SUPPLEMENTARY DATA

Supplementary Materials

The research complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All procedures were approved by the University of Barcelona Bioethics Committee, as stated in Law 5/21 July 1995 passed by the Generalitat de Catalunya.

Reagents

Control and HRI siRNA were purchased from Santa Cruz (Dallas, TX) and PPARβ/δ siRNA from GE Dharmacon (Lafayette, CO). Human and mouse FGF21 neutralizing antibodies were purchased from Antibody and Immunoassays Services (Hong Kong, China).

Plasma FGF21 was measured using a rat/mouse FGF21 ELISA kit (Millipore, Bedford, MA). Serum glucose (Bayer Iberia, Sant Joan Despí, Spain), triglyceride (Sigma) and free fatty acid (FFA) (Wako, Japan) levels were measured using commercial kits.

Cell culture

Human HepG2 and Huh-7 and mouse Hepa1c1c7 cells were purchased from the ATCC and cultured in DMEM supplemented with 10% serum, at 37°C/5% CO2. siRNA transfections were performed with Lipofectamine 2000 (Life Technologies).

Synthesis of N,N'-diarylureas

Step 1. (2-Amino-5-nitrophenyl) disulphide 2

15 g (83.4 mmol) 6-nitrobenzothiazole 1 was suspended in abs. ethanol (300 mL). Hydrazine hydrate (30 mL, 600 mmol) was added and the mixture was refluxed for 2 h, converting from a yellow mixture to a dark red solution. The reaction was cooled to 30 °C and 50% hydrogen peroxide (16.2 mL) was added in small portions, maintaining the temperature with an ice water bath. On completion, the red color disappeared and a yellow precipitate formed. The suspension was stirred for 1 h, the precipitate was collected, washed with water and diethyl ether, and dried to give 11.71 g of product 2. Yield: 83.5%. 1H-NMR (400 MHz, DMSO) δ: 6.85 (d, J = 9.2 Hz, 2 H, 3-H), 7.21 (b. s., 4 H, NH2), 7.51 (d, J = 2.6 Hz, 2 H, 6-H), 7.99 (dd, J = 9.2 Hz, J' = 2.6 Hz, 2 H, 4-H).

Step 2. 6-Nitro-1,2,3-benzothiadiazole 3

11.68 g (34.5 mmol) (2-amino-5-nitrophenyl) disulphide 2 was dissolved in concentrated sulfuric acid (96 mL), chilled to 0 °C, and 5.45 g (86.3 mmol) sodium nitrite was added in small portions, while the temperature was kept under 10 °C. The reaction was allowed to warm to room temperature and stirred for 18 h. The reaction mixture was poured into a mixture of 650 g ice and water (65 mL), stirred for 1 h, and the pale brown precipitate was collected, then washed with water and diethyl ether. The crude product was taken up in dichloromethane (1 x 300 mL), filtered, and the filtrate was treated with a small amount of charcoal (1 g), filtered, and evaporated to give 5.29 g of a brown solid. Yield: 42.3% NMR: 90%. Column chromatography on alumina (Hexane 95%/Ethyl acetate 5% mixture) gave product 3 as a yellow solid (3.45 g). 1H-NMR (400 MHz, CDCl3) δ: 8.51 (dd, J = 9.2 Hz, J' = 2.0 Hz, 1 H, 5-H), 8.80 (d, J = 9.2 Hz, 1 H, 4-H), 9.04 (d, J = 2.0, 1 H, 7-H). 13C-NMR (100.5 MHz, CDCl3) δ: 115.7 (CH,

C7), 122.0 (CH, C5), 124.8 (CH, C4), 141.1 (C, C3a), 147.7 (C, C7a), 159.5 (C, C6).

Step 3. 6-Amino-1,2,3-thiadiazole 4

2 g (11.0 mmol) 6-nitro-1,2,3-benzothiadiazole 3 was added to a solution of 10.6 g tin (II) chloride in concentrated hydrochloric acid (16 mL) at 55 °C, using a water bath to maintain that temperature. The reaction mixture was heated to 70 °C for 10 min, then cooled to 4 °C and let stand for 18 h. The crystalline precipitate was collected and washed with ice cold water. The solid was dissolved in water (40 mL) and 10 N sodium hydroxide (10 mL) was added followed by ethyl acetate (30 mL) and the mixture was stirred for 15 min. The layers were separated; the aqueous layer was extracted with, then ethyl acetate (1 x 30 mL, 1 x 20 mL). The organic layers were combined, washed with saturated brine (1 x 20 mL), water (1 x 20 mL) and evaporated to give 0.97 g of product 4. Yield: 58%. 1H-NMR (400 MHz, CDCl3) δ: 4.20 (b. s., 2 H, NH2), 6.92 (dd, J = 9.2 Hz, J' = 2.4 Hz, 1 H, 5-H), 7.13 (d, J = 2.4 Hz, 1 H, 7-H), 8.34 (d, J = 9.2, 1 H, 4-H). 13C-NMR (100.5 MHz, CDCl3) δ: 100.4 (CH, C7), 117.0 (CH, C5), 124.5 (CH, C4), 143.8 (C, C3a), 148.1 (C, C7a), 152.9 (C, C6).

