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Supplemental Information

Amino Acids Rather than Glucose Account for the Majority of Cell Mass in Proliferating Mammalian Cells

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Inventory of Supplementary Materials

Supplementary Figures

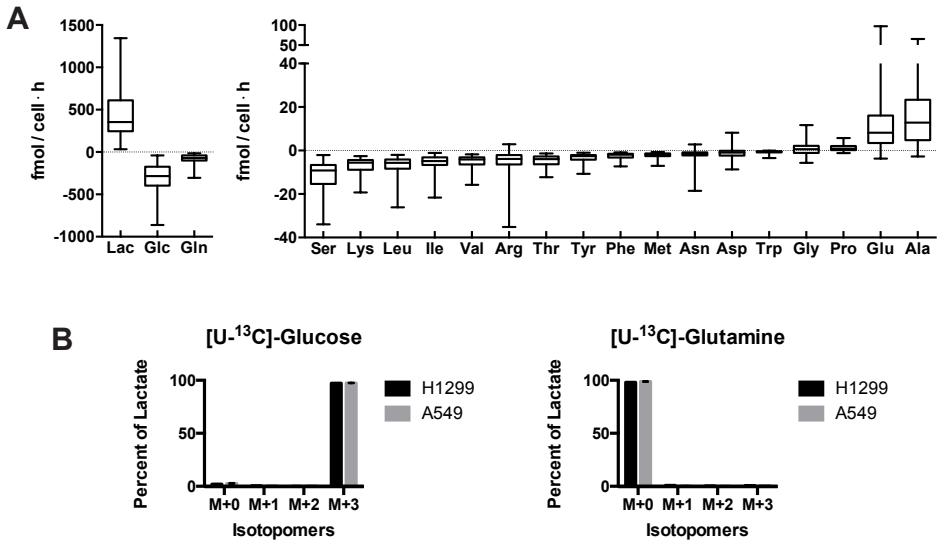

Figure S1, relating to Figure 1. Rapidly proliferating cells have common metabolic features. (A) Consumption and excretion rates (CORE) of glucose, lactate, and amino acids for the NCI-60 cell lines as observed by Jain *et al*. (Jain et al., 2012). Boxes indicate the mean and middle two quartiles, and whiskers indicate the minimum and maximum values. (B) Fractional labeling of lactate by H1299 and A549 cells grown in medium containing [U-^{13}C]-glucose or glutamine. Each bar represents the average of N=3, ±S.D. Standard three-letter abbreviations are used for amino acids; Glc, glucose; Lac, lactate.

