Supplementary Material and Methods

Cells culture and virus. The Huh7.5.1 cell line was provided by Francis Chisari (The Scripps Research Institute, La Jolla, CA). Cells were maintained in complete Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37℃ with 5% CO2. Primary human hepatocytes were obtained from TRL (Morrisville, NC) and maintained in Williams medium E containing cell maintenance supplement (Invitrogen, Carlsbad, CA). The HCV JFH-1 strain was used.[1]

siRNA-mediated knockdown and plasmid transfection. Non-Targeting (NT), TM6SF2, PNPLA3, CD81, ApoE, MTP, SREBF-1 or SREBF-2 ON-TARGETplus small interfering RNA (siRNA) SMARTpool were purchased from Dharmacon (Lafayette, CO). The siRNAs were transfected into Huh7.5.1 cells or PHH at a final concentration of 100 nM using a reverse transfection protocol with Oligofectamine (Invitrogen, Carlsbad, CA) as previously described.[2] To monitor efficient siRNA-mediated knockdown, total RNA (RNeasy, Qiagen, Hilden, Germany) was collected at 72 hours post-infection (p.i.). Knockdown was confirmed by qRT-PCR and normalized to 18S RNA. The TM6SF2 (entire ORF) vector and the empty parental vector were obtained commercially (Origene, Rockville, MD). We generated the pE167K plasmid using Q5® Site-Directed Mutagenesis Kit (NEB, Ipswich, Massachusetts) according to manufacturer's instructions. Plasmid transfection was performed using FuGENE 6 transfection reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions.

Viral RNA isolation and quantification. Total RNA was isolated with RNeasy Mini Kit (Qiagen, Hilden, Germany) from cells, or with QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) from supernatants. Copy numbers of intracellular and extracellular HCV RNA were determined by qRT-PCR with the probe, primers, and parameters described previously.[3] The relative amount of intracellular HCV RNA was normalized to the internal control human 18S rRNA (Applied Biosystems, Foster City, CA).

Western blot. Automated quantitative Western blots were performed using the Wes simple western instrument (Proteinsimple, San Jose, CA). The assays were carried out using the manufacturers protocol and the data was analyzed using the Compass software (Proteinsimple, San Jose, CA). The instrument quantified the chemiluminescent signal and the software extrapolated the bands. The following antibodies were obtained commercially: anti-TM6SF2 (MaxPab, Abnova, Taipei City, Taiwan), anti-Actin (ab8227, Abcam, Cambridge, UK), anti-NS3 (8G-2, Abcam, Cambridge, UK).

Gene expression assay. Total cellular RNA was prepared with the RNeasy Mini Kit (Qiagen, Hilden, Germany). The mRNA expression levels of target genes were quantified by quantitative real-time reverse transcriptase-PCR (qRT-PCR) using gene-specific primers and probes (IDT, Coralville, IA) and verso 1-step qRT-PCR Mix (Thermofisher, Waltham, MA) on an ABI 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). Relative mRNA levels were

calculated with the ΔΔ*CT* method, using 18S rRNA as the internal control for normalization.

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Intracellular and extracellular infectious particles. The number of HCV infectious particles from 48 h-infected and/or transfected Huh7.5.1 cells was determined. For intracellular infectivity assays, infected cells were washed with PBS, and lysed by incubation in 500 µL of water to induce hypo-osmotic shock. Lysates were clarified by centrifugation at 10,000 x g for 10 minutes at 4℃.[4] Cell lysates or supernatants were diluted in DMEM–10% FBS, and 100 µL was used to inoculate Huh7.5.1 cells. Infection was examined at 48 h p.i. by immunofluorescence, using the anti-HCV core monoclonal antibody (produced from the α-core 6G7 hybridoma cells provided by Harry Greenberg and Xiaosong He, Stanford University, Stanford, CA) with the Alexa Fluor 488 goat α-mouse IgG (A11001; Life Technologies, Carlsbad, CA) and viral foci were counted manually. The percentage of infection was calculated by comparing levels with those for the control during the assay.

