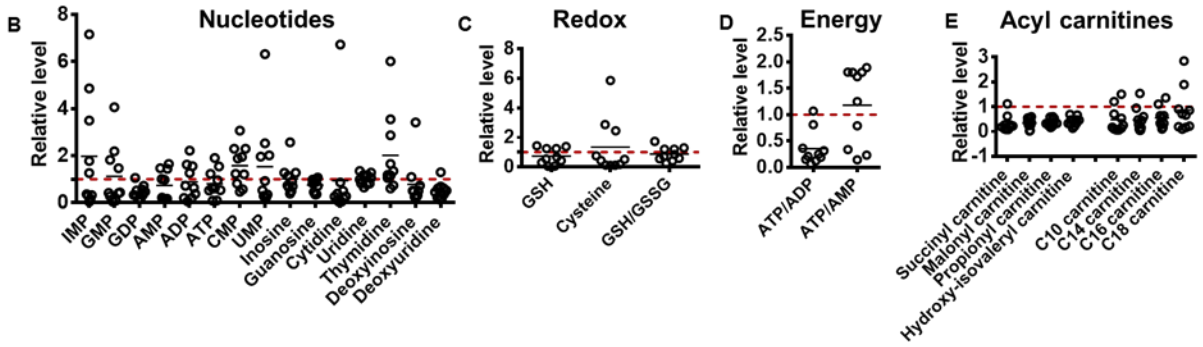

Figure S1. Related to Figure 1. Ovarian cancer patient information. **A.** Clinical information of the patients considered. **B to E**. Representative metabolite levels in patients receiving metformin treatment. Relative level was calculated by dividing MS intensity of each metabolite by corresponding MS intensity in samples without metformin treatment.

