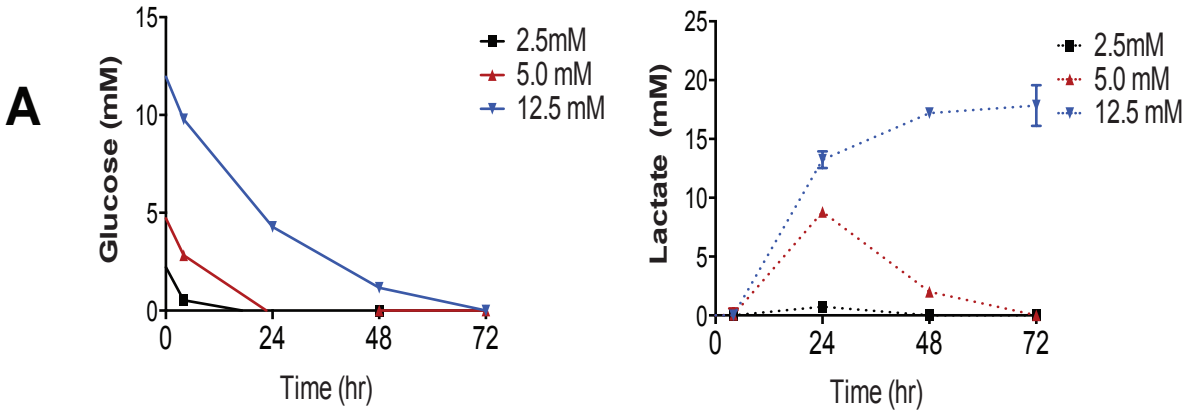
Supplemental Information

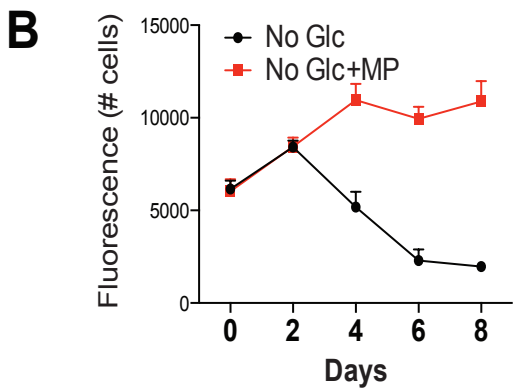
ERRα Regulated Lactate Metabolism Contributes to Resistance to Targeted Therapies in Breast Cancer

Sunghee Park, Ching-yi Chang, Rachid Safi, Xiaojing Liu, Robert Baldi, Jeff S. Jasper, Grace R. Anderson, Tingyu Liu, Jeffrey C. Rathmell, Mark W. Dewhirst, Kris C. Wood, Jason W. Locasale, and Donald P. McDonnell

Supplemental Figures

Figure S1, related to Figure 1

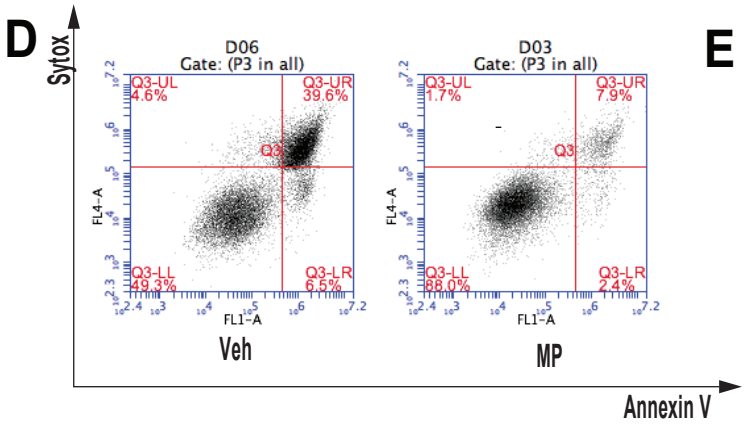




C

		Veh	Lac
TNBC	HCC1937	19	174
	MDAMB436	30	109
	Hs578T	69	101
	HCC1806	56	73
	HCC1143	89	121
	MDAMB435s	76	124
	MDAMB231	60	130
	DU4475	3	20
	MDAMB453	100	144
	SUM149PT	40	97
Luminal	MCF7	58	161
	T47D	78	178
	MDAMB361	58	155
Her2	SKBR3	40	120
	HCC1954	28	64
	HCC1569	67	143

