A Novel Regulatory Role of AATF in the Pathogenesis of NAFLD and HCC

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Supplementary Methods

Generation of Stable Cell Lines. As a first step for stable cell line generation, the optimal antibiotic concentration for selecting the stable cell colonies was determined by doing a dose-response experiment. QGY-7703 cells were treated with different concentrations of Puromycin (Santa Cruz Biotechnology, Dallas, TX) ranging from 1-10 μg/ml (1, 2, 4, 6, 8 and 10 μg/ml) and found that 1 μg/ml was the minimum Puromycin concentration needed to kill the cells over the course of a week.

AATF ShRNA plasmid (Santa Cruz Biotechnology, Dallas, TX) containing Puromycin resistance gene was transfected to QGY-7703 cells according to the manufacturer's protocol. Cells were then selected for 2 weeks in 1 μg/ml Puromycin and individual colonies were isolated, expanded and maintained in 1 μg/ml Puromycin. The knockdown of AATF in these clones were confirmed by RT-PCR (Santa Cruz) and immunobloting. Control ShRNA plasmids were used to similarly establish control QGY-7703 clones.

Transient Transfection. Cells were cultured in 6 well plate (SREBP1 SiRNA, Santa Cruz) or 96 well plate (AATF SiRNA, Santa Cruz Biotechnology, Dallas, TX) to 90% confluence. One day before transfection, the media were changed to antibiotic free medium. Transfection was carried out using Lipofectamine 2000 (Thermo Scientific, Waltham, MA) following the manufacturer's instructions.

Enzyme-linked Immunosorbent Assay. Concentrations of TNF-α and MCP-1 were measured in the serum samples of each group in both humans and mice by using ELISA kits (Thermo Scientific, Waltham, MA) according to manufacturer's protocol.

Proliferation Assay. Cells were seeded in 96 well plates at a density of 10^4 cells/well in 100 µl of culture medium. The cell numbers were determined every 24 hours for 3 consecutive days after transfection by using WST-1 cell proliferation assay kit (Clontech Laboratories Inc., Mountain view, CA) accordingly to the manufacturer's protocol.

Immunostaining. The tissues were fixed with 10% formalin, and the paraffin-embedded tissues were sectioned using a microtome. The tissue sections were deparaffinised and rehydrated following the treatment with xylene and series of ethanol concentrations. After antigen retrieval with citrate buffer (pH 6) at 94°C for 15 min followed by washing with water, the sections were incubated with 3% hydrogen peroxide (Amresco, Solon, OH) for 10 min. The sections were blocked with normal donkey serum and incubated with primary antibody (AATF- 1:100; Sigma-Aldrich, St. Louis, MA) overnight at 4°C. The sections were then incubated with SignalStain boost detection reagent for 30 min followed by SignalStain DAB (Cell Signaling Technology, Danvers, MA). After rinsing with water, the sections were counterstained with hematoxylin. The sections were then dehydrated and mounted, and the slides were visualized using AxioVision Zeiss microscope. For Ki67 and CD31 staining, the tissue sections were deparaffinised and rehydrated as described above and incubated with Ki67 (1:100; Abcam, Cambridge, UK) or CD31 (1:50; Abcam, Cambridge, UK) antibody followed by Alexa Fluor secondary antibody (Thermo Scientific, Waltham, MA). In addition, the cell nuclei were stained with DAPI (1:1000; Cell Signaling Technology) and the slides were visualized using Zeiss LSM 700 confocal microscope.

For immunostaining of *in vitro* cultured cells, the cells were plated on 4 well chambers and fixed in 4% formaldehyde. The cells were permeabilized with 0.5% Triton X-100 in PBS for 30 min. Further, the cells were blocked for 2 hour at room temperature with 5% donkey/goat serum (Thermo Scientific, Waltham, MA) and 1% BSA in PBS and incubated with anti AATF antibody overnight at 4°C. The slides were then rinsed in PBS and incubated with Alexa Fluor secondary antibody for 1 hour at room temperature. In addition, the cell nuclei were stained with DAPI and mounted, and the slides were visualized using Zeiss LSM 700 confocal microscope.

Immunoprecipitation. Cells were grown in 10 cm^2 culture plates to 90-95% confluence. The plates were washed with PBS, harvested with trypsin-EDTA (Invitrogen, Carlsbad, CA) and then centrifuged at 500×g for 5 minutes. Nuclear extracts were prepared following the manufacturer's

instructions (NE-PER nuclear and cytoplasmic extraction reagents; Thermos Scientific, Waltham, MA). The nuclear extracts were incubated with 1 mg AATF and STAT3 antibodies overnight at 4°C with 30 µL of protein A/G Agarose beads (Thermo Scientific, Waltham, MA) slurry added to the lysate antibody solution during the last 2 hours of incubation. After incubation, beads were washed 5 times with 600 µl lysis buffer and boiled in SDS sample buffer containing β-mercaptoethanol. The samples were loaded on SDS-PAGE gel and further analyzed by western blotting.

