Supplementary information

Supplementary experimental procedures

Glucose and insulin tolerance tests

Mice were fasted for 6 hours and then given an i.p. injection of glucose 2 g/kg of body weight of glucose (G8270, Sigma Aldrich) or 0.7 U/kg of body weight of insulin (Actrapid, Novo nordisk). Blood glucose was measured from the tail vein at the time points indicated in the figure. Area under curve (AUC) values were determined.

Oil Red O, hematoxylin&eosin and sirius red staining.

Liver biopsies were frozen at -80°C, and 8μm sections cut with a cryostat and stained in filtered oil Red O for 10 minutes. Sections were washed in distilled water, counterstained with Mayer´s hematoxylin for 3 minutes, and mounted in aqueous mounting (glycerin jelly).

Liver samples were fixed in 10% buffered formalin for 24 h and then dehydrated and embedded in paraffin by a standard procedure. Sections of 4μm were cut with a microtome and stained using a standard hematoxylin&eosin (H&E) alcoholic procedure according to the manufacturer´s instructions (BioOptica). Sections were then rinsed with distilled water, and dried for 30 minutes at 37°C. They were then mounted with permanent (non-alcohol, non-xylene based) mounting media, and evaluated and photographed using a BX51 microscope equipped with a DP70 digital camera (Olympus).

For Sirius Red staining, samples were fixated in paraffin. These sections were de-waxed, hydrated and stained in PicroSirius staining red for one hour. After two washes with acidified water, samples were dehydrated in three changes of 100% ethanol,

cleared in xylene and mounted in a resinous medium. Collagen depositions in Sirius Red-stained sections were analyzed using FRIDA software (Framework for Image Dataset Analysis) http://bui3.win.ad.jhu.edu/frida/.

Liver triglyceride content

Frozen liver samples (0.5g) were homogenized for 3 minutes in 500 µl of chloroform-methanol (2:1, vol/vol). Lipids were extracted during 3-h shaking at room temperature. For phase separation, 300µl of milli-Q water was added, samples were centrifuged and the organic bottom layer was collected. The organic solvent was dried overnight and triglyceride content of each sample was measured in duplicate with a colorimetric assay (1001314, Spinreact).

Levels of serum metabolites and hormones

Mice whole trunk blood was collected in a 1.5mL-tube. The blood was left at room temperature15-30 minutes and then was centrifuged for 15 minutes at 3000xg at 4°C to remove the clot; for serum collection, the supernatant was transferred to a new tube and stored at -20°C until analyze metabolites and hormones. Serum cholesterol (1001093, Spinreact), free fatty acids (436-91995,434-91795, WAKO), glucose (1001191, Spinreact), ALT (41283, Spinreact), AST (41273, Spinreact), leptin and insulin (MMHMAG-44K-0.2, Merk-Millipore), adiponectin (EZMADP-60K) were measured by spectrophotometry in a ThermoScientific Multiskan GO spectrophotometer. Troponin levels were measured using RF421C Troponin I (CTNI) from Siemens and the Siemens Dimension RxL Max analyzer.

RNA isolation and quantitative real-time PCR (qRT-PCR) analysis.

Total liver RNA was extracted with Trizol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions, and quantified with Nanodrop 1000 (Thermo Fisher Scientific) followed by cDNA synthesis. For each reverse transcriptase (RT) reaction we used 100ng of total RNA, and the total volume of the reaction mix was 30µl in the subsequent proportions: 6µl buffer (5x first strand buffer), 6µl dNTPs mixtures 2.5mM, 3µl $MgCl_2$ 25mM, 0.17µl random primers (3µg/µl), 0.25 RNaseOUT®, 40U/µl), 1µl m-MLV reverse transcriptase (200U/µl), corresponding µl of RNA sample and H_2O DEPC to complete final volume. The reagents for RT were purchased from Invitrogen. The RT reactions were carried out using the following cycling parameters: 37°C for 1h, 42°C for 15 min and 95°C for 5 min.