Proteolytic digestion protection assay. At 48 h following HCV infection, cells seeded in 6-well plates were collected into 170 µl of PK buffer (50 mM Tris-HCl [pH 8.0], 10 mM $CaCl_2$, 1 mM dithiothreitol [DTT]) and subjected to 5 freeze-thaw cycles. Samples (50 µL) were either left untreated or treated with 1% Triton X-100 for 1 h at room temperature and with 50 g/mL PK (Roche, Basel, Switzerland) for 1 h at 4°C. PK digestion was terminated by addition of 5 mM phenylmethylsulfonyl fluoride (PMSF) and 10 min of incubation at 4°C. The level of residual core was determined by traditional western blotting as described previously[5] and using anti-core antibody (C7-50, Thermofisher, Waltham, MA).

Immunofluorescence Labeling. Infected and siRNA transfected cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.1% Tween 20 –2% bovine serum albumin (BSA) in PBS. Cells were double-labeled by incubation overnight in permeabilization buffer with the primary antibody: mouse monoclonal anti-core Ab (C7–50, Thermo Scientific, Waltham, MA), HCS LipidTOX (Thermo Scientific, Waltham, MA) and/or rabbit monoclonal anti-calnexin Ab (C5C9, Cell Signaling, Danvers, MA). Secondary Abs were incubated in the same buffer followed by Hoechst dye. Confocal microscopy was performed with an Zeiss confocal microscope (Carl Zeiss, Thornwood, NY). Percentages of colocalization were determined by the Zen Software (Carl Zeiss, Thornwood, NY).

Supplementary references

1. Wakita T, Pietschmann T, Kato T, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 2005;11:791–796.

2. Li Q, Pène V, Krishnamurthy S, et al. Hepatitis C virus infection activates an innate pathway involving IKK-α in lipogenesis and viral assembly. Nat Med 2013;19:722–729.

3. **Li Q, Brass AL**, Ng A, et al. A genome-wide genetic screen for host factors required for hepatitis C virus propagation. Proc Natl Acad Sci 2009;106:16410–16415.

4. Etienne L, Blanchard E, Boyer A, et al. The Replacement of 10 Non-Conserved Residues in the Core Protein of JFH-1 Hepatitis C Virus Improves Its Assembly and Secretion. PLOS ONE 2015;10:e0137182.

5. Boyer A, Dumans A, Beaumont E, et al. The Association of Hepatitis C Virus Glycoproteins with Apolipoproteins E and B Early in Assembly Is Conserved in Lipoviral Particles. J Biol Chem 2014;289:18904–18913.

Author names in bold designate shared co-first authorship

Supplementary figures

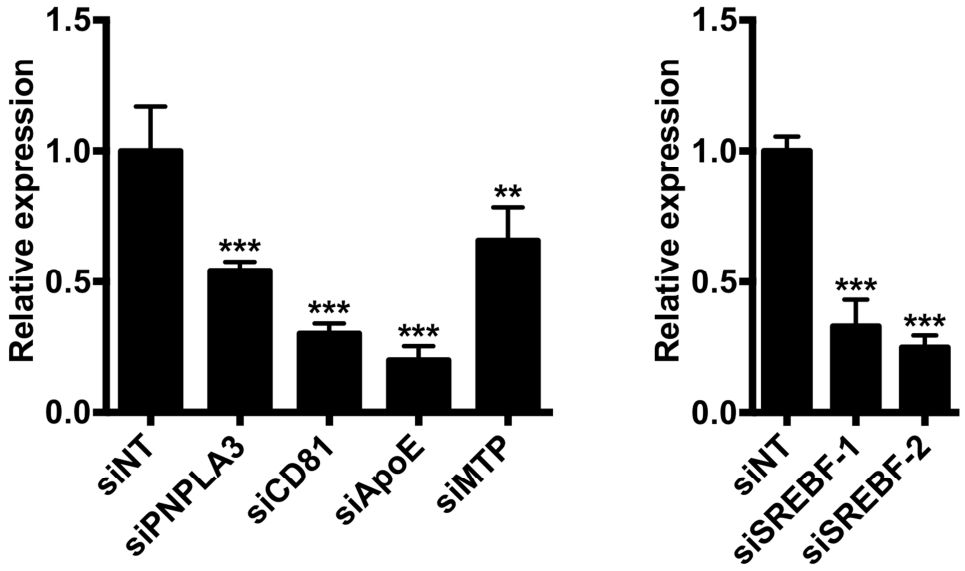

Figure S1: Relative expression of mRNA after siRNA transfection. 72 h post-transfection of siRNAs as indicated on the graph, the corresponding mRNA levels were measured by qRT-PCR and standardized with 18S RNA. The mRNA level of each gene is shown as a relative expression unit with the siNT level set as 1. The data represent means of 3 independent experiments in triplicates (n=9). **P < .01; ***P < .001 (Mann Whitney's test).

