Supplemental Information (Nadtochiy *et al.*)

Description of terms (glossary)

- Langendorff perfused heart – Ex-vivo retrograde perfusion of the heart.
- Steady-state metabolomics – Simultaneous measurement of metabolites levels (pool sizes) in biological samples
- ^{13}C – Heavy stable isotope of carbon
- ^{13}C-labeled substrates – Substrates (e.g. glucose, palmitate) where one or more carbons are substituted with ^{13}C
- Isotopologues – Molecules that contain at least one isotope labeled (^{13}C) atom
- Fractional saturation (F-SAT) – Fraction of all the ^{13}C isotopologues in a metabolite, versus the total number of carbons (labeled or unlabeled) in that metabolite.

Supplemental Experimental Procedures

Animals & materials

Male 2 month old mice on a C57BL/6J background were maintained in a pathogen-free vivarium under recommendations of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (2011 revision) with a 12 hr. light-dark cycle and food and water available *ad libitum*. All experimental protocols were approved by the AAALAC-accredited University of Rochester Committee on Animal Resources. All chemicals were the highest grade obtainable from Sigma (St. Louis, MO), unless otherwise specified.

In-vivo cardiac metabolomics

For *in-vivo* studies, 2 mo. old C57BL/6J mice were anesthetized and intubated, as for left anterior descending (LAD) coronary artery occlusion, as previously described [1,2]. After a 35 min. stabilization period, the thorax was opened wide, and the heart clamped in pre-cooled Wollenberger tongs and rapidly transferred into liquid N_2. Samples were processed for metabolomics as detailed below.

Ex-vivo heart perfusion

Following anesthesia with Avertin (tribromoethanol), hearts were perfused via the Langendorff (retrograde) method, in constant flow mode (4 ml/min.) as described previously [1,2] with minor modifications. Krebs-Henseleit (KH) buffer comprised 118mM NaCl, 4.7mM KCl, 25mM $NaHCO_3$, 1.2mM $MgSO_4$, 1.2mM KH_2PO_4, 2.5mM $CaCl_2$, and was supplemented with 5mM glucose and/or 100 µM palmitate conjugated to BSA in a 6:1 molar ratio. Note: lactate and pyruvate were omitted from KH buffer in order to be able to measure these metabolites in the steady-state experiments. A membrane oxygenator was used to saturate KH with carbogen (95/5 O_2/CO_2). A water filled balloon was inserted into the LV, and was inflated to set the initial $LVP_{diastolic}$ between 4-8 mmHg. Hearts were equilibrated for 20 min., and those with LVDP less than 100 mmHg were discarded from the experimental group. Coronary root pressure was monitored throughout the perfusion, and initial values were 109.8±6.7 mmHg. IR injury comprised 25 min. global ischemia followed by 60 min. reperfusion. IPC comprised 3 cycles of 5 min. ischemia plus 5 min. reperfusion. Where indicated, the SIRT1 inhibitor splitomicin was infused for 20 min. at a final concentration of 10 µM. Schematics showing all perfusion protocols are shown in Fig. S1A/B.

The following groups were tested: (i) IR with glucose plus palmitate, (ii) IPC+IR with glucose plus palmitate, (iii) IPC+IR +splitomicin with glucose plus palmitate, (iv) IR with palmitate alone, (v) IPC+IR with palmitate alone. Cardiac function was monitored throughout via a pressure transducer linked to a left ventricular balloon, with data sampling at 1 KHz. Heart rate (HR=Beats/min), LVDP (left ventricular developed pressure, $LVDP=LVP_{systolic} - LVP_{diastolic}$) and RPP (rate pressure product, $RPP=LVDP \cdot HR$) were measured. Infarct size was measured at the end of reperfusion by tetrazolium chloride staining and planimetry as previously described [2].