Each quantitative RT-PCR (qRT-PCR) was performed in duplicate using Brilliant III Ultra-Fast SYBR Green master mix (Agilent) in a 12µl reaction containing 1:3 diluted cDNA and 0.3µM of each primer, using a fluorescent temperature cycler (Applied Biosystems) following the manufacturer's instructions and the following conditions: 93°C for 3 min, 40 cycles of 95°C for 5 s, 60°C 32 s and melt curve 95°C 15 s, 60°C 1 min. Quantification was performed by the 2ΔΔCT method. The primers sequence used are shown in supplementary table 2.

Western blot analysis

Total protein lysates from liver (20µg), were subjected to SDS-PAGE, transferred onto a polivinylidene difluoride membrane (BioRad) and probed with antibodies indicates in supplementary table 3. For protein detection, we used horseradish peroxidase-conjugated secondary antibodies and chemiluminiscence (Amersham Biosciencies). Afterwards, membranes were exposed to radiograph film (Super RX Fuji Medical X-Ray Film, Fujifilm) and developed with developer and fixing liquids (AGFA) under

appropriate dark room conditions. Protein expression was quantified by densitometric analysis with Image J software (http://rsbweb.nih.gov/ij/). Protein levels were normalized to GAPDH or α-tubulin.

Cell culture

The THLE2 human liver cell line was purchased from ATCC (The Global Bioresource Center) and grown in bronchial epithelial cell basal medium (BEBM), supplemented with a growth factor BulleKit (Lonza/Clonetics Corporation), 70ng/mL phosphoethanolamine, 5 ng/mL epidermal growth factor, 10% fetal bovine serum (FBS) and 1% Glutamine-Penicillin-Streptomycin solution (Sigma Aldrich).

HepG2 cells, a cell line derived from a human hepatocellular carcinoma, were obtained from ECACC (European Collection of Cell Cultures) and maintained as a monolayer culture in Minimum Essential Medium Eagle (Sigma Aldrich) supplemented with 10% FBS, 1% Glutamine-Penicillin-Streptomycin solution and 1% Non-Essential Amino acid (NEAA) (growth medium) (Sigma Aldrich). Both cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Doxorubicin treatment in p53-silenced THLE2

THLE2 cells were seeded at a a density of $3\times10^4/cm_2$ in 6-well plates, after 24 h in culture, cells were transfected with specific small-interference RNA (siRNA) to knock down the expression of p53 (L-003329-00, Dharmacon). The transfection was performed using Dharmafect 1 reagent (Dharmacon) following the protocol: 50 or 100 pmol of the sip53 diluted in 200μl of optiMEM (Life Technologies) was mixed with 6.5 μl of Dharmafect 1 diluted in 193.5 μl of optiMEM; the mixture was added into each well, resulting in a final volume of 1.5ml with BEBM complete medium supplemented

with 5% FBS for 8 h. After that, the medium was replaced with fresh complete medium another 16 h until 25nM Doxorubicin (Tedec-Feji Farma) or vehicle was added. Cells were exposed to Doxorubicin for 48 h. THLE2 cells were collected for protein extraction and coverslips were placed in a 24-well with 300 µl of BEBM and incubated at 37ºC for 40 minutes with 2,4 µl of BODIPY 493/503 (green) to label lipid droplets (Thermo Fisher). Coverslips were washed with phosphate-buffered saline (PBS) and fixed with 4% formaldehyde for 10 minutes. The coverslips were mounted in aqueous medium (Fluoro-Gel 17985-10, Etectron Microscopy Sciences) with 4,6-diamino-2-phenylindole (DAPI) 1:1000 (blue). Confocal images were taken with a Leica confocal microscope (Leica A0B5-SP5) equipped with a high-grade color corrected plan apochromat lens for confocal scanning 63x/1.32 objective. Leica Confocal software was used for acquisition and analysis. Images are combinations of optical sections taken in the z axis at 0.5 mm intervals. To BODIPY 493/503 staining, cells excited at 492 nm with the laser and fluorescence emission was acquired in a 414-479-nm band. Silencing was checked by western blotting.

Doxorubicin treatment in hepatocyte cell lines incubated with oleic acid.