Western Blots. Tissues or cells were solubilized in RIPA buffer containing protease/phosphatase inhibitor cocktail (Cell Signaling Technology, Danvers, MA). The lysates were centrifuged at 10,000×g for 15 min at 4°C and the supernatant collected. Protein concentration was measured by DC protein assay kit (Bio-Rad). Equivalent amounts of protein were resolved by SDS-PAGE and transferred onto nitrocellulose membrane. Blots were blocked in 5% nonfat dry milk for 1 h followed by immunoblotting with specific primary antibodies [anti AATF (1:1000, Sigma-Aldrich); anti β-actin (1:50,000, Sigma-Aldrich); anti lamin A/C (1:1000, Cell Signaling Technology); anti STAT3 (1:1000, Cell Signaling Technology); anti SREBP1 (1:1000, Santa Cruz Biotechnology)] at 4°C overnight and with secondary antibodies for 2 hours at room temperature. Antigen-antibody complexes were detected using Pierce™ ECL western blotting substrate (Thermo Scientific, Waltham, MA). Western blot images were analyzed with ImageJ software for densitometric measurements. The average intensity for each band was normalized to its respective band of β-actin.

Quantitative PCR. Total RNA was extracted from tissues and cells by using TRIzol reagent (Thermos Scientific, Waltham, MA). The extracted RNA was purified and reverse-transcribed using high-capacity cDNA reverse transcription kit (Applied Biosystems™, Foster city, CA) following the manufacturer's instructions. Real time PCR was carried out using CFX96 Touch™ Real-Time PCR system (Bio-Rad) accordingly to the manufacturer's protocol. Cycle threshold (Ct) values were obtained and the relative fold change in gene expression was calculated as $2^{-\Delta\Delta Ct}$. The change in mRNA expression was calculated using differences of Ct values compared to internal control, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All the reactions were performed in triplicates. The sequences of primers used are as follows: AATF (Taqman Gene Expression Assays, Applied Biosystems, Hs00201719_m1 and Mm00498158_m1); Gapdh

(Hs02786624_g1 and Mm99999915_g1); SerpinF1 (Mm00441270_m1); IGFBP3 (Mm01187817_m1); TNF-α (human: forward 5'- ATGGCCTCCCTCTCATCAGT-3' and reverse 5'-TTTGCTACGACGTGGGCTAC-3'; mouse: forward 5'-CCCAGGGACCTCTCTCTAATC-3' and reverse 5'-ATGGGCTACAGGCTTGTCACT-3'); SREBP1-c (human: forward 5'-GCAGATCGCGGAGCCATGGATTGC-3' and reverse 5'-GAGGTGGAGACAAGCTGCCTGG-3'; mouse: forward 5'-GATCAAAGAGGAGCCAGTGC-3' and reverse 5'-TAGATGGTGGCTGCTGAGTG-3'); MCP-1 (human: forward 5'-CCCCAGTCACCTGCTGTTAT-3' and reverse 5'-TGGAATCCTGAACCCACTTC-3'; mouse: forward 5'-CCACTCACCTGCTGCTACTCAT-3' and reverse 5'-TGGTGATCCTCTTGTAGCTCTCC-3'); Gapdh (human: forward 5'-AGGTGAAGGTCGGAGTCAACGG-3' and reverse 5'-TTCCCGTTCTCAGCCTTGACGGT-3'; mouse: forward 5'-AGAAACCTGCCAAGTATGATG and reverse 5'-GGAGTTGCTGTTGAAGTCG).

Luciferase assay. HepG2 and Huh7 cells were plated at a density of 5×10^4 cells in 96 well plates and cultured until 80-90% confluent. One day before transfection, the DMEM media were changed to antibiotic free medium. The cells were then transiently transfected using Lipofectamine 3000 with empty vector (pGL4-basic), AATF wild type plasmid or AATF deletion 1 plasmid (still containing SREBP1 binding site, SREBP1- 0.92) or AATF deletion 2 plasmid (SREBP1 binding site deleted) along with renilla luciferase expression plasmid for transfection control. All transfections were done in triplet. After 24 hours of transfection, cells were treated with or without TNF-α (10 ng/ml) for 12 hours. Luciferase activity was measured using dual luciferase reporter kit (Promega, Madison, WI) according to the manufacture's protocol, and firefly luciferase activity was normalized by renilla luciferase activity.

Supplementary Figure Legends

Supplementary Figure 1. Effect of western diet sugar water on DIAMOND mice

(A) Heat map demonstrating high expression levels of AATF in WDSW fed DIAMOND mice (10). (B) Gross liver from DIAMOND mice fed with CDNW or WDSW for 8, 24 and 52 weeks.

Supplementary Figure 2. Analysis of AATF expression in the liver tissues

(A and B) Analysis of AATF expression by immunohistochemistry in the liver tissue of DIAMOND mice (A) and human normal, NASH and HCC subjects (B). (C) AATF protein expression by western blot in the cytosolic and nuclear extracts of mouse and human HCC cells. (D) Kaplan-Meier plot demonstrating the analysis of correlation between AATF mRNA expression levels and patient survival in liver cancer (https://www.proteinatlas.org/ENSG00000275700-AATF/pathology/tissue/liver+cancer).