Labeling with ^{13}C substrates

We analyzed the transmission of isotopic labeling from substrates to downstream metabolites in Langendorff perfused hearts subjected to the following experimental conditions: (i) control perfusion, (ii) perfusion plus splitomicin, (iii) IPC, (iv) IPC plus splitomicin. Perfusion

protocols were performed as described above, with KH buffer containing both unlabeled (^{12}C) glucose and palmitate. At the end of the perfusion protocols, one substrate (either glucose or fat) was replaced with its U^{13}C-labeled equivalent (i.e., ^{13}C-glucose or ^{13}C-palmitate), with continued presence of the other unlabeled substrate. The perfusion system was designed with narrow tubing and minimal mixing (e.g. small air bubble trap) to yield a dead volume of 12 ml, such that a perfusion rate of 4 ml/min. yielded a changeover time of 3 min. following substrate switching. Upon labeled substrate reaching the heart, perfusion was continued for 5 min., which was empirically determined to be sufficient for 40-70% fractional saturation of key metabolic pathways (Fig. 2, Fig. S3).

Sample preparation for metabolomics.

The heart was immediately clamped in pre-cooled Wollenberger tongs while still on the perfusion rig, and the tongs immediately plunged into liquid N_2. Tissue was ground in a pre-cooled pestle and mortar, and the resulting powder suspended in 3 ml 80% MeOH. The mixture was vortexed for 1 min. and centrifuged at 1,000 x *g* for 5 min. The supernatant was collected and the pellet further extracted in 2 x 1 ml. 80% MeOH. Combined extracts (5 ml) were dried under N_2 stream, and resuspended in 200 µl 50% MeOH for loading to LC-MS. For amino acid detection, samples were derivatized with triethylamine (5 % v/v) and benzyl chloroformate (1% v/v). Samples were centrifuged at 14,000 x *g* for 10 min., and supernantants transferred to autosample vials for LC-MS.

LC/MS-MS measurements and data analysis

Extracts were analyzed using reverse phase chromatography with an ion-paring reagent in a Shimadzu LC-20AD HPLC system coupled to a Thermo Quantum Ultra triple-quadrupole mass spectrometer. The liquid chromatography separation employed a Phenomenex Synergi 4u Hydro-RP 80A column (150 mm × 2 mm, 4 µm), and the mobile phase comprised 10 mM tributylamine, 15 mM acetic acid in 97:3 H_2O/MeOH. Flow rate: 0.2 ml/min. Gradient: 0–5 min. 100%, 6-20 min. 80%, 21-35 min. 35%, 36-42 min. 5%, 43-50min. 100%. Electrospray ionization spray voltage was 3000 V in the negative mode. Nitrogen was used as sheath gas at 30 psi and

as the auxiliary gas at 10 psi, and argon as the collision gas at 1.5 mTorr, with the capillary temperature 325 °C. Scan time for each SRM transition was 0.05s with a scan width of 1 m/z [3,4]. Data were analyzed using publicly available MzRock machine learning tool kit (http://code.google.com/p/mzrock/), which automated the identification of targeted metabolites based on their chromatographic retention time, whole molecule mass, collision energy, and resulting fragment mass (daughter ions). Metabolites with close retention times, such as pyruvate and lactate, were also analyzed using XCalibur Qual Browser (Thermo Scientific). Metabolite peak shapes and retention times were compared to previously run chemical standards. Further, sample dilution analysis was performed to ensure the signal linearity at the analyzed dilutions.

Data for each run were median-normalized. Overall, 81 metabolites were identified with high confidence, so between the 4 experimental groups (control, IPC, ±splitomicin) and 8 independent replicates, a theoretical 2592 individual data points could be obtained. There were 140 blanks (no value obtained) representing 5.4% of the data. An additional 63 data points (2.4%) were identified as outliers (>1 standard deviation from the mean) and also removed. Missing values were imputed as weighted medians [5], i.e. the median of the remaining values for the specific metabolite, normalized by the median of all metabolites in the particular run.

For ^{13}C labeling studies there was sufficient isotopologue information to calculate fractional carbon saturation (F-SAT) for 15 metabolites with ^{13}C glucose, and 12 metabolites with ^{13}C palmitate (Table S2). ^{13}C saturation was corrected for the natural abundance of ^{13}C at 1.1% [6].

