Supplemental Experimental Procedures:

Materials

LPS from E. coli 055:B5 (#L2880), TO901317 (#T2320), GGTI-2133 (#G5294), SIGMA*FAST* Protease Inhibitor Tablets (#S8820) and recombinant insulin from bovine pancreas (#I6634) were purchased from Sigma-Aldrich (St. Louis, MO). Atorvastatin hemicalcium salt (#3776), LB42708 (#4294), zVAD-FMK (#2163) were purchased from Tocris Biosciences (Bristol, United Kingdom). Rat tail collagen type 1 (#354557) was purchased from BD Biosciences (Franklin Lakes, NJ). Recombinant murine TNFα (#315-01/CF) was purchased from Peprotech (Rocky Hill, NJ) and reconstituted in PBS+10% FBS. H-1152 (#555550), Y-27632 (#688000), and GST-Rhotekin Agarose Beads (#14-383) were purchased from EMD Millipore (Billerica, MA) and reconstituted in PBS. Adenoviruses expressing dominant negative IκBα (S32A/S36A) and empty vectors (#1028 and #1300, respectively) were purchased from Vector Biolabs (Malvern, PA). Protein G Dynabeads (#10003D) and Williams' E Media (#12551-032) were purchased from Life Technologies (Grand Island, NY). Recombinant human insulin (Novolin) was purchased from Novo Nordisk (Plainsboro, NJ). Triglyceride SL kit was purchased from Genzyme Diagnostics (Cambridge, MA). NEFA-HR and Cholesterol E kits were purchased from Wako (Richmond, VA). Total Bile Acid Kit and Ultra Sensitive Mouse Insulin ELISA Kit were purchased from Crystal Chem (Downers Grove, IL). Micro BCA Protein Assay Kit (#23235) was purchased from ThermoScientific (Grand Island, NY). RNA-Bee (#CS-501B) was purchased from Tel-Test, Inc (Friendswood, TX).

Antibodies

Phospho-AKT(Ser473) (#4058), AKT (#9272), phospho-JNK(Thr183/Thr185) (#9251), JNK (#9252), RHOC (#3430), phospho-MLC2(Ser19) (#3671), MLC2 (#3672) were purchased from Cell Signaling Techology (Danvers, MA). IRS-1 (#06-248), phospho-IRS-1(Ser307) (#07-247), CYP7A1 (#MABD42) were purchased from Millipore (Billerica, MA). ßACTIN produced in ascites fluid (#A5316) was purchased from Sigma-Aldrich (St. Louis, MO). Secondary antibody staining was performed using HRP conjugated goat anti-rabbit IgG (#111-035-144) and anti-mouse IgG (#115-035-146) purchased from Jackson ImmunoResearch (West Grove, PA).

Animal Care and *In Vivo* Experimental Design

All mice used in these studies were on the C57Bl/6J background. Wild type mice were obtained from Jackson Laboratories (Bar Harbor, ME), ApoE-Cyp7a1 transgenic mice have been described previously (Miyake et al., 2001) and were obtained from the University California Davis Mutant Mouse Regional Resource Center. IRS-1 serine 307 to alanine mutants were a kind gift of Morris White and Kyle Copps (Copps et al., 2010). Mice were maintained on regular chow (Harlan, 2018S) and in a specific pathogen free facility on a 12-hour light/dark cycle with continuous access to food and water. All animal studies were approved by the Yale University Institutional Animal Care and Use Committee.

Sustained inflammation was modeled using daily intraperitoneal injection of LPS at 1.5mg/kg, or PBS as vehicle only control, for up to 7 days. Mice receiving either PBS or LPS were cohoused. Experiments involving co-treatment with inhibitors, or vehicle, were performed simultaneously with LPS injection. Atorvastatin hemicalcium was used at 10mg/kg in 5% DMSO/10% Ethanol/85% PBS as vehicle. Y-27632 was used at 30mg/kg in PBS as vehicle.

Obesity was modeled by feeding mice a high fat diet (60% kCal from fat, Research Diets D12492i) for 12-weeks. Mice were maintained on the high fat diet while given daily injections of Y-27632 at 30mg/kg or PBS as control for 2 weeks, after which they were subjected to metabolic studies.