Supplementary Figure 3. Upregulation of AATF and SREBP1c in HepG2 and Huh7 cells upon TNF-α treatment

(A and B) HepG2 and Huh7 cells were treated with TNF-α (10 ng/ml) for 0, 12 or 24 hours. The relative AATF (A) and SREBP1c (B) mRNA levels were determined by real time RT-PCR and normalized using Gapdh as an internal control. (C) SREBP1 protein expression in HepG2 and Huh7 cells transfected with control SiRNA or SREBP1 SiRNA with or without TNF-α treatment. Data are expressed as the mean ± SEM of three experiments (*$p<0.05$; **$p<0.001$).

Supplementary Figure 4. AATF expression is transcriptionally induced by TNF-α via SREBP1

(A and B) HepG2 (A) and Huh7 cells (B) were transfected with AATF wild type or SREBP1c binding site deleted luciferase promoter construct along with renilla luciferase expression vector. Luciferase assay was performed after 48 hours with or without TNF-α treatment (12 hours) and firefly luciferase activity was normalized by renilla luciferase activity. Data are expressed as the mean ± SEM of three experiments (**$p<0.001$).

Supplementary Figure 5. Stable knockdown of AATF in QGY-7703 cells

(A-C) AATF expression was analyzed by PCR (A), western blot (B) and immunofluorescence (C) in control and AATF knockdown clones of QGY-7703 cells.

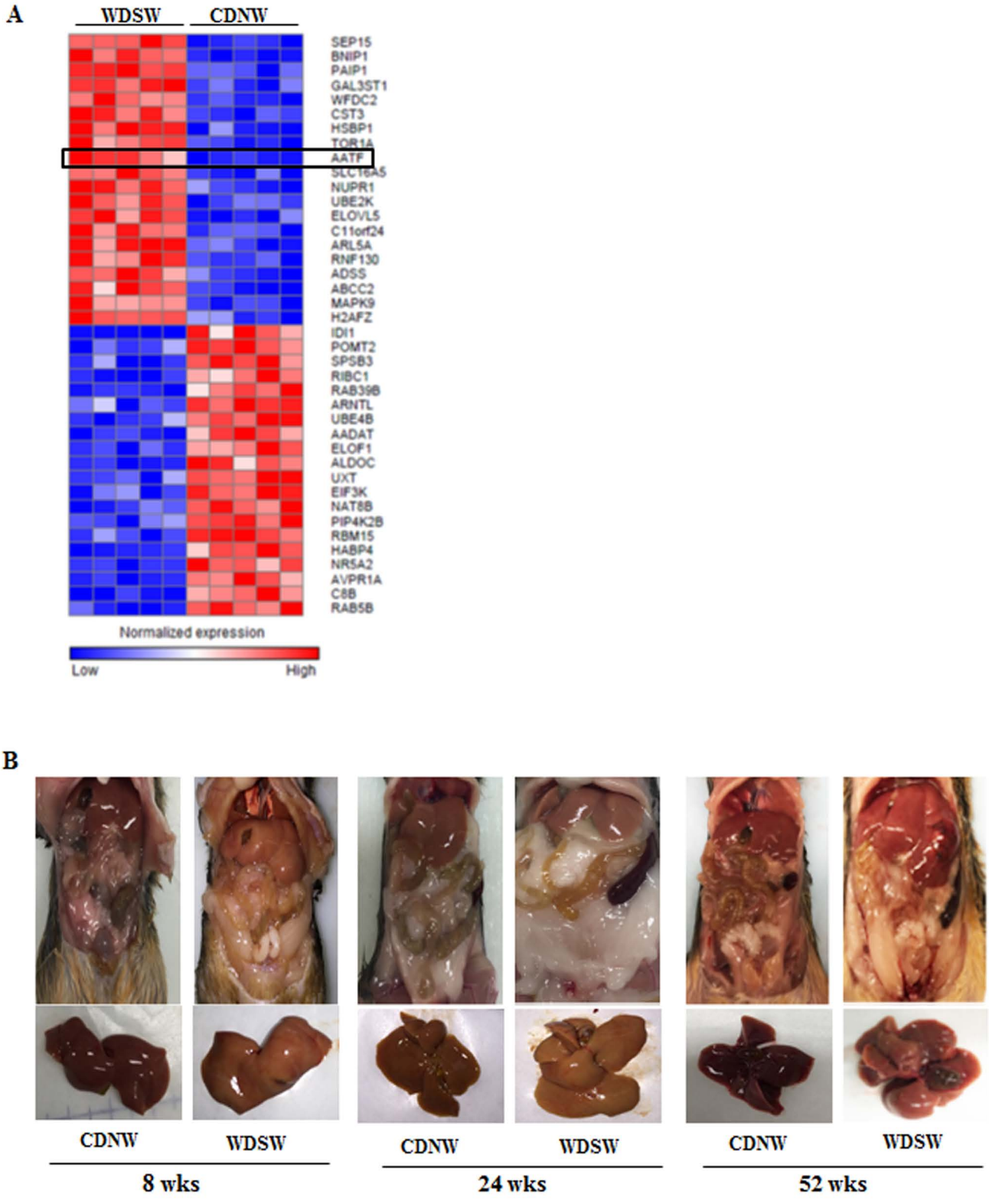
Supplementary Figure 6. Regulation of MCP-1 by AATF-STAT3 complex

