# SUPPLEMENTARY METHODS

## GLOBAL METABOLOMIC ANALYSIS.

### Sample Preparation for Global Metabolomics

Sample preparation was carried out as described previously [1] at Metabolon, Inc. Briefly, recovery standards were added prior to the first step in the extraction process for quality control purposes. To remove protein, dissociate small molecules bound to protein or trapped in the precipitated protein matrix, and to recover chemically diverse metabolites, proteins were precipitated with methanol under vigorous shaking for 2 min (Glen Mills Genogrinder 2000) followed by centrifugation. The resulting extract was divided into five fractions: two (i.e., early and late eluting compounds) for analysis by ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS; positive ionization), one for analysis by UPLC-MS/MS (negative ionization), one for the UPLC-MS/MS polar platform (negative ionization), and one sample was reserved for backup.

Three types of controls were analyzed in concert with the experimental samples: samples generated from a pool of human plasma extensively characterized by Metabolon, Inc. or generated from a small portion of each experimental sample of interest served as technical replicate throughout the data set; extracted water samples served as process blanks; and a cocktail of standards spiked into every analyzed sample allowed instrument performance monitoring. Instrument variability was determined by calculating the median relative standard deviation (RSD) for the standards that were added to each sample prior to injection into the mass spectrometers (median RSD typically = 4-6%; $n \geq 30$ standards). Overall process variability was determined by calculating the median RSD for all endogenous metabolites (i.e., non-instrument standards) present in 100% of the pooled human plasma or client matrix samples (median RSD = 10-14%; n = several hundred metabolites). Experimental samples and controls were randomized across the platform run.

### Mass Spectrometry Analysis

Non-targeted MS analysis was performed at Metabolon, Inc. Extracts were subjected to UPLC-MS/MS [2]. The chromatography was standardized and, once the method was validated no further changes were made. As part of Metabolon's general practice, all columns were

purchased from a single manufacturer's lot at the outset of experiments. All solvents were similarly purchased in bulk from a single manufacturer's lot in sufficient quantity to complete all related experiments. For each sample, vacuum-dried samples were dissolved in injection solvent containing eight or more injection standards at fixed concentrations, depending on the platform. The internal standards were used both to assure injection and chromatographic consistency. Instruments were tuned and calibrated for mass resolution and mass accuracy daily.

The UPLC-MS/MS platform utilized a Waters Acquity UPLC with Waters UPLC BEH C18-2.1×100 mm, 1.7 µm columns and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. The sample extract was dried then reconstituted in acidic or basic LC-compatible solvents, each of which contained 8 or more injection standards at fixed concentrations to ensure injection and chromatographic consistency. One aliquot was analyzed using acidic, positive ion-optimized conditions and the other using basic, negative ion-optimized conditions in two independent injections using separate dedicated columns (Waters UPLC BEH C18-2.1x100 mm, 1.7 µm). Extracts reconstituted in acidic conditions were gradient eluted using water and methanol containing 0.1% formic acid, while the basic extracts, which also used water/methanol, contained 6.5mM ammonium bicarbonate. A third aliquot was analyzed via negative ionization following elution from a HILIC column (Waters UPLC BEH Amide 2.1x150 mm, 1.7 µm) using a gradient consisting of water and acetonitrile with 10mM Ammonium Formate. The MS analysis alternated between MS and data-dependent $MS^2$ scans using dynamic exclusion, and the scan range was from 80-1000 *m/z*.

**Compound Identification, Quantification, and Data Curation**

Metabolites were identified by automated comparison of the ion features in the experimental samples to a reference library of chemical standard entries that included retention time, molecular weight (m/z), preferred adducts, and in-source fragments as well as associated MS spectra and curated by visual inspection for quality control using software developed at Metabolon [3]. Identification of known chemical entities was based on comparison to metabolomic library entries of purified standards.

1. Evans AM, DeHaven CD, Barrett T, Mitchell M, Milgram E (2009) Integrated, nontargeted ultrahigh performance liquid chromatography/electrospray ionization tandem mass spectrometry platform for the identification and relative quantification of the small-molecule complement of biological systems. Anal Chem 81: 6656–6667.
2. Evans AM, Bridgewater BR, Liu Q, Mitchell MW, Robinson RJ, Dai H, Stewart SJ, DeHaven CD, Miller LAD (2014) High resolution mass spectrometry improves data quantity and quality as compared to unit mass resolution mass spectrometry in high-throughput profiling metabolomics. Metabolomics 4: 132.
3. DeHaven C, Evans A, Dai H, Lawton K (2010) Organization of GC/MS and LC/MS metabolomics data into chemical libraries. Journal of Cheminformatics 2: 9.