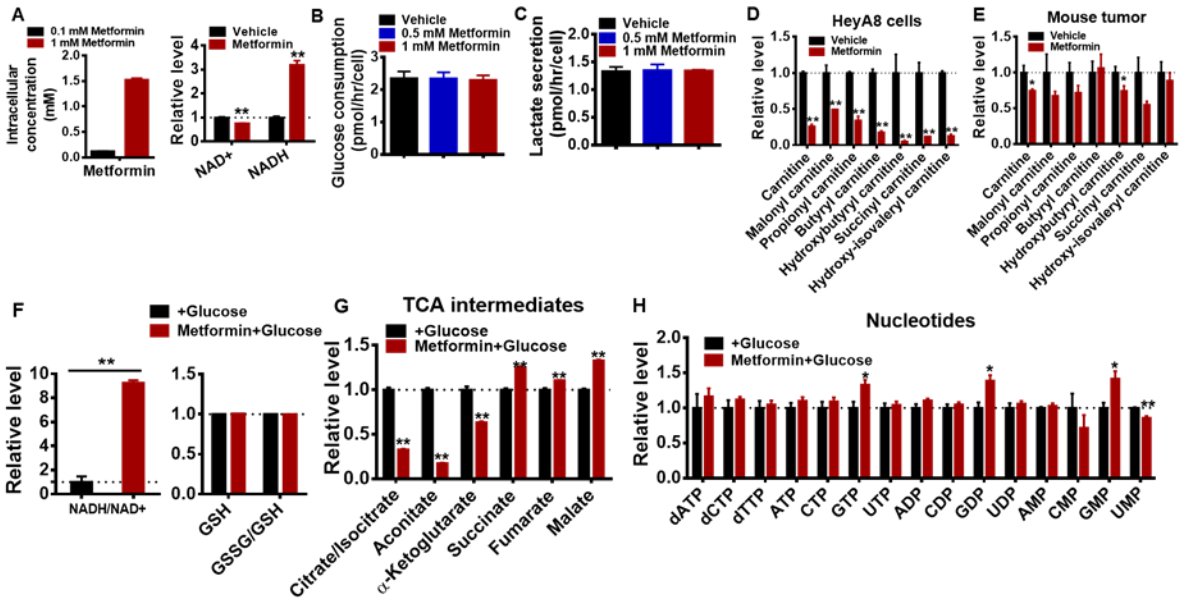

Figure S2. Related to Figure 4. Intracellular metformin concentration, glucose consumption, lactate secretion rate, and NAD+/NADH levels in ovarian cancer cells with or without metformin treatment. **A.** Intracellular metformin concentration, NAD+ and NADH in ovarian cancer cells cultured in low glucose medium with or without metformin. **B.** Glucose consumption rate upon 6 hours of vehicle, 0.5 or 1 mM metformin treatment. **C.** Lactate secretion rate in the first 6 hours of vehicle, 0.5 or 1 mM metformin treatment. **D.** Relative levels of short acyl chain carnitine in HeyA8 cells with or without metformin treatment. **E.** Relative levels of short acyl chain carnitine in HeyA8 cells with or without metformin treatment. **F to H.** Representative metabolite levels in HeyA8 cells treated in the presence of sufficient glucose (11 mM) and with or without metformin (1 mM). Relative levels were calculated by dividing MS intensity of each metabolite by the corresponding MS intensity in samples without metformin treatment. Error bars were obtained from SEM of n=3 independent measurements. * $p<0.05$, ** $p<0.01$

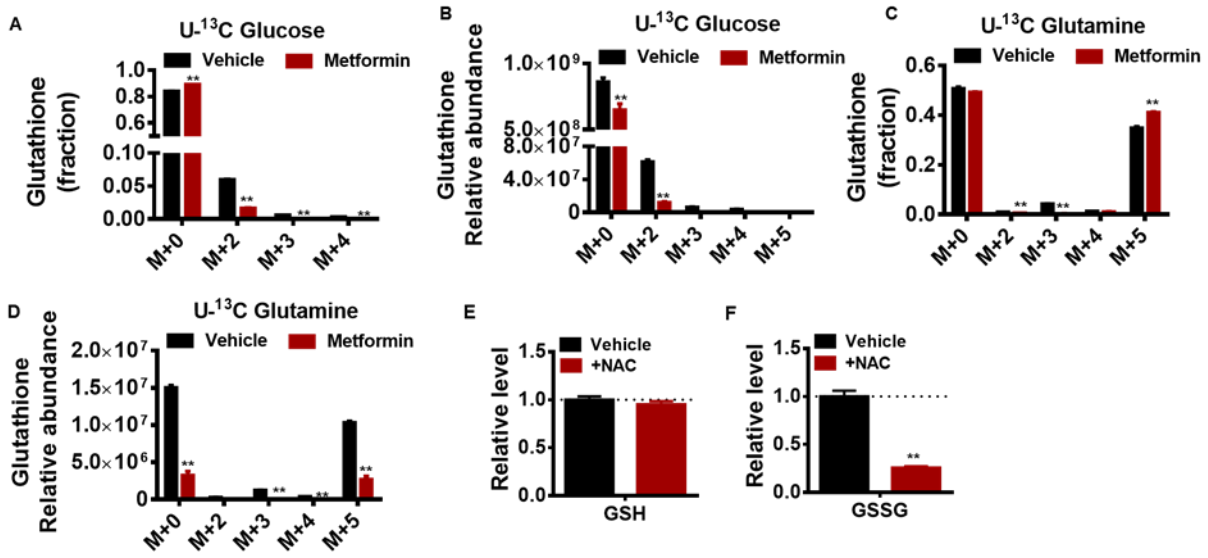

Figure S3. Related to Figure 5. Glutathione biosynthesis in the presence of metformin. **A**. ^{13}C isotopologue distribution of glutathione from cells treated with U-^{13}C labelled glucose. **B.** Relative abundance of ^{13}C isotopologues of glutathione in the presence of U-^{13}C glucose **C**. Same as A, except that the ^{13}C tracer is U-^{13}C glutamine. **D**. Relative abundance of ^{13}C isotopologues of glutathione in the presence of U-^{13}C glutamine. **E.** GSH levels in cells incubated in the absence or presence of NAC (1mM) for 24 hours. F. GSH levels in HEYA8 cells incubated in the absence or presence of NAC (1mM) for 24 hours. Error bars were obtained from SEM of n=3 independent measurements. * $p<0.05$, ** $p<0.01$