Metabolic Studies

For a glucose tolerance test, mice were fasted overnight on wire bottom cages after which they were injected intraperitoneally with 1g/kg (for high fat fed mice) or 2g/kg (for LPS treated mice) glucose. For insulin tolerance tests, mice were fasted for 4 hours on wire bottom cages and then injected intraperitoneally with 1U/kg (for high fat fed mice) or 0.75U/kg (for LPS treated mice) of human recombinant insulin. Blood was sampled from the retroorbital plexus before injection and at the indicated times after injection, and plasma glucose was measured using a OneTouch Ultra glucometer (LifeScan).

Hyperinsulinemic-Euglycemic Clamp Study

Hyperinsulinemic-Euglycemic clamp studies were performed by the Yale Mouse Metabolic Phenotyping Center. Briefly, mice had surgery performed 7 days prior to the hyperinsulinemic-euglycemic

clamp to establish a chronic catheter for intravenous infusion of substances (e.g., glucose, insulin) during the clamp. For this, a mouse is anesthetized with an intraperitoneal injection of ketamine and xylazine, and a catheter is inserted in the right jugular vein. Mice were then treated with PBS or LPS, as described above. On the day prior to the experiment, mice were fasted overnight in clean cages. On the day of the clamp experiment, an overnight-fasted mouse is placed in an over-sized restrainer (i.e., rat-sized) for the experiment to be conducted in an awake and minimally-stressed state. The tail is tethered using a tape for 2 hours prior to the start of experiment for acclimatization. A 3-way connector is attached to the jugular vein catheter for intravenous infusion, and the blood samples are obtained from the tail vessels requiring a small tail cut. A 2-hour hyperinsulinemic-euglycemic clamp is conducted with a primed-continuous infusion of human insulin at a rate of 15 pmol/kg/min to raise plasma insulin within a physiological range (~300 pM). Blood samples are collected at 10-20 min intervals for the immediate measurement of plasma glucose concentration, and 20% glucose is infused at variable rates to maintain glucose at basal concentrations (~6 mM). Insulin-stimulated whole body glucose metabolism is assessed with a continuous infusion of [3-3H]glucose (0.1 mCi/min) throughout the clamps. Basal rates of whole body glucose turnover are assessed using a primed-continuous infusion of [3-3H]glucose for 2 hours prior to the start of clamp. All infusions are performed using microdialysis pumps. To estimate insulin-stimulated glucose uptake in individual tissues, 2-deoxy-D-[1-14C]glucose (2-[14C]DG) is administered as a bolus (10 mCi) at 75 min after the start of clamp. Blood samples are taken at -5, 80, 85, 90, 100, 110, and 120 min of clamp for the measurement of plasma [3H]glucose, $[3H]_2O$, and/or 2-[14C]DG concentrations. Additional blood samples are collected before and at the end of clamp for the measurement of plasma insulin concentrations. At the end of clamp, mouse is anesthetized with sodium pentobarbital injection, and tissues are taken and stored for biochemical/molecular analysis. (Kim et al., 2004a; 2004b)

Serum Analysis

Serum was harvested from overnight fasted or ad libitum fed mice and analyzed for triglycerides, NEFAs, cholesterol, and bile acids using enzymatic kits. Insulin was measured using the Ultra Sensitive Mouse Insulin ELISA Kit.

In Vivo Tissue Analysis

For insulin signaling studies, animals were fasted overnight and then injected intraperitoneally with recombinant human insulin at 1U/kg. Animals were sacrificed 15 minutes after injection and tissues were harvested and snap frozen on liquid N_2. For all other tissue analysis, animals were treated as indicated in the figure legend, sacrificed by CO_2 asphyxiation, tissues harvested and snap frozen on liquid N_2. Harvested tissues were stored at -80˚C until further processing. Tissues were pulverized in liquid N_2 and either immediately subjected to further analysis or stored at -80˚C.