(A and B) mRNA detection of MCP-1 in DIAMOND mice fed with CDNW or WDSW at 8, 24 and 52 weeks and in human normal, NASH and HCC subjects. (C) Immunoprecipitation of the nuclear extracts of control and AATF KD QGY-7703 cells using anti AATF and anti STAT3 antibody. Data are expressed as the mean ± SEM (n= 6-8) (*$p<0.05$; **$p<0.001$).

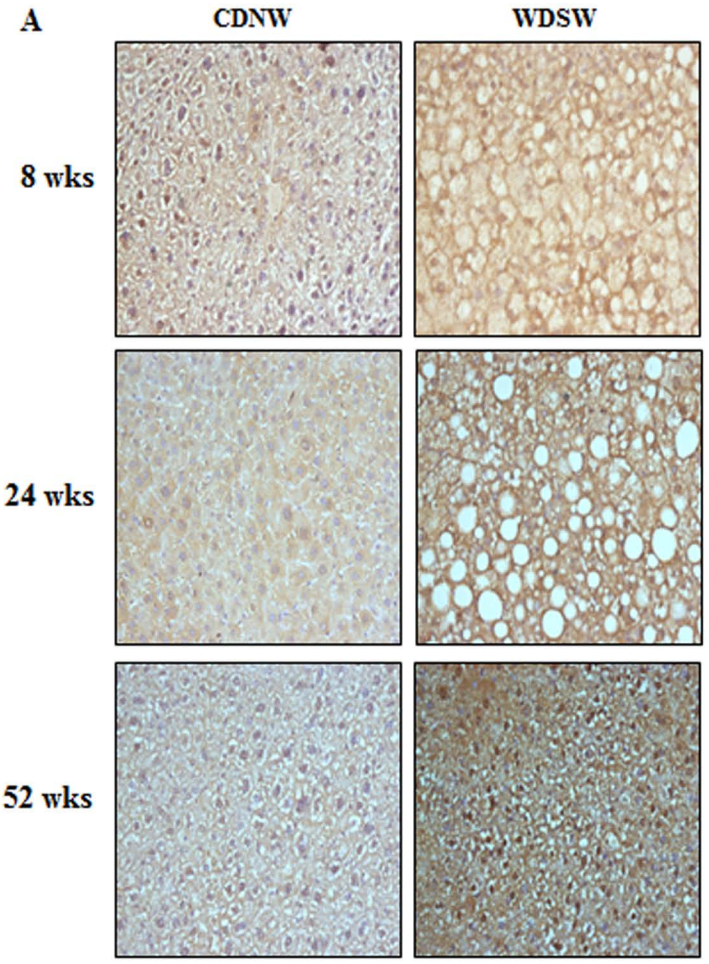
Supplementary Figure 7. Analysis of AATF downstream target genes

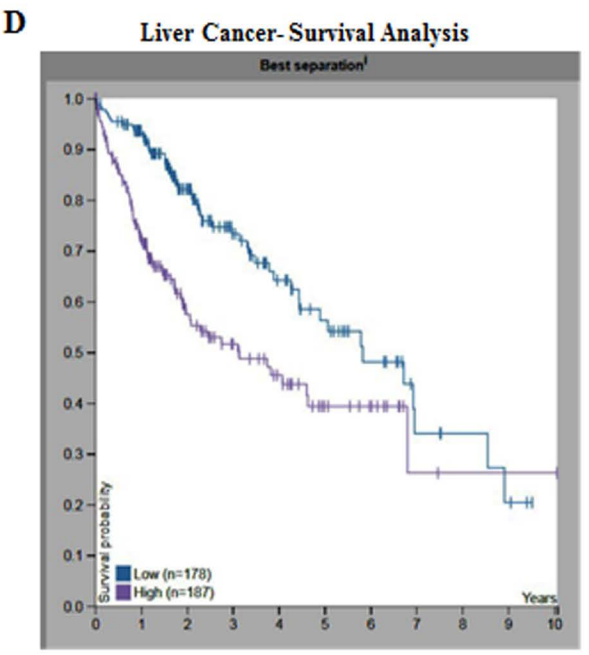
(A-C) mRNA expression of MCP-1 (A), Serpin F1 (B) and IGFBP3 (C) was determined by qRT-PCR in control and AATF depleted tumors. Data are expressed as the mean ± SEM (n= 6) (**$p<0.001$).