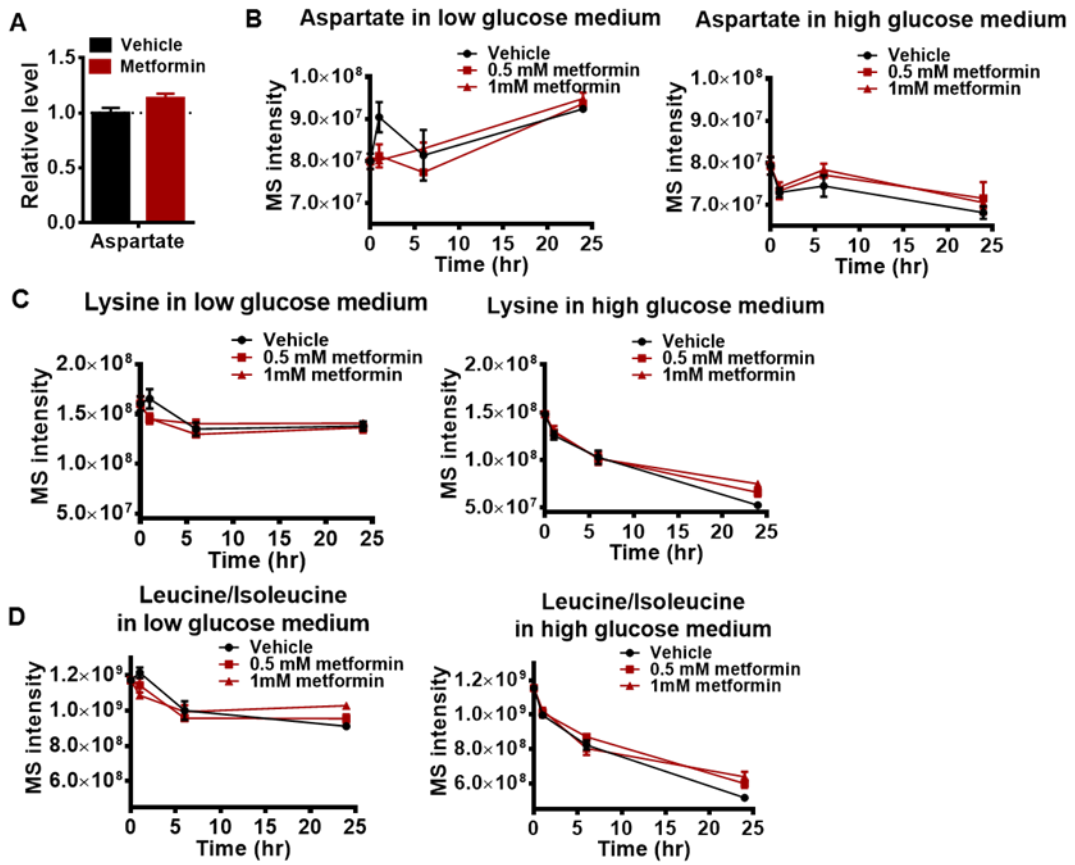

Figure S4. Related to Figure 5. Amino acid excretion and uptake kinetics after addition of metformin. A. Intracellular aspartate levels in the absence and presence of metformin. **B.** Aspartate levels in low and high glucose cell culture medium. **C.** Lysine levels in low (1 mM) and high (11 mM) glucose cell culture medium. **D.** Leucine/Isoleucine levels in low and high glucose medium. Y axis is MS intensity at time 0, 1, 6 and 24 hours. Error bars were obtained from SEM of n=3 independent measurements.* $p<0.05$, ** $p<0.01$

