Supplementary Methods:

Cell culture and treatment

The HSC-T6 cell line was purchased from Shanghai FuMeng Gene Biotechnology corporation (Shanghai, China). HSC-T6 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Keygen, China) containing 5 % fetal bovine serum (FBS, Biological Industries, Israel) at 37 °C in a humidified atmosphere containing 5 % CO_2.

Blood Measurements

Blood samples collected from mice were used to measure alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Blood samples were drawn from mice 4 weeks after with or without intraperitoneal injection of 10 % CCl_4. Serum ALT and AST levels were measured by alanine aminotransferase/aminotransferase Assay Kits (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions. The absorbance at 510 nm was obtained with a Multiskan MK3 (Biotek, USA).

Histopathology

The middle portion of the left lobe of the liver of C57BL/6J mice was excised and then perfused in 4 % paraformaldehyde for 24-48 h. After fixation, the tissue was embedded in paraffin, and 5μm sections were stained with hematoxylin and eosin (H&E) for morphological analysis and sirus-red and Masson’s trichrome staining for collagen expression by using the standard protocols. The stained tissues were scanned by Panoramic MIDI (3D HISTECH, Hungary) and viewed by CaseViewer slice software.

Oil-Red O staining

Fresh liver tissues were immersed in freezing medium and stored at -80 °C. The liver tissues were sliced, washed with PBS three times (x5min) and stained with Oil Red O agent according to the manufacturer's protocol (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Immunohistochemistry

Immunohistochemistry (IHC) was performed on paraffin sections to examine the expression of α-SMA, RCAN1, CD8, Ly6G, DNMT1, DNMT3a and DNMT3b using a microwave-based antigen retrieval technique. Sections were incubated with rabbit anti-α-SMA (1:50 dilution), anti-RCAN1 (1:200 dilution), anti-CD8 (1:50 dilution), anti-Ly6G (1:50 dilution), anti-DNMT1 (1:200 dilution), anti-DNMT3a (1:200 dilution), and anti-DNMT3b (1:200 dilution) for overnight at 4°C. After incubation with the secondary antibody and chromagen liquid DAB (3, 30-diaminobenzidine tetrahydrochloride), the slides were counterstained with hematoxylin. Non-immune rabbit IgG instead of the primary antibody was used as a negative control. The stained tissues were examined with an inverted fluorescence microscope (OLYMPUS IX83, Tokyo, Japan), or scanned by Panoramic MIDI (3D HISTECH, Hungary) and viewed by CaseViewer slice software. The characteristics of antibodies used are listed in **Table S1**

Immunofluorescence

To determine the colocalization of α-SMA and RCAN1 *in vivo*, double immunofluorescent staining was performed. Briefly, freshly dissected liver tissues were OCT-embedded, and the sections (10 μm in thickness) were cut with a cryotome Cryostat (Leica, CM 1950, Germany). Frozen tissue sections were incubated with 10% BSA for one hour at room temperature to block

unspecific staining. After rinsing, the slides were treated with Cy3 (red)-conjugated anti-α-SMA antibody (1:50 dilution) in combination with FITC (green)-conjugated anti-RCAN1 (1:200 dilution) overnight at 4 ℃. The stained sections were scanned by Panoramic MIDI (3D HISTECH, Hungary) and viewed by CaseViewer slice software.

HSC-T6 cells were fixed and permeabilized in 4 % paraformaldehyde, 0.2 % TritonX-100 in PBS for 10 min. Nonspecific binding was blocked with 10 % BSA for 1 h at RT. Subsequently, the cells were incubated with primary antibodies for RCAN1 (1:200 dilution), α-SMA (1:50 dilution), DNMT1 (1:200 dilution), DNMT3a (1:200 dilution), and DNMT3b (1:200 dilution) overnight at 4 ℃. Sections were washed twice with PBS and incubated with fluorescein-labeled secondary antibody at a dilution of 1:50 for 1.5 h at RT in the dark. Slides were mounted in mounting media with DAPI for 5 min at RT. After washing twice with PBS, the slides were covered with DABCO and the stained tissues were examined with an inverted fluorescence microscope (OLYMPUS IX83, Tokyo, Japan). The characteristics of antibodies are listed in **Table S1**.

TUNEL Staining

To visualize apoptotic bodies, liver tissue sections were fixed in 10 % buffered formalin at room temperature for 25 min and then washed with PBS containing 0.2 % Triton X-100 at room temperature. Subsequently, the sections were covered with 100 μL equilibration buffer for 10 min and rTdT reaction was added. The reaction was terminated using saline-sodium citrate. Next, liver tissue sections were immersed in 0.3 % H_2O_2 for 3-5 min, washed three times with PBS, immersed in 50 μL TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) detection solution at 15–25 °C for 1 h. Finally, slides were incubated with DAPI (Bi

Yuntian Biological Technology, China) for 10 min and scanned by Panoramic MIDI (3D HISTECH, Hungary) and viewed by CaseViewer slice software.

Western blot analysis

Total Proteins from cultured HSC-T6 cells and primary hepatic stellate cells were extracted with RIPA lysis buffer (contained 1 % PMSF) (Beyotime, China). Nuclear proteins and cytoplasmic proteins were extracted using CelLytic™ NuCLEAR™ Extraction Kit (BestBio, Shanghai, China) according to the manufacturer's instructions. The concentration of protein was determined by the BCA protein assay kit (Beyotime, Jiangsu, China). Proteins of each sample 30–50 μg were separated by SDS-PAGE gel (10 % or 12 %) and then transferred onto PVDF membranes (Millipore Corp, Billerica, MA, USA). The membranes were blocked in 5 % skim milk for 3 h to block nonspecific binding. Subsequently, membranes were incubated with the primary antibodies against RCAN1, COL1a1, α-SMA, β-actin, C-myc, Cyclin D1, Bax, Bcl2, cleaved-caspase3, DNMT1, DNMT3a, DNMT3b, calcineurin (CaN), NFAT1, NFAT2, NFAT3, and NFAT4 overnight at 4 °C, followed by incubation with secondary antibody at room temperature for 1 h. Signals were captured with BioshineChemiQ image system. The intensities of Western blotting bands were quantified and analyzed by using the Image J software (NIH, Bethesda, MD, USA). The characteristics of antibodies are listed in **Table S1.**

RNA extraction and RT-qPCR analysis

Total RNA was extracted from primary HSCs isolated from a fibrotic mouse model and cultured HSC-T6 cells using TRIzol (Invitrogen, USA) according to the manufacturer's instructions. The total RNA was uniformly quantified to 1000 ng/μl by the Thermo Scientific

NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA). Single-stranded cDNA was synthesized from total RNA using the PrimeScript ® RT reagent kit (Takara, JAP) according to the manufacturer's instructions. SYBR-Green real-time quantitative PCR (RT-q-PCR) analysis was performed for RCAN1.1, RCAN1.4, α-SMA, Col1a1, GAPDH and β-actin mRNAs by using QuantiNova SYBR Green PCR Kit (Takara, Japan) and the Pikoreal 96 real-time PCR system (Thermo Scientific, USA). The ratio for the mRNA of interest index was normalized to GAPDH (in mice) or β-actin (in rat) and expressed as the mean ± s.e.m. The sequences of primers used for RT-qPCR are listed in **Table S2**.