% cell survival



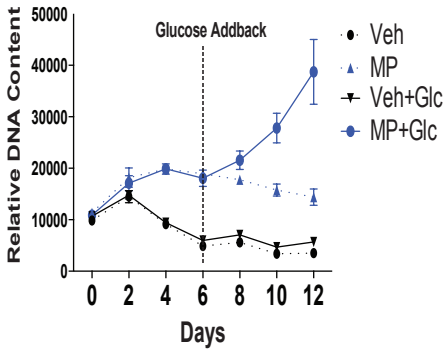


Figure S2, related to Figure 2

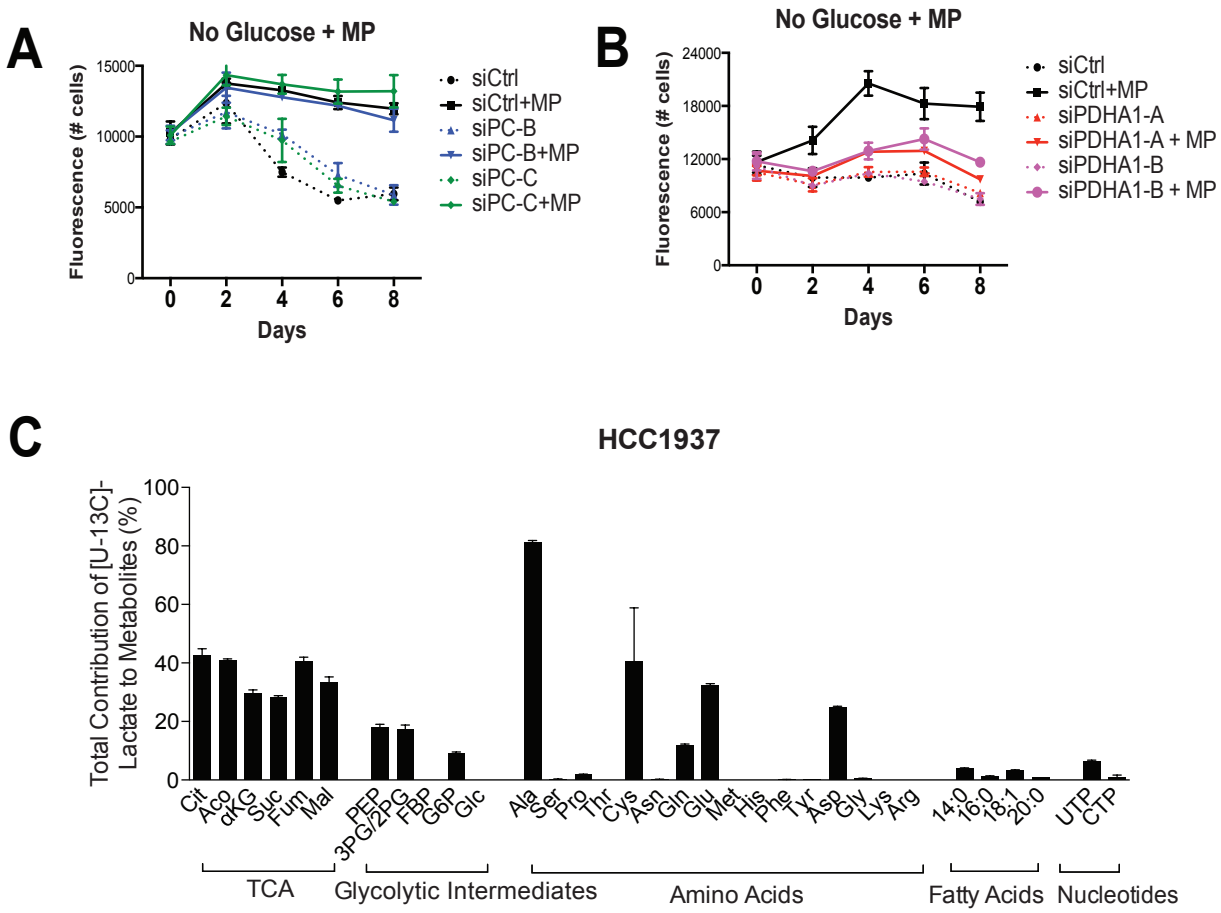


Figure S3, related to Figure 3