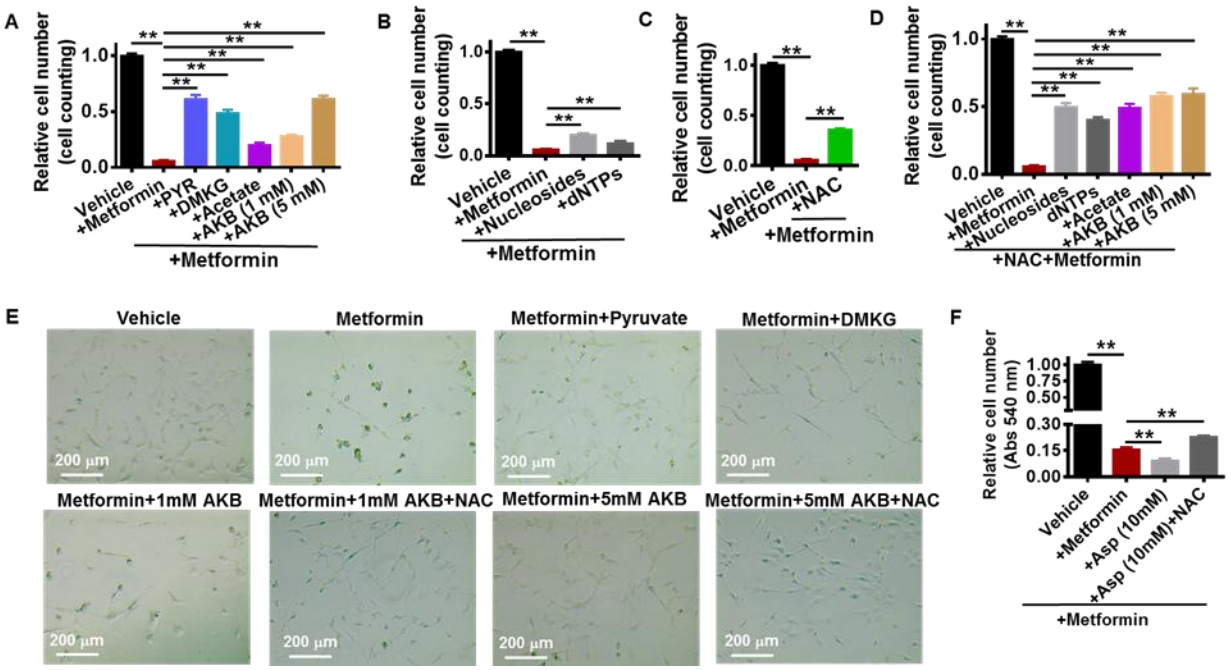

Figure S5. Related to Figure 6. Relative cell numbers under different treatments. A to D. HeyA8 cells were cultured in similar medium conditions as in figure 6, and the annotations of all medium conditions are the same as in figure 6. After 48 hours of treatment, all cells were counted and normalized to the cell number in Vehicle condition. **E.** Pictures of HeyA8 cells treated with or without metformin and in the absence or presence of rescue nutrients for 48 hours. Cell photos were taken with a Leica DMIL microscope (×10). **F.** Cell viability for metformin-treated HeyA8 cells with the indicated treatments. Error bars were obtained from SEM of n=3 independent measurements. * $p<0.05$, ** $p<0.01$