Supplementary Figure 1

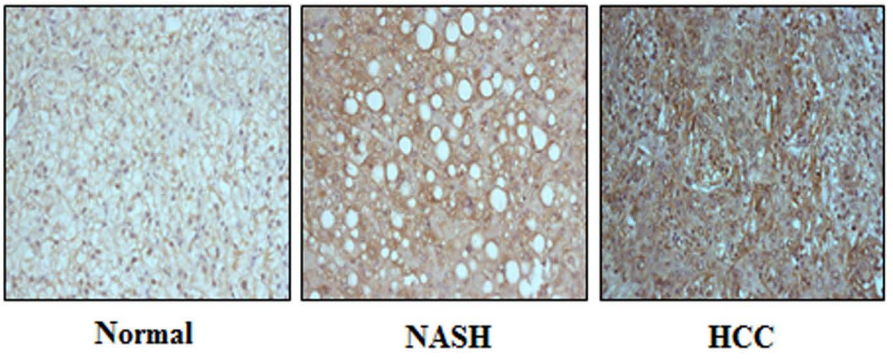


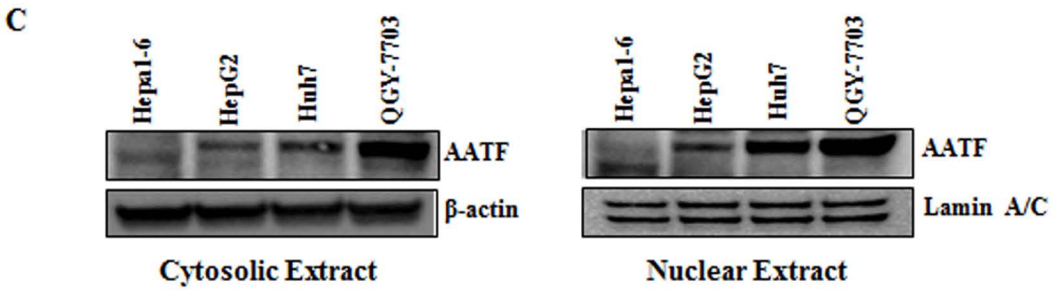
Supplementary Figure 2

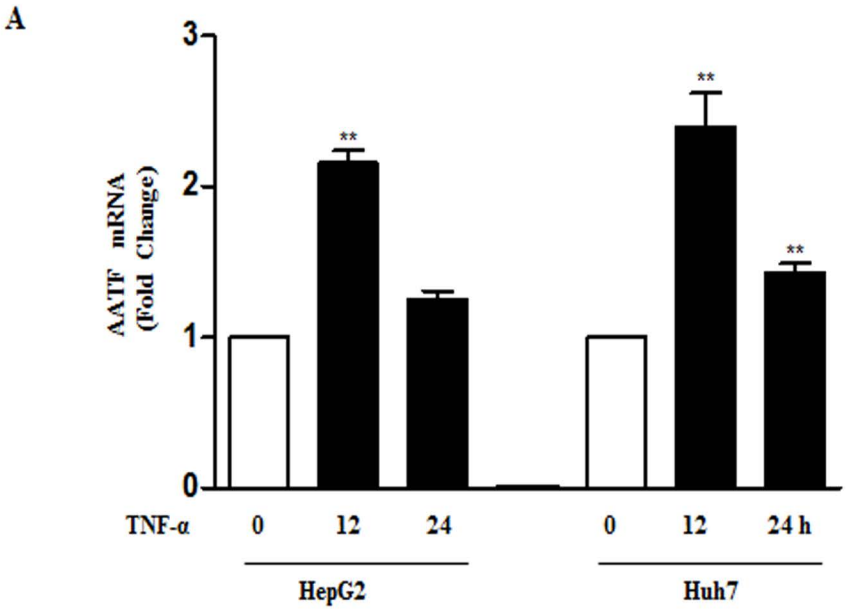


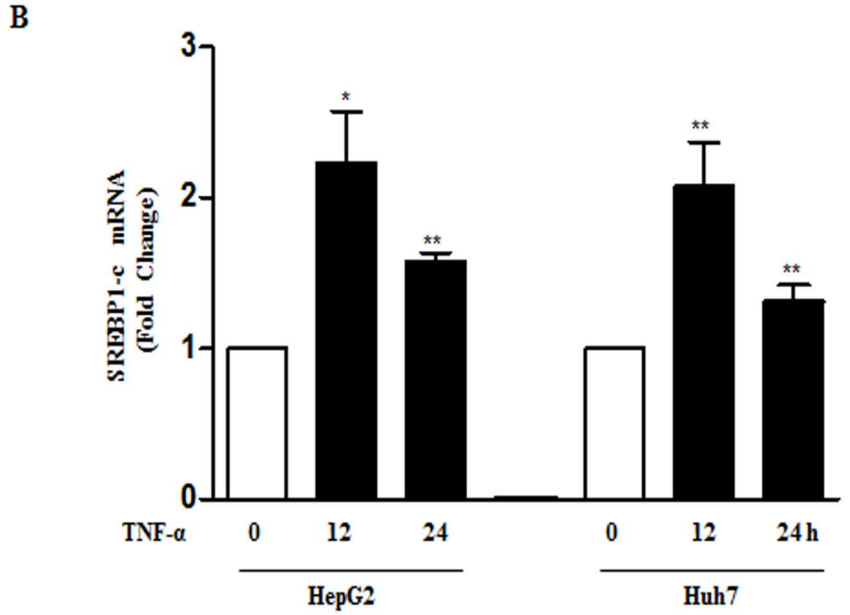


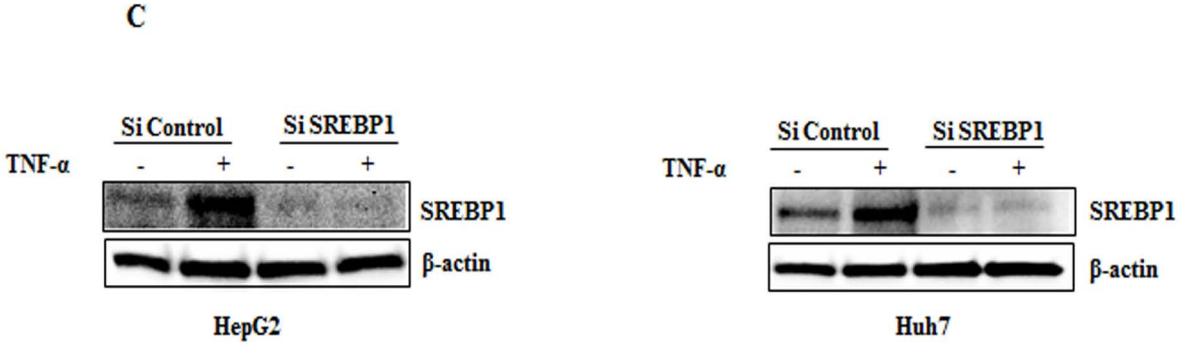
AATF Expression cutoff	10.2 FPKM
5- year survival high	39%