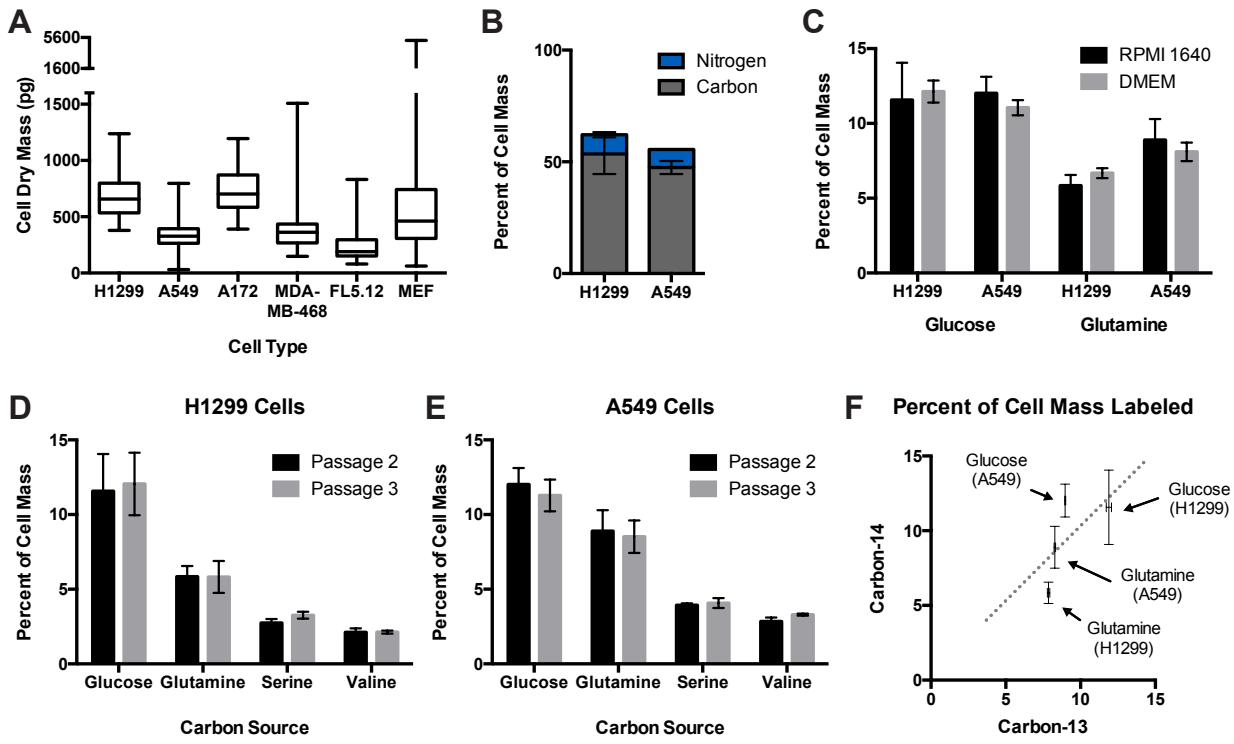

Figure S2, relating to Figure 2. Validation of carbon-14 incorporation measurement to quantify nutrient contribution to cell mass. (A) Cell dry mass of H1299, A549, A172, MDA-MB-468, FL5.12, and MEF cells as measured using a suspended microchannel resonator (SMR) (Feijo Delgado et al., 2013). (B) Carbon and nitrogen elemental composition of H1299 and A549 cell dry mass as determined by elemental analysis. (C) Incorporation of glucose and glutamine carbon into dry mass of H1299 and A549 cells cultured in DMEM with 10% FBS, or RPMI 1640 with 10% FBS. (D and E) Incorporation of carbon from glucose, glutamine, serine, or valine into H1299 and A549 cell mass after two and three passages in medium containing carbon-14-labeled nutrients. (F) Percent of cell dry mass labeled by [U-^{14}C]- or [U-^{13}C]-glucose or glutamine in H1299 and A549 cells. Carbon-14 incorporation was measured by scintillation counting and normalized relative to culture mass. Carbon-13 incorporation was measured as a fraction of total cellular carbon by EA-IRMS and was adjusted to percentage of cell mass by multiplying by the fraction of cell dry mass consisting of carbon (see S2A). The dotted line represents equal labeling by carbon-14 and carbon-13. In A, boxes indicate the mean and middle two quartiles, and whiskers indicate the minimum and maximum values measured; single cell dry masses of 60-200 cells are presented for each cell type assessed. In B-F, each point represents the average of N=3, ±S.D.