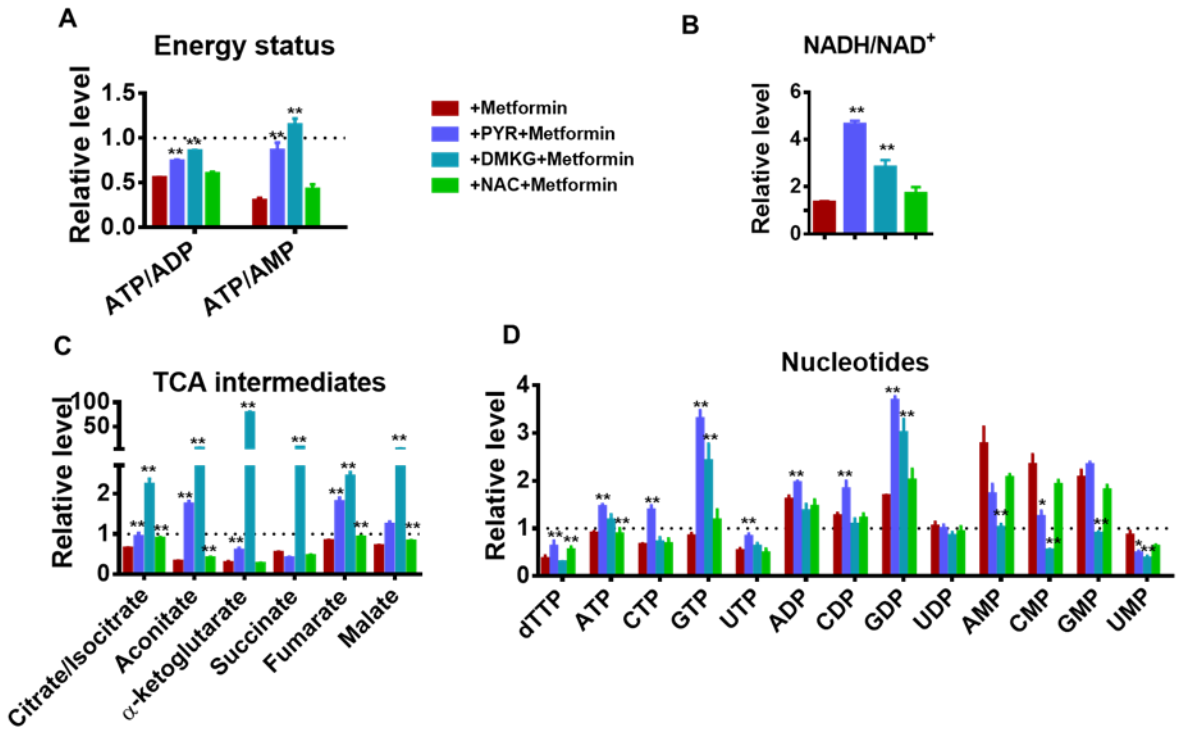

Figure S6. Related to Figure 6 and 7. Metabolite levels during sensitivity and resistance to metformin treatment. A. ATP/ADP and ATP/AMP ratios in different cell culture conditions. **B**. NADH/NAD$^+$ ratio in cells with different cell culture conditions. **C**. TCA intermediates in different cell culture conditions. **D**. Nucleotides levels in different cell culture conditions. The fold change is calculated by diving the MS intensity values of metabolites in different treatment conditions by those in control medium (Vehicle denotes low glucose medium in the absence of metformin). Error bars were obtained from SEM of n=3 independent measurements. * $p<0.05$, ** $p<0.01$

Supplemental Experimental Procedures

Reagents

RPMI 1640 medium was purchased from Cellgro. Fetal Bovine Serum (FBS), penicillin and streptomycin were purchased from Hyclone Laboratories. Dialyzed FBS was obtained from Life Technologies. Optima ammonium acetate, ammonium hydroxide, Optima LC-MS grade, acetonitrile, methanol and water were purchased from Fisher Scientific. U-^{13}C glucose, U-^{13}C glutamine, and U-^{13}C sodium pyruvate were obtained from Cambridge Isotope Laboratories. Metformin hydrochloride, [$^{2}H_6$]-metformin hydrochloride, sodium pyruvate, sodium citrate, sodium acetate, and N-acetyl cysteine (NAC) were purchased from Santa Cruz biotechnology. Dimethyl α-ketoglutarate, α-ketobutyrate, U-^{13}C Palmitate and DMSO were purchased from Sigma. Nucleosides (100X) were obtained from Millipore. MTT and dNTP mix were purchased from Life Technologies. Inorganic salts and amino acids were purchased from BDH Chemicals.