Cardiomyocyte isolation and Seahorse XF24 measurements

Adult mouse cardiomyocytes (AMC) were isolated using a previously established protocol [7]. Briefly, the protocol included tissue digestion with trypsin/collagenase (Roche Blendzyme) followed by step-wise Ca^{2+} introduction to make Ca^{2+}-tolerant cardiomyocytes. Overall, the protocol yields ~$6x10^5$ cells per heart, with ~85% cell viability. Immediately upon isolation, cells were plated on laminin-coated V7-PS plates (Seahorse Bioscience, North Billerica, MA). An hour later AMC were supplied with either glucose (GLU, 5mM), palmitate/fat-

free BSA (FAT, 100µM); or GLU+FAT. Previously, using a Seahorse XF24 we developed a protocol for *in-vitro* simulated ischemic conditions [7,8]. For simulated IPC (sIPC), AMC were exposed to 20 min. ischemia, followed by 60 min. of reperfusion. Note… due to the logistics of gas-flow manipulation inside the XF apparatus, nominal ischemia takes ~5 min. to fully develop, such that the actual time spent in ischemia for a 20 min. exposure, is ~15 min. In separate experiments cells were subjected to simulated IR injury (60 min. ischemia, 60 min. reperfusion, with or without prior sIPC, and cell death was assayed by LDH release (schematic, Fig. 5 main manuscript). Oxygen consumption rates (OCR, mitochondrial respiratory activity) were measured pre- and post-sIPC to investigate metabolic changes.

Statistical analysis

Significance between two groups was determined using Student's t-test. ANOVA was used for comparisons among multiple groups with Tukey's multiple comparison test (Prism 6.0 for Windows; GraphPad, La Jolla, CA). Pathway impact analysis and principle component analysis (PCA) for the steady state metabolomic data were performed using publicly available software MetaboAnalyst 3.0 (www.metaboanalyst.ca) [9].

Supplemental Literature Cited

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[9] Xia J, Mandal R, Sinelnikov IV, Broadhurst D, Wishart DS. MetaboAnalyst 2.0 – a comprehensive server for metabolomic data analysis. Nucl Acids Res 2012:40:W127-33.