5- year survival low	54%
Log-rank P value	6.62e-5

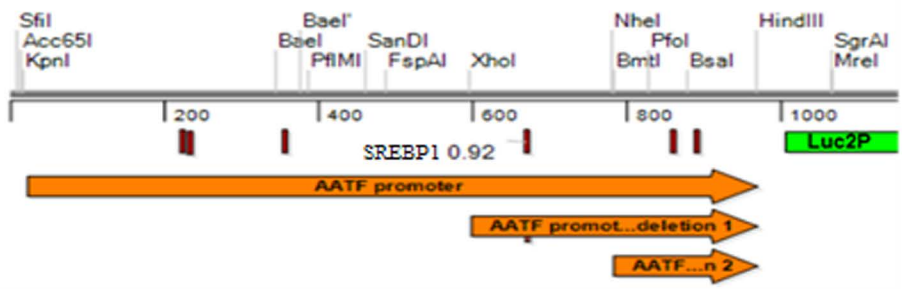




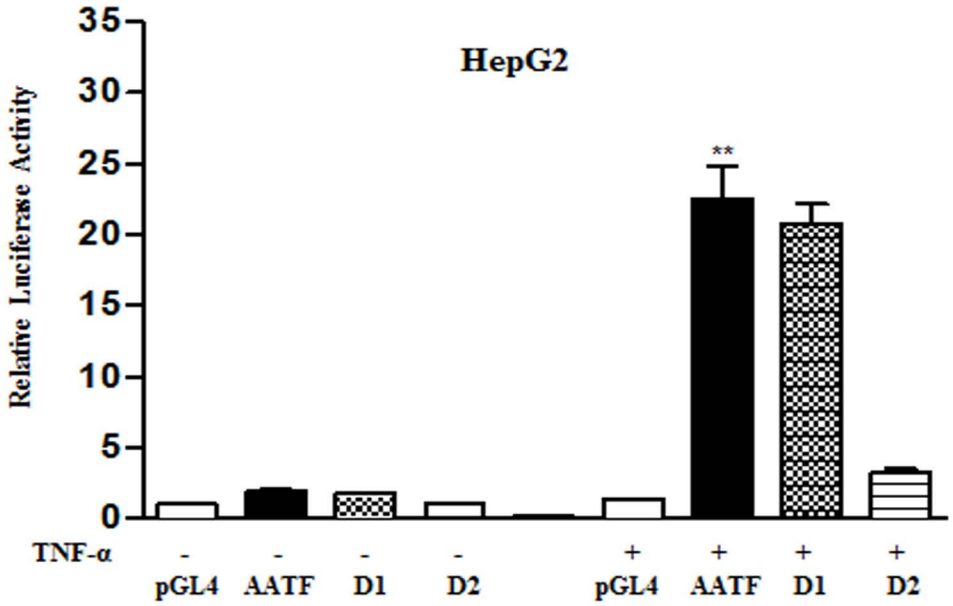

A
AATF mRNA (Fold Change)
3
2
1
0
**
**
**
TNF-α
0
12
24
0
12
24 h
HepG2
Huh7


B
SREBP1-c mRNA (Fold Change)
3
2
1
0
*
**
**
**
TNF-α
0
12
24
0
12
24 h
HepG2
Huh7