THLE2 cells were seeded at a density of $3\times10^4/cm^2$ in 6-well plates. Cells were exposed to either 25nM Doxorubicin or the vehicle for 48 h. Next, the medium was removed and replaced with cell culture medium supplemented with 1 mM of oleic acid (Sigma Aldrich) bound to fatty-acid-free bovine serum albumin (BSA) (Capricorn Scientific) (2:1 molar ratio) for 24 h to induce triglyceride accumulation. Controls were supplemented with BSA alone. After treatments, coverslips were placed in another 24-well plate to be stained with BODIPY and DAPI. The confocal images were taken and analyzed as described above. The same protocol was followed for the HepG2 cell line,

adjusting the initial cell-seeding density to 1×10^5 cells/cm^2 and using Doxorubicin at a dose of 50nM. Additionally, HepG2 cells were collected for protein extraction.

De novo lipogenesis

HepG2 were seeded at a density of 3x104/cm^2 in the plate and incubated with Minimum Essential Medium Eagle (EMEM) supplemented with 10% FBS (v/v) and 1% Glutamine-Penicillin-Streptomycin-non-essential amino acids (v/v) (growth medium) until 40% of cell confluence was achieved. Then, the medium was removed and replaced by fresh growth medium supplemented with or without 50 nM Doxorubicin and cells were incubated with this media for 48 hours. After that, control or doxorubicin medium was replaced by new FBS-free control or doxorubicin medium supplemented with 0.5 mM fatty acid free BSA complexed with or without 1mM oleic acid. The cells were, then, incubated for another 24 hours with these media.

For de novo lipogenesis the FBS-free medium with Doxorubicin or the vehicle and/or BSA:oleic complex was supplemented with glucose to a final concentration of 22 mM overnight. Then, cells were incubated for 4 hours with fresh EMEM supplemented with 84 nM insulin, 75 µM fatty acid-free BSA, 1% glutamine (v/v) and 20 µM acetate containing 20 µCi/ml (^{3}H) -acetate (Perkin Elmer (Boston, MA)) (29). After the incubation, the cells were harvested and washed twice with ice cold phosphate buffered saline (PBS) (pH 7.4). Cells and medium were separated, lipids were extracted and separated by thin layer chromatography (5). Lipid classes were visualized by exposure to iodine vapor, the corresponding bands were scraped, and the label incorporated into lipids was determined by scintillation counting and expressed relative to the cell protein.

Fatty acid oxidation

The rate of fatty acid oxidation was determined by measuring the amount of $^{14}CO_2$ (complete oxidation) and the amount of ^{14}C labeled acid-soluble metabolites (incomplete oxidation) released, as described by others with minor modifications (5, 30). Briefly, cells were incubated for 4 hours with EMEM supplemented with 1% glutamine (v/v) and 75 μM fatty acid-free BSA complexed with 0.2 mM palmitate containing 0.5 μCi/ml (1-^{14}C)-palmitate (Perkin Elmer Inc (Waltham, MA)). Medium was then collected in a tube containing Whatman filter paper soaked with 0.1 M NaOH in the cap and 500 μl of 3 M perchloric acid were added to release the CO_2, which was captured in the filter paper. The acidified medium was centrifuged at 21,000 xg for 10 min to remove particulate matter. The radioactivity of CO_2 captured by the filter papers and the radioactivity in acid-soluble metabolites (the supernatants of the culture media) was measured by a scintillation counter.

Supplementary figure legends

Supplementary figure 1. Dose-response effects of doxorubicin on Swiss mice fed a chow diet. Swiss mice were randomly treated with either saline or doxorubicin at different doses injected intraperitoneally daily for 5 days. Cumulative body weight (A), food intake (B) and body composition (C). Values are mean ± standard error of the mean of 10 animals per group. *p<0.05, **p<0.01, ***p<0.001 versus control group.

Supplementary figure 2. Doxorubicin treatment ameliorates high fat diet-induced liver steatosis. C57BL6 mice fed a high fat diet (HFD) (60% fat) for 2 months were randomly treated with either water or doxorubicin (10 and 20 mg/Kg) co-administrated orally with quercetin (15 mg/Kg) twice a week for 50 days. Cumulative body weight (A), food intake (B) and body composition (C). Representative photomicrographs of hematoxylin&eosin (upper panel) and oil red O staining (lower panel) of liver sections (D) and liver TG content (E). Serum levels of AST and ALT (E). Glucose tolerance test 24 hours after a single oral administration of doxorubicin (F). Values are mean ± standard error of the mean of 6-8 animals per group. *p<0.05, **p<0.01, ***p<0.001 versus control group.