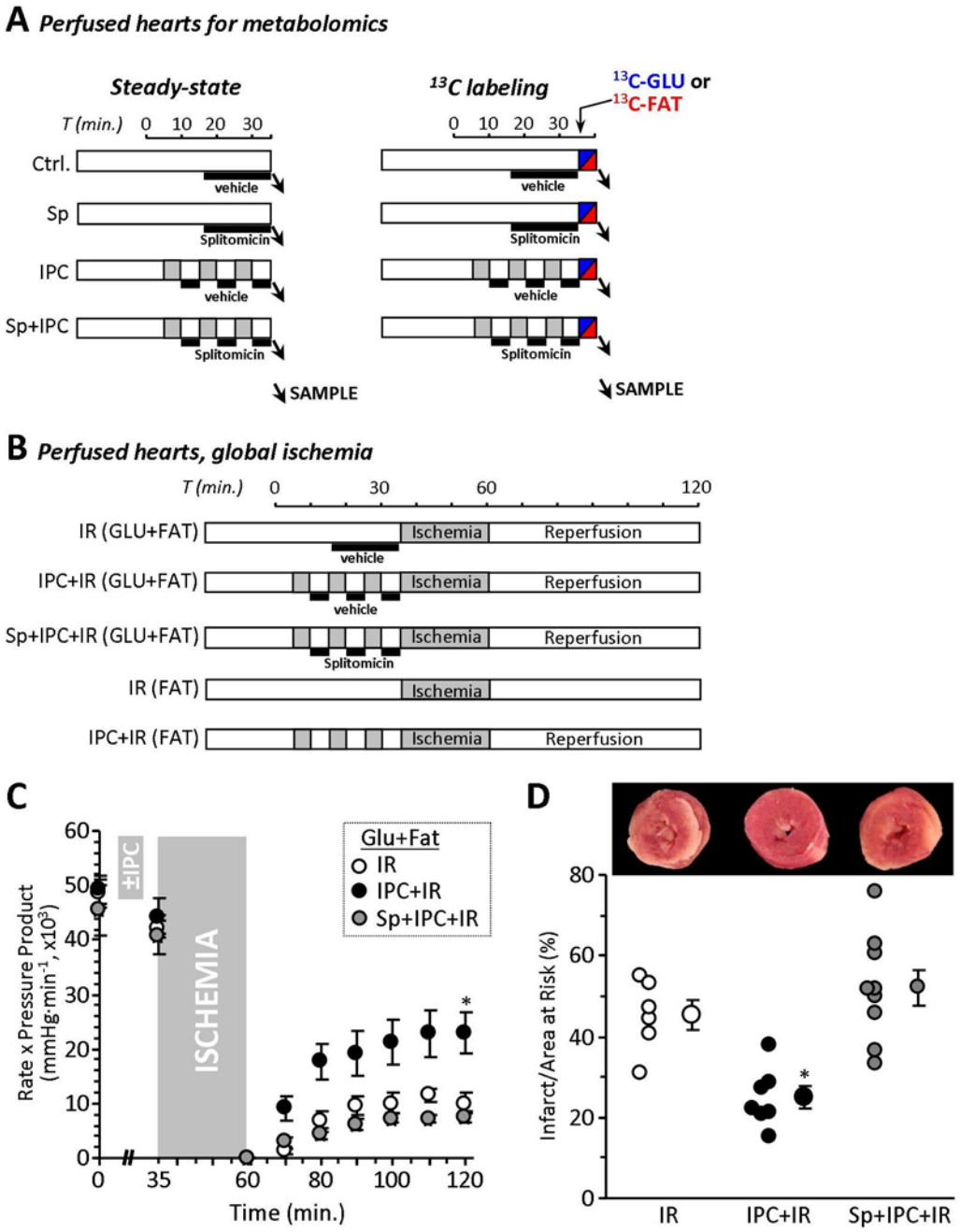

Figure S1. Schematics of perfusion protocols, and model validation. **(A):** Schematics of perfusion protocols for metabolomics studies. Left = steady state, Right = ^{13}C label incorporation. Downward arrow at the end of each schema represents sampling time. In each schema, white represents normoxic perfusion, gray represents ischemia. Where present, black bars beneath the schema indicate infusion of vehicle or splitomicin. In right panel, red and blue represent infusion of either ^{13}C-glucose or ^{13}C-palmitate. **(B):** Schematics of perfusion protocols

for ischemia reperfusion injury and protective effects of IPC. White/gray represent normoxia/ischemia as in panel A. **(C/D):** Hearts perfused with glucose plus fat were subjected to IR injury as described in the methods. Optionally, hearts were also subjected to IPC prior to IR injury, or treated with the SIRT1 inhibitor splitomicin (Sp) during IPC. Panel C shows cardiac function (rate pressure product) in IR, IPC, and Sp+IPC hearts. Data are means ± SEM. Panel D shows myocardial infarct, plotted as individual points (left) and means ± SEM (right). Images above graph show representative TTC-stained cardiac cross-section slices (white = infarct, red = live myocardium). For panels B & C, n = 6, 7, and 9 for IR, IPC, and Sp+IPC groups respectively. *$p<0.05$ vs. IR (ANOVA).