1.44 g (7.9 mmol) 6-nitro-1,2,3-benzothiadiazole 3 was added to a solution of 7.6 g tin (II) chloride in concentrated hydrochloric acid (12 mL) at 55 °C, using a water bath to maintain that temperature. The reaction mixture was heated to 70 °C for 10 min, then cooled to 4 °C and let stand for 18 h. The crystalline precipitate was dissolved in water (20 mL) and 10 N sodium hydroxide (5 mL) was added followed by ethyl acetate (20 mL) and the mixture was stirred for 15 min. The layers were separated; the aqueous layer was extracted with, then ethyl acetate (1 x 20 mL, 1 x 10 mL). The organic layers were combined, washed with saturated brine (1 x 20 mL), water (1 x 20 mL) and evaporated to give 0.84 g of product 4. Yield: 70%

Step 4a. 1-(1,2,3-Benzothiadiazol-6-yl)-3-(4-chloro-3-(trifluoromethyl)phenyl)urea 5

0.5 g (3.31 mmol) 6-Amino-1,2,3-thiadiazole 4, and 0.81 g (3.64 mmol) 4-chloro-3-(trifluoromethyl)phenyl isocyanate were dissolved in tetrahydrofuran (13 mL), and stirred at room temperature for 2.5 h; a white precipitate formed. IR (ATR) ν: 659, 679, 720, 749, 782, 812, 840, 863, 875, 910, 949, 1026, 1130, 1173, 1220, 1249, 1268, 1321, 1352, 1403, 1469, 1484, 1545, 1573, 1708, 3094, 3132, 3266, 3302, 3339. 1H-NMR (400 MHz, CDCl3) δ: 7.63-7.67 (c. s., 2 H, 5'-H, 6'-H), 7.69 (dd, J = 9.2 Hz, J' = 2.0 Hz, 1 H, 5-H), 8.17 (d, J = 2.0 Hz, 1 H, 2'-H), 8.59 (d, J = 9.2 Hz, 1 H, 4-H), 8.63 (d, J = 2.0, 1 H, 7-H), 9.38 (b. s., 1 H, NH), 9.52 (b. s., 1 H, NH). 13C-NMR (100.5 MHz, CDCl3) δ: 106.8 (CH, C7), 117.0 (q, J = 6.1 Hz, CH, C2'), 119.8 (CH, C5), 122.76 (q, J = 273.2 Hz, C, CF3), 122.83 (q, J = 2.0 Hz, C, C4'), 123.3 (b. s., CH, C5'), 123.6 (CH, C4), 126.7 (q, J = 31.0 Hz, C, C3'), 132.0 (CH, C6'), 138.9 (C, C1'), 140.8 (C, C3a), 142.2 (C, C7a), 152.2 (C, C6), 153.8 (C, CO). Calcd for C14H8ClF3N4OS · 2/3 H20: C 43.70, H 2.45, N 14.56. Found: C 43.75, H 2.43, N 14.60.