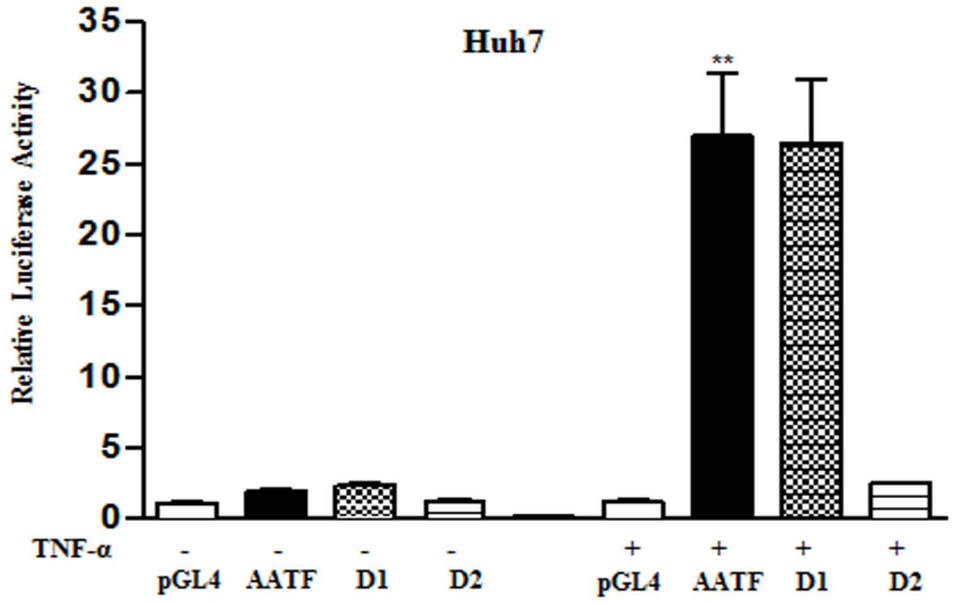

C
Si Control
Si SREBP1
TNF-α
-
+
-
+
SREBP1
β-actin
HepG2
Si Control
Si SREBP1
TNF-α
-
+
-
+
SREBP1
β-actin
Huh7


SfiI
Acc65I
KpnI
BaeI'
BaeI
PflMI
SanDI
FspAI
XhoI
NheI
PfoI
BmtI
BsaI
HindIII
SgrAI
MreI
200
400
600
800
1000
SREBP1 0.92
Luc2P
AATF promoter
AATF promot...deletion 1
AATF...n 2

A

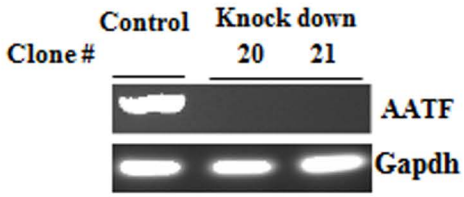

HepG2
Relative Luciferase Activity
35
30
25
20
15
10
5
0
**
TNF-α
-
-
-
-
+
+
+
+
pGL4
AATF
D1
D2
pGL4
AATF
D1
D2

B


Huh7
Relative Luciferase Activity
35
30
25
20
15
10
5
0
**
TNF-α
-
-
-
-
+
+
+
+
pGL4
AATF
D1
D2
pGL4
AATF
D1
D2

Supplementary Figure 5

A



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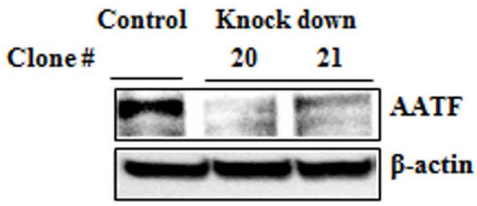
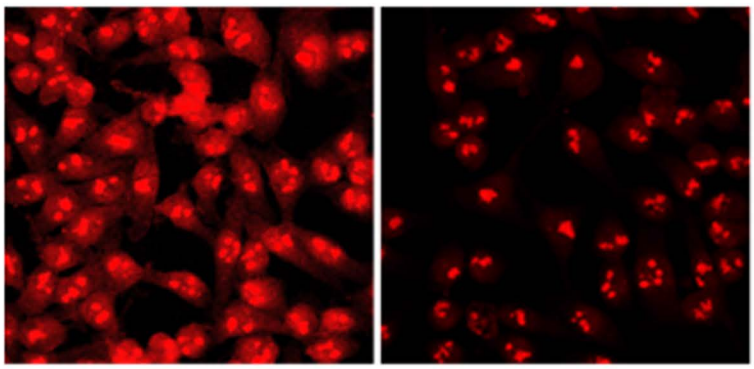