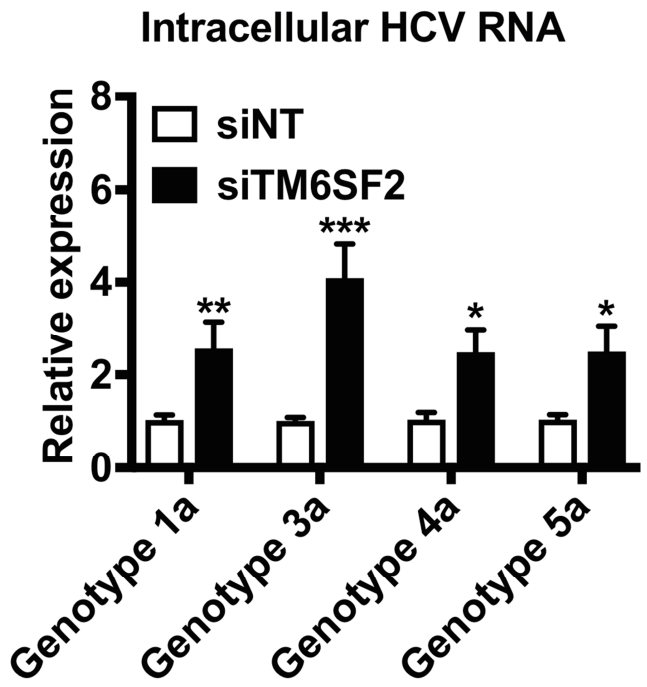

Figure S2: The effect of TM6SF2 knockdown on HCV is not HCV genotype-dependent. Huh7.5.1 cells were transfected with siRNA for 72 h and then infected with chimeric HCV. Viral particles were produced from chimeric JFH1 construct: non-structural proteins were from JFH1 (genotype 2a) and structural proteins were from different genotype 1a, 3a, 4a and 5a. Total RNAs were extracted from cells at 48 h p.i. Intracellular HCV RNA levels were quantified by qRT-PCR (standardized to 18S RNA) and converted to relative expression units (siNT as 1). Plotted data represent means from at least 2 independent experiments in triplicates. *P < .05; **P < .01; ***P < .001 (Mann Whitney's test).