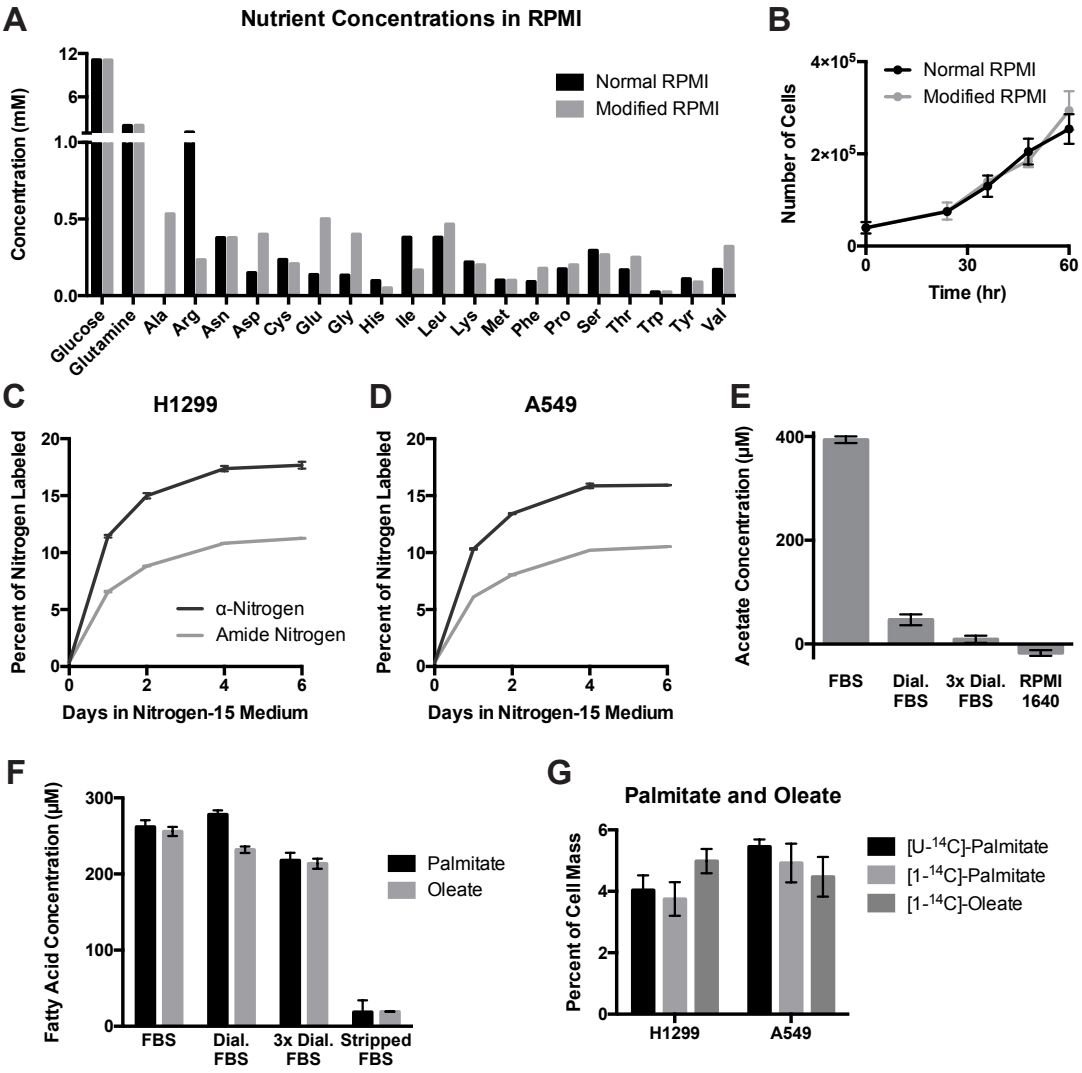

Figure S3, relating to Figure 3. (A) Concentrations of glucose and amino acids in normal RPMI 1640 and modified medium used to measure incorporation of amino acids into cell mass (see Table S2). (B) Growth of A549 cells in normal RPMI 1640 medium and in medium with modified amino acid concentrations. Nitrogen-15 incorporation into (C) H1299 and (D) A549 cells from [amide-^{15}N]- or [α-^{15}N]-glutamine. Concentrations of (E) acetate and (F) palmitate and oleate in FBS, commercial dialyzed FBS (dial. FBS), FBS dialyzed three times against saline (3x dial. FBS), FBS extracted with 2:1 diisopropyl-ether:butanol (stripped FBS), and RPMI 1640 medium (without serum). (G) The fraction of H1299 and A549 cell dry mass derived from serum palmitate and oleate. The amount of carbon labeled by [1-^{14}C]-palmitate and [1-^{14}C]-oleate was multiplied by 16 and 18, respectively, to estimate the contribution from the full-length fatty acid. This calculation makes the assumption that like palmitate, oleate is also fully incorporated into cell mass. Each point represents the average of N=3, ±S.D.

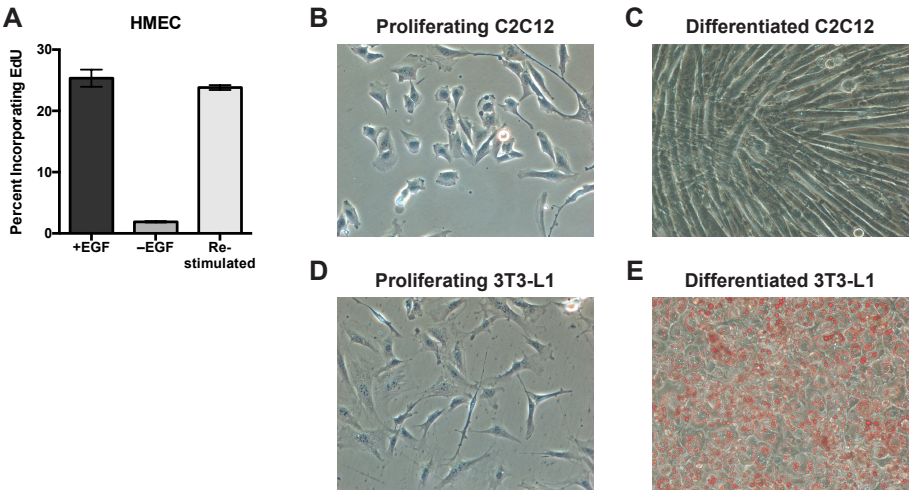

Figure S4, relating to Figure 4. Validation of non-proliferating cell models. (A) Human mammary epithelial cell (HMEC) proliferation is reversibly arrested when epidermal growth factor (EGF) is withdrawn, as measured by 5-ethynyl-2-deoxyuridine (EdU) incorporation. N=3, ±S.D. Representative phase contrast images of (B) proliferating C2C12 myoblasts, (C) differentiated C2C12 myocytes, (D) proliferating 3T3-L1 fibroblasts, and (E) 3T3-L1 differentiated into adipocytes. Cells in (D) and (E) are stained with oil red O.