Cell culture medium effect on metformin response

For MTT assay, HeyA8 cells were plated in 96 well plate at a density of 3000 cells per well, and after incubation overnight, the full growth media were replaced with 100 μl low nutrient medium or cell death rescue medium in the absence or presence of 1.5 mM metformin. For cell counting, HeyA8 cells were plated in a 24 well plate at a density of 12000 cells per well, and medium volume was 400 μl per well. For cell apoptosis assays, HeyA8 cells were cultured in a 6-well plate at a density of 48000 cells per well. After overnight incubation, the media were replaced with 2 ml of treatment medium. The treatment medium contains complete or low nutrient RPMI 1640, 10% dialyzed FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were grown at 37 °C with 5% CO_2. High glucose medium (HGLC) contains 11 mM glucose, while low glucose medium (LGLC) contains 1mM glucose. Low glutamine medium contains 0.1 mM glutamine. Serine, glycine and methionine (Ser/Gly/Met) deprivation medium contains no supplemented serine/glycine, and 0.01 mM methionine. Arginine and asparagine (Arg/Asn) deprivation medium contains 0.057 mM arginine and 0.019 mM asparagine. Hydrogen peroxide (H_2O_2) was used at final concentration of 30 μM together with metformin treatment. Cell death rescue medium was prepared from LGLC medium with the addition of pyruvate (5 mM), citrate (5 mM), acetate (5mM), dimethyl α-ketoglutarate (5mM), α-ketobutyrate (1 and 5 mM), nucleosides(1X), dNTP mix (0.1 mM), aspartate (10 mM) or NAC (1 mM). Cells were further incubated for 48 hours before MTT assay. All cell culture assays were done in triplicate.

Cell viability assays

Cell viability was assessed using a tetrazolium-based MTT assay and cell counting. To perform the MTT assay, at the experimental endpoint, all media were replaced with the same 100 μl phenol free RPMI medium to minimize the potential nutrient deprivation effect on MTT readout. Cell plates were then placed in cell incubator for 1 hour before the addition of 10 μl of MTT stock solution (5 mg/ml PBS, filtered with 0.22 μm filter). 96 well plates were then wrapped with aluminum foil and placed in cell incubator for additional 4 hours. MTT solution was gently removed and 100 μl of DMSO was added to dissolve the crystal on the bottom of each well. The absorbance at 540 nm was used to reflect cell number in each well. For cell counting, HeyA8 cells were cultured in 24 well plate, and in the end of treatment, cells were first harvested by trypsinization, and then counted using Moxi™ Z Mini Automated Cell Counter (ORFLO Technologies).

HPLC method

Ultimate 3000 UHPLC (Dionex) is coupled to Q Exactive-Mass spectrometer (QE-MS, Thermo Scientific) for metabolite separation and detection. For additional polar metabolite analysis, a hydrophilic interaction chromatography method (HILIC) with an Xbridge amide column (100 x 2.1 mm i.d., 3.5 μm; Waters) is used for compound separation at room temperature. The mobile phase and gradient information were described previously (Liu et al., 2014).

Mass spectrometry

The Q Exactive mass spectrometer (QE-MS) is equipped with a HESI probe, and the relevant parameters are as listed: heater temperature, 120 °C; sheath gas, 30; auxiliary gas, 10; sweep gas, 3; spray voltage, 3.6 kV for positive mode and 2.5 kV for negative mode. Capillary temperature was set at 320 °C, and S-lens was 55. A full scan range was set at 60 to 900 (*m/z*) when coupled with the HILIC method, or 300 to 1000 (*m/z*) when low abundance metabolites were measured. The resolution was set at 70 000 (at *m/z* 200). The maximum injection time (max IT) was 200 ms. Automated gain control (AGC) was targeted at 3×10^6 ions. For targeted MS/MS analysis, the isolation width of the precursor ion was set at 1.5 (*m/z*), high energy collision dissociation (HCD) was 35%, and max IT is 100 ms. The resolution and AGC were 35 000 and 200 000, respectively.

