Supplementary Data

Supplementary Materials and Methods

Reagents, Plasmids and Adenovirus vectors. The reagents fenofibrate, bezafibrate, clofibrate, GW7647, WY14643, Wortmannin and H89 were obtained from Sigma; PD98059, SP600125 and Compound C from Calbiochem; TGFβ, TNFα, HGF and IL-1α from R&D systems; and metformin from Wako Chemicals. Human and mouse SHP promoter luciferase reporter, human PAI-1 promoter luciferase reporter, Sft4 luciferase reporter and PPRE luciferase reporter constructs were described previously [4, 5, 16]. Smad 3, Smad 4, LRH-1, SF-1, PPARα, SHP, Nur77, dn-Nur77, ca-AMPK, dn-AMPK, dn-USF-1, NF-κBp65 subunit, IκBα, p300 and CBP expression vectors were described previously [4, 5, 17 and Supplementary ref. 3-5]. The Smad7 (inhibitor of Smad, iSmad) expression vector was a kind gift from Dr CH Heldin. The USF-1 expression vector was a kind gift from Dr. IK Chung, the pSuper vector and pSuper siSHP were kind gifts from Dr. JK Kemper. Ad-SHP, Ad-AMPKα, Ad-siSHP and Ad-dnAMPKα constructs were described [4, 5].

Cell Culture and transient transfection assays. HepG2, H4IIE and AML12 cells were obtained from the American Type Culture Collection. Maintenance of cell lines and transient transfections were performed as described [4, 5].

RNA isolation and analysis. Total RNA was isolated for Northern blot analysis and RT-PCR analysis using primers for PAI-1, SHP, α(1) I collagen, TNFα, IL-6, TIMP-1, TGFβ, SREBP-1c, FAS and β-actin as described [4, 5, 23, 26 and Supplemetary ref. 6, 7].

Immunoblotting analysis. Cell lysate preparation and immunoblotting analysis were performed as described [4]. Mouse monoclonal anti-PAI-1 antibody and rabbit polyclonal SHP antibody (H-160) was from SantaCruz (Santa Cruz, CA, USA).

Measurement of ATP concentration. ATP concentrations were estimated via luciferase acitivity using ATP bioluminescence assay kit (Roche Applied Bioscience, Switzerland) as described previously [4, Supplementary ref. 12].

Isolation of mitochondria. Healthy C57BL/6 mice were asphyxiated and liver tissues were rinsed twice with ice-cold buffer A (320mM sucrose, 1mM EDTA, 10mM Tris, pH 7.5). Livers were finely minced and homogenized in 4 ml of buffer AT (75mM sucrose, 225mM mannitol, 1mM EGTA, 0.01% BSA, pH 7.4) per gram of liver using a glass-teflon homogenizer. The resultant homogenate was centrifuged at 1,000 x *g* for 5 min at 4 °C.

The 1,000 x *g* supernatant was centrifuged twice at 13,000 x *g* for 10 min [Supplementary ref. 8]. The mitochondria-enriched pellet was used for measuring mitochondrial respiratory chain activity.

Assay of respiratory enzyme activities. To prepare submitochondrial particles, mitochondrial pellets were suspended in buffer AT, then freezen and thawed three times. Mitochondrial protein concentration was measured by using BSA as standard [Supplementary ref. 9]. Complex I activity (NADH CoQ oxidoreductase) was measured in the presence of decylubiquinone as the rotenone-sensitive decrease in NADH at 340nm. The activity of complex II (succinate: DCIP oxireductase) was measured in the presence decylubiquinone plus rotenone as the antimycin A-sensitive reduction of 2,6-DCIP at 600nm with 520nm as reference wavelength. Complex III activity (ubiquinol:

cytochrome c oxireductase) was measured in the presence of rotenone and decylubiquinone following the rate of reduction of cytochrome c at 550nm with 580nm as the reference wavelength. Complex IV activity (cytochrome c oxidase) was measured as the disappearance of reduced cytochrome c at 550nm [Supplementary ref. 10-11]. All absorbance measurements were performed in a Beckman DU650 (Beckman coulter fullerton, CA) spectrophotometer.

MTT cell viability assay. Cell viability was evaluated in HepG2 cells as described previously [Supplementary ref. 14].

Statistical analyses. Data are means ± SD. Analysis of variance (ANOVA) was used to determine significant differences, followed by Duncan's multiple comparison tests. All experiments were performed at least three times. Data calculation and statistical analysis were performed using GraphPad Prism 4.0 software. Two-way ANOVA analysis for repeated measures and Student's t-test for unpaired data were used as appropriate to detect any significant differences. Significance was accepted at the $P < 0.05$ level.

Supplementary Figure Legends

Supplementary Figure 1. Induction of SHP and activation of AMPK by fenofibrate.

A: H4IIE and AML12 cells were treated with fenofibrate (50μM) or vehicle (DMSO) for the time indicated and in the concentrations indicated for 24 h. Total RNA was isolated for Northern blot analysis of SHP mRNA expression and was normalized to GAPDH expression. Data represent mean ± SD of 3 individual experiments. **B:** HepG2 cells were treated with various PPARα agonists, bezafibrate (Beza, 100 μM), clofibrate (Clo, 250 μM), WY14643 (WY, 100 μM) and fenofibrate (Feno, 50 μM) for 12 h (left) or for 3 h (right) under serum-starved conditions. Total RNA was isolated for Northern blot analysis of SHP and Trb3 mRNA expression and was normalized to GAPDH expression or whole cell extracts (50 μg/lane) were analyzed by immunoblotting with phospho-AMPKα (p-AMPKα), total-AMPKα (t-AMPKα), phospho-ACC (p-ACC) and total-ACC (t-ACC) antibodies. Data represent mean ± SD of 3 individual experiments. *P < 0.05 compared to untreated control. **C:** HepG2 cells were treated with fenofibrate (50μM) for the indicated time and at indicated concentrations for 3 h and with various PPARα agonists for 3 h under serum-starved conditions. Total intracellular ATP was quantified by luminescence according to manufacturer's protocol. Results are expressed as the % decrease in levels of intracellular ATP, setting untreated cells as 100, and are normalized to the total protein level. Data represent mean ± SD of 3 individual experiments. *P < 0.05 compared to untreated cells. **D:** HepG2 cells were treated with fenofibrate or clofibrate at indicated concentrations for 24 h and MTT assay was performed according to the manufacturer's protocol to determine cell viability. Data represent mean ± SD of 3 individual experiments.