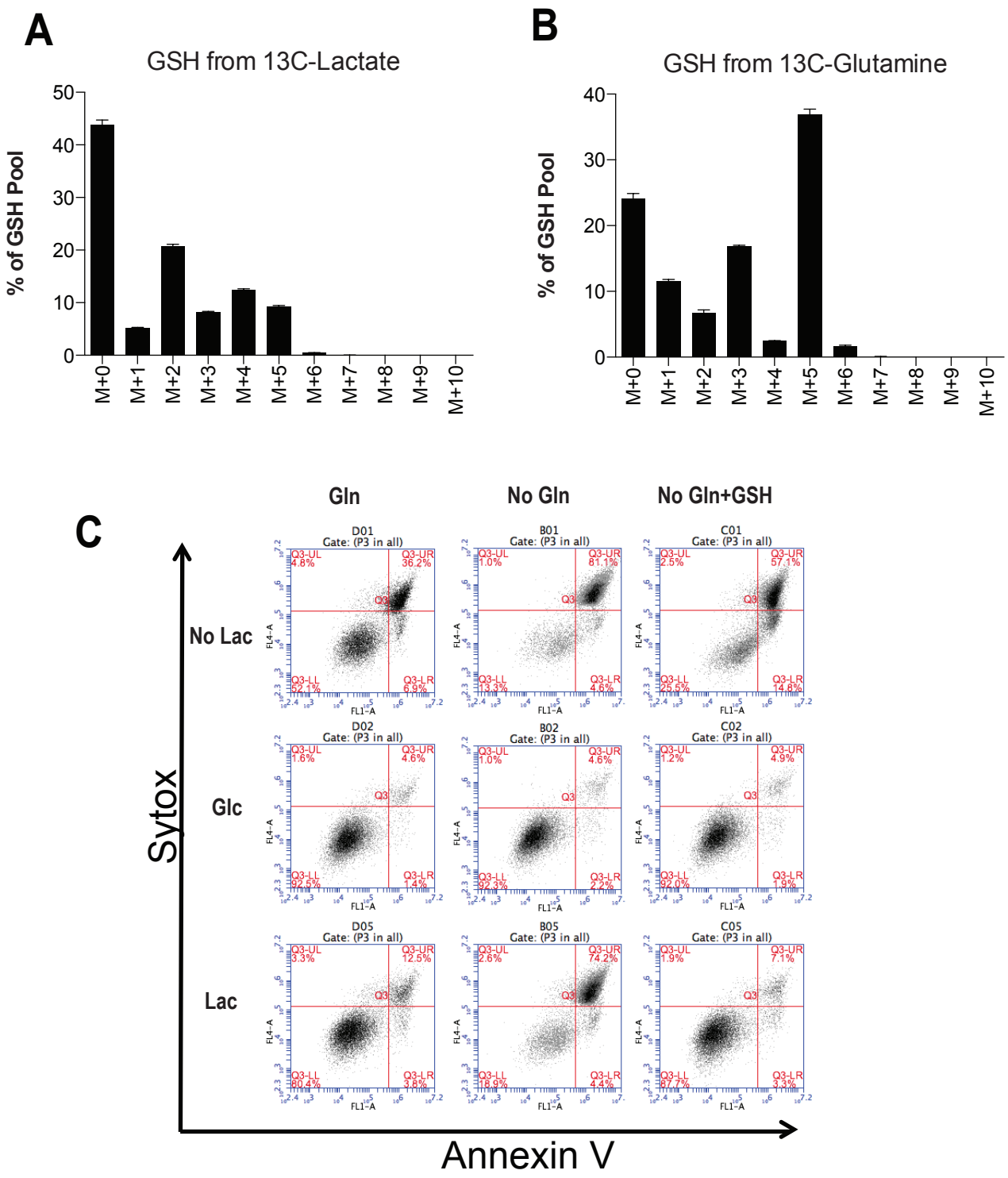


Figure S4, related to Figure 4

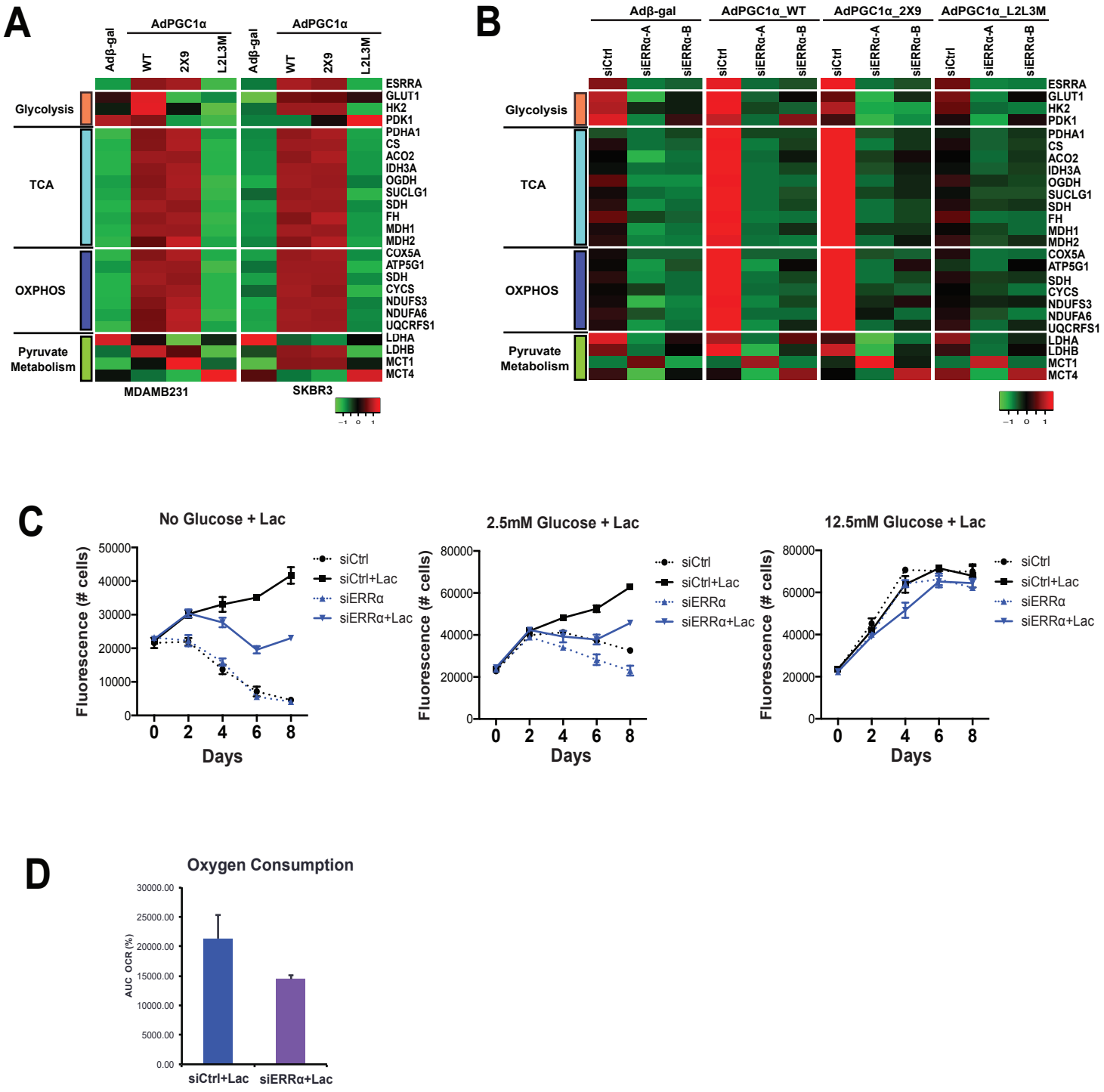


Figure S4, related to Figure 4