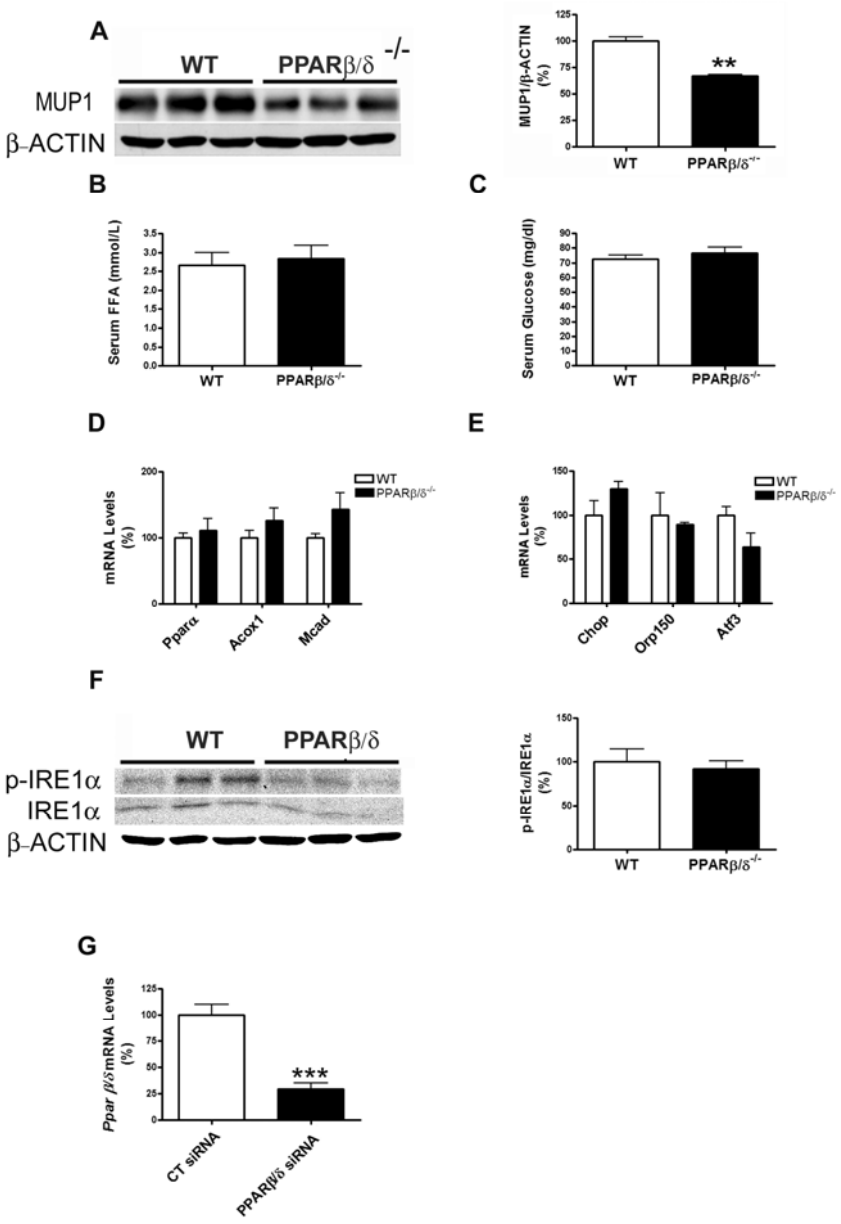
Step 4b. 1-(1,2,3-Benzothiadiazol-6-yl)-3-(3,4-dichlorophenyl)urea 6

0.5 g (3.31 mmol) 6-Amino-1,2,3-thiadiazole 4, and 0.68 g (3.64 mmol) 3,4dichlorophenyl isocyanate were dissolved in tetrahydrofuran (13 mL), and stirred at room temperature for 2.5 h; a white precipitate formed. Methanol (0.3 mL) was added and the mixture was stirred for 15 min, cooled to 0 °C in an ice-water bath, stirred for 30 min, and the precipitate was collected and washed with ice cold tetrahydrofuran to give 0.71 g of product 6 (68% yield), mp 267 – 269 °C (dec). IR (ATR) ν: 685, 731, 746, 805, 856, 881, 910, 1026, 1060, 1129, 1220, 1265, 1304, 1351, 1385, 1417, 1455, 1467, 1531, 1567, 1673, 3098, 3171, 3306. 1H-NMR (400 MHz, CDCl3) δ: 7.38 (dd, J = 9.2 Hz, J' = 2.4 Hz, 1 H,

6’-H), 7.55 (d, J = 9.2 Hz, 1 H, 5’-H), 7.69 (dd, J = 9.2 Hz, 1 H, J’ = 2.0 Hz, 1 H, 5-H), 7.91 (d, J = 2.4 Hz, 1 H, 2’-H), 8.56-8.60 (c. s., 2 H, 4-H, 7-H), 9.20 (b. s., 1 H, NH), 9.47 (b. s., 1 H, NH). 13C-NMR (100.5 MHz, CDCl3) δ: 106.7 (CH, C7), 118.6 (CH, C6’), 119.6 (CH, C2’), 119.7 (CH, C5), 123.58 (C, C4), 123.64 (C, C4’), 130.6 (CH, C6’), 131.1 (C, C3’), 731, 746,139.5 (C, C1’), 140.8 (C, C3a), 142.2 (C, C7a), 152.1 (C, C6), 153.8 (C, CO). Calcd for C13H8Cl2N4OS: C 46.03, H 2.38, N 16.52. Found: C 45.89, H 2.36, N 16.10.