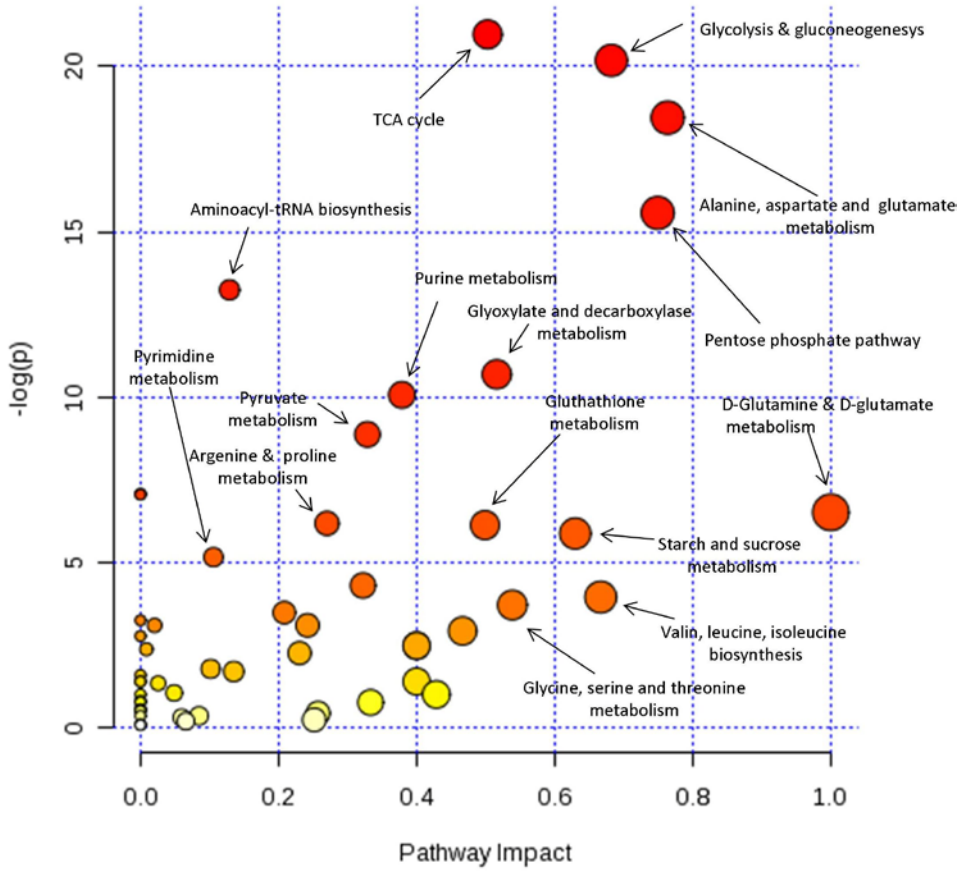

Figure S2. Representation of metabolic pathways based on detected metabolites. Pathway analysis was performed by the open source software MetaboAnalyst 3.0 software (www.metaboanalyst.ca) [19], using all detected metabolites from the steady state analysis (Table S1).

