

Supplemental Methods

Generating HepG2-CERS2^{-/-} cells

HepG2-CERS2^{-/-} cells were generated using two CRISPR/Cas9 knockout kits. Initially, HepG2 cells were transfected with *CERS2* gRNA and donor vector containing a GFP-puromycin functional cassette (KN200145, Origene, Rockville, MD) using Turbofectin 8.0 (Origene). The cells were then cultured according to the manufacturer's protocol. To help ensure the generation of *CERS2* biallelic knockouts, puromycin-resistant cells were subjected to a second round of CRISPR/Cas9 knockout using the same *CERS2* gRNA along with a new donor vector containing mBFP and neomycin (G418) selection genes (KN200145BN, Origene). After antibiotic selection using medium containing 2 µg/ml puromycin (Sigma-Aldrich, St. Louis, MO) and 1000 µg/ml G418 (EMD Millipore, Burlington, MA), the puromycin/G418 resistant cells were single-cell sorted using a Cytena CloneSelect single-cell printer (Molecular Devices, San Jose, CA). The single cells were then allowed to expand using DMEM complete media containing the two antibiotics, and 2 puromycin/G418 resistant clones were confirmed by RT-qPCR and immunofluorescence staining and were used for subsequent experiments.

Ceramide measurements

To examine the effects of the *CERS2* E115A variant on the synthesis of ceramides in HepG2 cells with and without palmitate treatment, a QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) was used to insert a point mutation into a *CERS2* expression plasmid (Origene) that changes the E115 codon (GAG) to an A115 codon (GCG). The A to C point mutation was confirmed by sequencing. HepG2-CERS2^{+/+} and HepG2-CERS2^{-/-} cells were transfected with either the pCMV6-AC empty vector (Origene) or pCMV6-

AC-*CERS2* expression plasmids carrying either the E115 (A) allele (*pCERS2^A*) or A115 (C) allele (*pCERS2^C*) using lipofectamine 3000 (Thermo Fisher Scientific). Forty-eight hours post-transfection, the untreated and palmitate treated (500 μ M final concentration) HepG2 cells were collected and ceramide levels were analyzed at the UCSD Lipidomics Core (1). Data are expressed as normalized intensities and constitute relative abundances per 1×10^6 cells (relative to exactly measured internal standards). To account for transfection efficiencies for the *pCERS2^A* and *pCERS2^C* plasmids, intensities were normalized with *CERS2* gene expression levels.

Differential gene expression analysis

HepG2-*CERS2*^{+/+} and HepG2-*CERS2*^{-/-} cells were transfected with either the pCMV6-AC empty vector or pCMV6-AC-*CERS2* expression plasmids containing either the E115 (A) allele (*pCERS2^A*) or A115 (C) allele (*pCERS2^C*) using lipofectamine 3000 (Thermo Fisher Scientific). Forty-eight hours post-transfection, the cells were collected for RNA isolation. Total RNA was extracted using a RNeasy Mini Kit (Qiagen, Hilden, Germany) and treated with DNase using RNase-free DNase set (Qiagen). The total RNA was sent to SeqMatic (Fremont, CA) for sequencing and featureCount was used to count the number of reads mapping to known genes as defined by Ensembl gene annotations. Differential expression comparisons were performed using DESeq2. Differentially expressed genes with ≥ 2 -fold change (\log_2 values ≥ 1.0 and ≤ -1.0) and $P_{\text{FDR}} < 0.04$ (FDR, false discovery rate by Benjamini-Hochberg method) were classified into KEGG pathways using the web-based David Functional Annotation tool, David Bioinformatics Resources 6.8 (2; 3). Selected differentially expressed genes were verified by reverse transcription quantitative real-time PCR (RT-qPCR) using TaqMan gene expression assays (Thermo Fisher Scientific) on a Quantstudio 12K Flex Real-Time PCR System (Thermo Fisher Scientific). Each

sample was run in triplicate and transcript levels were normalized using *ABL1* as the endogenous control (Hs01104728_m1, Thermo Fisher Scientific).

Complex I and citrate synthase activity assays

Complex I and citrate synthase activities were measured using Complex I Enzyme Activity Microplate Assay and Citrate Synthase Assay kits (Abcam, Waltham, MA). Cell pellets were resuspended in cold PBS and proteins were extracted using a detergent solution. Lysates were centrifuged at 16,000g for 20 minutes at 4 °C to remove detergent-insoluble material. Total protein concentration was determined using a DC Protein Assay kit (Bio-Rad, Hercules, CA). For the complex I activity assays, equal amounts of protein were loaded in triplicate for each sample onto precoated microplate 8 well strips and incubated for 3 hours at room temperature. After the addition of Assay Solution, absorbance was measured at 450 nm in kinetic mode using a BioTek Synergy Neo2 plate reader. For the citrate synthase assays, equal amounts of protein for each sample were loaded in triplicate into wells of a Greiner white flat bottom 96 well plate. Reactions were started by adding Reaction Mix and absorbance was measured at 412 nm in kinetic mode using a BioTek Synergy Neo2 plate reader. Complex I and citrate synthase activities are expressed as mOD/min.