Metabolite extraction in tumor, serum and cell culture medium

Both patient and mouse tumors were extracted the same way as described previously (Liu et al., 2015). Briefly, the tumor sample was first homogenized in liquid nitrogen and then 5 to 10 mg was weighed in a new Eppendorf tube. Ice cold extraction solvent (250 μl) was added to tissue sample, and a pellet mixer was used to further break down the tissue chunk and form an even suspension. After incubation on ice for an additional 10 min, the tissue extract was centrifuged with the speed of 20 000 g at 4 °C for 10 min. 20 μl of serum or medium was added to 80 μl ice cold water in Eppendorf tube on ice, followed by the addition of 400 μl ice cold methanol and the tube was vortexed rigorously for 1 min before centrifuged at 20 000 g for 10 min at 4 °C. For absolute quantitation of metformin and glucose, [$^2H_6$]-metformin and U-^{13}C labeled standards were added to extraction solvent before centrifugation. The ^{13}C labeled standards were prepared from *E.coli* growing in U-^{13}C glucose medium, as described previously (Liu et al., 2015). The supernatant was transferred to a new Eppendorf tube and dried in vacuum concentrator. The dry pellets were reconstituted into 30 μl sample solvent (water:methanol:acetonitrile, 2:1:1, v/v) and further analyzed by LC-QE-MS.

Metabolite extraction in cell culture

HeyA8 cells were seeded at a density of 150,000 cells per well in 6 well plates. After overnight incubation in full growth medium, the old medium was removed and cells were washed with 1 ml PBS before the addition of 2 ml of treatment medium. To measure glucose and lactate in the media, at 1, 3 and 6 hours after replacing the fresh medium, 10 μl of media were collected and added with 10 μl water before the addition of 80 μl of ice cold methanol. After vigorous vortexing and centrifugation, the supernatant was directly injected to LC-HRMS. The calibration curves were generated by spiking in known concentrations of lactate to cell free RPMI medium. For intracellular metabolite analysis, after an incubation for 20 hours, metabolites were extracted as described in a previous study (Liu et al., 2014), except that for intracellular metformin measurement, cells were washed with 2 ml ice cold saline solution three times before metabolite extraction, and [$^2H_6$]-metformin was added as internal standard to quantify intracellular metformin. HeyA8 cell number and volume were measured using Moxi™ Z Mini Automated Cell Counter (ORFLO Technologies) after scraping cells into RPMI 1640 medium. For ^{13}C tracing, the cells were first incubated in full or low nutrient medium in the absence of presence of metformin for 16 hours, and then the old media were replaced with fresh media containing 1 mM U-^{13}C glucose, 2mM U-^{13}C glutamine, 100 μM U-^{13}C palmitate, or 5 mM U-^{13}C pyruvate. Metabolites were extracted either after 6 hours incubation (for U-^{13}C glucose, U-^{13}C glutamine and U-^{13}C pyruvate) or 24 hours incubation (for U-^{13}C palmitate).

Apoptotic cell death assay

Phosphatidylserine on the external surface and propidium iodide uptake were measured using the Annexin V Alexa Fluor® 488 & Propidium Iodide (PI) Dead Cell Apoptosis Kit (ThermoFisher Scientific) according to the manufacturer's recommendations. HeyA8 cells were cultured in 6-well plate for 40 hours in glucose limited medium in the presence and absence of 1.5 mM metformin. Before staining, old media were removed, and cells were washed with PBS. Therefore, the results presented are from adherent cells at the end of the treatment. We also thank Lifeng Yuan, Xiao-Fan Wang, Peter Winter, and Kris Wood (Duke University Department of Pharmacology and Cancer Biology) for assistance in the assay protocol and Dr. Michael Cook (Duke Cancer Institute) for help with the flow cytometry assay.

Data analysis and metabolic network construction

LC-MS peak extraction and integration were performed using commercially available software Sieve 2.0 (Thermo Scientific). The integrated peak intensity was used to calculate the fold changes between different treatments and ^{13}C enrichment. For human and mouse data, a quantile normalization was applied to the integrated peak intensity values. A natural abundance correction was performed as previously described (Yuan et al., 2008). The contribution of carbon was calculated using the method described in a previous study (Schoors et al., 2015).

Supplemental References

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