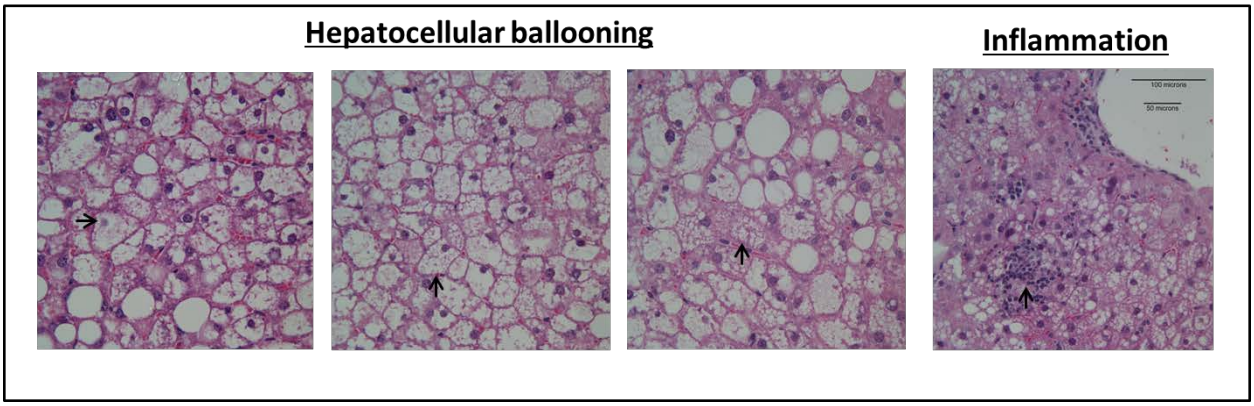
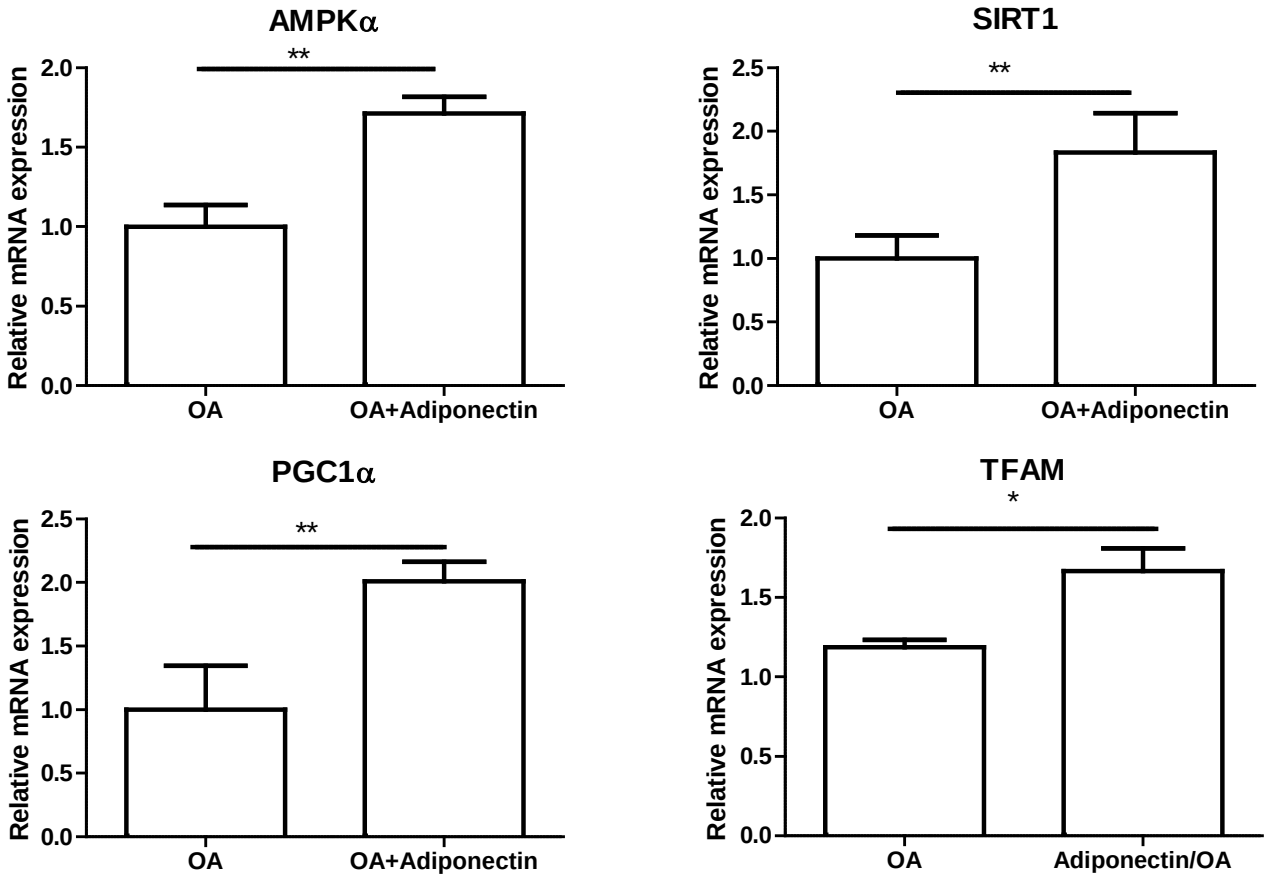
Supplementary Information