Supplementary figure 3. **Silencing of p53 in THLE2 cells inhibits the p53 signaling pathway.** p53 protein levels in THLE2 cells after p53 silencing with a siRNA (A). p21 and BAX protein levels in THLE2 cells after p53 silencing with a siRNA treated with vehicle or doxorubicin (B). GAPDH was used to normalize mRNA and protein levels. Values are mean ± standard error of the mean of 8 animals per group. *p<0.05, **p<0.01, ***p<0.001 versus control group.

Supplementary figure 4. **Topoisomerase II is not altered by low doses of doxorubicin.** Topoisomerase II protein levels in the liver of mice fed a HFD (60%) and treated intraperitoneally with vehicle or doxorubicin (0.3 and 0.6 mg/kg) twice a week during 60 days (A). Topoisomerase II protein levels in the liver of mice fed a CD-HFD and treated orally with vehicle or doxorubicin (20 mg/kg) co-administrated orally with quercetin (15 mg/kg) for 2 weeks (B).

Supplementary figure 5. Uncropped blots for Figure 1.

Supplementary figure 6. Uncropped blots for Figure 2E.

Supplementary figure 7. Uncropped blots for Figure 3.

Supplementary figure 8. Uncropped blots for Figure 4.

Supplementary figure 9. Uncropped blots for Figure 5.

Supplementary figure 10. Uncropped blots for Figure 6A.

Supplementary figure 11. Uncropped blots for Figure 6F.

Supplemental table 1. Serum troponin levels.

Diet	Treatment	Troponin (ng/mL)
HFD (45%)	Vehicle i.p.	0.0125
	Dox 0.6 mg/Kg i.p.	0
HFD (60%)	Vehicle i.p.	0.32 ± 0.18
	Dox 0.6 mg/Kg i.p.	0.22 ± 0.14
MCD	Vehicle orally	0.22 ± 0.15
	Quer + dox 20 mg/Kg orally	0.84 ± 0.84
CDHFD	Vehicle orally	0.063 ± 0.03
	Quer + dox 20 mg/Kg orally	0.03 ± 0.008
HFD (60%)	Vehicle i.p.	0.123 ± 0.02
	Quer + dox 20 mg/Kg orally	0.04 ± 0.03
Control +		102.5

Supplemental table 2. Serum cholesterol and free fatty acids (FFA) levels.

Diet	Treatment	Cholesterol (mg/dL)	p value	FFA (mg/dL)	p value
HFD (45%)	Vehicle i.p.	221.47 ± 19.26	*0,029*	206.22 ± 17.48	*0,022*
	Dox 0.6 mg/Kg i.p.	158,2 ± 22.9		143,01 ± 21,77	
HFD (60%)	Vehicle i.p.	182.12 ± 9.86		124.2 ± 17.4	n.s.
	Dox 0.15 mg/Kg i.p.	188.43 ± 14.84	n.s.	86.19 ± 10.12	
	Dox 0.3 mg/Kg i.p.	190.27 ± 11.26	n.s.	90.92 ± 5.49	n.s.
	Dox 0.6 mg/Kg i.p.	157.6 ± 15.35	n.s.	69.4 ± 10.42	*0.02*
MCD	Vehicle orally	13.41 ± 2.28		74.76 ± 6.92	n.s.
	Quer + dox 20 mg/Kg orally	20.93 ± 3.12	n.s.	60.3 ± 3.51	
CDHFD	Vehicle orally	68.36 ± 5.16		38.62 ± 2.12	n.s.
	Quer + dox 20 mg/Kg orally	77.57 ± 2.51	n.s.	41.62 ± 2.81	
HFD (60%)	Vehicle i.p.	143.92 ± 12.45		24.8 ± 1.34	n.s.
	Quer + dox 20 mg/Kg orally	151.52 ± 3.5	n.s.	28.5 ± 3.7	

Supplemental table 3. Primers used for gene amplification.