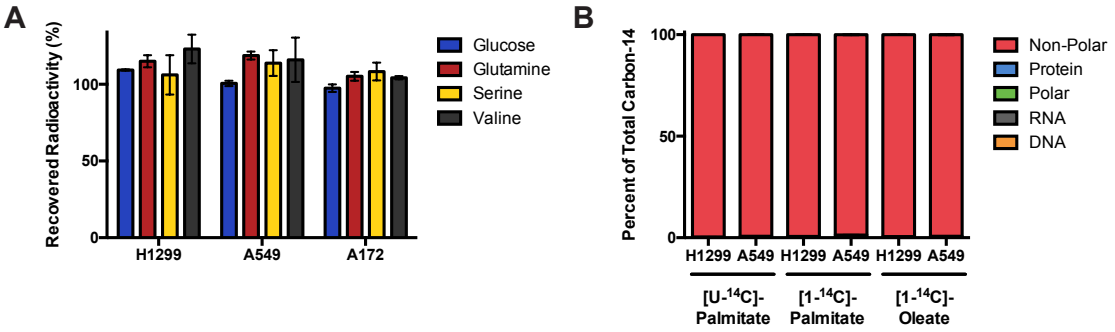

Figure S5, relating to Figure 5. (A) Recovery of input radioactivity after fractionation of H1299, A549, and A172 cells grown in the presence of [U-^{14}C]-glucose, glutamine, serine, or valine. The radioactivity recovered in the different macromolecule fractions (Figure 5A) was summed and is shown relative to the total radioactivity incorporated into parallel cultures. (B) Fate of exogenous lipids in H1299 and A549 cells. N=3, ±S.D.