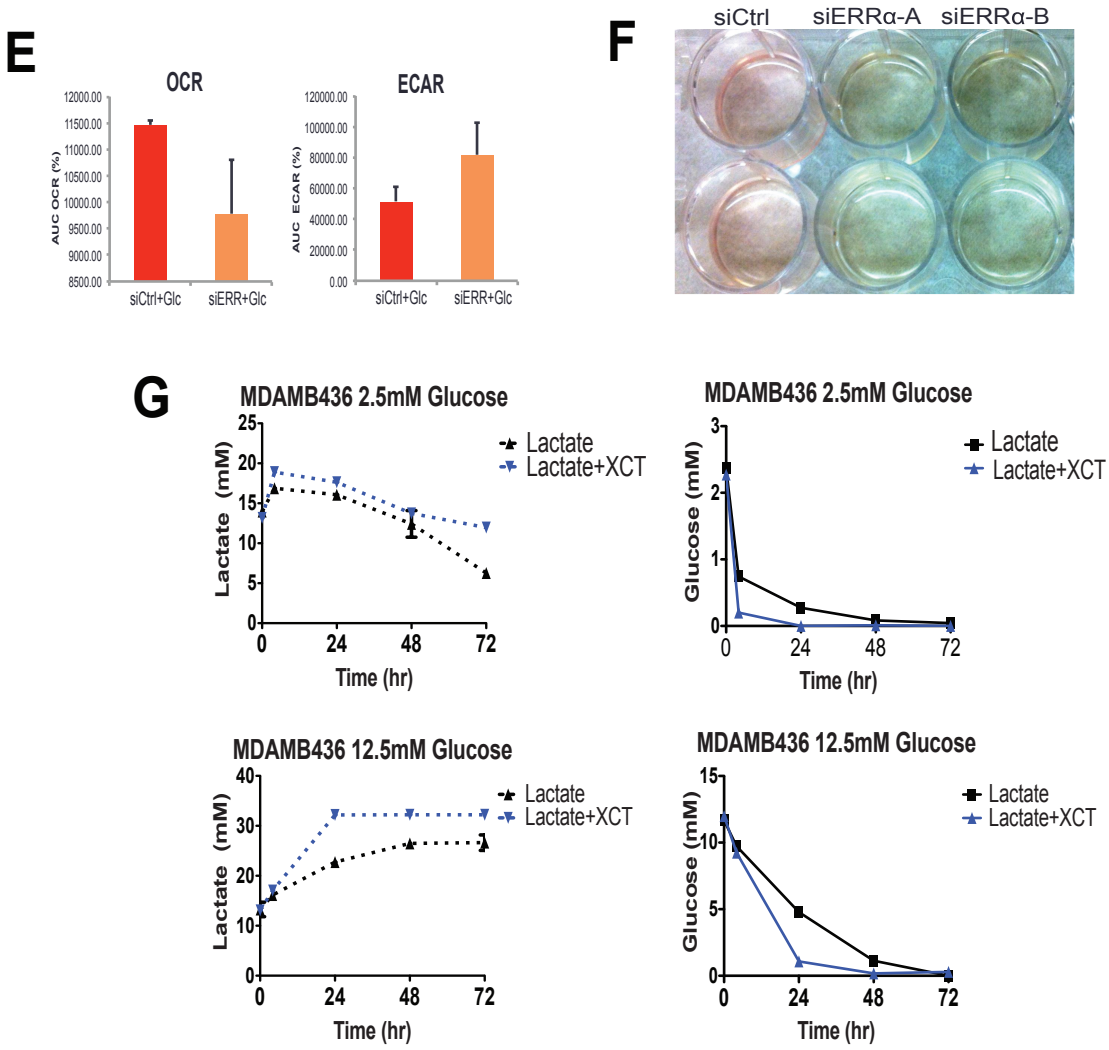


Figure S5, related to Figure 5

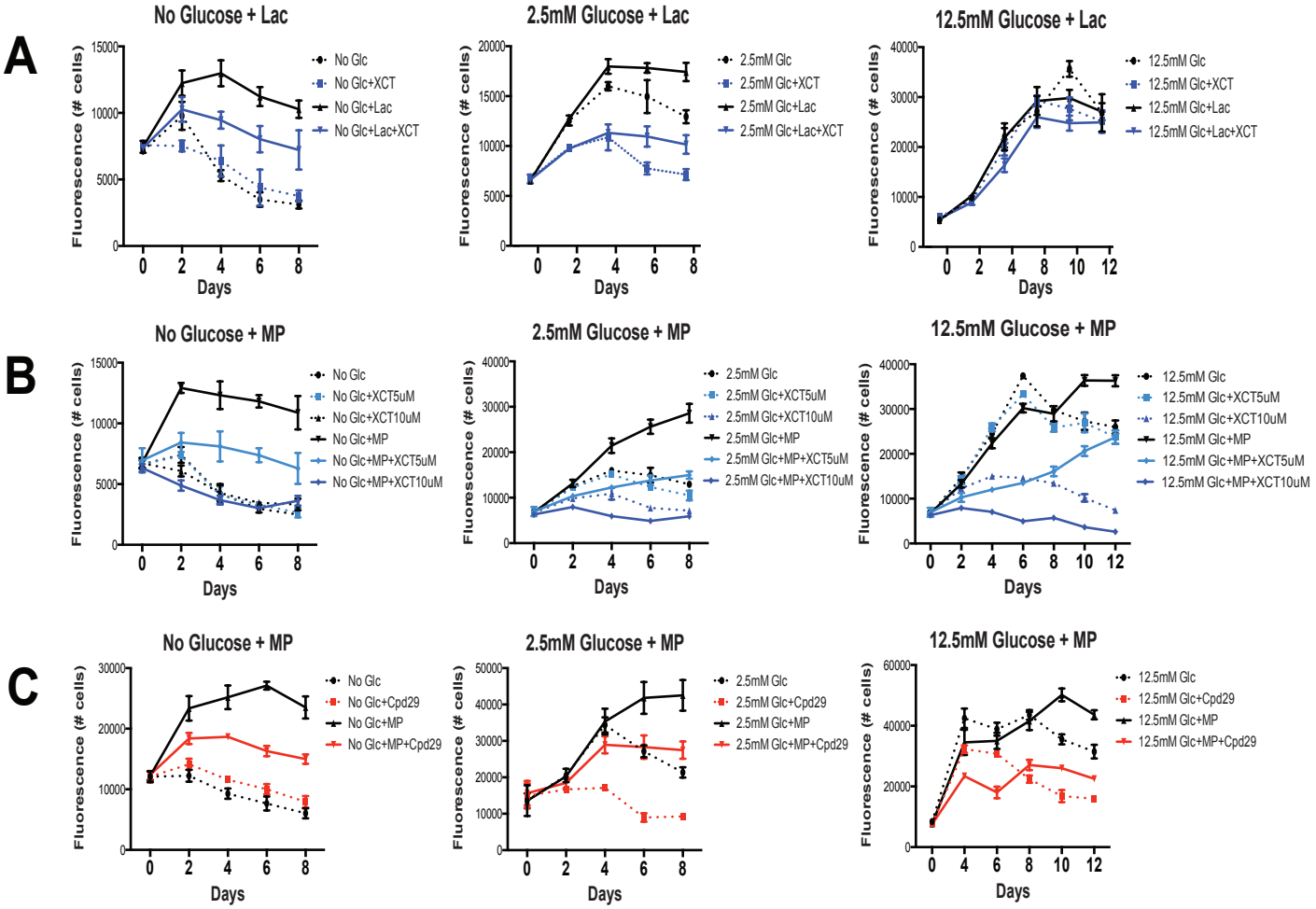


Figure S5, related to Figure 5