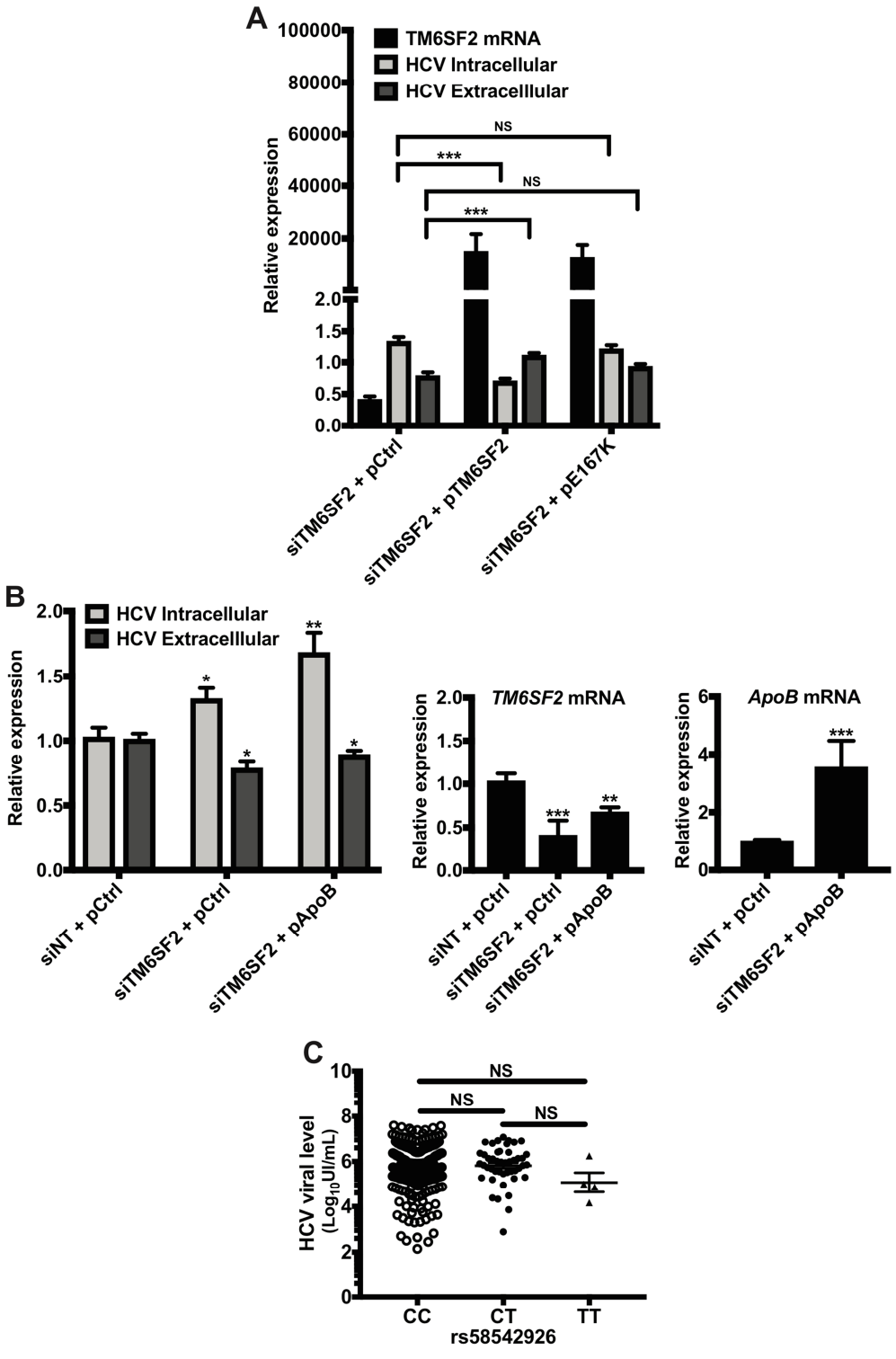

Figure S3: The effect of TM6SF2 knockdown is abrogated by ectopic expression of functional TM6SF2 but not by ApoB. Huh7.5.1 cells were transfected with siNT or siTM6SF2 then with *TM6SF2* (pTM6SF2), the mutant

TM6SF2-E167K (pE167K) (A) or *ApoB* (B) expression plasmids for 8 h and infected with HCV. Intra and extracellular total RNA were extracted at 48 h p.i. HCV RNA and mRNA levels were quantified by qRT-PCR (standardized to 18S RNA) and converted to relative expression units (siNT as 1). Shown values are means ± SEM. NS, Non-Significant; *P < .05; **P < .01; ***, *P*-value < .001 (Mann Whitney's test). (D) HCV viremia load was evaluated for each TM6SF2 genotype *rs58542926* (C>T; E167K variant) in 393 Caucasian CHC patients with *TM6SF2* genotype CC (n=343), CT (n=46), TT (n=4). NS: *P*-value not significant.