Transfection of RCAN1.4 and NFAT3 overexpression plasmid constructs and RCAN1.4 small hairpin RNA into HSC-T6 cells

Overexpression or knock-down of RCAN1.4 and knock-in of NFAT3 were assessed by Western blotting and RT-qPCR analysis after transfection of HSC-T6 cells with rat-derived RCAN1.4 and NFAT3 overexpression plasmids or RCAN1.4 shRNA,. All shRNAs and plasmid constructs were obtained from GenePharma Co., Ltd. Cells (3 x 10^5/mL) were seeded in 6-well plates and transfected with the RCAN1.4 plasmid or shRNA and control constructs mixed with Lipo2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Cells were incubated with Opti-MEM at 37 °C and 5 % CO_2 for 6 h. Subsequently, cells were cultured in DMEM containing 5 % FBS. The RCAN1.4 overexpressing or knockdown cells were treated with TGF-β1 (10 ng/mL) for 24h and then harvested for Western blotting, RT-qPCR or other analyses.

RNA interference (RNAi) analysis

For DNMT1, DNMT3a, and DNMT3b silencing, HSC-T6 cells were transfected with corresponding siRNAs (GenaPharma, China). The culture medium was replaced by DMEM containing 5 % FBS and TGF-β1 (10 ng/mL) was added 6 h after transfection. The following siRNA sequences were used: DNMT1-siRNA (sense: 5'-CCCAGAGUAUGCACCAAUATT-3' and antisense: 5'-UAUUGGUGCAUACUCUGGGTT-3'); DNMT3a-siRNA (sense: 5'-GCGUCACACAGAAGCAUAUTT-3' and antisense: 5'-AUAUGC UUCUGUGUGACGCTT-3'); DNMT3b-siRNA (sense: 5'-AGAUGACAGGUGCCCAGAGUU-3' and antisense: 5'-CUCUGGGCACCUGUCAUGUUU-3'); siRNA-control with scrambled sequence: (sense: 5'-UUCUCCGAACGUGUCACGUTT-3' and anti-sense: 5'-ACGUGACACGUUCGGAGAATT-3').

Scratch wound healing assay

Parallel were drawn behind a 6-well plate at a distance interval of 0.5-1 cm. RCAN1.4-RNAi transfection experiment was performed as previously described in this part. When the cells were about 90 % confluent, scratches perpendicular to the parallel were made using a spearhead. Following 12 h of culture, the images of cell motility close to the scratches were captured using Zeiss inverted microscope Primovert (Carl Zeiss, Jena, Germany). Cell migration ability was reflected by the width of scratch. Each experiment was repeated at least three times.

Transwell assay

Transwell chamber system (Costar 3422, Corning, USA) was used to investigate the effects of RCAN1.4 silencing on cell migration. 2×10^5 RCAN1.4-RNAi-transfected cells in serum-free DMEM were added to each upper inset pre-coated with 8.0 μm Pore Polycarbonate Membrane. DMEM containing 5 % FBS was added to the lower chamber. After 24 h of culture, the non-

invading cells were removed and stained with crystal violet. Finally, the images were captured using Zeiss inverted microscope Primovert (Carl Zeiss, Jena, Germany).

String database analysis

The protein interactions with RCAN1 were predicted by the online String database (Search Tool for the Retrieval of Interacting Genes, http://string-db.org/).

Cell cycle analysis

After 24 h of transfection, cells were trypsinized and washed three times with cold PBS, then fixed in 70 % ethanol in PBS at 4 °C overnight. Subsequently, cells were washed three times with cold PBS and incubated with propidium iodide (PI) staining buffer at 37 °C for 30 min in the dark. The cell cycle was analyzed on BD FACS Verse (BD Biosciences) and cell populations were quantified by the ModFit data analysis software package (Verity Software House, USA). All experiments were performed in triplicate and repeated at least three times.

Cell apoptosis analysis

The number of apoptotic and necrotic cells was detected by flow cytometry. Transfected HSC-T6 cells were cultured with TGF-β1 (10 ng/mL) for 24h, digested with trypsin, and centrifuged at 1500 r.p.m. for 5 min. Subsequently, 400 μL Annexin V binding fluid was added to about10^6 cells /mL and the cells were stained with 5 μL FITC for 15 min at 4 °C in the dark. Cells were re-stained with 10 μL PI for 5 min, placed at 4 °C in the dark and measured with a laser eight-color flow cytometer (FACSVerse, BD, USA). The results were quantified using the FlowJo 7.6 software.

Cell proliferation analysis

The proliferation of HSC-T6 cells was determined by Cell Counting Kit-8 (CCK-8) assay. 100 μL HSC-T6 cells were seeded in 96-well culture plates at a density of 5×10^3 per well, and the edge wells were filled with sterile PBS. After attachment, HSC-T6 cells were transfected with GV230-Control or GV230-RCAN1.4 plasmid or RCAN1.4-RNAi or scrambled-RNAi for 6h in Opti-MEM and cultured in DMEM containing 5 % FBS (Biological Industries, Israel). HSC-T6 cells in the model and transfected groups were cultured with TGF-β1 (10 ng/mL) for 24 h. Subsequently, 10 μL CCK-8 solution (Sigma, USA) was added to 6-well culture plates for 4 h. The value of absorbance (A) was measured at 490 nm. Cell viability = the A value of experimental group wells/the A value of blank group wells * 100%. All experiments were performed in triplicate and repeated at least three times.

Supplementary Figure Legends:

Figure S1. Liver fibrosis mouse model was established in C57BL/6j mice. (A) Gross pathology of liver tissues from the vehicle and CCl_4-stimulated groups. Representative pictures of **(B)** H&E, **(C)** Masson and Sirius-Red, **(D)** Oil-red O, **(E)** α-SMA, **(F)** CD8, **(G)** Ly6G, **(H)** TUNEL staining and **(J)** Desmin staining of liver tissue sections were shown. **(I)** Serum ALT and AST levels. Values represent the mean ±s.e.m. $^{***}p<0.001$ compared to Vehicle group.