For western blotting analysis, protein was extracted for 30 minutes on ice in RIPA buffer (150mM NaCl, 50mM Tris-HCl pH 8.0, 1% NP-40, 0.5% Sodium Deoxycholate, 0.1% SDS, 20mM NaF, 17.5mM ß-glycerophosphate, 1mM Na_3VO_4, 10% glycerol) with protease inhibitors followed by sonication 3 times for 20 second bursts at 2.0/10.0 power while maintained on ice. Samples were centrifuged at 14,000RPM for 10 minutes at 4˚C, supernatants collected, and protein quantified using the BCA Assay. Sample protein concentration was equilibrated and subjected to western blot analysis.

For RT-qPCR analysis, 50mg (liver) or 150mg (adipose tissue) of pulverized tissue was placed in 1mL of RNA-Bee and homogenized by pushing through a 25½ gauge needle > 30 times. Liver samples were diluted 1:10 and RNA was extracted per manufacturers' instructions. RNA was extracted from adipose tissue samples using twice as much chloroform as indicated in the manufacturers' instructions.

Primary Hepatocytes

Plasma-treated tissue culture plates were coated with type 1 collagen from rat-tail at 50µg/mL in 20mM acetic acid for 4 hours at 37˚C, washed three times with Hanks Balanced Salt Solution, air dried, and stored at 4˚C until further use. Primary hepatocytes were freshly isolated by collagenase digestion of livers from *ad libitum* fed mice and cultured on collagen-coated plates in Wlliams' E Medium (WEM) supplemented with 5% FCS, 2mM L-glutamine, 10mM HEPES, 200U/mL penicillin, 200µg/mL streptomycin, 8µg/mL gentamycin, 50nM dexamethasone, 1nM insulin (WEM complete). Cells were cultured in WEM complete + 100µg/mL chloramphenicol for 4 hours, washed 1x with PBS, and then cultured in WEM complete overnight. All treatments were performed in WEM complete starting on the morning after isolation of hepatocytes. For signaling studies, samples were cultured in WEM supplemented

with 2mM L-glutamine, 10mM HEPES, 200U/mL penicillin, 200µg/mL streptomycin, 8µg/mL gentamycin, 50nM dexamethasone for 1 hour prior to stimulation with 100nM bovine insulin for 5 minutes.

Western Blotting and Immunoprecipitation

Samples were cultured and treated as indicated in the figure legends. For western blotting from primary hepatocytes, protein was extracted in 1x SDS buffer (150mM NaCl, 45mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.2% ß-mercaptoethanol) at room temperature followed by heating at 95˚C for 10 minutes. Equal volume of lysate was loaded per well of a 4-12% Bis-Tris mini-gel (Invitrogen) and run in 1x MOPS or 1x MES (Invitrogen) buffer. For samples from in vivo analysis, equal amounts of protein were loaded per well. Protein was transferred onto activated PVDF membrane (Millipore), blocked in 5% BSA in TBST (20mM Tris, 150mM NaCl, 0.05% Tween 20) for 1 hour at room temperature followed by incubation with 1˚ antibody in 5% BSA in TBST at 4˚C overnight. All primary antibodies were used at dilutions of 1:1000 except IRS-1 at 1µg/mL and ß-actin at 1:10,000. Samples were washed 3x with TBST, incubated with 2˚ antibody at 1:5000 dilution in TBST for 1 hour, and finally washed 3x with TBST. Protein was visualized using enhanced chemiluminescence (ThermoScientific) and autoradiography film. Blots were stripped by boiling in TBST for 30 seconds and then reprobed following above procedure. Densitometry was calculated using ImageJ software. Numbers above blots for phosphorylated serine 473 on AKT represent a ratio between pS473-AKT and total AKT or ßACTIN.

For immunoprecipitation, primary hepatocytes were washed 3x with ice cold PBS, and then lysed on ice in RIPA buffer with protease inhibitors for 15 minutes. Lysate was centrifuged at 14,000 RPM for 10 minutes at 4˚C and supernatants were moved to a separate tube. Some sample was set aside for input control and the remaining lysate was immunoprecipitated overnight while rotating at 4˚C with 4µg anti-IRS-1. The next day, sample was added to 40µl recombinant Protein G Dynabeads and was rotated at 4˚C for 1-2 hours. Samples were washed 3x with ice cold RIPA buffer with protease inhibitors, protein eluted using 2X SDS buffer, and run alongside input on 4-12% Bis-Tris mini-gel as above.