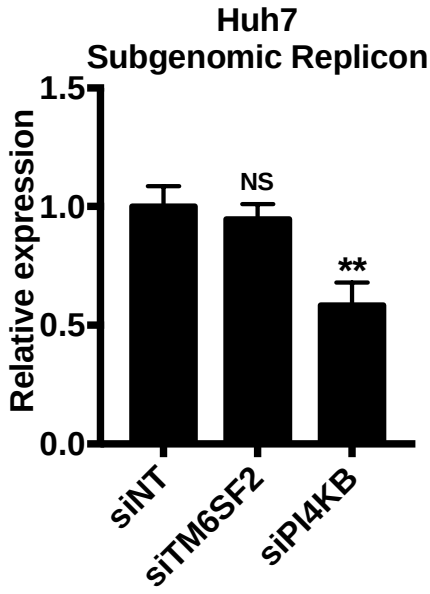

Figure S4: Effect of TM6SF2 knockdown on HCV replication. The siRNAs indicated on the graph were transfected into Huh7 cell line stably expressing HCV subgenomic replicon (SGR) luciferase reporter system. After 72 h, luciferase activity was measured and shown as relative expression units (non-targeting siRNA set as 1). Shown values are means ± SEM. NS, Non-Significant; *P < .05 (Mann Whitney's test).

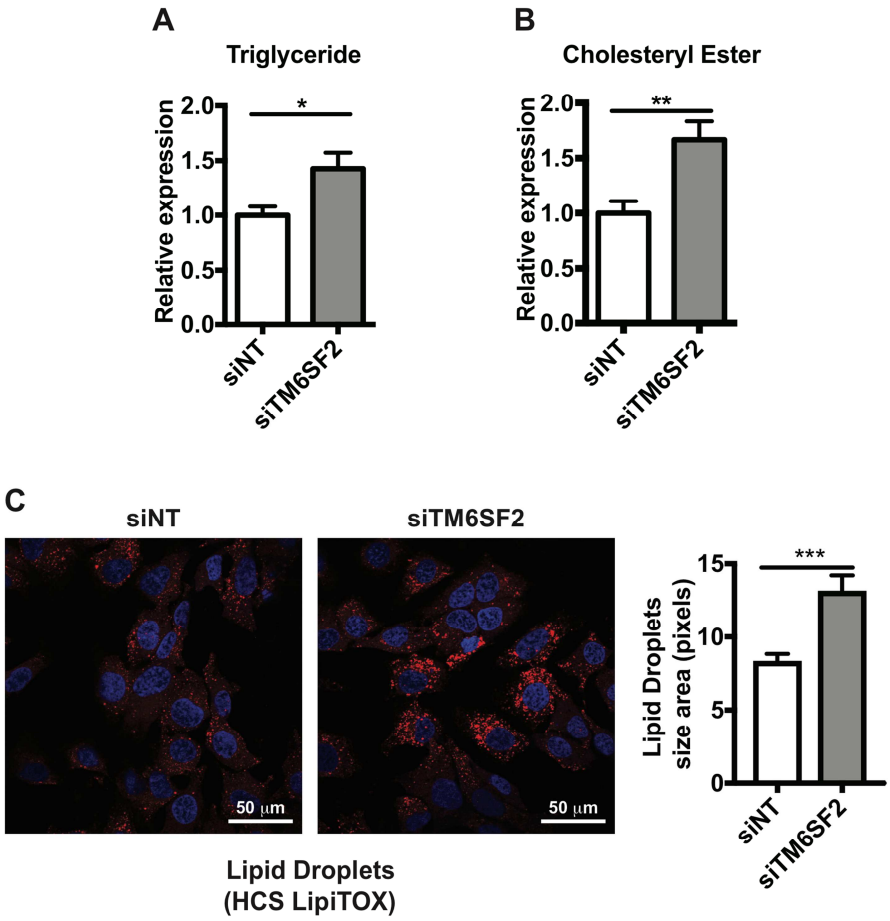

Figure S5: TM6SF2 knockdown induces lipid droplet accumulation in Huh7.5.1 cells. After siTM6SF2 transfection in Huh7.5.1 cells, the relative expression of (A) cellular triglycerides and (B) cholesteryl ester concentrations were measured with assays supplied by Biovision and Abcam according to the instructions of manufacturers. Data in A and B represent 3 independent experiments in triplicates (n=9). (C) After fixation of cells, LDs and nuclei were stained with respectively HCS LipidTOX and Hoechst dye. Average of LDs (size area) were quantified, using Image J software, for the siNT and siTM6SF2 samples (siNT cells: n=1754; siTM6SF2 cells: n=1878). White bar scale, 50 µm. Shown values are means ± SEM. NS, Non-Significant; *P < .05; **P < .01; ***, *P*-value < .001 (Mann Whitney's test).