SUPPLEMENTARY DATA

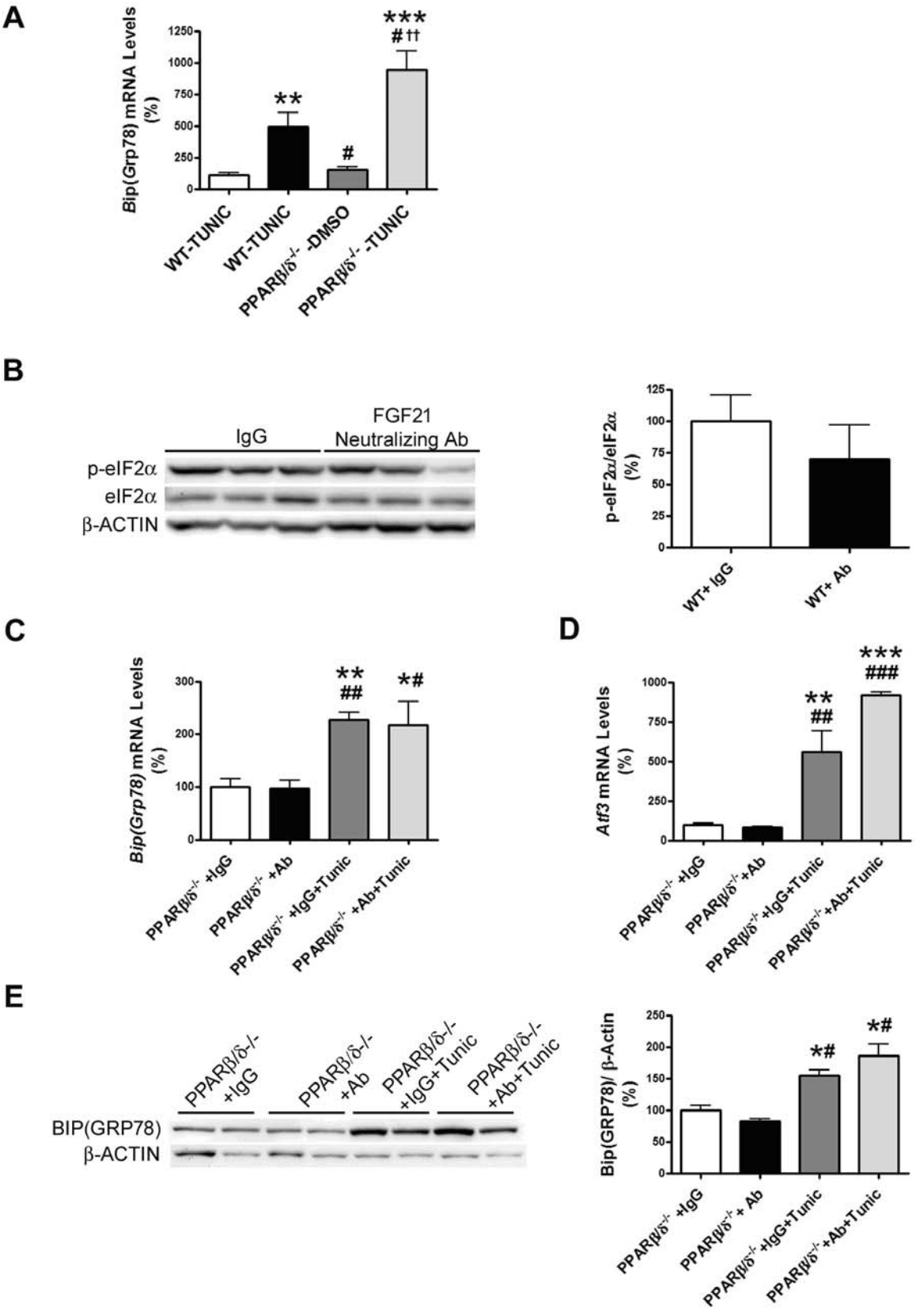
Supplementary Figure 1. A, liver cell lysates from male wild-type and PPARβ/δ-null mice were assayed for Western-blot analysis with antibodies against MUP-1 and β-actin. Data are presented as the mean ± S.D. (n=6 per group) relative to the wild-type mice. Serum free fatty acids (FFA) (B) and glucose (C) levels in wild-type and PPARβ/δ-null mice. D, mRNA levels of hepatic *Pparα*, *Acox1* and *Mcad*. E, mRNA abundance of *Chop*, *Orp150* and *Atf3*. F, immunoblot analyses of total and phospho-IRE1. Data are presented as the mean ± S.D. (n=6 per group). G, *PPARβ/δ* mRNA abundance in primary hepatocytes transfected with control siRNA or PPARβ/δ siRNA for 24 h. ***p<0.001 and **p<0.01 vs. wild-type mice.