Figure S2. (A) The α-SMA protein expression was detected by immunofluorescence. Representative picture (Original magnification was 400×).

Figure S3. (A) Macroscopic examination of fresh liver tissues without fixation by an inverted

fluorescence microscope. **(B)** Representative CD8, Ly6G, and TUNEL staining (Original magnification is indicated). **(C)** The proteins that interact with RCAN1.4 were predicted by online String database.

Figure S4. The mRNA levels of vimentin, S100A4, and fibronectin were examined. $^{***}p < 0.001$ as indicated.

Figure S5. (A) The efficiency of RCAN1.4-RNAi transfection was determined by RT-qPCR analysis. The influence of RCAN1.4 knock-down on HSC-T6 cell migration was assessed by **(B)** scratch wound healing assay and **(C)** Transwell experiment. Original magnification is indicated.

Figure S6. (A) The cytoplasmic proteins were extracted 24 h after GV230-RCAN1.4 plasmid or RCAN1.4-RNAi were transfected in activated HSC-T6 cells. The protein levels of NFAT1, NFAT2, NFAT3, and NFAT4 were detected by Western blotting analysis. Representative images of three independent experiments are shown. **(B)** Total proteins were isolated 24 h after GV230-RCAN1.4 plasmid or RCAN1.4-RNAi transfection in activated HSC-T6 cells. The protein level of NFAT3 was determined by Western blotting analysis. **(C)** The protein levels of CaN, NFAT1, NFAT2, NFAT3, and NFAT4 in liver fibrosis tissue were detected by Western blotting analysis. $^{\#\#}p<0.01$, $^{\$}p<0.05$, $^{**}P<0.01$, as indicated. **(D)** The transfection efficiency of NFAT3 plasmid was detected by Western blotting analysis, $^{*}p<0.05$ as indicated. **(E)** The protein levels of NFAT3, COL1a1, and α-SMA in the GV114-NFAT3 transfected group was examined by Western blotting analysis, $^{*}p<0.05$, $^{\#}P<0.05$, as indicated. **(F)** Expression of COL1a1 and α-SMA, $^{*}p<0.05$, $^{\#}P<0.05$, as indicated. For all panels, data represent the mean ± s.e.m. for 3–4 independent

experiments.

Figure S7. (A) Protein levels of DNMT1, DNMT3a, and DNMT3b were determined by immunohistochemistry staining *in vivo* and immuno-fluorescence analysis *in vitro*. Representative views from each group are presented. **(B)** The mRNA expression of RCAN1.4 in DNMT1-RNAi-, DNMT3a-RNAi-, DNMT3b-RNAi-, and scrambled-RNAi transfected groups was measured by RT-qPCR analysis. $^{\#\#}p<0.01$ as indicated. Values represent the mean ±s.e.m.

Table S1. The characteristics of antibodies

Protein	Application	Origin	Dilution
RCAN1	WB&IHC&IF	D6694, Sigma-Aldrich, USA	1:800&1:200&1:200
COL1a1	WB	bs10423R, Bioss, China	1:300
α-SMA	WB&IHC&IF	bs0189R, Bioss, China	1:300&1:50&1:50
DNMT1	WB&IHC&IF	ab13537, Abcam, UK	1:800&1:200&1:200
DNMT3a	WB&IHC&IF	ab13888, Abcam, UK	1:800&1:200&1:200
DNMT3b	WB&IHC&IF	ab2851, Abcam, UK	1:800&1:200&1:200
C-myc	WB	D3N8F, Cell Signaling Technology, USA	1:800
CyclinD1	WB	92G2, Cell Signaling Technology, USA	1:800
Bax	WB	2772s, Cell Signaling Technology, USA	1:800
Bcl2	WB	ab194583, Cell Signaling Technology, USA	1:800
Caspase3	WB	9662, Cell Signaling Technology, USA	1:800
CaN	WB	ab52761, Abcam, UK	1:800
NFAT1	WB	ab2722, Abcam, UK	1:800

NFAT2	WB	ab2796, Abcam, UK	1:800
NFAT3	WB	bs6461R, Bioss, China	1:300
NFAT4	WB	bs2952, Bioss, China	1:300
CD8	IHC	GB11068, Servicebio, China	1:500
Ly6G	IHC	GB11229, Servicebio, China	1:500
Desmin	IHC	GB12081, Servicebio, China	1:800

Table S2. The sequence of primers used for RT-qPCR

Gene (species)	Forward	Reverse
Rat		
RCAN1.1	5'-AGAAGTACG AGCCAC ACGCA-3'	5'-TTCCTCCTCCTCCTCTTGGT-3'
RCAN1.4	5'-AAGGCCTCCTCTCCTTGTTC-3'	5'-GTGTGATTGCCACAC ACTCA-3'
Colla1	5'-GATCCTGCCGATGTCGCTAT-3'	5'-TGTAGGCTACGCTGTTCTTGCA-3'
α-SMA	5'-CGAAGCGCAGAGCAAGAGA-3'	5'-CATGTCGTCCCAGTTGGTGAT-3'
Vimentatin	5'-ACCCTGCAGTCATTCAGACA-3'	5'- CTGCAGCTCCTGGATCTCTT-3'
Fibronectin	5'-AATGGTGACAGTTGGTTGCC-3'	5'- CATTGCATCGTGGTTGGCTA-3'
S100A4	5'-GCTGCATTCCAGAAGCTGAT-3'	5'- CATCATGGCAATGCAGGACA-3'
β-actin	5'-CCCATCTATGAGGGTTACGC-3'	5'-TTTAATGTCACGCACGATTTC -3'
Mouse		
RCAN1.1	5'-GATGGAGGAGGTGGA TCT GC-3'	5'-ATTCAAATTTGGCCCGGCAC-3'
RCAN1.4	5'-CGG TCTCCGTTGTTTCCT TC-3'	5'-CCTCTGCCCTCTACT TCCAC-3'
Colla1	5'-TGTAAACTCCCTCCACCCCA-3'	5'-TCGTCTGTTTCCAGGGTTGG-3'
α-SMA	5'-CGGGCTTTGCTGGTGATG-3'	5'-CCCTCGATGGATGGGAAA-3'
GAPDH	5'-GGACCTCATGGCCTACATGG-3'	5'-TAGGGCC TCTCTTGCTCAGT-3'