C



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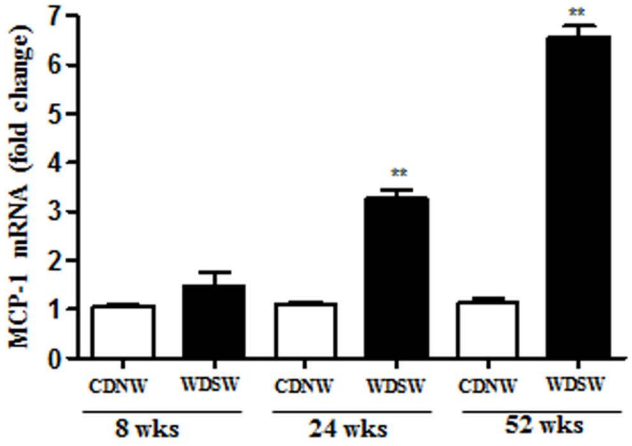
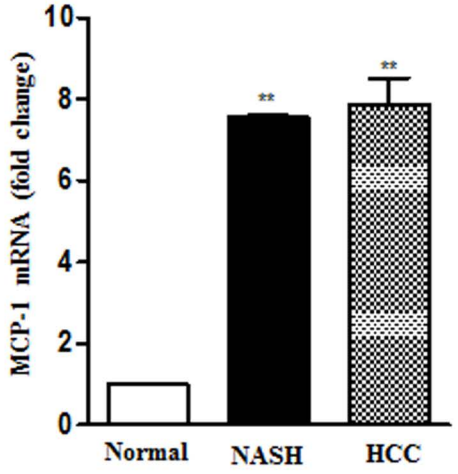
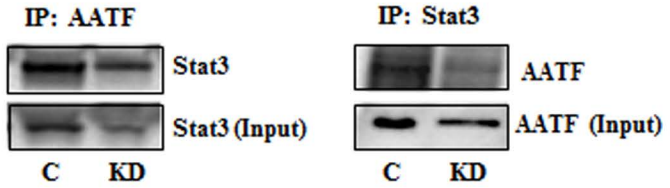

MCP-1 mRNA (fold change)
0
1
2
3
4
5
6
7
**
**
CDNW
WDSW
CDNW
WDSW
CDNW
WDSW
8 wks
24 wks
52 wks

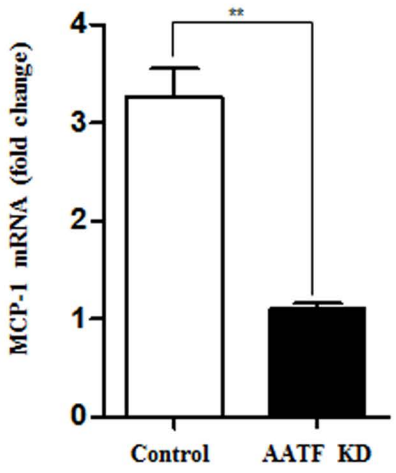
B


MCP-1 mRNA (fold change)
0
2
4
6
8
10
**
**
Normal
NASH
HCC

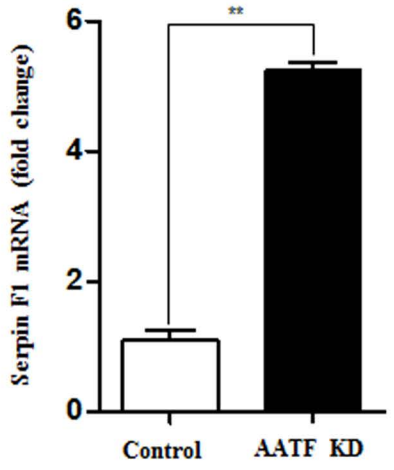
C


IP: AATF
Stat3
Stat3 (Input)
C
KD
IP: Stat3
AATF
AATF (Input)
C
KD

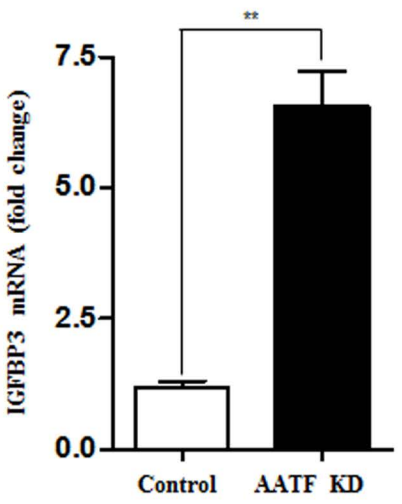
A


**
MCP-1 mRNA (fold change)
4
3
2
1
0
Control
AATF KD

B


**
Serpin F1 mRNA (fold change)
6
4
2
0
Control
AATF KD

C


**
IGFBP3 mRNA (fold change)
7.5
5.0
2.5
0.0
Control
AATF KD