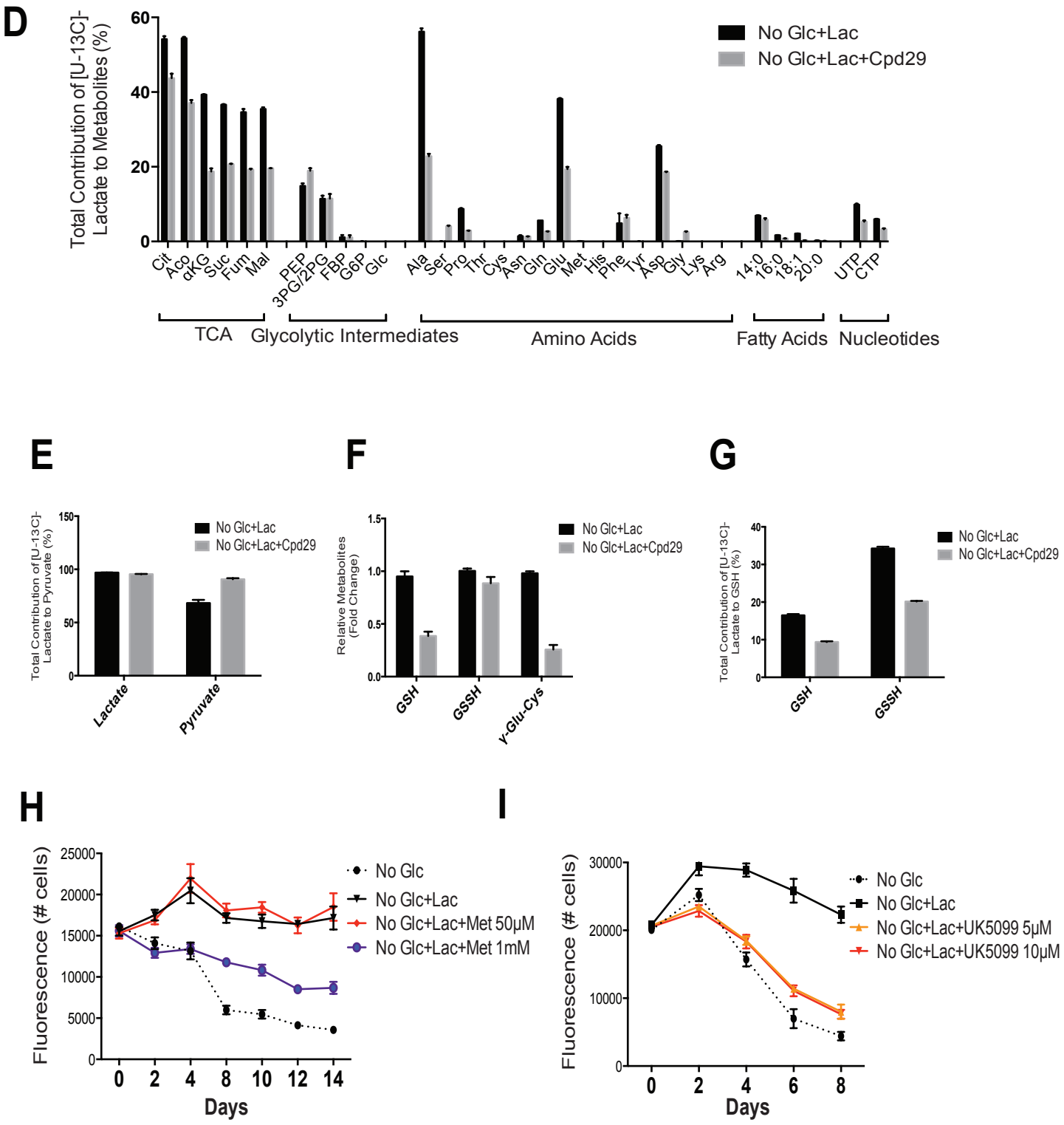


Figure S6, related to Figure 6

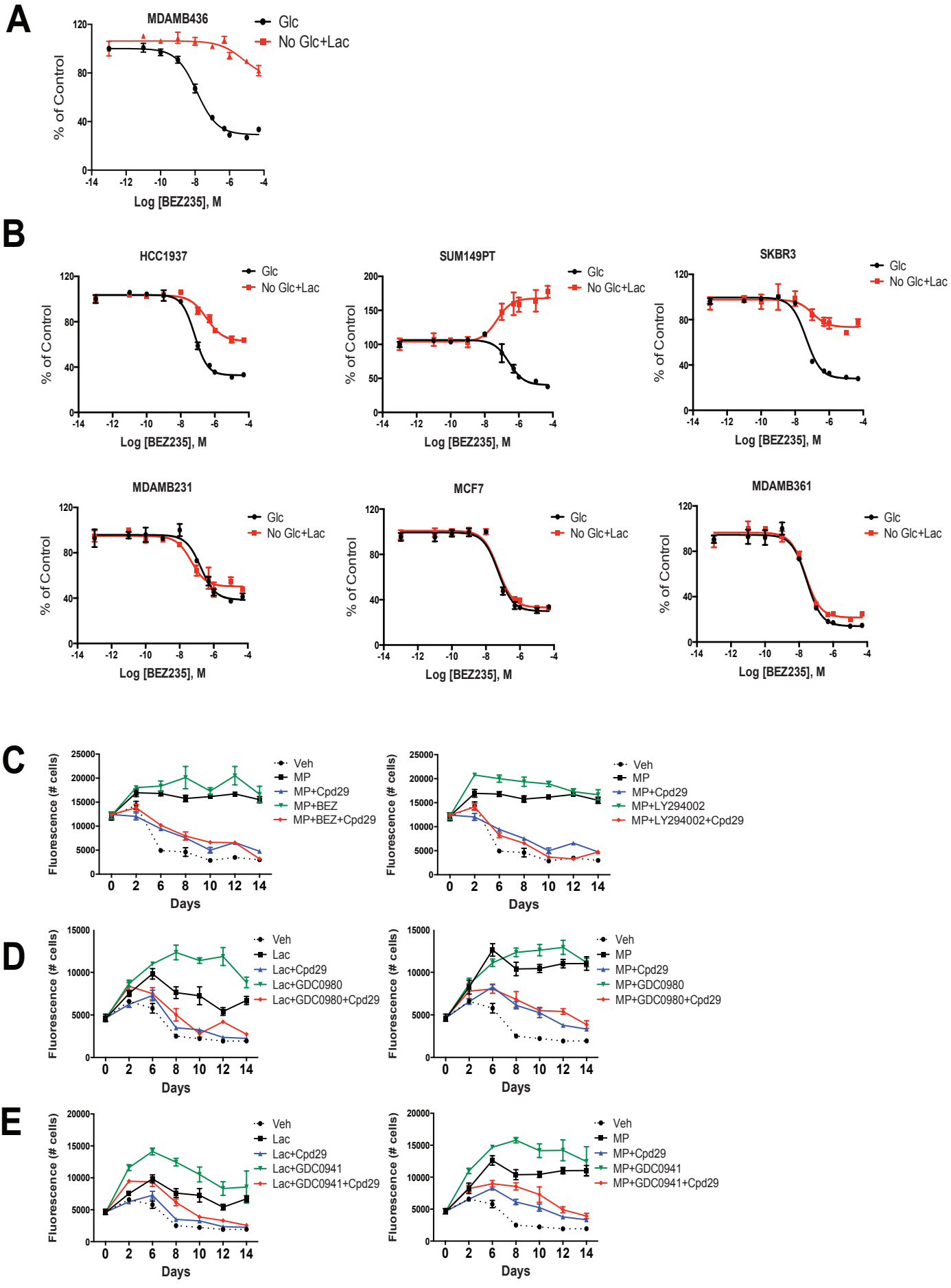
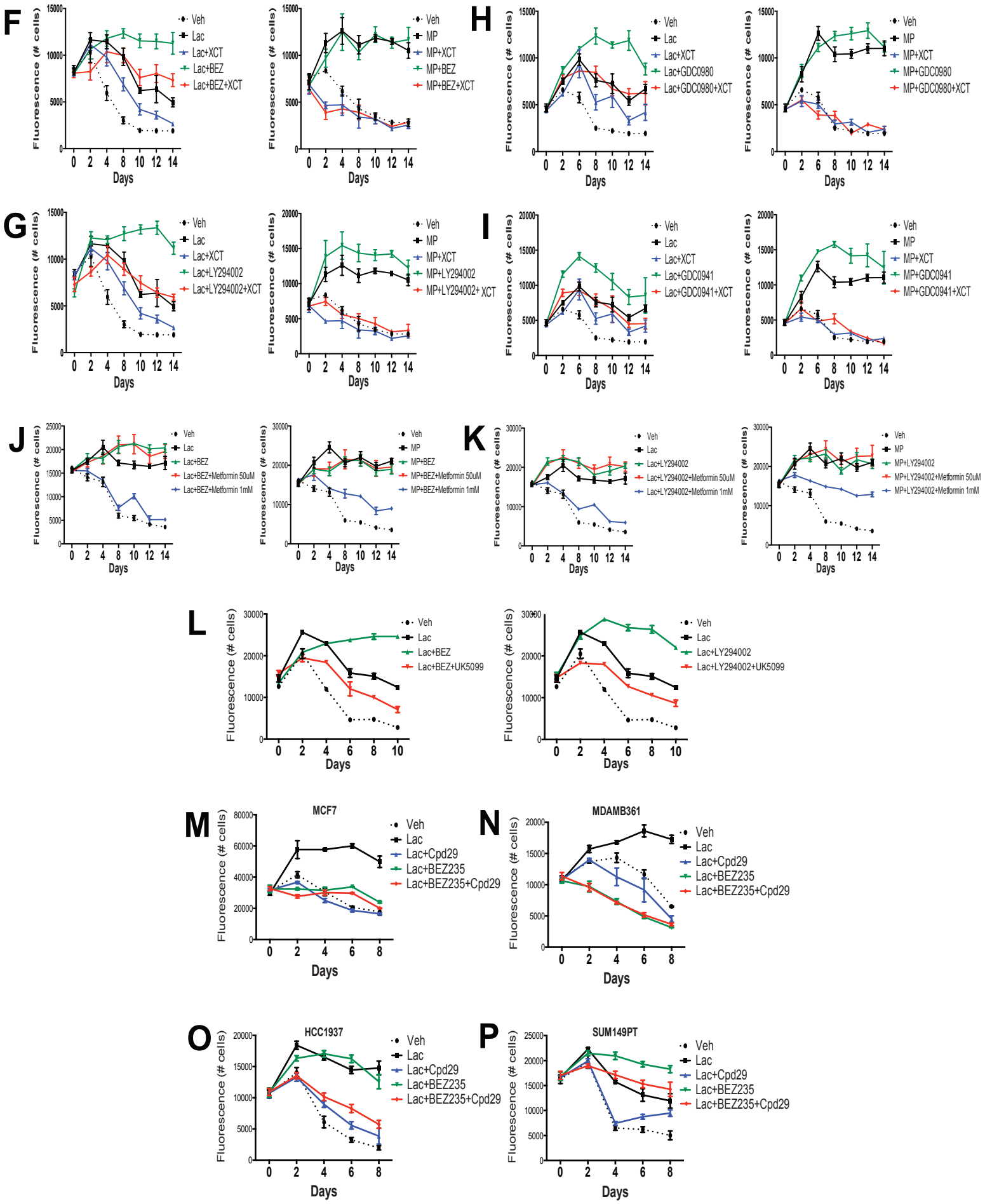