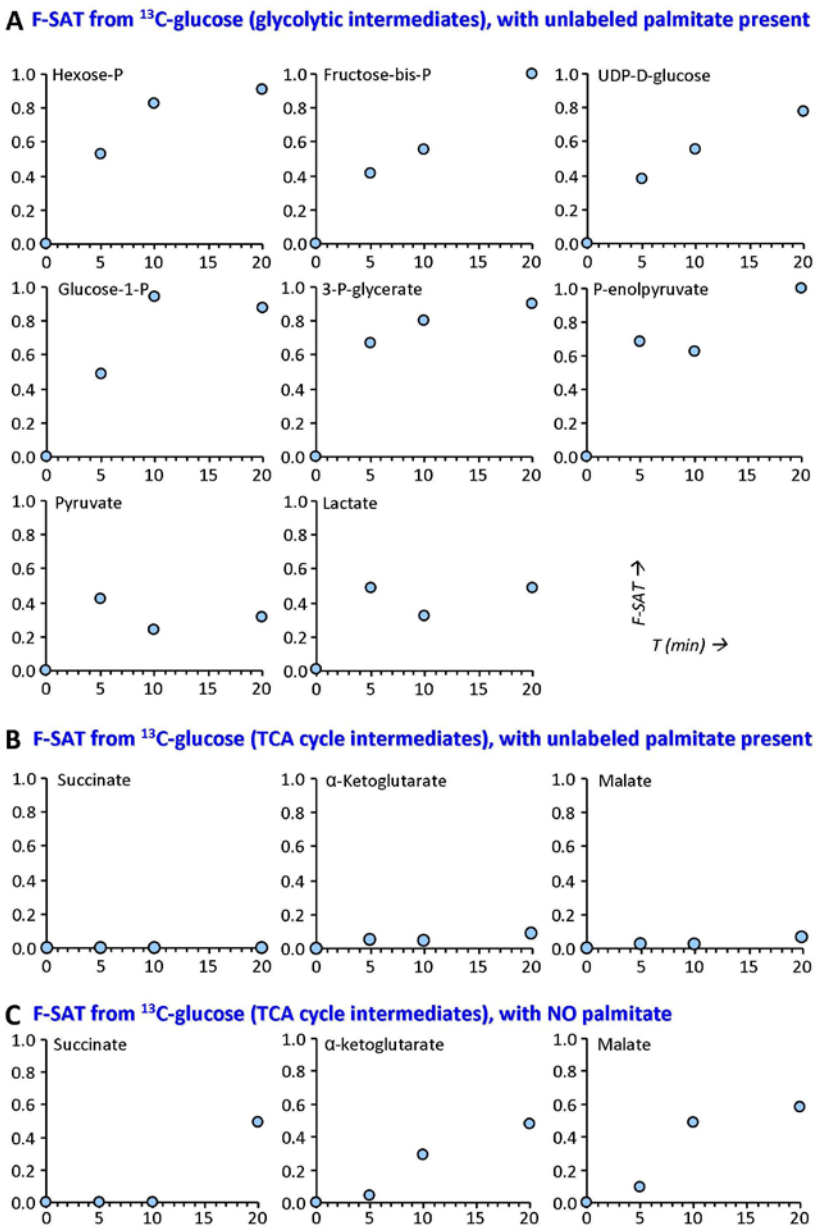

Figure S3. Time-dependent fractional carbon saturation of metabolites originating from ^{13}C-labeled glucose. (A): Glycolytic metabolites from ^{13}C-glucose in the presence of unlabeled palmitate). **(B):** TCA cycle metabolites from ^{13}C-glucose in the presence of unlabeled palmitate **(C):** TCA cycle metabolites from ^{13}C-glucose with no palmitate added. Hearts were perfused with ^{13}C-labeled glucose for 0, 5, 10 and 20 min. The levels of ^{13}C and ^{12}C isotopologues were measured for each individual metabolite and fractional saturation (F-SAT) was calculated.