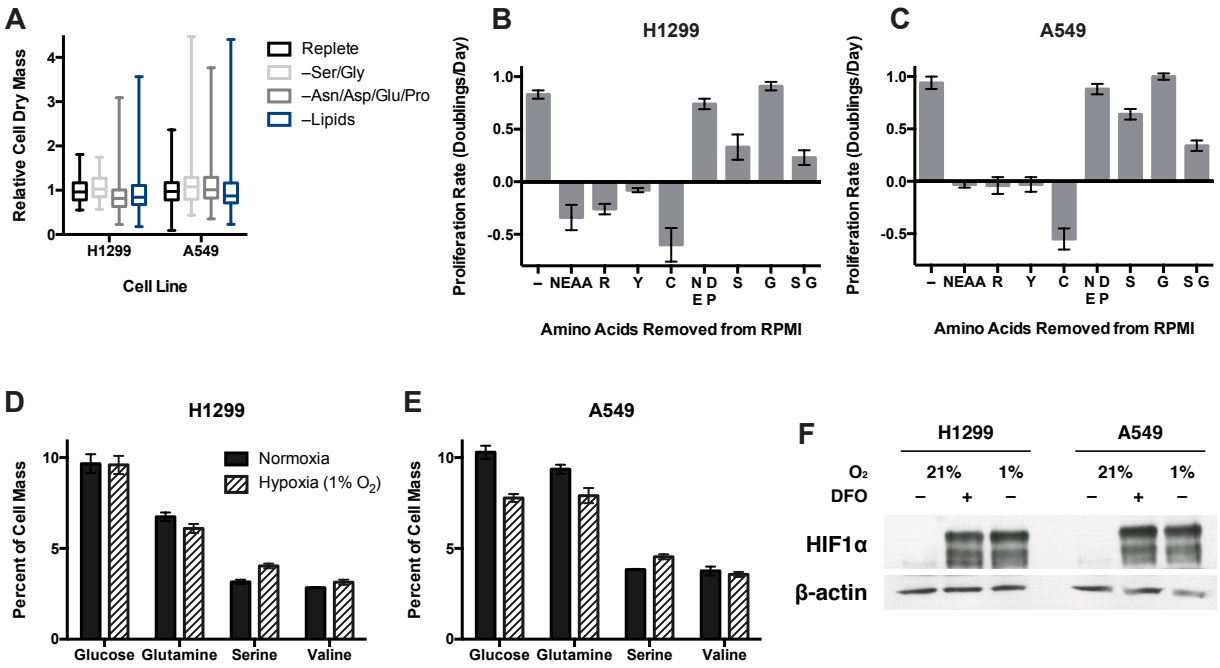

Figure S6, relating to Figure 6. Identification of non-essential medium components and analysis of nutrient incorporation by hypoxic cells. (A) Relative cell dry masses of H1299 and A549 cells cultured in RPMI 1640 with 10% dialyzed FBS (replete), medium lacking serine and glycine, medium lacking asparagine, aspartate, glutamate, and proline, or medium supplemented with 10% lipid-stripped FBS. Boxes indicate the mean and middle two quartiles, and whiskers indicate the minimum and maximum values measured; each represents single cell dry masses of 60-200 cells. Average cell mass in each condition did not differ significantly from that in replete medium according to a two-tailed Student's t-test ($p > 0.05$). Proliferation rates of (B) H1299 and (C) A549 cells in RPMI 1640 lacking all non-essential amino acids (NEAA), or specific non-essential amino acids (standard one-letter abbreviations are used). N=3, ±S.D. Carbon incorporation into proliferating (D) H1299 and (E) A549 cells cultured in 1% O_2. (F) Immunoblot for HIF1α in cells cultured in 21% oxygen with or without deferoxamine (DFO, a chemical inhibitor of HIF degradation), or 1% oxygen for 4 hours. A β-actin immunoblot is shown as a loading control.

Supplementary Tables

Table S1, relating to Figure 2: Characteristics of the cell lines used in this study.

Cell Type	Species	Known mutations*	Notes*
H1299	Human	N-Ras^{Q61K}, p53-null	Non-small cell lung cancer, lymph-node metastasis
A549	Human	K-Ras^{G12S}	Lung adenocarcinoma
A172	Human	PTEN- and p16/p19-null	Glioblastoma
MDA-MB-468	Human	p53$^{R273H/\ R273H}$; pRB-, PTEN-, and SMAD4-null	Breast adenocarcinoma metastasis
HMEC, 184A1	Human	p16-null	Human mammary epithelial cells, immortalized in culture
FL5.12	Mouse		Pro-B cells immortalized in culture, IL-3 dependent
L1210	Mouse	p53$^{M240I/\Delta}$, p16/p19-null	Lymphocytic leukemia
C2C12	Mouse	n/a	Myoblast, differentiates into myocytes
3T3-L1	Mouse		Fibroblast, differentiates into adipocytes
MEF	Mouse	n/a	Primary fibroblasts
T Cell	Mouse	n/a	Primary lymphocytes activated *ex vivo*
Hepatocyte	Mouse	n/a	Primary hepatocytes, non-proliferating

* Source: American Type Culture Collection, and (Bhadury et al., 2013; McKearn et al., 1985).

Table S2, relating to Figure 3: Concentrations (in mM) of glucose and amino acids in RPMI 1640, RPMI with modified amino acids, and DMEM.

Component	RPMI 1640*	Modified RPMI	DMEM*
Glucose	11.11	11.11	25
Alanine		0.534	
Arginine	1.149	0.234	.4
Asparagine	0.379	0.379	
Aspartate	0.150	0.401	
Cystine	0.208	0.208	.2
Glutamate	0.136	0.501	
Glutamine	2.055	2.055	4
Glycine	0.133	0.401	.4
Histidine	0.097	0.050	.2
Hydroxyproline	0.153		
Isoleucine	0.382	0.167	.8
Leucine	0.382	0.467	.8
Lysine	0.219	0.200	.8
Methionine	0.101	0.101	.2
Phenylalanine	0.091	0.178	.4
Proline	0.174	0.200	
Serine	0.286	0.267	.4
Threonine	0.168	0.250	.8
Tryptophan	0.025	0.025	.08
Tyrosine	0.111	0.089	.4
Valine	0.171	0.321	.8

* Source: Life Technologies.

Supplementary Experimental Procedures

Cell Culture: Cells were maintained in RPMI 1640 or DMEM (without pyruvate) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 µg/mL) using standard tissue culture techniques. FL5.12 cultures were always supplemented with 0.35 ng/mL IL-3 (Chemicon International). When cells were grown in the absence of specific amino acids, RPMI 1640 lacking these amino acids was supplemented with 10% heat-inactivated dialyzed FBS and all other medium components did not differ relative to normal RPMI 1640. RPMI containing modified amino acids was prepared by dissolving glucose and amino acids into RPMI 1640 base. The composition of this medium is detailed in **Table S2** and **Figure S3A**. Medium lacking lipids was RPMI 1640 supplemented with 10% lipid-stripped heat-inactivated FBS (see below). Sodium acetate was added to RPMI 1640 at various concentrations where indicated. Hypoxic cells were cultured in 1% O_2, 5% CO_2, and 94% N_2. Prototrophic SK1 *S. cerevisiae* were maintained on YEPD (1% yeast extract, 2% peptone, 1% glucose) agar plates and grown in liquid cultures of synthetic dextrose minimal medium (0.67% yeast nitrogen base without amino acids, 2% glucose) containing glucose as the sole carbon source.