SUPPLEMENTARY DATA

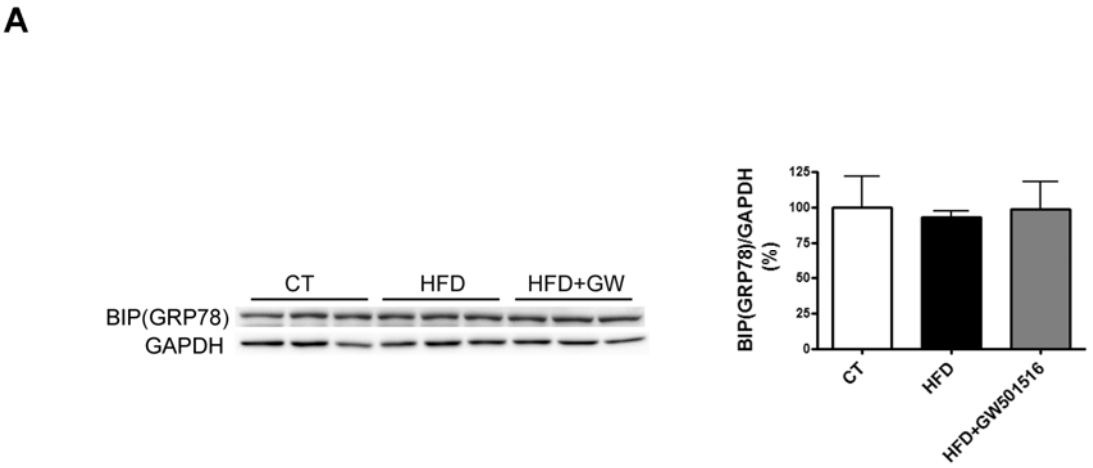
Supplementary Figure 2. A, Male wild-type and PPARβ/δ-null mice at 12 weeks of age were treated for 24 h through intraperitoneal injection with DMSO (vehicle) or tunicamycin (Tunic) (3 mg kg^{-1} body weight) and the mRNA abundance of hepatic *BiP* was determined. B, male wild-type mice were injected intraperitoneally with IgG or a neutralizing antibody (Ab) against FGF21. Immunoblot analyses of total and phospho-eIF2α. Data are presented as the mean ± S.D. (n=6 per group). Male wild-type and PPARβ/δ-null mice were injected intraperitoneally with IgG or a neutralizing antibody (Ab) against FGF21 together with DMSO or tunicamycin (Tunic) (3 mg kg^{-1} body weight). Mice were sacrificed at 14 h after treatment. mRNA abundance of hepatic *BiP* (C) and *Atf3* (D). Data are presented as the mean ± S.D. (n=6 per group). E, immunoblot analyses of hepatic BiP. Data are presented as the mean ± S.D. (n=4 per group). $^{***}p<0.001$, $^{**}p<0.01$ and $^{*}p<0.05$ *vs.* PPARβ/δ-null mice treated with IgG and DMSO. $^{###}p<0.001$, $^{##}p<0.01$ and $^{#}p<0.05$ *vs.* PPARβ/δ-null mice treated with neutralizing antibody against FGF21 and DMSO. $^{†††}p<0.001$ *vs.* PPARβ/δ-null mice treated with IgG and tunicamycine.