Figure S6, related to Figure 6



Supplemental Figure Legends

Figure S1. Confluent MDA436 cells received the indicated media and 100µL of media were taken at the times shown. (A) Glucose (solid lines) and lactate (dotted lines) concentrations in the culture media were measured by enzyme assays. (B) MDA436 cells were seeded in 96-well plates and 48 hrs later were changed to the media as indicated. Cells were then harvested on days as shown and cell numbers were determined by staining with the DNA dye Hoechst 33258. (C) The percent cell survival was calculated at day 6 by normalizing to the number of cells on day 0. (D) MDA436 cells were incubated with vehicle (Veh) or 10mM methyl pyruvate (MP) in glucose-free RPMI media for 24 hrs and cells were then harvested, stained with Alexa Fluor 488-AnnexinV+Sytox Red dye, and analyzed by flow cytometry. (E) MDA436 cells were cultured in glucose-free media supplemented with or without 10mM of methyl pyruvate (MP) for 6 days and vehicle (Veh; media) or glucose (Glc; final12.5mM) was added back to culture media at day 6 and harvested on days as indicated. Cell numbers were determined by staining with the DNA dye Hoechst 33258.

Figure S2. (A-B) MDA436 cells were transfected with control (siCtrl), PC (siPC-B and siPC-C), or PDHA-1 (siPDHA1-A and siPDHA1-B) siRNAs for 48hrs. Cells were then switched to glucose-free media with or without MP and harvested on days as indicated. Cell numbers were determined by staining with the DNA dye Hoechst 33258. PDHA1: pyruvate dehydrogenase alpha 1, PC: pyruvate carboxylase. (C) Relative metabolite abundance in HCC1937 cells grown in glucose-free media supplemented with 10mM of

[U-^{13}C]-lactate for 24 hrs. Data are presented as a relative amount of ^{13}C-labeled metabolite pool. Error bars represent S.D. of three experimental replicates.

Figure S3. (A and B) Mass isotopomer distributions of the GSH in MDA436 cells grown in glucose-fee media containing 10mM of [U-^{13}C]-lactate or 2mM of [U-^{13}C]-glutamine for 24 hrs. Error bars represent S.D. of three experimental replicates. (C) MDA436 cells were incubated with glucose-free DMEM media without glutamine (No Gln), supplemented with 10mM GSH (No Gln+GSH) or 2mM glutamine (Gln) in the presence or absence of 12.5mM glucose (Glc) or 10mM lactate (Lac) for 24 hrs and cells were then harvested, stained with Alexa Fluor 488-AnnexinV+Sytox Red dye, and analyzed by flow cytometry. The populations of AnnexinV$^-$/Sytox$^-$, AnnexinV$^+$/Sytox$^-$, and AnnexinV$^+$/Sytox$^+$ correspond to live cells, early apoptotic cells, and late apoptotic cells, respectively.

Figure S4. (A) The heatmap was constructed using qRT-PCR data obtained from MDA-MB-231 and SKBR-3 cells infected with adenoviruses expressing β-gal (negative control), wild-type PGC-1α, ERRα selective PGC-1α (2X9), or the nuclear receptor-binding deficient PGC-1α (L2L3M) for 48 hrs. RNA was harvested and the expression of ERRα target genes was analyzed by qRT-PCR. (B) MDA-MB-231 cells were transfected with either control (siCtrl) or ERRα (siERRα-A and siERRα-B) siRNAs for 24 hrs, followed by infection of adenoviruses expressing β-gal (negative control), wild-type PGC-1α, ERRα selective PGC-1α (2X9), or the nuclear receptor-binding deficient PGC-1α (L2L3M) for 48 hrs. The qRT-PCR data were normalized to expression of 36B4. Heat maps were derived from qRT-PCR data using R software. (C) HCC1937 cells were

transfected with either control (siCtrl) or ERRα (siERRα) siRNA for 48 hrs and cells were then changed to the indicated experimental media with or without 10mM sodium lactate. Cells were harvested on days as shown and cell numbers were determined by staining with Hoechst 33258. (D) Oxygen consumption rate (OCR) was assessed following the knockdown of either control (siCtrl) or ERRα (siERRα) in glucose-free media supplemented with 10mM lactate. (E) Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured by Seahorse XF24 analyzer after the knockdown of either control (siCtrl) or ERRα (siERRα) in 12.5mM glucose containing RPMI media. (F) Representative picture of typical culture dishes transfected with either control (siCtrl) or ERRα (siERRα) siRNAs in 12.5mM glucose containing RPMI media. (G) Lactate (dotted lines) and glucose (solid lines) concentration in the supernatant of cells cultured in the indicated media with or without XCT (10μM) were determined by enzymatic assays.