Activated Rho-GTPase Pulldown

Cells were treated as described, washed three times with ice cold PBS, and lysed in 300µl/well of lysis buffer (500mM NaCl, 50mM Tris HCl pH 7.2, 10mM $MgCl_2$, 0.1% SDS, 1% Triton X-100) + protease inhibitors. Lysate was centrifuged at 14,000RPM for 10 minutes at 4˚C and supernatant was transferred to new tube. 48µl of supernatant was taken for input analysis. 35µg of GST-Rhotekin Agarose Beads were added to remaining supernatant and samples were rotated end-over-end for 45 minutes at 4˚C. Samples were washed four times with wash buffer (150mM NaCl, 50mM Tris HCl pH 7.2, 10mM $MgCl_2$, 1% Triton X-100) + protease inhibitors, protein eluted from beads using 25µl of hot 2X SDS buffer, and samples resolved by SDS-Page and examined for expression of RHOC by western blot.

Lipid Extraction and Assays

Primary hepatocytes were treated as described, washed three times with ice cold PBS, and extracted using hexane/isopropanol (3:2) at room temperature for 1 hour. Solvent was blown off under N_2 and lipid residue was stored at -80˚C until it was assayed. Residual protein was solubilized in 10mM $NaBH_4$ + 1% SDS and quantified using the BCA assay. Tissue lipids were extracted from pulverized liver using the method of Bligh & Dyer and solvent was blown off under N_2 and lipid residue was stored at -80˚C until it was assayed. Tissue lipids were normalized to wet tissue weight.

Cholesterol was measured by dissolving pellet in Cholesterol E reagent and following manufacturer's instructions. Triglyceride and NEFA were measured by dissolving pellet in 1mL isopropanol and following manufacturer's instructions.

Dimethylallyl pyrophosphate (DMAPP), farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP) were assayed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Briefly, the lipid pellets were solubilized in 50% methanol/50% H_2O and analyzed by a Perkin Elmer Flexar UPLC System coupled to a 4000 Q-Trap LC-MS/MS system. The compounds were separated utilizing an Agilent Technologies ZORBAX Eclipse XDB-C18 (3.0 x 100mm, 3.5 micron pore size) column (p.n. 961967-302), coupled to an analytical Phenomenex SecurityGuard trap (C18, 4 x 3.0mm). The column and trap were kept at 40°C. Briefly, the samples were eluted at a flow rate of 500 µL/min using a Methanol:Water-based mobile phase. Buffer A is 100% Water and buffer B is 100% MeOH. The table below shows the gradient that was utilized.

Total Time (min)	**Flow Rate (uL/min)**	**A%**	**B%**
0.1	500	50	50
0.5	500	50	50
.8	300	5	95
2	300	5	95
2.2	300	0	100
4	300	0	100
4.1	300	50	50
10	500	50	50

A blank injection of 100% MeOH was run after each sample injection to ensure no carry over. Optimization of the differential potential (DP) and collision potential (CE) were carried out utilizing negative mode direct injection of standard compounds (Cayman Chemicals, Ann Arbor, MI). Mixture of the standard was then carried out to determine the best gradient (above) to use for the sample runs. Standard curves of individual standards were used to calibrate specific transitions for each of the molecules. Note that we have three transitions per compounds that we were monitoring, but only one transition (in **bold**) was utilized in our quantitation measurements. The table below summarizes the transitions utilized for each compounds and their corresponding DP and CE optimization parameters. HP mix standards were utilized as an external control to ensure instruments data acquisition stability and reproducibility for our specific gradient and instrument parameter settings.