Figure S1

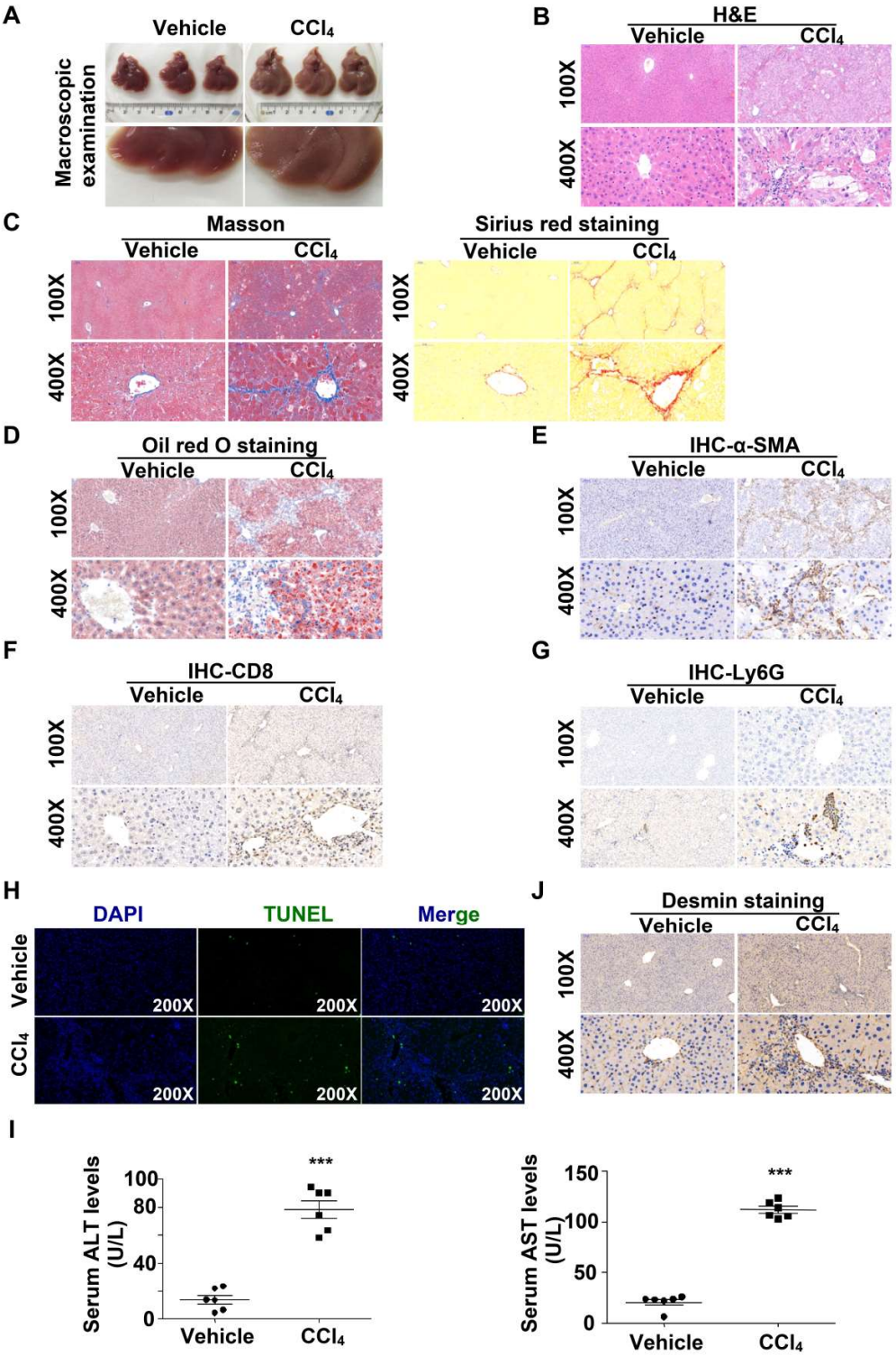

A
Vehicle
CCl4
Macroscopic examination
B
H&E
Vehicle
CCl4
100X
400X
C
Masson
Vehicle
CCl4
100X
400X
Sirius red staining
Vehicle
CCl4
100X
400X
D
Oil red O staining
Vehicle
CCl4
100X
400X
E
IHC-α-SMA
Vehicle
CCl4
100X
400X
F
IHC-CD8
Vehicle
CCl4
100X
400X
G
IHC-Ly6G
Vehicle
CCl4
100X
400X
H
DAPI
TUNEL
Merge
Vehicle
CCl4
200X
J
Desmin staining
Vehicle
CCl4
100X
400X
I
Serum ALT levels (U/L)
100
80
40
0
***
Vehicle
CCl4
Serum AST levels (U/L)
150
100
50
0
***
Vehicle
CCl4