Mitochondrial DNA copy number

Relative mitochondrial DNA (mtDNA) copy number was determined by duplex quantitative polymerase chain reaction (qPCR). DNA and RNA were isolated from HepG2-*CERS2*^{+/+}, HepG2-*CERS2*^{-/-}, HepG2-p*CERS2*^A, and HepG2-p*CERS2*^C cells using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen). The isolated DNA was evaluated and quantitated using a

NanoDrop One spectrophotometer (Thermo Fisher Scientific) and then diluted to 5 ng/μl which was used as the template for qPCR. Mitochondrial genome copy number was determined by simultaneous detection of mtDNA and nuclear genes in the same well using FAM-labeled TaqMan probes for MT-ND1 (Hs02596873_s1) or MT-ND6 (Hs02596879_g1) along with a VIC-labeled probe for the nuclear reference gene TERT (4401633) (Thermo Fisher Scientific). Each sample was run in triplicate and relative mtDNA copy number was determined using the $2^{-\Delta\Delta C_t}$ method.

Seahorse analyses

Real-time cellular respiration in response to fatty acid oxidation was measured using an XF Palmitate Oxidation Stress Test Kit (Agilent Technologies). HepG2-*CERS2*^{-/-} cells were seeded at a density of 2×10^6 in 25 cm² flasks cultured overnight and then transfected with 10 μg of the pCMV6-AC empty vector or pCMV6-AC-*CERS2* expression plasmids containing either the E115 (A) allele (p*CERS2*^A) or A115 (C) allele (p*CERS2*^C) using Lipofectamine 2000 (Thermo Fisher Scientific). The following day, the transfected cells were reseeded in a Seahorse XF96 cell culture microplate coated with Poly-L-Lysine and cultured overnight in substrate-limited growth media (RPMI 1640 plus 0.5 mM glucose, 1 mM L-glutamine, 1% fetal bovine serum, 0.5 mM L-carnitine). The next day, the substrate-limited growth medium was replaced with Seahorse assay medium (Agilent Seahorse XF RPMI Medium plus 2 mM XF glucose, 0.5 mM L-carnitine) and mitochondrial function was assessed using a Seahorse XFe96 analyzer (Agilent Technologies). Prior to starting the assay, Palmitate-BSA and BSA control were added to the XF96 microplate which was then loaded into an XFe96 analyzer. After baseline oxygen consumption rates (OCR) were measured, either assay media plus solvent (DMSO, control) or the fatty acid oxidation inhibitor etomoxir (4 μM) were added followed by a stepwise injection of mitochondrial

respiration inhibitors: 1.5 μ M oligomycin, 0.5 μ M FCCP, 0.5 μ M rotenone/antimycin A. After the assays were completed, cells were stained with Hoechst 33342 (1 μ g/ml) and cell number in each well was determined using a Celigo Image Cytometer (Nexcelom Biosciences, Lawrence, MA). OCR and extracellular acidification rates (ECAR) were normalized to 10,000 cells. Differences in mitochondrial respiration rates between the HepG2-pCERS2^A and HepG2-pCERS2^C cells were assessed via mixed model analyses of repeated measurements obtained during the Seahorse experiments using GraphPad Prism 9.1 (GraphPad Software, San Diego, CA).

Western blot analysis

To examine the effects of CERS2 E115A on AKT phosphorylation, 2 days post-transfection, HepG2-pCERS2^A and HepG2-pCERS2^C cells were serum starved for 6 hours and then treated with 100 nM of insulin (Sigma-Aldrich) for 15 minutes. Cell lysates were prepared in cell lysis buffer (Cell Signaling Technology, Danvers, MA) supplemented with a protease and phosphatase inhibitor cocktail (Abcam) and total protein concentration was determined using a DC Protein Assay kit (Bio-Rad). Equal amounts of protein were loaded onto 4–15% Mini-PROTEAN TGX gels (Bio-Rad) and separated by electrophoresis and then transferred to nitrocellulose membranes using a Trans-Blot Turbo Transfer System and Transfer packs (Bio-Rad) following the manufacturer's instructions. Nitrocellulose membranes were blocked with 5% BSA in tris buffered saline with Tween-20 (TBST) (Cell Signaling Technology) at room temperature for 1 hour. Primary antibodies were diluted in TBST containing 2% BSA and incubated with the nitrocellulose membranes overnight at 4°C. The next day, the membranes were washed 3 times with TBST and then incubated with HRP-conjugated secondary antibody diluted in TBST containing 2% BSA (Cell Signaling Technology) for 1 hour at room temperature.

Chemiluminescence was detected using either SignalFire ECL or SignalFire Elite ECL reagents (Cell Signaling Technology) and a ChemiDoc Touch Imaging System (Bio-Rad). Anti-Akt (#9272) and anti-phospho-Akt (Ser473) (#9271) antibodies were purchased from Cell Signaling Technology. Anti-phospho-Akt (Thr308) (600-401-2695) antibody was purchased from Thermo Fisher Scientific. Anti- β -actin (C4) (SC-47778) antibody was purchased from Santa Cruz Biotechnology (Dallas, TX).

Effects of insulin on gluconeogenic gene expression

Twenty-four hours post-transfection, HepG2-pCERS2^A and HepG2-pCERS2^C cells were serum starved for 24 hours and then treated with either 100 nM insulin (Sigma-Aldrich), 100 μ M dbcAMP (STEMCELL Technologies, Cambridge, MA) or 100 nM insulin plus 100 μ M dbcAMP for 6 hours. The HepG2 cells were collected for RNA isolation followed by cDNA synthesis using a High Capacity cDNA Reverse Transcription kit plus RNase Inhibitor (Thermo Fisher Scientific). RT-qPCR was performed using TaqMan gene expression probes for *G6PC1* and *PCK1* (Thermo Fisher Scientific). Each sample was run in triplicate and transcript levels were normalized using *ABLI* as the endogenous control.

References

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