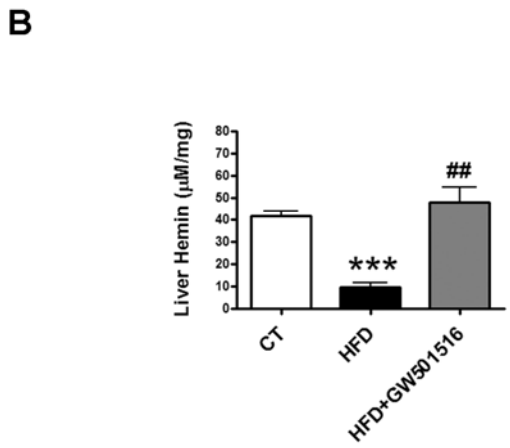
SUPPLEMENTARY DATA



SUPPLEMENTARY DATA

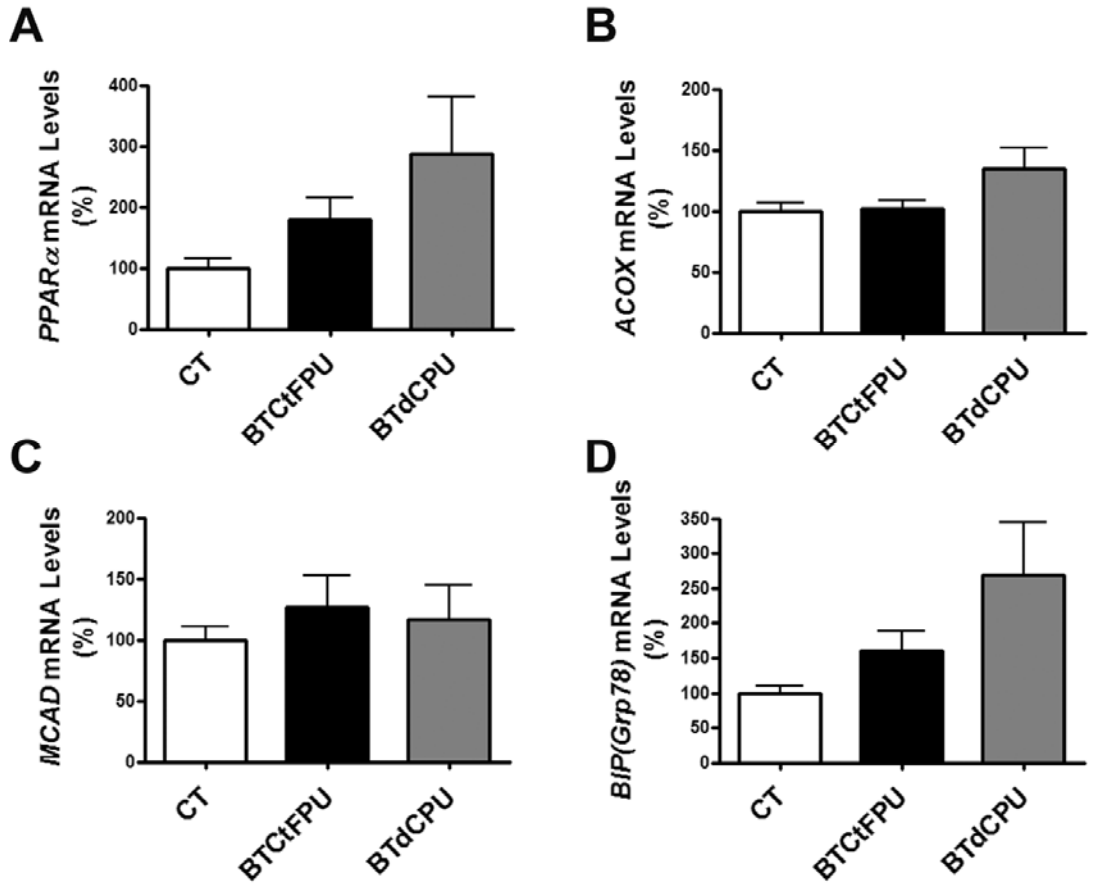
Supplementary Figure 3. Male mice were fed a standard chow or HFD with or without GW501516 (3 mg kg^{-1} day^{-1}). Animals were sacrificed after three weeks of treatment. A, Immunoblot analyses of BiP. B, hemin levels. Data are presented as the mean ± S.D. (n=6 per group).