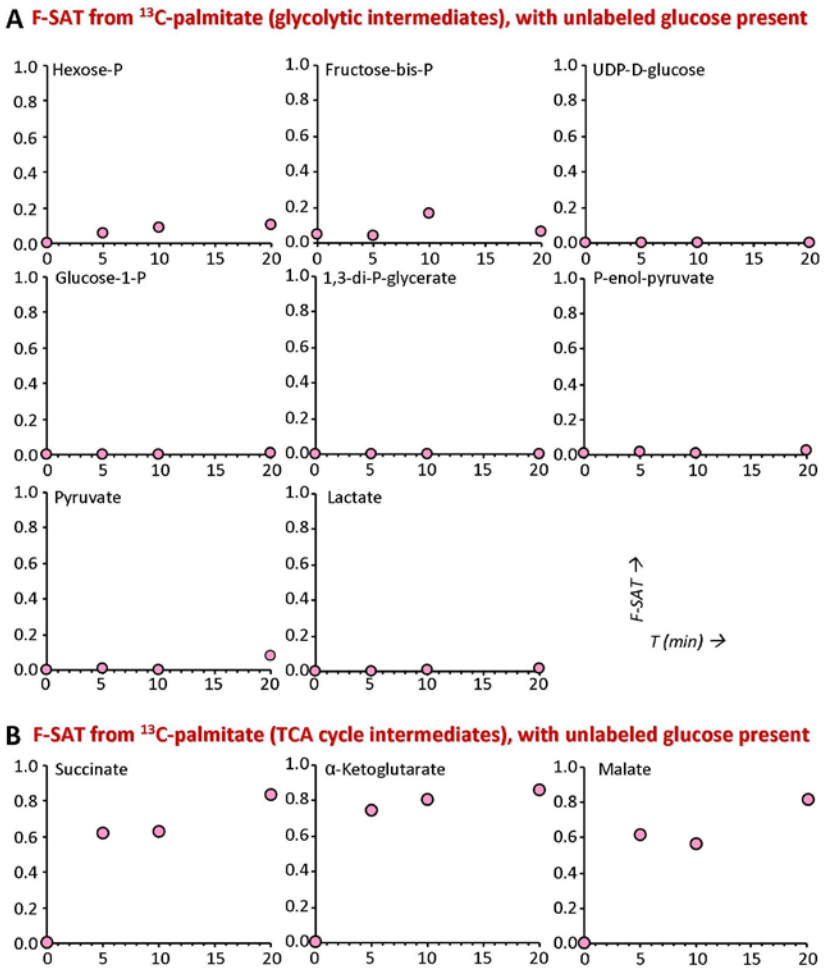

Figure S4. Time-dependent fractional carbon saturation of metabolites originating from ^{13}C-labeled palmitate. (A): Glycolytic metabolites from ^{13}C-palmitate in the presence of unlabeled glucose. **(B):** TCA cycle metabolites from ^{13}C-palmitate in the presence of unlabeled glucose. Hearts were perfused with ^{13}C-labeled palmitate for 0, 5, 10 and 20 min. The levels of ^{13}C and ^{12}C isotopologues were measured for each individual metabolite and fractional saturation (F-SAT) was calculated.