Figure S5. (A-C) MDA436 cells were cultured in the indicated media for up to 12 days and cell numbers were determined by staining with the DNA dye Hoechst 33258. (D, E, and G) Relative metabolite abundance in MDA436 cells pre-treated with DMSO or 5μM Cpd29 for 40 hrs in 12.5mM glucose containing media and changed to glucose-fee media containing 10mM of [U-^{13}C]-lactate for 24 hrs. Data are presented as the percentage of ^{13}C-labeled metabolite in the total metabolite pool. Error bars represent S.D. (n=3). (F) Total metabolite pool of GSH and GSH precursor in cells received the same treatment as in D but with 10mM of non-labeled lactate. (H) MDA436 cells were cultured in the indicated media in the presence of metformin and UK5099 (I) and

harvested on days as shown and cell numbers were determined by staining with Hoechst 33258.

Figure S6. (A-B) Indicated breast cancer cells were treated with escalating doses of BEZ235, as indicated, for 96hr. Cell viability in each treatment group was calculated relative to cells treated with vehicle control (% of Control). (C-I) MDA436 cells were cultured in glucose-free media either with no supplement (Veh), with 10 mM lactate (Lac) or with 10mM methyl pyruvate (MP) as indicated. Cell grown in different media were further treated with the ERRα antagonists Cpd29 or XCT790, the PI3K/mTOR inhibitors BEZ235 or GDC0980, the PI3K inhibitor LY294002 or GDC0941, either as single agent or in combination for 14 days. (J-L) MDA436 cells were cultured in indicated media and treated with two different concentrations of metformin (50μM and 1mM) or UK5099 (10μM) in combination with either BEZ235 (BEZ) or LY294002 for 14 days. (M-P) Four different breast cancer cell lines cultured in glucose-free media containing 10mM lactate were treated with the ERRα antagonist Cpd29 or the PI3K/mTOR inhibitor BEZ235 (BEZ) alone or in combination for 14 days. Cell numbers were determined by staining with the DNA dye Hoechst 33258.

Supplemental Experimental Procedures

Chemicals

XCT790 and Compound29 (Cpd29) were synthesized by the Small Molecule Synthesis Facility at Duke University. BEZ235, GDC0980, and GDC0941 were purchased from Selleck Chemicals (Houston, TX), LY294002 from Alexis Biochemicals (Plymouth Meeting, PA), metformin from Sigma (St. Louis, MO). Sodium L-lactate, Methyl Pyruvate, and L-Glutathione reduced were purchased from Sigma (St. Louis, MO). CM-H2DCFDA was purchased from Molecular Probes (Invitrogen). Lactic Acid, Sodium Salt, L-[14C(U)] and Glutamine, L-[14C(U)] were purchased from PerkinElmer (Waltham, MA). Sodium L-Lactate (13C3) was purchased from Cambridge Isotope Laboratories (Andover, MA). Oligonucleotides were synthesized by IDT DNA (Coralville, IA) and siRNAs were purchased from Invitrogen (San Diego, CA).

Cell Culture

All cell lines except SUM149PT (Aird et al., 2012) were obtained from ATCC (Manassas, VA) and maintained in a 37°C incubator with 5% CO_2. MDA-MB-436, HCC1937, HCC1806, HCC1143, HCC1954, HCC1659, SKBR3, and DU4475 were maintained in RPMI (Invitrogen), MDA-MB-231, Hs578T, MDA-MB-435s, T47D, MDA-MB-361 cells in DMEM (Invitrogen), MDA-MB-453, MCF-7 cells in DMEM/F12 (Invitrogen), and SUM149TP cells in F12 (Invitrogen) supplemented with insulin/hydrocortisone. All media were supplemented with 8% fetal bovine serum (Sigma), 1mM sodium pyruvate and 0.1mM non-essential amino acids (Invitrogen). For

glucose deprivation experiments, RPMI no glucose (Invitrogen; 11879-020) + 10% dialyzed FBS (Sigma) or DMEM no glucose, no glutamine, no phenol red (Invitrogen; A14430-01) + 10% dialyzed FBS (Sigma) was used. 2mM L-glutamine was present in RPMI no glucose media.

Quantitative PCR

Total DNA-free RNA was isolated using the Aurum RNA kit (Bio-Rad Laboratories, Hercules, CA). Five hundred nanograms of total RNA were reverse transcribed and analyzed by real-time PCR as described previously (Chang et al., 2011).

Western Blot Analysis

Immunoblotting was performed as previously described (Park et al., 2012) using the following antibodies: ERRα (ab76228; Abcam), PGC-1α (sc-13067; Santa Cruz Biotechnology), β-actin (A2228; Sigma), PDH-E1α pSer293 (AP1062; Calbiochem), and PDH-E1α (459400; Invitrogen).

Adenoviral Transduction

Adenovirus expressing β-gal, PGC-1α, PGC-1α 2X9, or PGC-1α L2L3M were generated as described previously (Gaillard et al., 2006). MDA-MB-436, MDA-MB-231, and SKBR3 cells were infected at multiplicity of infection (MOI) of 50 for 48 hours.

ATP Assay

MDA-MB-436 (9,000 cells/well) were seeded in 96-well plates containing regular RPMI. Forty-eight hours later, the cell media were replaced to glucose-free RPMI (10%

dialyzed FBS and 2mM glutamine) supplemented with 10mM sodium lactate. Time zero was designated at the addition of 10mM exogenous lactate. Cells were then harvested 4, 8, 18, or 24 hrs. after the treatment. Total ATP content was determined over time using the PerkinElmer ATPlite luminescence Assay System Kit according to the manufacturer's protocol.