Precursor Ion(Da)	Product Ion(Da)	Time (msec)	Target Compound (ID)	DP	CE
381.2	363.1	40	FPP381A	-85	-26
381.2	159.2	40	FPP381B	-80	-28
381.2	**78.9**	**40**	**FPP381C**	**-60**	**-60**
245.2	227.1	40	DMAPP245A	-50	-18
245.2	159.1	40	DMAPP245B	-50	-20
245.2	**78.9**	**40**	**DMAPP245C**	**-45**	**-34**
449.2	431.2	40	GGPP449A	-85	-30
449.2	159.1	40	GGPP449B	-80	-36
449.2	**78.9**	**40**	**GGPP449C**	**-90**	**-74**

Source and gas conditions are presented in the table below and were optimized based on the standard direct injection runs. Data were acquired on the 4000 QTRAP instrument utilizing Analyst 1.5.2 and resulting collected raw data were processed utilizing Multiquant software (v. 2.0).

SOURCE / GAS Conditions	
Curtain gas (CUR)	10
Collision gas (CAD)	low
Ion Spray Voltage (IS)	-3700
Temperature (TEM)	400
Ion Source Gas1 (GS1)	15

Ion Source Gas2 (GS2)	0
Interface Heater Temperature (IHT)	ON

RNA Analysis

RNA was extracted from primary hepatocytes using RNA-Bee according to manufacturer's instructions. cDNA was synthesized from equal amounts of RNA using SMART MMLV Reverse Transcriptase (Clontech #639524). RT-qPCR was run using Low ROX PerfeCTa SYBR Green SuperMix (Quanta #95056) according to manufacturer's instructions on a CFX96 Real-Time PCR C1000 Thermal Cycler (BioRad) and analyzed using the ΔCt or ΔΔCt method. Primer sequences used for each gene are available upon request.

Microarray Analysis

RNA was extracted as described above and then subjected to a cleanup step using an RNeasy Mini Kit with on-column DNase digestion (Qiagen). Expression profiles were then assayed on an Illumina MouseWG-6 v2.0 Expression BeadChip according to manufacturer's protocols. Hybridized BeadChips were scanned using an Illumina BeadArray reader and .IDAT files were processed using GenePattern software (Broad Institute) according to established protocols. Relative expression changes were determined using established pipelines made available on GenePattern. Pathway analysis was conducted using the NIH's Database for Annotation, Visualization and Integrated Discovery (Huang et al., 2009a; 2009b) on genes with expression changes > 1.5 fold compared to untreated. Raw data files have been deposited in the NCBI Gene Expression Omnibus under accession number GSE67422.

RNA Sequencing Analysis

RNA was extracted from 30mg of pulverized liver tissue. Tissue was homogenized in 1mL of RNA Bee in FastPrep Lysing Matrix tubes using a FastPrep-24 5G homogenizer (MP Biomedicals) and samples were then extracted per manufacturer's instructions, followed by a cleanup step with RNeasy Mini Kit with on-column DNase treatment (QIAGEN). cDNA libraries were prepared using TruSeq Stranded mRNA Library Prep Kit (Illumina). Paired-end RNA sequencing was performed on a NextSeq 500 (Illumina) using a NextSeq 500 High Output Kit at 150 cycles. FastQ files were processed using TopHat (v2.1.0) and Cufflinks (v2.2.1) (Trapnell et al., 2012) and aligned to the mm10 build from UCSC. Data was analyzed using the cummeRbund (v2.12.0) and WGCNA (v1.48) packages in R (Langfelder and Horvath, 2008; 2012). Gene Ontology and KEGG Pathway analysis were performed using the NIH's DAVID software on pathway specific genes (for WGCNA) and genes with expression change > 1.5 fold compared to untreated. Raw data files have been deposited in the NCBI Gene Expression Omnibus under accession number GSE75477.

Statistics

Data are represented as mean ± SEM. Statistical analysis was performed in Graph Pad PRISM 6. Data is judged to be statistically significant when $p < 0.05$ by two-tailed Student's T-Test or 2-way ANOVA, where appropriate. Outliers were excluded based upon analysis by the ROUT Test with Q=0.2% (selecting for only definitive outliers). In figures, asterisks denote statistical significance as calculated by Student's T-test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$) while pound signs denote statistical significance by 2-way ANOVA (#, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$; ####, $p < 0.0001$) as compared to untreated controls, unless otherwise specified by lines connecting the compared pieces of data. Pearson correlation coefficients were calculated with PRISM and statistical significance is based upon the assumption that values exhibit a Gaussian distribution.

Supplementary References

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