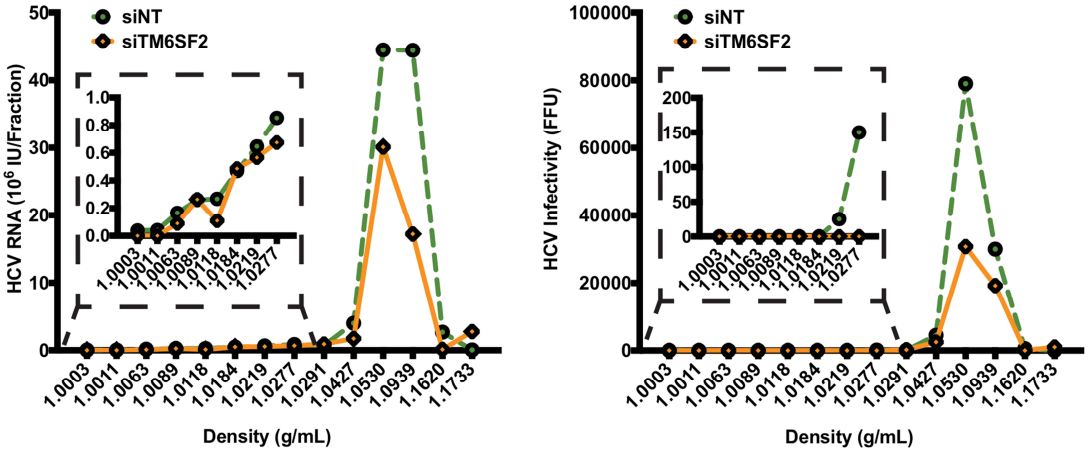

Figure S6: TM6SF2 knockdown reduces the lipidation of lipoviroparticles. The supernatants from HCV-infected Huh7.5.1 cells overexpressing TM6SF2 or control cells were subjected to isopycnic centrifugation through iodixanol gradients, as described in Materials and Methods. Each fraction was used to infect Huh7.5.1 cells. At 48 h p.i., cells were then stained with anti-HCV core Ab and viral focus forming units (FFU) were counted manually.

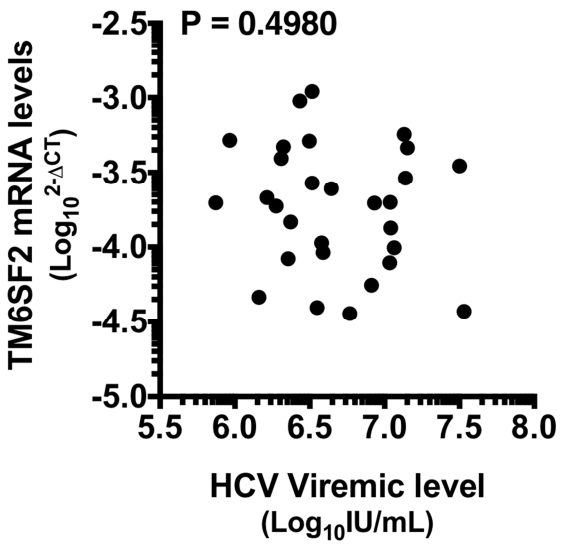

Figure S7: Lack of correlation between hepatic *TM6SF2* mRNA and HCV viremic levels. Total RNA extractions were obtained from 29 liver biopsies of HCV infected patients. The *TM6SF2* mRNA levels ($2^{-\Delta CT}$) were quantified by qRT-PCR (standardized to 18S RNA) and correlated with the HCV viremic levels at the time of liver biopsy (Spearman rank correlation's test).

Table S1: Baseline characteristics of chronic hepatitis C patients.

	CHC Patients (n = 446)
Age (mean ± SD), y	46 ± 10.4
Female (% patients)	172 (39)
ALT (mean ± SD)	112 ± 139
Viral load (mean ± SD), log IU/mL	5.65 ± 0.9
HCV Genotype (% patients)	
1	301/408 (74)
2	63/408 (15)
3	36/408 (9)
4	8/408 (2)
TM6SF2 rs58542926 genotype (% patients)	
CC	343/393 (87)
CT	46/393 (12)
TT	4/393 (1)