Global untargeted lipidomics.

Standards.

Standards for quality assurance and control (to assess process variability and instrument variability) were procured from Avanti Polar Lipids (Birmingham, AL) and included deuterated SPLASH LIPIDOMIX mass spectrometry standard and exogenous lipid standards: N-heptadecanoyl-D-erythro-sphingosine (C17 ceramide (d18:1/17:0)) and D-glucosyl-β1-1'-D-erythro-sphingosine (glucosyl (β) sphingosine (d18:1)).

Sample Preparation and Lipid Extraction.

The iBAT and pWAT was harvestedat sacrifice from mice at W24. Samples were immediately frozen in liquid nitrogen followed by -80˚C storage before analysis. Both WT (n=4) and cKO (n=4) mice were analyzed. Adipose tissue samples, ranging from 10-30 mg, were thawed on ice and mixed with 1 mL of cold 1:1:2 (v:v:v) methanol MeOH:ACN:H_2O with 50 mM ammonium bicarbonate lysis buffer. Samples were homogenized using a tissue homogenizer operated at 20,000 rpm for 10 seconds to break the tissue, then vortex mixed for 10 seconds. An appropriate volume of lysate was transferred from each sample such that individual samples were normalized based on tissue amount. Following volume adjustment to 200 μL, 800 μL of cold MeOH was added to the samples. Individual samples were vortexed for 30 seconds and incubated overnight at -80°C for protein precipitation. Following incubation, samples were centrifuged for 15 min at 15,000 rpm at 4°C and the supernatant was transferred to a new labeled tube and dried down using a cold vacuum centrifuge.

Samples were reconstituted in 100 μL H_2O, 100 μL MeOH, and 10 μL of SPLASH LIPIDOMIX with vortex mixing after each addition. Samples were incubated at room

temperature for 10 min followed by liquid-liquid extraction. For liquid-liquid extraction (LLE), 800 µL MTBE was added with vortex mixing for 30 seconds followed by incubation on ice for 10 min andcentrifugation at 15,000 rpm for 15 minutes at 4°C. An 800 µL portion of the upper (hydrophobic) fraction was transferred and dried down using cold vacuum centrifuge. Extracts were stored, dry, at 4°C for four days. Prior to analysis, dried extracts were reconstituted in 100 µL reconstitution solvent consisting of 9:1 (v:v) methanol:$CHCl_3$ containing exogenous sphingolipid standards (n=2) to assess instrument variability. A pooled quality control (QC) sample was prepared by pooling equal volumes (15 µL) from each individual sample following reconstitution.

Liquid Chromatography-Mass Spectrometry Data Acquisition.

Prepared samples were analyzed by LC-IM-MS/MS in the Center for Innovative Technology (CIT) at Vanderbilt University. Chromatographic separation was performed using an Agilent 1290 LC system fitted with a Hypersil Gold column (1.9 µM, 2.1 mm x 100 mm) held at 40°C. Mobile phase A was H_2O with 10 mM ammonium acetate and 0.1% formic acid additives. Mobile phase B consisted of 60:36:4 (v:v:v) ACN:IPA:H_2O with 10 mM ammonium acetate and 0.1% formic acid additives. The flow rate was kept at 0.25 mL/min across the 30 min LC gradient. The autosampler was held at 4°C, and the injection volume was 5 µL with needle wash enabled. The Agilent 6560 instrument was controlled by MassHunter Acquisition software (B.09, Agilent) and tuned in low (1700 *m/z*) mass range. Electrospray ionization (Dual AJS, Agilent) source conditions were optimized for lipidomic analyses and operated in positive ionization mode with gas temperature of 280°C, drying gas flow of 5 L/min, nebulizer at 10 psi, sheath gas

temperature of 300°C, sheath gas flow at 11.8 L/min, capillary at 3500 V, nozzle at 2000 V, and octupole RF at 750 V_{pp}.

Data acquisition included calibration solution (Agilent Tuning Mixture) acquired at the beginning and end of analysis and used for mass error quality assurance. Solvent blanks and standard blanks were acquired at the beginning of the analysis to ensure there were no spectral contaminants and to generate an exclusion list for the MS/MS method. A QC sample, was injected four times immediately preceding the first sample to condition the LC column, and the QC was injected again after every four samples to assess instrument repeatability through principle component analysis. Samples were injected in randomized order, and a randomly selected 10% of samples were reinjected at the end of the worklist for quality control assessment. Fragmentation spectra were acquired via three iterative, top two, data dependent analysis MS/MS acquisitions.

Data Analysis.

Data analysis was performed using Progenesis QI software (version 3.0, Nonlinear Dynamics, Newcastle, UK). Retention time alignment, peak picking, and peak deconvolution used default parameters. Spectra were normalized to all compounds, and data were filtered for coefficients of variance < 25% in QC technical replicate injections. A prioritized compound list was generated via a one-factor ANOVA, with four experimental groups for comparison including wild type and Vps34 knockout for both brown and visceral adipose tissue. Lipids were considered to be differentially altered if the p-value < 0.05 and the fold change was greater than I2I. Significantly changed compounds were selected for annotation.

Lipidomic annotations were performed using a previously described classification system with compounds being assigned a confidence level of 1 to 5 (1 being the highest confidence) with improved confidence requiring more supporting evidence such as accurate mass, MS/MS fragmentation, and retention time matching to standards. Lipid annotated were performed with reference to in-house and online databases (MS-DIAL, LipidMatch, and Lipid Annotator). Differentially abundant lipids (DALs) were uploaded into the LIPEA algorithm for pathway enrichment analysis. Corrected p-values were calculated using Benjamini correction and a p-value <0.05 was used to determine significantly affected pathways.