Primary Cell Culture: Mouse embryonic fibroblasts (MEFs) were derived from C57BL/6J embryos at day E14.5. Embryos were dissected and digested in 0.25% trypsin/EDTA, and cells were passed through an 18-gauge needle and then incubated in HBSS with 5 µg/mL DNase at 37 ºC. Cells were plated in DMEM with 10% heat-inactivated FBS, and non-adherent cells were washed away with PBS, leaving adherent proliferating cells referred to as MEFs in this study. Primary T cells were derived from the spleens of 6-week old C57BL/6 mice, and pan T cells were isolated using the mouse Pan T cell Isolation Kit II (Miltenyi Biotec) according to the manufacturer's protocol. Cells were resuspended in RPMI 1640 with 10% FBS and activated in

CD3/CD28-coated 24-well culture plates and cultured for 48 hours before analysis. Primary hepatocytes were isolated from adult female C57BL/6J mice using the Hepatocyte Isolation System (Worthington Biochemical) according to the manufacturer's instructions. Briefly, livers were perfused with HBSS followed by collagenase, elastase, and DNase I, and hepatocytes were released by dissecting the digested livers. Cells were filtered through a 100 μm filter, washed and plated in hepatocyte adherence medium (Medium 199 with 0.1% BSA, 2% FBS, 100 nM dexamethasone, and 100 nM insulin) on plates coated with type I collagen. The following day, medium was changed to hepatocyte basal medium (Medium 199 with 2% dialyzed FBS, 100 nM dexamethasone, and 1 nM insulin) for carbon-14 labeling.

Differentiation Models: Proliferating C2C12 and 3T3-L1 cells were maintained in DMEM with 10% FBS. Confluent C2C12 cells were differentiated for six days in DMEM with 2% horse serum refreshed daily before initiating carbon-14 labeling in that medium. Confluent 3T3-L1 cells were differentiated by incubating for two days in DMEM with 10% FBS, 0.5 mM IBMX, 4 μg/mL insulin, 2 μM rosiglitazone, and 100 nM dexamethasone, followed by two days in DMEM with 10% FBS and 4 μg/mL insulin. The resulting adipocytes were returned to DMEM with 10% FBS for one day prior to carbon-14 labeling in that medium. Adipocyte differentiation was confirmed by oil red O staining: cells were fixed with 4% formalin, washed with 60% isopropanol, air dried, and stained for 10 min in 60% isopropanol containing 0.3% oil red O. Cells were washed multiple times with water prior to imaging.

HMEC Culture and Proliferation: Human mammary epithelial cell (HMEC) line 184A1 (Stampfer et al., 1993) was maintained in RPMI 1640 supplemented with 5 μg/mL insulin, 0.5% (v/v) bovine pituitary extract, 5 μg/mL transferrin, 0.5 μg/mL hydrocortisone, 10 μM isoproterenol, and 5 ng/mL EGF. Cells were arrested by incubating in this medium lacking EGF for three days prior to carbon-14 labeling in the absence of EGF. Proliferation was assessed by

incorporation of 5-ethynyl-2-deoxyuridine (EdU), a nucleotide analogue, using the Click-iT EdU Flow Cytometry Kit (Life Technologies). Cells were pulsed with 10 μM EdU for 2 hours and fixed and stained according to the manufacturer's instructions. The proportion of cells incorporating EdU was analyzed using a BD FACSCanto II using FACS Diva Software.

Measurement of nutrient consumption and excretion: Cells number was monitored and medium collected from cultures of exponentially proliferating cells. Glucose, glutamine, and lactate concentrations were measured on a YSI-7100 MBS (Yellow Springs Instruments). Medium amino acids were quantified by gas chromatography-mass spectrometry (GC-MS, see below) by comparison to isotopically labeled internal standards of known concentrations. To calculate consumption rate per cell for a given nutrient, its concentration over time was plot relative to the area under the curve (units: cells · time) of an exponential function fit to the number of cells. Linear regression analysis was performed, and the calculated slope is the consumption rate.

Mass Spectrometry Analysis: Samples were analyzed by gas-chromatography coupled to mass spectrometry (GC-MS) as described previously (Lewis et al., 2014). Polar metabolites were extracted from medium with 15 volumes of cold acetone containing norvaline and isotopically labeled amino acid standards (Cambridge Isotopes MSK-A2-1.2) and dried under nitrogen gas. Samples were derivitized with MOX reagent (Thermo Scientific) and *N*-tert-butyldimethylsilyl-*N*-methyltrifluoroacetamide with 1% tert-butyldimethylchlorosilane (Sigma Aldrich). Fatty acids were extracted from serum with 9 volumes of 4:5 methanol:chloroform, dried under nitrogen gas, and derivitized to fatty acid methyl esters in methanol with 2% sulphuric acid. After derivitization, samples were analyzed by GC-MS, using a DB-35MS column (Agilent Technologies) in an Agilent 7890A gas chromatograph coupled to an Agilent 5975C

mass spectrometer. Data were analyzed using in-house software described previously (Lewis et al., 2014).