Supplementary Figure 1:



Supplementary Figure legend 1: Representative micrographs of ballooning (630 X magnification, arrows) and inflammation (400X) in different NASH mice showing the extent of liver injury. Arrows indicate ballooned hepatocytes and inflammatory infiltrates.

Supplementary figure 2:



Supplementary figure legend 2: Freshly isolated rat hepatocytes were serum starved for 24 hours. They were then either treated with 400 uM oleic acid (OA) alone or OA in the presence of recombinant adiponectin (20 μg/ml) for 24 hours. After the treatment period, RNA was isolated and gene expression analysis performed. Relative gene expression levels of AMPKα, SIRT1, PGC1α and TFAM are shown. Representative data obtained from three separate experiments with similar outcomes is shown. *P<0.05, **P<0.01. n=3.

Supplementary Table 1. Mouse Primer sequences used for RT-qPCR.

Gene	Forward Primer Sequence	Reverse Primer Sequence
Gapdh	GCCCTTCCACAATGCCAAAG	TGATGGGTGTGAACCACGAGA
Il6	AAGTCCGGAGAGGAGACTTC	CCTCCGACTTGTGAAGTGGT
Tnfα	CTCTTCAAGGGACAAGGCTG	GGAGGTTGACTTTCTCCTGG
Crp	GCAGAGTCAAAGAAGCCACTG	AGAGAAGACACTGAAGCTGCG
Nfkb1	GCCTGCAAAGGTTATCGTTC	GTCCTTGGGTCCTGCTGTTA
Dgat2	CTCTCCAAGAATGGGAGTGG	TCTTCAGGGTGACTGCGTTC
Adipoq	GACAAGGCCGTTCTCTTCAC	CCAGATGGAGGAGCACAGA
Hif1α	CACCAGACAGAGCAGGAAAG	GACAGCTTAAGGCTCCTTGG
C/ebpα	CAAGAACAGCAACGAGTACCG	GTCACTGGTCAACTCCAGCAC
Tfam	CCTTCGATTTTCCACAGAACA	GCTCACAGCTTCTTTGTATGCTT
Nrf1	CCATCTATCCGAAAGAGACAGC	GGGTGAGATGCAGAGTACAATC
Pgc1α	AAACTTGCTAGCGGTCCTCA	TGGCTGGTGCCAGTAAGAG
Cox4	AGTTCAGTTGTACCGCATCCAG	GGGCCATACACATAGCTCTTCT
Sirt1	GGGTGAGATGCAGAGTACAATC	TTGTGGTTTTTCTTCCACACA
Adipor2	GGATGTGGAAGTCGTGTGTG	ACCTGGTCAAACGAGACACC
Cd68	ACTTCGGGCCATGGTTCTCT	GGCTGGTAGGTTGATTGTCGT
Emr1/F480	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
PGC1β	AGGAAGCGGCGGGAAA	CTACAATCTCACCGAACACCTCAA
SCD1	CCGGAGACCCCTTAGATCGA	TAGCCTGTAAAAGATTTCTGCAAACC
Ccl2/Mcp1	GGCTGGAGAGCTACAAGAGG	TCTTGAGCTTGGTGACAAAAAC
FAS	GCTGCGGAAACTTCAGGAAAT	AGAGACGTGTCACTCCTGGACTT

Primers for genes discussed in the manuscript are presented above in the 5'- to 3'-direction.

Supplementary Table 2. Correlation coefficients for serum adiponectin and *Adipoq* gene expression versus expression of inflammatory genes *(Il6, Tnfα, Nfkb1, Crp)* and *C/ebpα* in epididymal white adipose tissue.

vs.	Serum Adiponectin		Log *Adipoq*	
	NASH	NAFL	NASH	NAFL
Log *Il6*	-0.68	0.53	-0.89*	-0.07
Log *Tnfα*	-0.94*	0.47	-0.55	-0.28
Log *Nfkb1*	-0.92*	0.29	-0.60	-0.10
Log *Crp*	-0.59	0.32	-0.92*	-0.18
Log *C/ebpa*	0.95*	-0.64	0.17	0.61

Pearson correlation coefficients (r) are presented for associations of the given variables within the NAFL and NASH groups. *Adipoq*, adiponectin; *Il6*, interleukin-6; *Tnfα*, tumor necrosis factor alpha; *Nfkb1*, Nuclear factor NF-kappa-B p50; *Crp*, C-reactive protein; *C/ebpα*, CCAAT/enhancer-binding protein alpha . *P<0.05.