SUPPLEMENTARY DATA

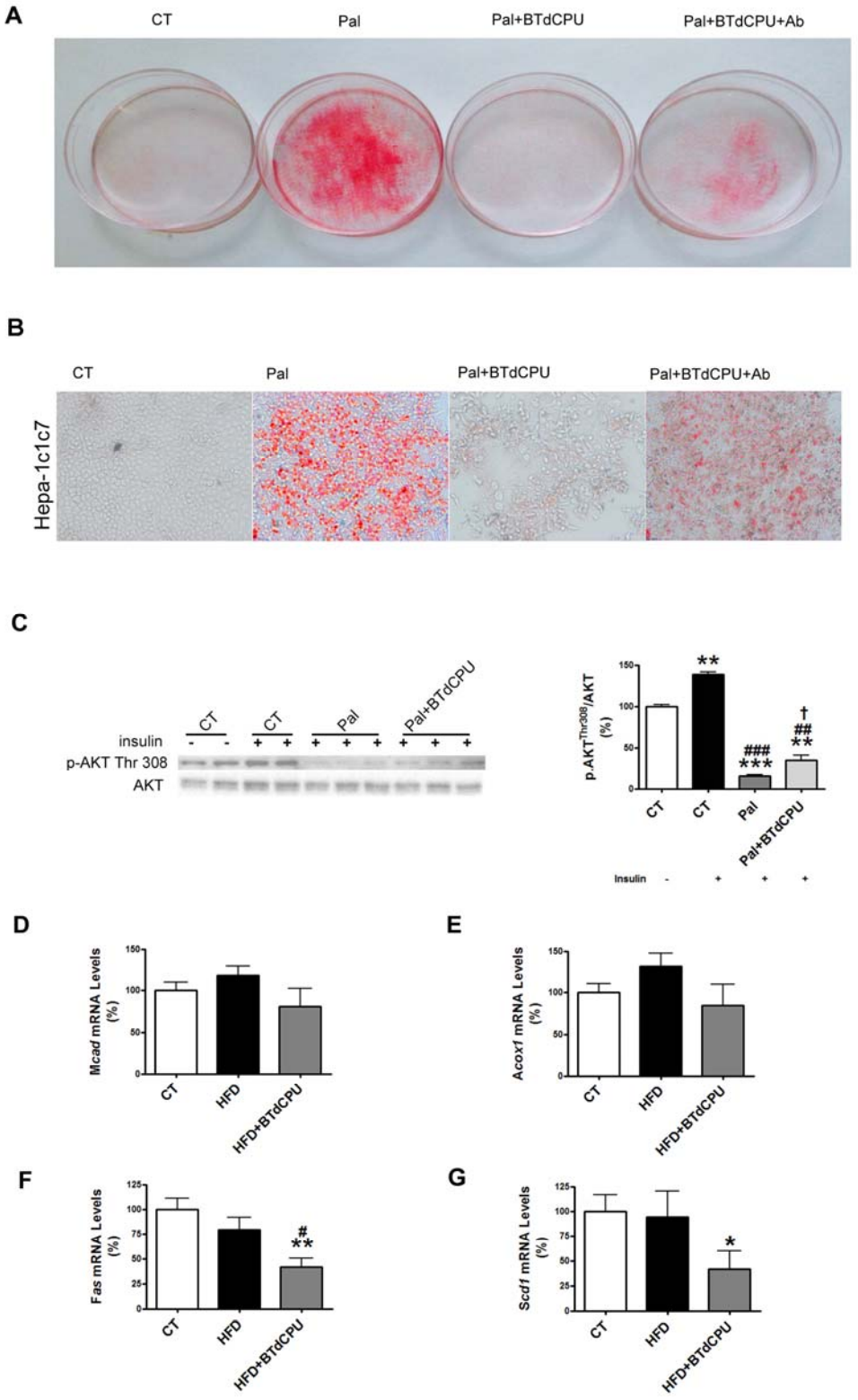
Supplementary Figure 4. Huh-7 hepatocytes were incubated for 24 h in the absence (Control, CT) or in the presence of 10 μmol/L of either BTdCPU or BTCtFPU. mRNA abundance of PPARα (A), ACOX (B), MCAD (C) and BiP (D Data are presented as the mean ± S.D. (n=5 per group).



SUPPLEMENTARY DATA

Supplementary Figure 5. Oil Red O staining of Huh-7 (A) and Hepa1c1c7 (B) hepatocytes. Cells were incubated for 24 h with BSA (Control, CT), 0.75 mmol/L palmitate (Pal) conjugated with BSA, 0.75 mmol/L palmitate plus 10 μmol/L BTdCPU and IgG (3 μg/ml) or 0.75 mmol/L palmitate plus 10 μmol/L BTdCPU and a FGF21 neutralizing antibody (Ab) (3 μg/ml). C, immunoblot analyses of total and phosphorylated Akt at Thr^{308}. When indicated, cells were incubated with 100 nmol/L insulin for the last 10 min. Data are presented as the mean ± S.D. (n=4 per group). $^{***}p<0.001$ and $^{**}p<0.01$ vs. control cells not exposed to insulin. $^{###}p<0.001$ and $^{##}p<0.01$ vs. insulin-stimulated control cells. $^{†}p<0.05$ vs. insulin-stimulated cells incubated with palmitate. mRNA abundance of MCAD (D), ACOX (E), FAS (F) and SCD1 (G) in the livers of mice fed a standard chow, a HFD for three weeks or a HFD for three weeks plus BTdCPU during the last week. Mice fed a standard chow and half of the mice fed the HFD received one daily i.p. administration of DMSO (vehicle) for the last week. The rest of the mice fed the HFD received one daily i.p. administration of BTdCPU (70 mg kg-1 day-1) for the last week. Data are presented as the mean ± S.D. (n=5 per group). $^{**}p<0.01$ and $^{*}p<0.05$ vs. mice fed a standard diet (CT). $^{#}p<0.05$ vs. mice fed a HFD.