Carbon-14 labeling studies: Unless otherwise indicated, mammalian cells were grown in RPMI 1640 containing 10% dialyzed heat-inactivated FBS supplemented with carbon-14 tracer. Media were supplemented with one of the following tracers: 0.2 µCi/mL [U-^{14}C]-glucose, 0.05 µCi/mL [U-^{14}C]-glutamine, 0.05 µCi/mL sodium [1,2-^{14}C]-acetate, 0.05 µCi/mL [U-^{14}C]-palmitic acid, or 0.05 µCi/mL L-[U-^{14}C]-amino acid mixture (all from American Radiolabeled Chemicals); or 0.05 µCi/mL [U-^{14}C]-serine, 0.05 µCi/mL [U-^{14}C]-valine, 0.05 µCi/mL [1-^{14}C]-palmitic acid, or 0.05 µCi/mL [1-14C]-oleic acid (all from Perkin Elmer). Before addition to the medium, fatty acids were first conjugated to serum proteins by mixing 0.55 µCi/mL of tracer with dialyzed FBS overnight at 37 ºC. To ensure labeling steady state was reached, cells were grown in the presence of carbon-14 until >95% of cellular material had turned over. Cells were washed three times with phosphate-buffered saline (PBS) to remove unincorporated carbon-14, collected by trypsinization, and counted. Cells were lysed in ≥10 volumes Emulsifier-Safe liquid scintillation cocktail (Perkin Elmer), and incorporated carbon-14 was quantified by liquid scintillation counting by comparison to a carbon-14 standard curve. Overnight cultures of SK1 yeast grown in SD (2% glucose) with 0.2 µCi/mL [U-^{14}C]-glucose were diluted into the same medium and grown to mid-log phase. Approximately 20 million cells were washed three times with cold deionized water and lysed with cold trichloroacetic acid on ice. Incorporated carbon-14 was quantified by scintillation counting as above.

To determine the fraction of cell dry mass derived from a specific nutrient, the ratio of concentrations of carbon-14 tracer ($^{14}C_{medium}$) to carbon-12 nutrient ($^{12}C_{medium}$) in the medium was first calculated. The amount of carbon-14 present in the medium was verified by scintillation counting. The amount of carbon-14 incorporated into cells ($^{14}C_{cells}$) was divided by this ratio to

determine the total amount of carbon incorporated from the nutrient, and this was converted to mass by multiplying by 12 g/mol. Finally, this number was normalized to cell mass by dividing by the number of cells in the culture (N) and the average mass of a single cell (m) (see below). The reported fraction of cell mass derived from a given nutrient is therefore equal to: $^{14}C_{cells}(^{12}C_{medium}/^{14}C_{medium})(12Nm)^{-1}$.

Measurement of cell dry mass: Dry mass of individual cells was determined by a method described previously (Feijo Delgado et al., 2013). Cells were trypsinized and resuspended in PBS at a concentration of 200,000 cells/mL. The cells were then loaded into a 25 × 25 μm cross-section suspended microchannel resonator (SMR), and the buoyant mass of single cells was measured first in PBS and then in D_2O-PBS. Each experiment lasted around 60 min at room temperature. Dry mass values for cells grown in different conditions were compared using an unpaired Student's t-test, assuming unequal variances. Where cell dry mass was not significantly different between conditions, average cell dry mass was taken to be equal among these conditions for the calculations in the carbon-14 labeling studies.

Stable isotope labeling studies: Cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated dialyzed FBS and containing either [U-^{13}C]-glucose or [U-^{13}C]-glutamine (Cambridge Isotopes Laboratories) in place of the corresponding unlabeled nutrient. After cells were cultured in this medium for 24 hours, polar metabolites were obtained by 80% methanol extraction, dried under nitrogen gas, and analyzed by GC-MS to determine lactate labeling (see above). Contribution of glucose or glutamine to total cellular carbon was measured after multiple passages in medium containing carbon-13. Contribution of glutamine to total cellular nitrogen was measured at the times indicated for cells grown in RPMI 1640 supplemented as above but containing [amide-^{15}N]- or [α-^{15}N]-glutamine (supplemented at 4% of total glutamine). In either case, approximately ten million cells per replicate were trypsinized, washed three times with

cold PBS, and dried. Carbon-13 and nitrogen-15 enrichment (i.e. ^{13}C:^{12}C and ^{15}N:^{14}N) were measured by isotope ratio mass spectrometry (IRMS) (see below).