Supplementary Table 3. Rat primer sequences used for RT-qPCR.

Name	**Sequence**
Rat SIRT1 F	GATCTCCCAGATCCTCAAGCC
RAT SIRT1 R	CACCGAGGAACTACCTGAT
RAT AMPKα F	GGGATCCATCAGCAACTATCG
RAT AMPKα R	GGGAGGTCACGGATGAGG
RAT TFAM1 F	GCTTCCAGGAGGCTAAGGAT
RAT TFAM1 R	CCCAATCCCAATGACAACTC
RAT PGC1α F	AGGTCCCCAGGCAGTAGAT
RAT PGC1α R	CGTGCTCATTGGCTTCATA
β-ACTIN F	AGGGAAATCGTGCGTGAC
β-ACTIN R	CGCTCATTGCCGATAGTG

Supplementary materials and methods

Details of the diet for the animal studies involving mice

The 71% fat content of the high-fat diet consists of approximately 13% saturated fatty acids (SFAs), 31% mono-unsaturated fatty acids (MUFAs), and 56% poly-unsaturated fatty acids(PUFAs) by mass of which the major species were linoleic acid (PUFA, 55.7%), oleic acid (MUFA, 30.6%), palmitic acid (SFA, 10.7%), and stearic acid (SFA, 2.2%).

Histological analysis of liver tissue

Scores of steatosis, lobular inflammation and ballooning were used to give the mice the diagnosis of "DM," "NAFL", or "NASH." All mice scored with steatosis, inflammation, and ballooning were given a diagnosis of NASH (n=6) (3 from each the 5 and 10 week Lepr$^{db/db}$ HF mice). Mice with only steatosis were classified as NAFL (n=8, all Lepr$^{db/db}$ mice, 5 HF/3 N). All remaining Lepr$^{db/db}$ mice fed normal chow that lacked steatosis were classified as diabetes mellitus (DM, n=8, Lepr$^{db/db}$ N).

Animal studies involving rats

8 week old male Sprague Dawley rats (~250 g) were purchased from Charles River Laboratories Inc (Wilmington, MA, USA). Rats were housed under standard laboratory conditions with free access to standard laboratory chow and water. Prior to isolation, rats were anesthetized using a combination of xylazine/ketamine (i.p. injection). Experiments were

approved by the Institutional Animal Care and Use Committee of the Benaroya Research Institute and were performed following their guidelines.

Rat Hepatocyte Isolation

Hepatocytes were isolated from rats by a two step collagenase perfusion method. Briefly, the liver is perfused with HBSS (Invitrogen) followed by 0.5% collagenase (Sigma) until the liver is optimally digested. The hepatocytes are removed by low speed centrifugation (50xg). After isolation, hepatocytes were plated on collagen (Invitrogen)-coated 12-well or 24-well dishes in William's E medium (Invitrogen) with 0.005 mg/mL insulin, 0.005 mg/ml transferrin, 5 ng/ml selenium (Invitrogen), 40 ng/ml dexamethasone (Sigma), Sodium pyruvate and 10% FBS. Cells were cultured in a humidified incubator at 37°C and 5% CO2. Confluent cultures were used for experiments as described in the supplementary figure legend 2. Briefly, cells were grown in complete rat hepatocyte media till 80% confluent. Primary cells were treated with isopropanol (0.8% IPA, data not shown) as a control (since the 50 mM oleic acid stocks were made in 100% isopropanol) or lipid-loaded with 400 μM oleic acid (OA) in the presence or absence of adiponectin (20 μg/ml) for 24 hours. Prior to treatment with IPA, OA alone, or adiponectin and OA, cell cultures were treated with 1% FBS containing rat hepatocyte media for 24 hours.