Metabolic Assays

Lactate and glutamine oxidation assays were performed as described previously (Wang et al., 2011). Briefly, MDA-MB-436 cells were plated in 10 cm plates using regular RPMI media and allowed to reach 90% confluency. At this time, cells were incubated with glucose-free RPMI (10% dialyzed FBS) supplemented with 10mM sodium lactate and 2mM glutamine for 24 hrs. Cells were then trypsinized and resuspended in glucose-free RPMI (10% dialyzed FBS) supplemented with unlabeled sodium lactate (10mM) or/and glutamine (2mM). 1mL of cells (1×10^6) were transferred into 7mL of glass vial (TS-13028, Thermo) containing a PCR tube with 50µL of 0.2M KOH. After adding 1µCi of L-[14C(U)]-lactate or 0.5µCi of L-[14C(U)]-glutamine, the vials were capped using a screw cap with rubber septum (TS-12713, Thermo). The assay was quenched 4hr later by injection of 200µL 0.2N HCl and the vials were kept at room temperature overnight to trap the $^{14}CO_2$. The 50µL KOH in the PCR tube was then transferred to scintillation vials containing 5mL of scintillation solution. The ^{14}C contents of the vials were then evaluated using a liquid scintillation counter (Beckman LS 6000 SC). A cell-free sample containing 1µCi of L-[14C(U)]-lactate or 0.5µCi of L-[14C(U)]-glutamine were included as negative control. OCR and ECAR were analyzed using the XF24 Extracellular Flux

Analyzer (Seahorse Bioscience). OCR and ECAR values were normalized to cell number.

Lactate and Glucose Measurements

MDA-MB-436 and SKBR3 cells were plated in 10 cm plates using regular RPMI media and allowed to reach 90% confluency. At this time, the media were replaced with glucose-free RPMI (10% dialyzed FBS) supplemented with 2.5, 5, and 12.5mM glucose. 100µL of media were then taken at 0, 24, 48, and 72 hrs. after the treatment for lactate and glucose measurements. Lactate and glucose concentrations were determined over time using the L-lactate Assay Kit or Glucose Assay Kit (Eton Bioscience, San Diego, CA) according to the manufacturer's protocol.

Apoptosis Assay

MDA-MB-436 cells were collected and double stained with Alexa Fluor 488 Annexin V and Sytox (Invitrogen) according to the manufacturer's instruction. Annexin V–positive cells were considered apoptotic, and their percentage of the total number of cells was calculated. Ten thousand events were collected for each sample using a BD Accuri C6 flow cytometer (BD), and data were analyzed using the CFlow Plus program software (BD).

ROS Generation Assay

Treated cells were stained with the oxidation-sensitive dye CM-H_2DCFDA (5µmol/L) for 60 minutes in PBS at 37°C, and the treatment was terminated by ice-cold PBS.

Reactive oxygen species (ROS) generation was determined by FACS as described (Safi et al., 2014).

LC-MS analysis

LC equipped with an Xbridge amide column (Waters) was coupled to Q Exactive mass spectrometer (Thermo Fisher Scientific) (Liu et al., 2014; Shestov et al., 2014). Cell extract were reconstituted into 30µL water:methanol:acetonitrile (2:1:1), and 3µL was injected to LC-MS. The peak area integration of every metabolite was done with manufacturer's software SIEVE (Thermo Fisher Scientific), and used to calculate isotopomer distribution. The natural abundance was corrected based on previously published method (Yuan et al., 2008). The contribution of carbon is calculated using the method described in previous study (Schoors et al., 2015)

Supplemental References

Aird, K. M., Allensworth, J. L., Batinic-Haberle, I., Lyerly, H. K., Dewhirst, M. W., and Devi, G. R. (2012). ErbB1/2 tyrosine kinase inhibitor mediates oxidative stress-induced apoptosis in inflammatory breast cancer cells. Breast cancer research and treatment *132*, 109-119.

Chang, C. Y., Kazmin, D., Jasper, J. S., Kunder, R., Zuercher, W. J., and McDonnell, D. P. (2011). The metabolic regulator ERRalpha, a downstream target of HER2/IGF-1R, as a therapeutic target in breast cancer. Cancer Cell *20*, 500-510.

Gaillard, S., Grasfeder, L. L., Haeffele, C. L., Lobenhofer, E. K., Chu, T. M., Wolfinger, R., Kazmin, D., Koves, T. R., Muoio, D. M., Chang, C. Y., and McDonnell, D. P. (2006). Receptor-selective coactivators as tools to define the biology of specific receptor-coactivator pairs. Mol Cell *24*, 797-803.

Liu, X., Ser, Z., and Locasale, J. W. (2014). Development and quantitative evaluation of a high-resolution metabolomics technology. Anal Chem *86*, 2175-2184.

Martz, C. A., Ottina, K. A., Singleton, K. R., Jasper, J. S., Wardell, S. E., Peraza-Penton, A., Anderson, G. R., Winter, P. S., Wang, T., Alley, H. M.*, et al.* (2014). Systematic identification of signaling pathways with potential to confer anticancer drug resistance. Sci Signal *7*, ra121.

Park, S., Yoon, S., Zhao, Y., Park, S. E., Liao, L., Xu, J., Lydon, J. P., DeMayo, F. J., O'Malley, B. W., Bagchi, M. K., and Katzenellenbogen, B. S. (2012). Uterine development and fertility are dependent on gene dosage of the nuclear receptor coregulator REA. Endocrinology *153*, 3982-3994.

Safi, R., Nelson, E. R., Chitneni, S. K., Franz, K. J., George, D. J., Zalutsky, M. R., and McDonnell, D. P. (2014). Copper signaling axis as a target for prostate cancer therapeutics. Cancer Res *74*, 5819-5831.

Schoors, S., Bruning, U., Missiaen, R., Queiroz, K. C., Borgers, G., Elia, I., Zecchin, A., Cantelmo, A. R., Christen, S., Goveia, J.*, et al.* (2015). Fatty acid carbon is essential for dNTP synthesis in endothelial cells. Nature *520*, 192-197.

Shestov, A. A., Liu, X. J., Ser, Z., Cluntun, A. A., Hung, Y. P., Huang, L., Kim, D., Le, A., Yellen, G., Albeck, J. G., and Locasale, J. W. (2014). Quantitative determinants of aerobic glycolysis identify flux through the enzyme GAPDH as a limiting step. Elife *3*.

Wang, R. N., Dillon, C. P., Shi, L. Z., Milasta, S., Carter, R., Finkelstein, D., McCormick, L. L., Fitzgerald, P., Chi, H. B., Munger, J., and Green, D. R. (2011). The Transcription Factor Myc Controls Metabolic Reprogramming upon T Lymphocyte Activation. Immunity *35*, 871-882.

Yuan, J., Bennett, B. D., and Rabinowitz, J. D. (2008). Kinetic flux profiling for quantitation of cellular metabolic fluxes. Nature protocols *3*, 1328-1340.