Figure S2

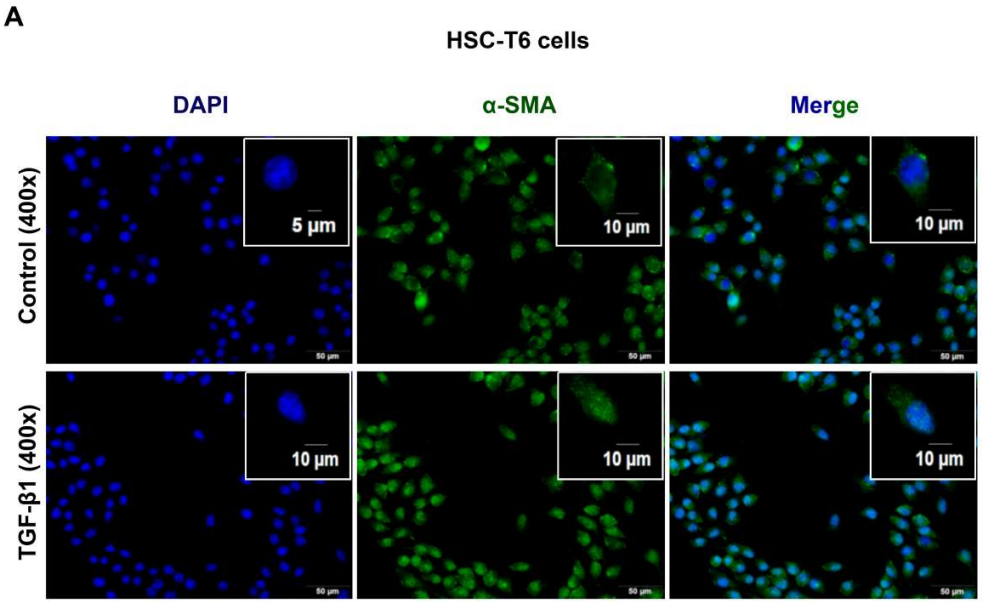
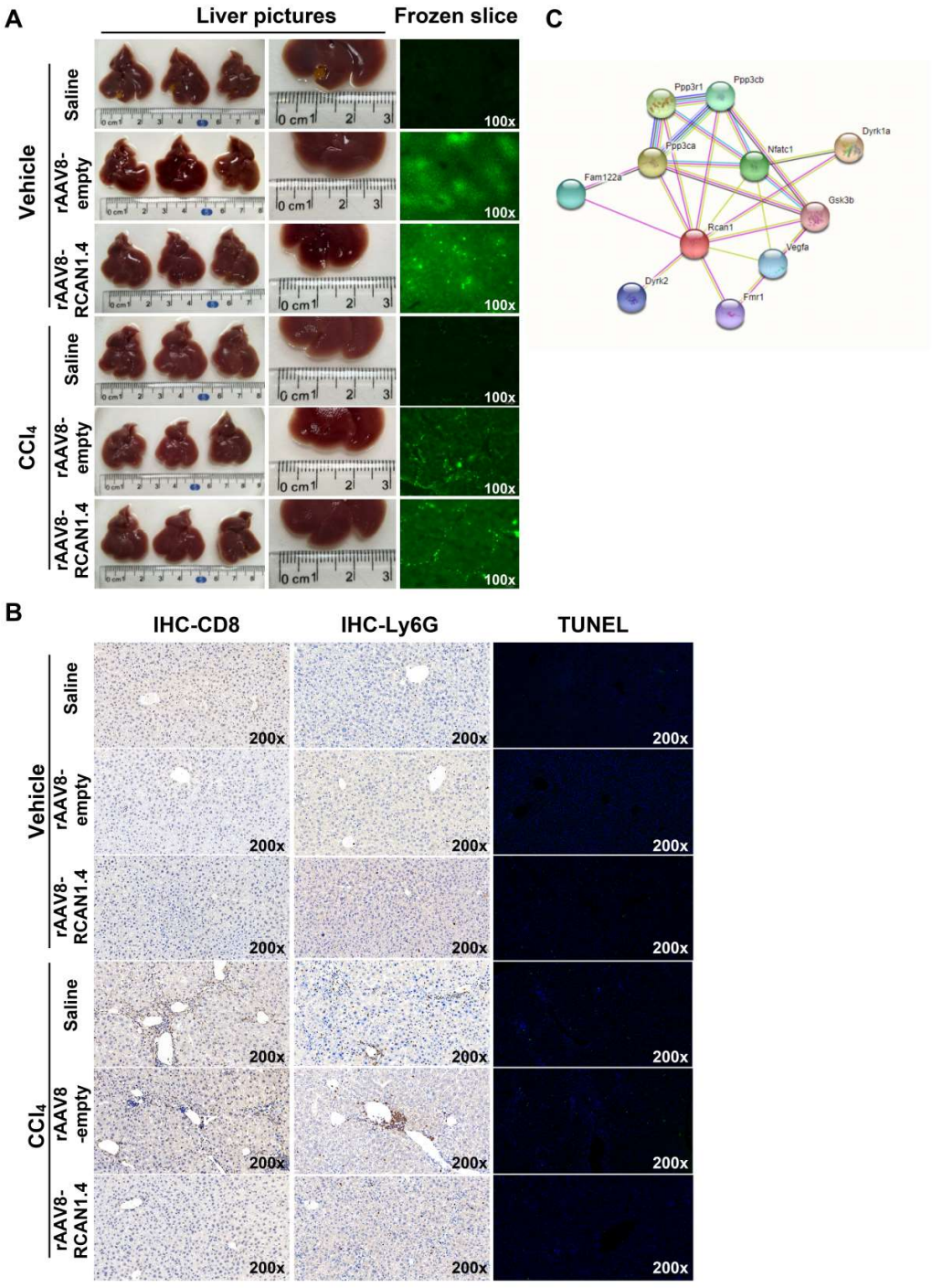

A
HSC-T6 cells
DAPI
α-SMA
Merge
Control (400x)
TGF-β1 (400x)
5 μm
10 μm
10 μm
10 μm
10 μm
10 μm

Figure S3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Frozen slice
Vehicle
CCl4
Saline
rAAV8-empty
rAAV8-RCAN1.4
100x
C
Ppp3r1
Ppp3cb
Dyrk1a
Ppp3ca
Nfatc1
Fam122a
Gsk3b
Rcan1
Vegfa
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Fmr1
B
IHC-CD8
IHC-Ly6G
TUNEL
rAAV8-empty
200x

Figure S4

A

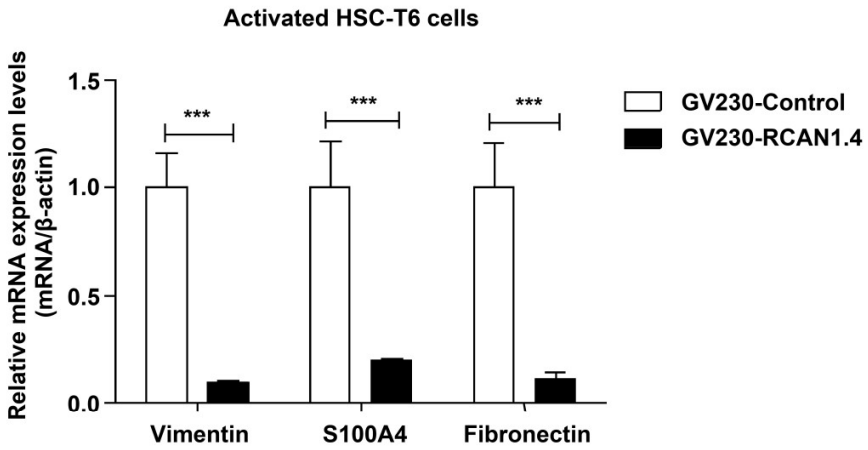

Activated HSC-T6 cells
Relative mRNA expression levels (mRNA/β-actin)
1.5
1.0
0.5
0.0
***
***
***
GV230-Control
GV230-RCAN1.4
Vimentin
S100A4
Fibronectin