Cell culture and reagents

Alpha mouse liver cells (AML-12) cells are well characterized, non–malignant hepatocytes of murine origin[1-3]. AML-12 cells were obtained directly from the originating Fausto laboratory[1,2] and cultured in Dulbecco's modified Eagle's medium/F-12 50/50 (Invitrogen, cat. no. 10-090-CV; Cellgro) with 0.005 mg/mL insulin, 0.005 mg/ml transferrin, 5 ng/ml selenium (Invitrogen), 40 ng/ml dexamethasone (Sigma), and 10% FBS[3]. Recombinant mouse adiponectin protein (prepared in HEK cells) was obtained from Enzo Lifesciences, MA. Cells were grown in complete AML-12 media till 80% confluent. Just before the experiment, they were treated

overnight in AML-12 media containing 1% FBS with or without adiponectin supplementation(20 µg/ml). We used reduced serum containing media for AML-12 cells to determine the effect of the exogenously supplemented adiponectin on mitochondrial biogenesis and minimize the effect of the substantial amount of circulating adiponectin in Fetal Bovine Serum (FBS). In separate experiments, AML-12 cells were treated with 0.5% IPA as a control or lipid-loaded with 250 µM OA in the presence or absence of adiponectin (20 µg/ml) for 24 hours. Prior to treatment with adiponectin and OA, cells were treated with 1% FBS containing AML-12 media for 48 hours.

Apoptosis Quantification

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was performed using ApopTag Plus Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon, Billerica, MA) per manufacturer's instruction with the following exceptions. A 2-Solution DAB Kit (Invitrogen, Carlsbad, CA) was substituted for the Peroxidase Substrate. DAB was prepared just prior to use and 5 µl of $NiCl_2$ (8% in dH_2O)/ml of DAB was added to enhance visualization of dye. Specimens were counterstained using 2% methyl green for 4 min at room temperature followed by three washes each of dH_2O, absolute ethanol, and xylene. Quantification of apoptosis was determined by counting apoptotic cells in five random fields per animal at 200X magnification. Because the number of cells in each field varies widely between normal, non-diseased mice and those with NAFL or NASH due to the presence of steatotic hepatocytes, and therefore may influence the estimation of apoptosis, it was important to obtain a measure of cellularity for each field. To estimate the total number of cells/field, one representative field per mouse was selected and overlaid with a 10x10 grid in ImageJ (NIH, Bethesda, MD). Ten squares within the grid were analyzed for the number of cells and this number was multiplied by 10 to approximate the total number of cells per field. Percent apoptosis was expressed as the average number of apoptotic cells/average total number of cells for each group.

Gene expression analyses

Total RNA was isolated (from AML-12 cells, primary hepatocytes liver tissue, EWAT), using the Qiagen RNeasy kit (Valencia, CA). RNA was reverse-transcribed using a cDNA synthesis kit (Applied Biosystems) and analyzed by quantitative real-time PCR (RT-PCR) using an ABI 7900HT instrument. Levels of the target mRNAs were calculated relative to the reference gene, Glyceraldehyde-3 Phosphate dehydrogenase (GAPDH) for the mouse-related studies (AML-12, mouse liver and EWAT) and β-actin for rat hepatocytes, using the ΔΔCt formula. RT-PCR primer sequences are shown in the supplementary Table 1 and 3.

Western blot analysis

Protein was extracted from liver or EWAT samples and quantified (Micro BCA Protein Assay Kit; Pierce, Rockford, Il). Equal amounts of protein lysates were used for immunoblotting using gradient 4-20% Tris-Glycine gels (Biorad, Hercules, CA). Blots were probed using the following primary antibodies: phospho- (thr-172)-AMPKα, phospho-(Ser72) -ACC α, SIRT1, LKB1 or GAPDH at 1:1000 dilution (Cell Signaling Technologies, Danvers, MA), adiponectin receptor-2 , PGC1α antibodies (1:500, Abcam, Cambridge, MA) and mac-2 (1:1000, Cedarlane). Corresponding secondary antibodies were obtained from Santa Cruz Biotechnology, CA. Densitometric quantitation of the blots were performed using Image J analysis software (NIH, Bethesda, MD).

Biochemical assays

Serum and plasma were separated by centrifugation at 4°C and stored at -80°C until analyzed. Serum glucose and ALT levels were determined using the Beckman DXC 800 Analyzer (Brea, CA). Insulin, TNFα, IL-6, leptin, and adiponectin were measured in plasma using a Millipore multiplex panel (Billerica, MA) following the manufacturer's instructions.

References:

1. Wu JC, Merlino G, Fausto N. Establishment and characterization of differentiated, nontransformed hepatocyte cell lines derived from mice transgenic for transforming growth factor alpha. Proc Natl Acad Sci U S A. 1994; 91:674-8.
2. Messner DJ, Rhieu BH, Kowdley KV. Iron overload causes oxidative stress and impaired insulin signaling in AML-12 hepatocytes. Dig Dis Sci**.** 2013; 58:1899-908.
3. Tateya S, Rizzo-De Leon N, Handa P, Cheng AM, Morgan-Stevenson V, Ogimoto K, Kanter JE, Bornfeldt KE, Daum G, Clowes AW, Chait A, Kim F. VASP increases hepatic fatty acid oxidation by activating AMPK in mice. Diabetes. 2013; 62:1913-22.