Name	5´3´	Sequence
Acc	FW RV	TGGGCGGGATGGTCTCTTT AGTCGCAGAAGCAGCCCATT
Acox	FW RV	CAGGAAGAGCAAGGAAGTGG CCTTTCTGGCTGATCCCATA
Acadm	FW RV	AGGTTTCAAGATCGCAATGG CTCCTTGGTGCTCCACTAGC
Acadl	FW RV	GCATCAACATCGCAGAGAAA GGCTATGGCACCGATACACT
Abcd1	FW RV	CCTGTCTGGAGGTGAGAAGC TGCAGCAAGTGTGTGTGGTA
Fasn	FW RV	TGGGTTCTAGCCAGCAGAGT ACCACCAGAGACCGTTATGC
Fatp2	FW RV	ATGCCGTGTCCGTCTTTTAC GACCTGTGGTTCCCGAAGTA
Pparα	FW RV	TCTGTGGGCTCACTGTTC AACTACCTGGTCAGGGCTCA
ApoB	FW RV	TCAGAGGGAACTGGAGCACT CGGAGACTGACCTCAAAAGC
Tnfα	FW RV	AGCCCCCAGTCTGTATCCTT CTCCCTTTGCAGAACTCAGG
Il6	FW RV	AGTTGCCTTCTTGGGACTGA TCCACGATTTCCCAGAGAAC
Arginase	FW RV	GTGAAGAACCCACGGTCTGT CTGGTTGTCAGGGGAGTGTT
F480	FW RV	TGCATCTAGCAATGGACAGC GCCTTCTGGATCCATTTGAA
Collagen III	FW RV	GCACAGCAGTCCAACGTAGA TCTCCAAATGGGATCTCTGG
Hprt	FW RV	TGCTGACCTGCTGGATTACATT CCCCGTTGACTGATCATTACAGTA

Supplemental table 4. Antibodies used for western blot.

Name	Manufacter Catalog number	Species reactivity	Dilution used
Anti-Acetyl CoA Carboxylase 1, ACC	Millipore 04-322	Rabbit monoclonal	1:5000
Phospho-Acetyl-CoA Carboxylase (Ser 79), pACC	Cell Signaling 3661S	Rabbit polyclonal	1:1000
Bax	Cell Signaling 2772	Rabbit polyclonal	1:1000
Anti-p53 (1C12)	Cell Signaling 2524	Mouse monoclonal	1:1000
Phospho-p53 (Ser15)	Cell Signaling 9284	Rabbit polyclonal	1:1000
Anti-Fatty Acid Syntase, FAS	Abcam Ab128870	Rabbit monoclonal	1:5000
Phospho-SAP/JNK (Thr183/Tyr185) (81E11), pJNK	Cell Signaling 4668	Rabbit monoclonal	1:1000
c-Jun N-terminal kinase 1/3 (C-17), JNK	Santa Cruz Biotechnology sc-474	Rabbit polyclonoal	1:1000
IRE 1 alpha phosphospecific [Ser 724], pIRE1	Novus Biologicals NB100-2323	Rabbit polyclonal	1:1000
X-Box binding protein 1, XBP1	Santa Cruz Biothecnology sc-7160	Rabbit polyclonal	1:1000
CHOP (GADD R-20)	Santa Cruz Biothecnology sc-793	Rabbit polyclonal	1:1000
IL6	Abcam ab6672	Rabbit polyclonal	1:1000
IL1β	Novus Biologicals NBP1-19775	Rabbit polyclonal	1:1000
IL10	Abcam ab9969	Rabbit polyclonal	1:1000
NFκβ p65	Santa Cruz Biotechnology sc-372	Rabbit polyclonal	1:1000
p21 (C19)	Santa Cruz Biotechnology sc-397	Mouse polyclonal	1:1000
α-tubulin	Sigma Aldrich T5168	Mouse monoclonal	1:5000
Topoisomerase	Abcam ab52934	Rabbit monoclonal	1:1000
α-tubulin	Sigma Aldrich T5168	Mouse monoclonal	1:5000
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Merck Millipore CB1001	Mouse monoclonal	1:5000