Supplementary Figure 2. Inhibition of PAI-1 gene expression and activation of AMPK signaling by fenofibrate *in vivo.*

A: C57BLKS-Leptin receptor deficient db/db mice (BKS-*Lepr*$^{db/db}$, n=4 per group) were fed with fenofibrate (100mg/kg/day) for the indicated time period and liver samples were obtained for semiquantitative RT-PCR analysis of PAI-1 and SHP mRNA expression and was normalized to actin expression (left) and tissue extracts (100 µg/lane) were analyzed by immunoblotting with PAI-1 and α-tubulin antibodies (middle) or with phospho-AMPKα (p-AMPKα), total-AMPKα (t-AMPKα), phospho-ACC (p-ACC) and total-ACC (t-ACC) antibodies (right). Data represent mean ± SD.*$P < 0.05$ compared to untreated control. **B-C:** C57BL/6 mice (B6, n=4 per group) and SHP null mice (SHPKO, n=4 per group) were fed with chow (C) or injected with TGFβ (T, 25 µg/kg) intraperitoneally followed by treatment with fenofibrate (100mg/kg/day) (T+F) for an additional 3 days (panel B) or a methionine and choline deficient diet (M) for 4 weeks and compared to diet-fed mice treated with fenofibrate (100mg/kg/day) (M+F) for the final 3 days of the experimental period (panel C). Liver tissue extracts (100 µg/lane) were analyzed by immunoblotting with PAI-1, α-tubulin, phospho-AMPKα (p-AMPKα), total-AMPKα (t-AMPKα), phospho-ACC (p-ACC) and total-ACC (t-ACC) antibodies. Data represent mean ± SD. *$P < 0.05$ compared to chow-fed mice.

Supplementary Figure 3. Inhibition of cytokine-induced PAI-1 mRNA levels by fenofibrate is mediated by AMPK-dependent induction of SHP in AML12 cells.

A-B: HepG2 cells were treated with fenofibrate (Feno) at indicated concentrations for 24 h after which cells were treated with TGFβ (panel A) and TNFα (panel B) at indicated

concentrations for further 4 h under serum starved conditions. Total RNA was isolated for Northern blot analysis of PAI-1 and SHP mRNA expression and was normalized to GAPDH expression. Data represent mean ± SD of 3 individual experiments. *$P < 0.05$ and $^{\&}P < 0.005$ compared to untreated control and cytokine treated cells. nd, non-detectable. **C-D:** AML12 cells were treated with mock virus, adenovirus siRNA SHP (Ad-siSHP) or adenovirus dominant negative AMPKα (Ad-dnAMPKα) for 48 h, followed by fenofibrate (Feno) treatment at indicated concentrations for an additional 24 h, after which cells were treated with TGFβ (panel A) or HGF (panel B) at indicated concentrations for further 4 h under serum starved conditions. Total RNA was isolated for semiquantitative RT-PCR analysis of PAI-1 and SHP mRNA expression and was normalized to actin expression. Data represent mean ± SD of 3 individual experiments. *$P < 0.05$, $^{\&}P < 0.005$ and **$P < 0.005$, compared to untreated control, cytokine treatment and fenofibrate treated cells.

Supplementary Figure 4. Repression of LRH-1 but not SF-1 transcriptional activity by fenofibrate. A: HepG2 cells were transfected with the human PAI-1 gene promoter (-800bp) for 18 h followed by treatments with TGFβ, TNFα, HGF and IL-1α at indicated concentrations for 24 h in the presence or absence of cotransfected Smad7 (200 μg), dominant negative Nur77 (dn-Nur77, 200 μg), dominant negative USF1 (dn-USF1, 200 μg) or IκB respectively under serum-starved conditions. All experiments were done in triplicate, and data are expressed in relative luciferase units (RLU) and as the fold activation relative to the control, representing mean ± SD of 3 individual experiments.*$P < 0.05$, $^{\&}P < 0.05$, compared to untreated control and individual cytokine treatments. **B:** HepG2 cells were transfected with pSuper vector (pSuper only, 200μg)

or pSuper siRNA SHP (p-siSHP, (200μg) for 48 h. Total RNA was isolated for semiquantitative RT-PCR analysis of SHP mRNA expression and was normalized to actin expression. Data represent mean ± SD of 3 individual experiments. .*$P < 0.05$ compared to untreated control and pSuper only treated cells. **C:** HepG2 cells were co-transfected with Sft4-luc along with LRH-1 (200 μg) or SF-1 (200 μg) in the presence or absence of SHP (200 μg), pSuper siRNA SHP (p-siSHP, 200 μg) for 18 h followed by fenofibrate and metformin treatments as indicated for a further 24 h under serum-starved conditions. All experiments were done in triplicate, and data are expressed in relative luciferase units (RLU) and as the fold activation relative to the control, representing mean ± SD of 3 individual experiments. *$P < 0.05$, $^{\&}P < 0.05$ and **$P < 0.005$, compared to untreated control, individual transcription factor cotransfection and fenofibrate or metformin treatment.

Supplementary References

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