Figure S5

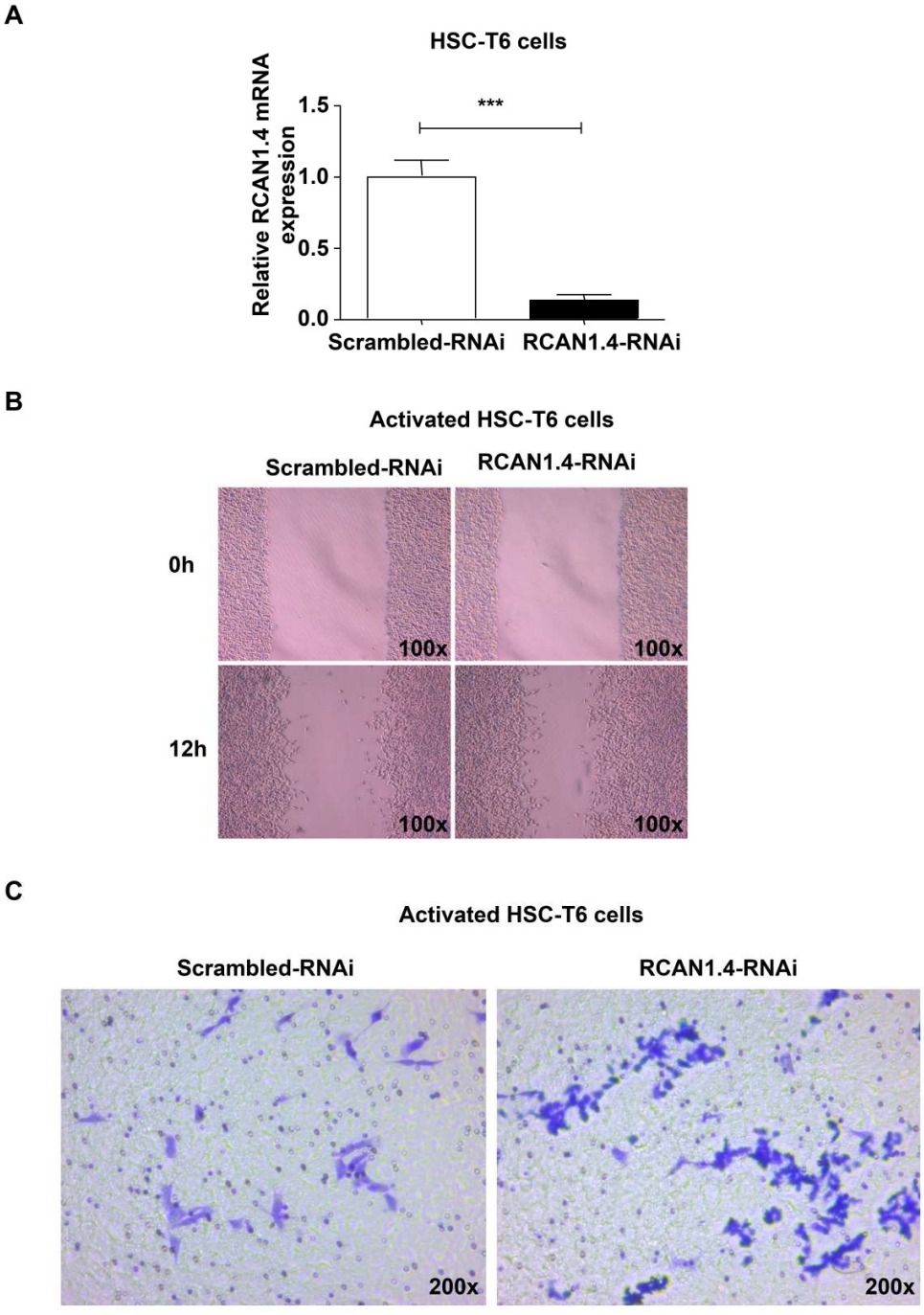

A
HSC-T6 cells
Relative RCAN1.4 mRNA expression
1.5
1.0
0.5
0.0
***
Scrambled-RNAi
RCAN1.4-RNAi
B
Activated HSC-T6 cells
Scrambled-RNAi
RCAN1.4-RNAi
0h
12h
100x
100x
100x
100x
C
Activated HSC-T6 cells
Scrambled-RNAi
RCAN1.4-RNAi
200x
200x