Elemental analysis and IRMS: To determine the elemental composition of mammalian cells, approximately ten million cells per replicate were trypsinized and washed twice with cold PBS and once with 154 mM ammonium bicarbonate. Approximately 100 million SK1 yeast cells were washed three times with cold deionized water. After washing, cells were flash frozen and lyophilized. Samples of known mass (determined by analytical balance) were wrapped in tin capsules, and total carbon and nitrogen content were determined by continuous-flow direct combustion mass spectrometry on a Europa 20/20 IRMS (Sercon Limited) at the Utah State University Stable Isotope Laboratory (as in (Herron et al., 2009)). This mass spectrometer was calibrated with glucose and ammonium sulfate standards. Mammalian cells labeled with carbon-13 were analyzed by this method.

For nitrogen-15 labeled cells, IRMS was conducted as previously described (Kim et al., 2014). Approximately 10 million cells for each replicate were dried and aliquoted to tin cups prior to analysis. Samples were analyzed using an elemental analyzer (Vario Pyrocube, Elementar) coupled to an IRMS (Isoprime 100, Elementar). Prior to analysis, instrumental tuning was confirmed using urea standards and standard replicates were analyzed at regular intervals to confirm absence of instrumental drift.

Cell fractionation: Cells were lysed using the TRIzol reagent (Life Technologies) and RNA, DNA, and protein were extracted and purified by bi-phasic extraction according to the manufacturer's instructions (Chomczynski, 1993). Briefly, following initial lysis, insoluble material was considered to be DNA; RNA was precipitated from the aqueous phase, and the remaining soluble material was termed the "polar fraction"; protein was precipitated from the organic

phase, and the remaining soluble material was termed the “non-polar fraction”. Radioactivity in each fraction was quantified by liquid scintillation counting.

To evaluate the efficacy of this purification scheme, radioactive molecules of a defined macromolecular class were spiked into a TRIzol lysate of unlabeled HEK293 cells prior to fractionation, and radioactivity in the resulting fractions measured. For this analysis, radioactive lipids were obtained from HEK293 cells grown in medium containing [U-^{14}C]-glucose or [^{32}P]-orthophosphate (Perkin Elmer) with 2:1 chloroform:methanol extraction. Non-lipid contaminants were removed by further extracting with 0.2 volumes of saline. Radioactive protein was obtained by extensively dialyzing a RIPA lysate of HEK293 cells grown in medium containing [^{35}S]-methionine (Perkin Elmer). Radioactive DNA and RNA were respectively purified with the PureLink Genomic DNA Mini Kit (Life Technologies) and the RNeasy Mini Kit (Qiagen) from HEK293 cells grown in medium containing [methyl-^{3}H]-thymidine (Perkin Elmer) or [^{32}P]-orthophosphate. Radioactive small molecule metabolites were obtained by 80% methanol extraction of HEK293 cells grown with various carbon-14 nutrients. Yield from fractionation was assessed by comparing the sums of the quantities of radioactivity in each fraction to the amount of input radioactive tracer (**Figure 5B,C**) or to a culture grown in parallel (**Figure S5A**).

Cell proliferation. One day after cells were sparsely seeded, they were washed extensively with PBS and changed into different media prior to cell counting over time. To calculate proliferation rate, cells were counted over a period of 96 hours, and cell counts fit to the function $N(t) = N_0 \cdot 2^{kt}$ to determine k, the proliferation rate (in units of doublings per time).

Generation of lipid-stripped serum: Lipids were extracted from FBS without denaturing serum protein according to a previously established method (Cham and Knowles, 1976). Briefly, a 5:4:2 mixture of serum:diisopropyl-ether:*n*-butanol was stirred at room temperature for 30 min.

Phases were separated by centrifugation, and the aqueous phase was re-extracted with an equal volume of diisopropyl ether. The resulting aqueous phase was dialyzed three times against saline at 4 ºC and filter sterilized prior to use. Control (not stripped) serum was prepared by dialyzing FBS in this manner without organic extraction. Serum fatty acids were quantified by GC-MS (see above).

Measurement of acetate concentration: Polar metabolites were extracted from serum by adding four volumes of cold methanol to precipitate protein. Following centrifugation, the supernatant was dried under nitrogen gas and resuspended in ultrapure water. Acetate concentration in this extract was determined using the Acetate Colorimetric Assay Kit (Sigma Aldrich).

Immunoblot Analysis: H1299 and A549 cells were incubated for 4 hrs in hypoxia (1% O_2) or in normoxia with or without the addition of 100 μM deferoxamine (DFO) to chemically inhibit HIF1α degradation. Cells were lysed in RIPA buffer containing cOmplete Mini Protease Inhibitors (Roche) and 100 μM DFO. Samples were analyzed by SDS-PAGE and proteins were detected by immunoblot and ECL assay. Antibodies used: HIF1α (BD Biosciences, 610958) and β-actin (Abcam, 8227).

Supplementary References

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