**GLOBAL LIPIDOMIC ANALYSIS**

Lipids were extracted from samples via a modified Bligh-Dyer extraction using methanol/water/dichloromethane in the presence of deuterated internal standards. The extracts were dried under nitrogen and reconstituted in ammonium acetate dichloromethane:methanol. The extracts were transferred to vials for infusion-MS analysis, performed on a Shimadzu LC with nano PEEK tubing and the Sciex SelexIon-5500 QTRAP. The samples were analyzed via both positive and negative mode electrospray. The 5500 QTRAP was operated in MRM mode with a total of more than 1,100 MRMs. Individual lipid species were quantified by taking the ratio of the signal intensity of each target compound to that of its assigned internal standard, then multiplying by the concentration of internal standard added to the sample. Lipid class concentrations were calculated from the sum of all molecular species within a class, and fatty acid compositions were determined by calculating the proportion of each class comprised by individual fatty acids.

**STATISTICAL ANALYSIS:** Following normalization to Bradford protein concentration, log transformation and imputation of missing values, if any, with the minimum observed value for each compound, Welch’s two-sample t-test were used to identify biochemicals that differed significantly between the experimental groups.

# SUPPLEMENTARY FIGURE LEGENDS

**Figure S1.** Overview of the results from the global metabolomic and complex lipid profiling studies. The numbers of biochemicals reaching statistical significance ($p \leq 0.05$) and approaching statistical significance ($0.05 \leq p \leq 0.1$) are shown.

**Figure S2.** Statistical heat map displaying the fold change values observed when dipeptide abundance was compared between the skeletal muscles of 24 month old WT and 4E-BP1/2 DKO mice. The dark green color is used to indicate statistically significant decreases $p<0.05$ while the light green indicates differences that trended towards significance with a $0.05<p<0.1$.

**Figure S3.** (A) Potential fates of glucose utilization in the 4E-BP1/2 DKO mice are highlighted. (B) Statistical heat map displaying the fold change values observed when carbohydrate abundance was compared between the skeletal muscles of 24 month old WT and 4E-BP1/2 DKO mice. The dark green and red color are used to indicate statistically significant decreases $p<0.05$ while the light green and pink indicate differences that trended towards significance with a $0.05<p<0.1$.

**Figure S4.** (A) Statistical heat map showing the fold change values observed when total neutral lipid abundance was compared between the WT and 4E-BP1/2 DKO mice. (B) Statistical heat map showing the fold change values observed in select diacylglycerol species observed when the skeletal muscle of the WT and 4E-BP1/2 DKO mice were compared. (C) Statistical heat map showing the fold change values observed in select triacylglycerol species (TAG) when the skeletal muscle of the WT and 4E-BP1/2 DKO mice were compared. Each species measured is represented as TAG(a):(b)-FA(c) where: a= The total number of carbons that are present within the three acyl chains esterified to the glycerol backbone of the TAG molecule, b= The total number of double bonds amongst the three acyl chains esterified to the glycerol backbone of the TAG molecule, c= The carbon length and saturation status of *one* of the three acyl chains. The dark red color is used to indicate statistically significant increases $p<0.05$ while the light pink indicates differences that trended towards significance with a $0.05<p<0.1$.

**Figure S1.**

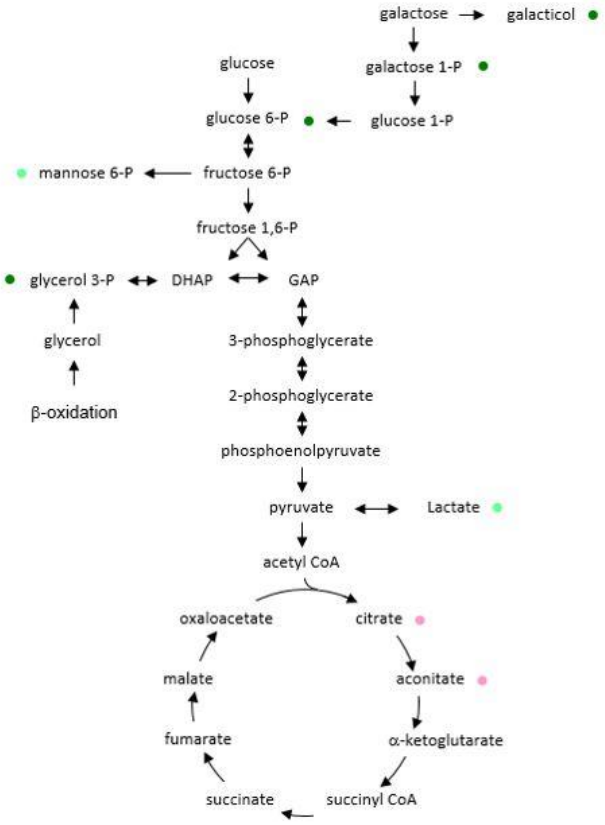
| | Total Biochemicals ($p \leq 0.05$) | Biochemicals (↑/↓) | Total Biochemicals ($0.05 < p < 0.1$) | Biochemicals (↑/↓) |
|---|---|---|---|---|
| Metabolomic | 59 | 17 / 42 | 53 | 24 / 29 |
| Lipids | 31 | 31 / 0 | 35 | 34 / 1 |

**Figure S2.**

| | | DKO/WT | |
|---|---|---|---|
| Pathway | Biochemicals | Fold change | p value |
| Dipeptides | 3-methylhistidine | **0.63** | 0.072 |
| | alanylleucine | **0.44** | **0.0182** |
| | glycylleucine | 0.72 | NS |
| | glycylvaline | **0.8** | 0.053 |
| | leucylalanine | **0.57** | 0.0543 |
| | leucylglycine | **0.6** | 0.0799 |
| | phenylalanylglycine | 0.8 | NS |
| | prolylglycine | 0.92 | NS |
| | valylglycine | **0.47** | **0.0123** |
| | valylleucine | **0.73** | 0.0872 |
| | leucylglutamine | **0.62** | **0.0208** |

**Figure S3.**

A.