Figure S6-1

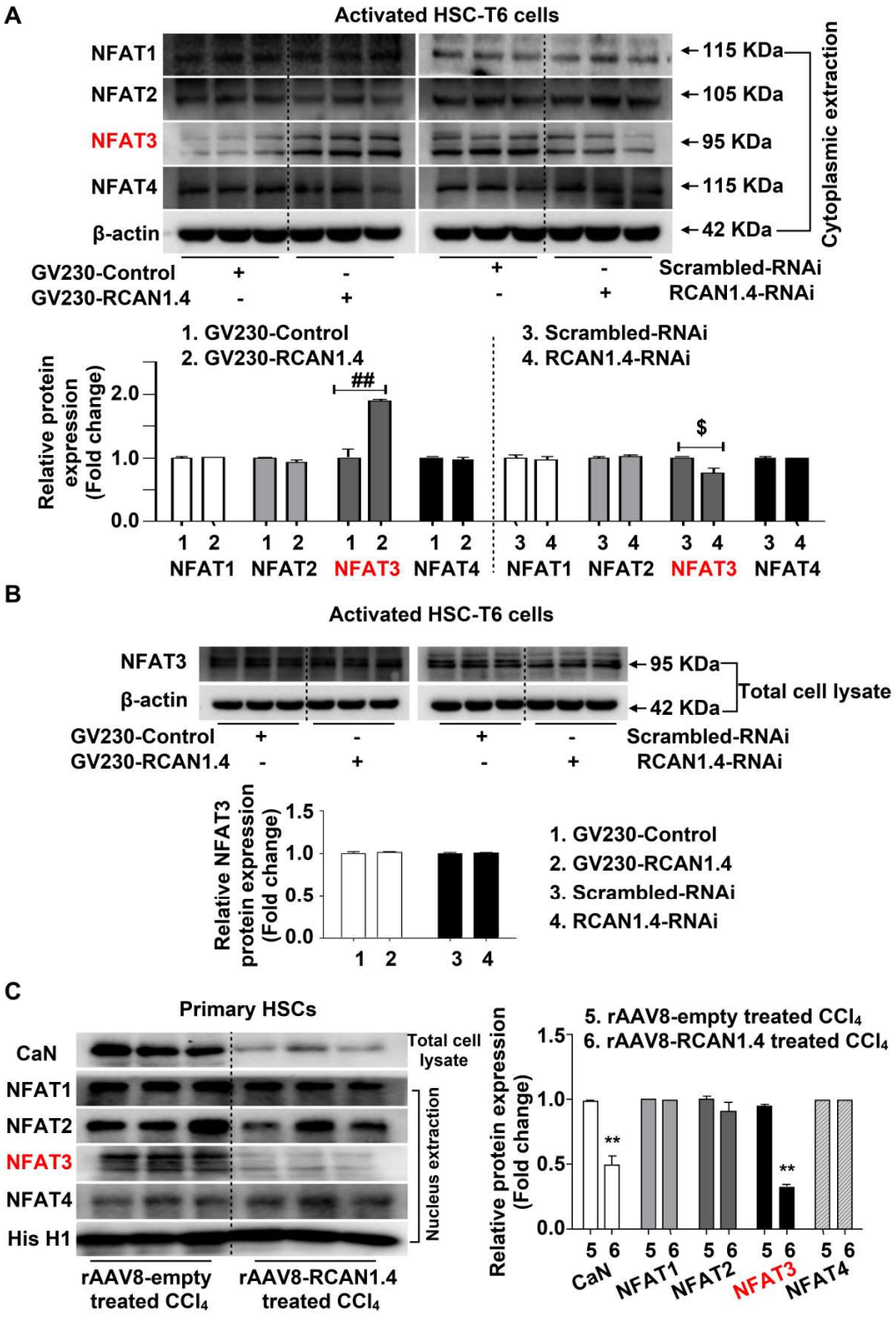
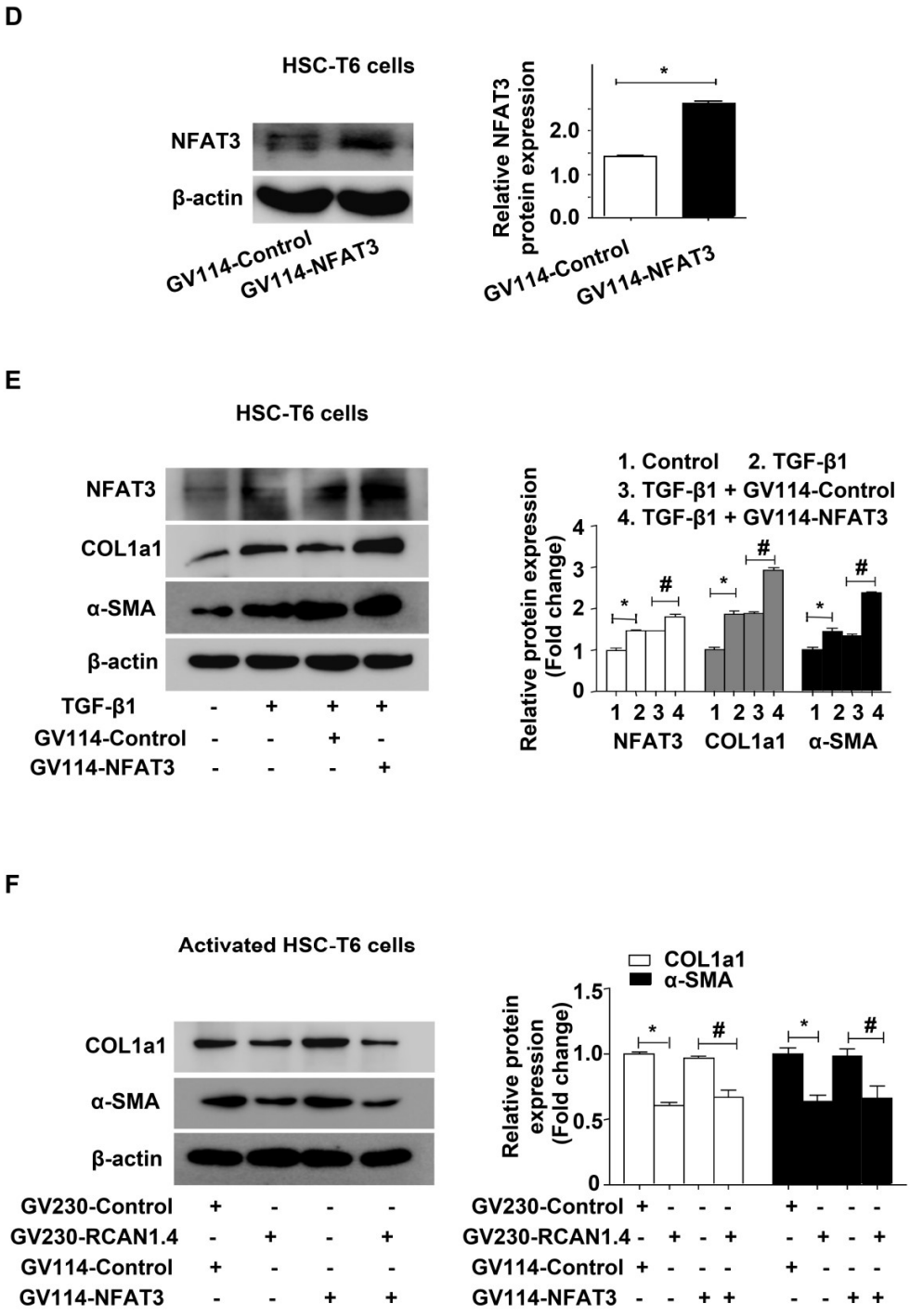

A
Activated HSC-T6 cells
NFAT1
NFAT2
NFAT3
NFAT4
β-actin
115 KDa
105 KDa
95 KDa
115 KDa
42 KDa
Cytoplasmic extraction
GV230-Control
GV230-RCAN1.4
Scrambled-RNAi
RCAN1.4-RNAi
1. GV230-Control
2. GV230-RCAN1.4
3. Scrambled-RNAi
4. RCAN1.4-RNAi
Relative protein expression (Fold change)
##
$
B
Activated HSC-T6 cells
NFAT3
β-actin
95 KDa
42 KDa
Total cell lysate
Relative NFAT3 protein expression (Fold change)
C
Primary HSCs
CaN
NFAT1
NFAT2
NFAT3
NFAT4
His H1
Total cell lysate
Nucleus extraction
rAAV8-empty treated CCl4
rAAV8-RCAN1.4 treated CCl4
5. rAAV8-empty treated CCl4
6. rAAV8-RCAN1.4 treated CCl4
Relative protein expression (Fold change)