SUPPLEMENTARY DATA



SUPPLEMENTARY DATA

Supplementary Table 1. Primer sequences used for real-time RT- PCR

Gene	Primers	
mAprt	for	5'-CAGCGGCAAGATCGACTACA-3'
	rev	5'-AGCTAGGGAAGGGCCAAACA-3'
mAtf3	for	5'-CTGGAGATGTCAGTCACCAAGTCT-3'
	rev	5'-TTTCTCGCCGCCTCCTTT-3'
mAtf4	for	5'-AGCAAAACAAGACAGCAGCC-3'
	rev	5'-ACTCTCTTCTTCCCCCTTGC-3'
hATF4	for	5'-GCGGGCTCCTCCGAAT-3'
	rev	5'-ATCCTCCTTGCTGTTGTTGGA-3'
mAcox	for	5'-TCTGGAGATCACGGGCACTT-3'
	rev	5'-TTTCCAAGCCTCGAAGATGAG-3'
hACOX	for	5'-GGAAAAAAACTCGGGCAGAAC-3'
	rev	5'-TGGCGAGGAACTCTGACCTT-3'
mβ-Klotho	for	5`-TGGGGTCCCATTGGATAGAG-3`
	rev	5`-ACTCAGGGTAGTCGCCGTC-3`
mBip	for	5`-CAGATCTTCTCCACGGCTTC-3`
	rev	5`-GCAGGAGGAATTCCAGTCAG-3`
hBIP	for	5`-ACTATTGCTGGCCTAAATGTTATGAG-3`
	rev	5`-TTATCCAGGCCATAAGCAATAGC-3`
mBmal1	for	5`-ACGACATAGGACACCTCGCAGA-3`
	rev	5`-CGGGTTCATGAAACTGAACCATC-3`
mChop	for	5`-CGAAGAGGAAGAATCAAAAACCTT-3`
	rev	5`-GCCCTGGCTCCTCTGTCA-3`
hCHOP	for	5`- GGAAATGAAGAGGAAGAATCAAAAAT-3`
	rev	5`-GTTCTGGCTCCTCCTCAGTCA-3`
mFas	for	5`-CATTGGTGGTGTGGACATGGT -3`
	rev	5`-GACCGCTTGGGTAATCCATAGA-3`
hFGF21	for	5`-ACCAGAGCCCCGAAAGTCT-3`
	rev	5`-CTTGACTCCCAAGATTTGAATAACTC-3`
mFgfr1c	for	5`-TGTTTGACCGGATCTACACACA-3`
	rev	5`-CTCCCACAAGAGCACTCCAA-3`
mGlut1	for	5`-GCCCCCAGAAGGTTATTGA-3`
	rev	5`-CGTGGTGAGTGTGGTGGAT-3`
hGAPDH	for	5`-GGCCTCCAAGGAGTAAGACC-3`
	rev	5`-AGGGGTCTACATGGCAACTG-3`
mHmgcs2	for	5`-TCTTTTCATTCCGAGTGTCCA-3`
	rev	5`-ATCTGACACACTAGACACCAGTTTCTC-3`
mHsd3b5	for	5`-GCTCTTGGAAACCACAAGGAAC-3`
	rev	5`-GACAATCCTCTGGCCAAGAAAC-3`
mL-cpt1α	for	5`-GCAGAGCACGGCAAAATGA-3`
	rev	5`-GGCTTTCGACCCGAGAAGAC-3`
mMcad	for	5`-TGACGGAGCAGCCAATGA-3`
	rev	5`-ATGGCCGCCACATCAGA-3`
hMCAD	for	5`-CCCAGTGGCTGCAGAATATGAT-3`

SUPPLEMENTARY DATA

	rev	5`-AAACCAAGTTCCCAGGCTCTTC-3`
mMup1	for	5`-CAAAACAGAAAAGGCTGGTGA-3`
	rev	5`-TTGTGCAAACCTTTCCTTGA-3`
mOrp150	for	5`-CACTGCACAGAACGTCATGTTCT-3`
	rev	5`-GGTGACGATGGTGCACACA-3`
mRev-Erb α	for	5`-GGACAACCAGCCCTCAGTTC-3`
	rev	5`-GCAGCTTCGGACCCATGTT-3`
hREV-ERB α	for	5`-ATGACCAAGTCACCCTGCTTAG-3`
	rev	5`-TCTGGTCCTTCACGTTGAACAA-3`
mPparα	for	5`-CAAGGCCTCAGGGTACCACTAC-3`
	rev	5`-GCCGAATAGTTCGCCGAAA-3`
*h*PPARα	for	5`-TGAAGTTCAATGCACTGGAACTG-3`
	rev	5`-GGACGATCTCCACAGCAAATG-3`
mPparβ/δ	for	5`-GCCACAACGCACCCTTTG-3`
	rev	5`-CCACACCAGGCCCTTCTCT-3`
mScd1	for	5`-CTGTACGGGATCATACTGGTTC-3`
	rev	5`-GCCGTGCCTTGTAAGTTCTG-3`