B.

| | | DKO/WT | |
|---|---|---|---|
| Pathway | Biochemicals | Fold change | p value |
| Glycolysis, Gluconeogenesis, and Pyruvate Metabolism | 1,5-anhydroglucitol (1,5-AG) | 1.01 | NS |
| | glucose | 0.81 | NS |
| | glucose 6-phosphate | 0.48 | **0.0137** |
| | fructose 1,6-bisphosphate | 0.76 | NS |
| | dihydroxyacetone phosphate (DHAP) | 1.08 | NS |
| | 2-phosphoglycerate | 1.41 | NS |
| | 3-phosphoglycerate | 1.22 | NS |
| | phosphoenolpyruvate (PEP) | 1.01 | NS |
| | pyruvate | 0.86 | NS |
| | lactate | 0.87 | 0.0809 |
| | glycerate | 0.93 | NS |
| Fructose, Mannose and Galactose Metabolism | fructose | 1.05 | NS |
| | mannitol/sorbitol | 0.86 | NS |
| | mannose | 0.66 | NS |
| | mannose-6-phosphate | 0.53 | **0.0344** |
| | galactitol (dulcitol) | 0.86 | **0.0462** |
| | galactose 1-phosphate | 0.53 | **0.0118** |
| | galactonate | 0.96 | NS |
| Disaccharides and Oligosaccharides | sucrose | 2.28 | 0.0505 |
| | maltitol/lactitol/cellobiotol/palatinol | 33.57 | **0,000** |
| TCA Cycle | citrate | 1.14 | 0.076 |
| | aconitate [cis or trans] | 1.21 | 0.0819 |
| | isocitric lactone | 1.3 | 0.064 |
| | alpha-ketoglutarate | 0.9 | NS |
| | succinylcarnitine (C4-DC) | 0.89 | NS |
| | succinate | 0.9 | NS |
| | fumarate | 0.92 | NS |
| | malate | 0.96 | NS |
| | 2-methylcitrate/homocitrate | 0.7 | NS |

**Figure S4.**

A.

| | | DKO/WT | |
|---|---|---|---|
| Pathways | Biochemicals | Fold change | p value |
| Neutral Complex Lipids SUM | Free Fatty Acids | 1.38 | NS |
| | Cholesteryl Esters | 1.09 | NS |
| | Diacylglycerols | 1.3 | NS |
| | Triacylglycerols | 1.28 | NS |
| | Monoacylglycerols | 1.02 | NS |

B.

| | | DKO/WT | |
|---|---|---|---|
| Pathways | Biochemicals | Fold change | p value |
| Diacylglycerol | DAG(14:0/16:0) | 1.42 | 0.0019 |
| | DAG(14:0/16:1) | 1.53 | 0.3619 |
| | DAG(16:0/16:0) | 1.5 | **0.0003** |
| | DAG(16:0/16:1) | 1.6 | **0.1086** |
| | DAG(16:0/18:1) | 1.41 | **0.4381** |
| | DAG(16:0/20:3) | 1.49 | **0.0343** |
| | DAG(16:0/20:4) | 1.26 | **0.0034** |

C.

| | | DKO/WT | |
|---|---|---|---|
| Pathways | Biochemicals | Fold change | p value |
| Triacylglycerol | TAG40:0-FA16:0 | 1.86 | 0.0666 |
| | TAG42:1-FA16:0 | 2.2 | 0.0934 |
| | TAG42:1-FA16:1 | 2.51 | 0.0764 |
| | TAG44:0-FA16:0 | 1.56 | 0.0993 |
| | TAG44:1-FA14:0 | 1.7 | 0.0905 |
| | TAG44:1-FA14:1 | 1.74 | 0.0831 |
| | TAG44:1-FA16:1 | 1.7 | 0.0873 |
| | TAG45:0-FA16:0 | 1.74 | **0.0446** |
| | TAG46:1-FA14:1 | 1.68 | 0.071 |
| | TAG46:1-FA16:0 | 1.61 | 0.0781 |
| | TAG46:1-FA16:1 | 1.65 | 0.0904 |
| | TAG46:2-FA14:1 | 1.82 | 0.0875 |
| | TAG46:2-FA16:1 | 1.78 | 0.0954 |
| | TAG48:1-FA16:1 | 1.62 | 0.0881 |