Figure S6-2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HSC-T6 cells
NFAT3
β-actin
GV114-Control
GV114-NFAT3
Relative NFAT3 protein expression
2.0
1.0
0.0
*
GV114-Control
GV114-NFAT3
E
HSC-T6 cells
NFAT3
COL1a1
α-SMA
β-actin
TGF-β1 - + + +
GV114-Control - - + -
GV114-NFAT3 - - - +
1. Control 2. TGF-β1
3. TGF-β1 + GV114-Control
4. TGF-β1 + GV114-NFAT3
Relative protein expression (Fold change)
4
3
2
1
0
* # * # * #
1 2 3 4 1 2 3 4 1 2 3 4
NFAT3 COL1a1 α-SMA
F
Activated HSC-T6 cells
COL1a1
α-SMA
β-actin
GV230-Control + - - -
GV230-RCAN1.4 - + - +
GV114-Control + - - -
GV114-NFAT3 - - + +
COL1a1
α-SMA
Relative protein expression (Fold change)
1.5
1.0
0.5
0
* # * #
GV230-Control + - - - + - - -
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Figure S7-1

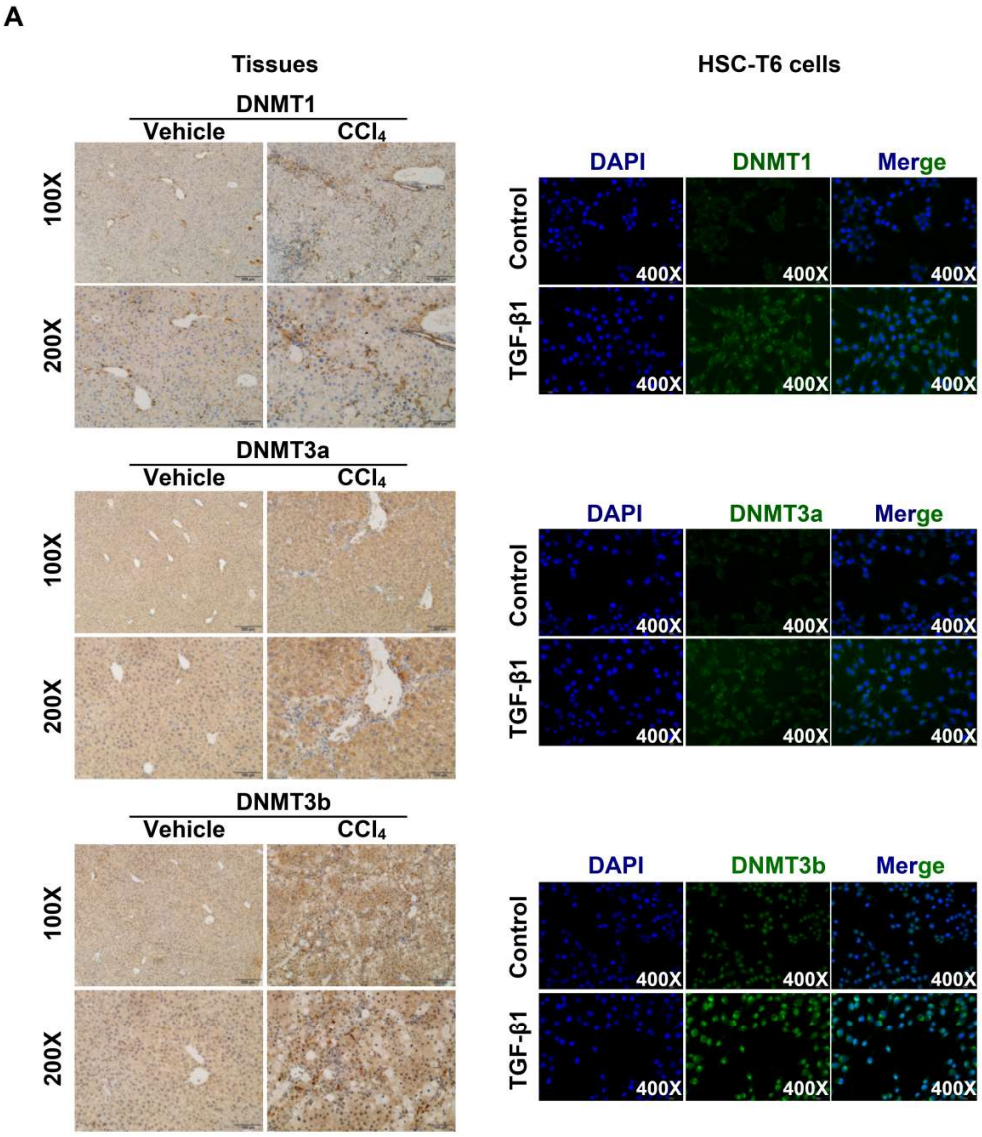
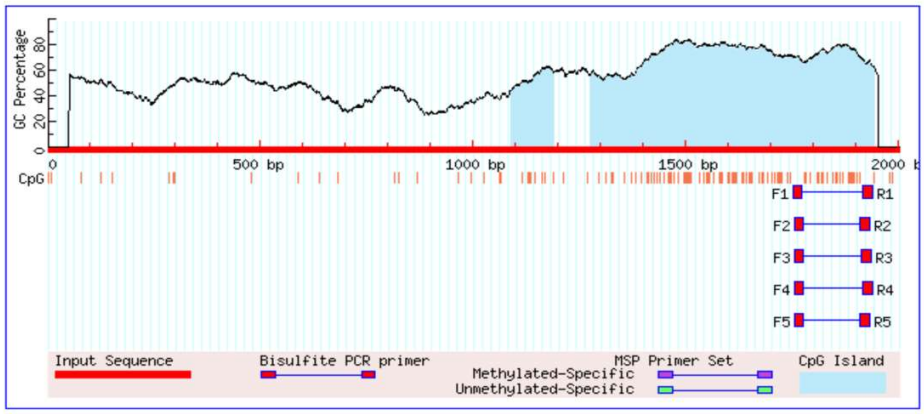

A
Tissues
HSC-T6 cells
DNMT1
Vehicle
CCl4
100X
200X
DAPI
DNMT1
Merge
Control
TGF-β1
400X
DNMT3a
DAPI
DNMT3a
Merge
DNMT3b
DAPI
DNMT3b
Merge

Figure S7-2

B



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Sequence Length: 2001

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(Criteria used: Island size > 100, GC Percent > 50.0, Obs/Exp > 0.6)
2 CpG island(s) were found in your sequence

	Size	(Start - End)
Island 1	101 bp	(1089 - 1189)
Island 2	667 bp	(1277 - 1943)

C