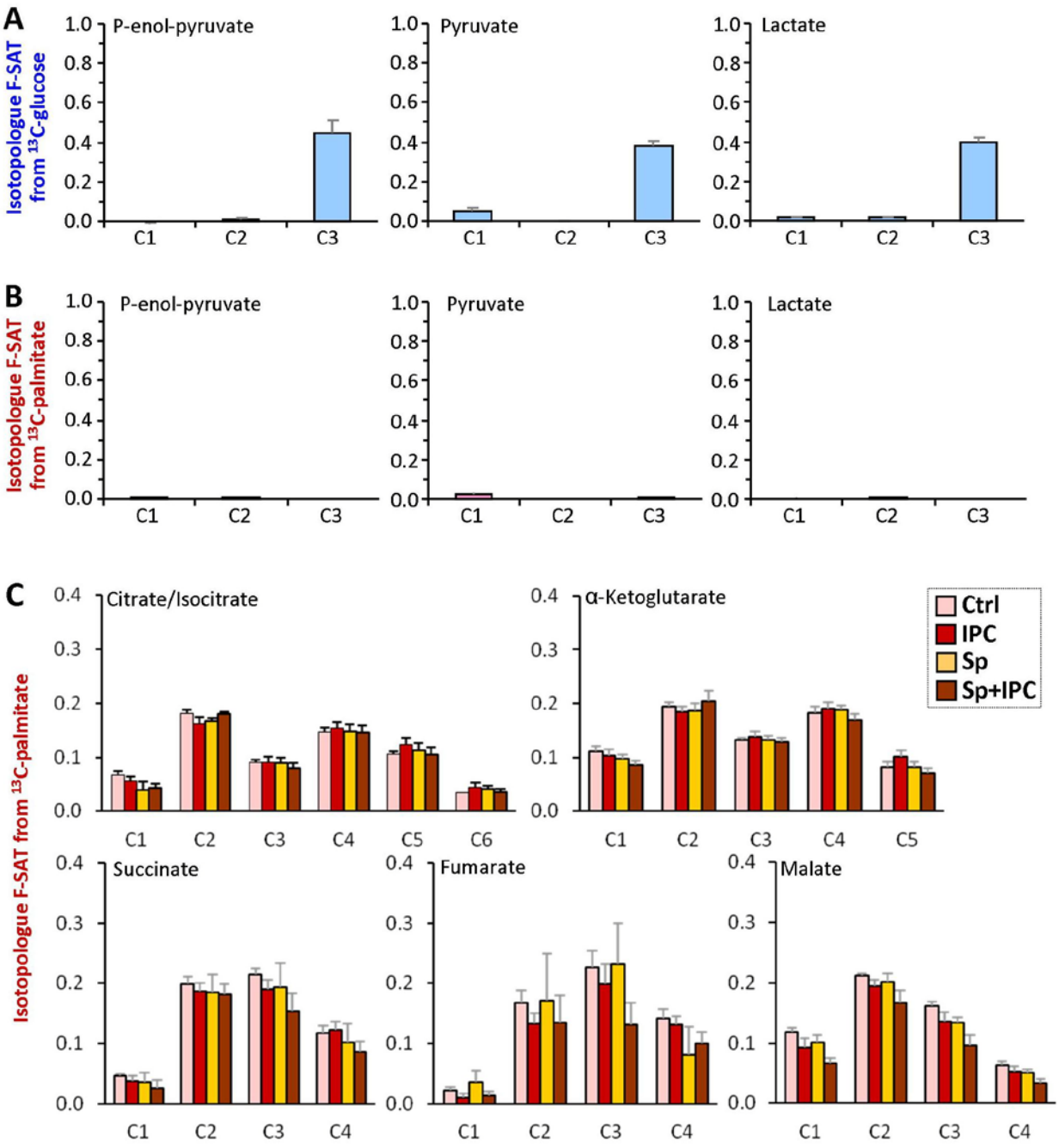

Figure S5. Isotopologue analysis of metabolites. Isotopologues were calculated as individual fractions of labeled carbons. For example, pyruvate, which contains 3 carbons, has 3 isotopologues (C1, C2, C3). **(A):** Glycolytic metabolite isotopologues originating from ^{13}C-labeled glucose in the presence of unlabeled palmitate. **(B)**: Glycolytic metabolite isotopologues originating from ^{13}C-labeled palmitate in the presence of unlabeled glucose. **(C):** TCA cycle isotopologues originating from ^{13}C-labeled palmitate in the presence of unlabeled glucose,

under control perfusion or IPC conditions. Experiments were performed in the absence or presence of splitomicin. Color scheme for bars is the same as Figs. 3 and 4 in the main manuscript. Following control or IPC treatments, hearts were perfused with ^{13}C-labeled glucose or palmitate for 5 min and the levels of ^{13}C and ^{12}C isotopologues were measured for each individual metabolite and fractional saturation (F-SAT) was calculated. Data are means ± SEM, N=6.

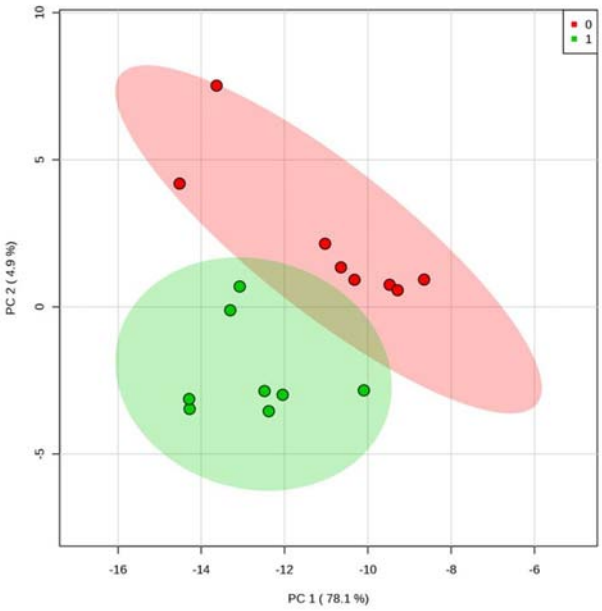

Figure S6. PCA analysis. Principle component analysis (PCA) comparing the effects of IPC on global changes in steady state levels of 81 metabolites, either alone (green), or in the presence of splitomicin (red). PCA was conducted using open source MetaboAnalyst 3.0 software (www.metaboanalyst.ca) [9].

Supplemental Tables 1, 2, and 3 – See separate Microsoft Excel (.xls) files