Cell Reports, Volume *17*

Supplemental Information

Solute Carrier NTCP Regulates Innate Antiviral Immune Responses Targeting Hepatitis C Virus Infection of Hepatocytes

Eloi R. Verrier, Che C. Colpitts, Charlotte Bach, Laura Heydmann, Laetitia Zona, Fei Xiao, Christine Thumann, Emilie Crouchet, Raphaël Gaudin, Camille Sureau, François-Loïc Cosset, Jane A. McKeating, Patrick Pessaux, Yujin Hoshida, Catherine Schuster, Mirjam B. Zeisel, and Thomas F. Baumert

Supplemental Figures

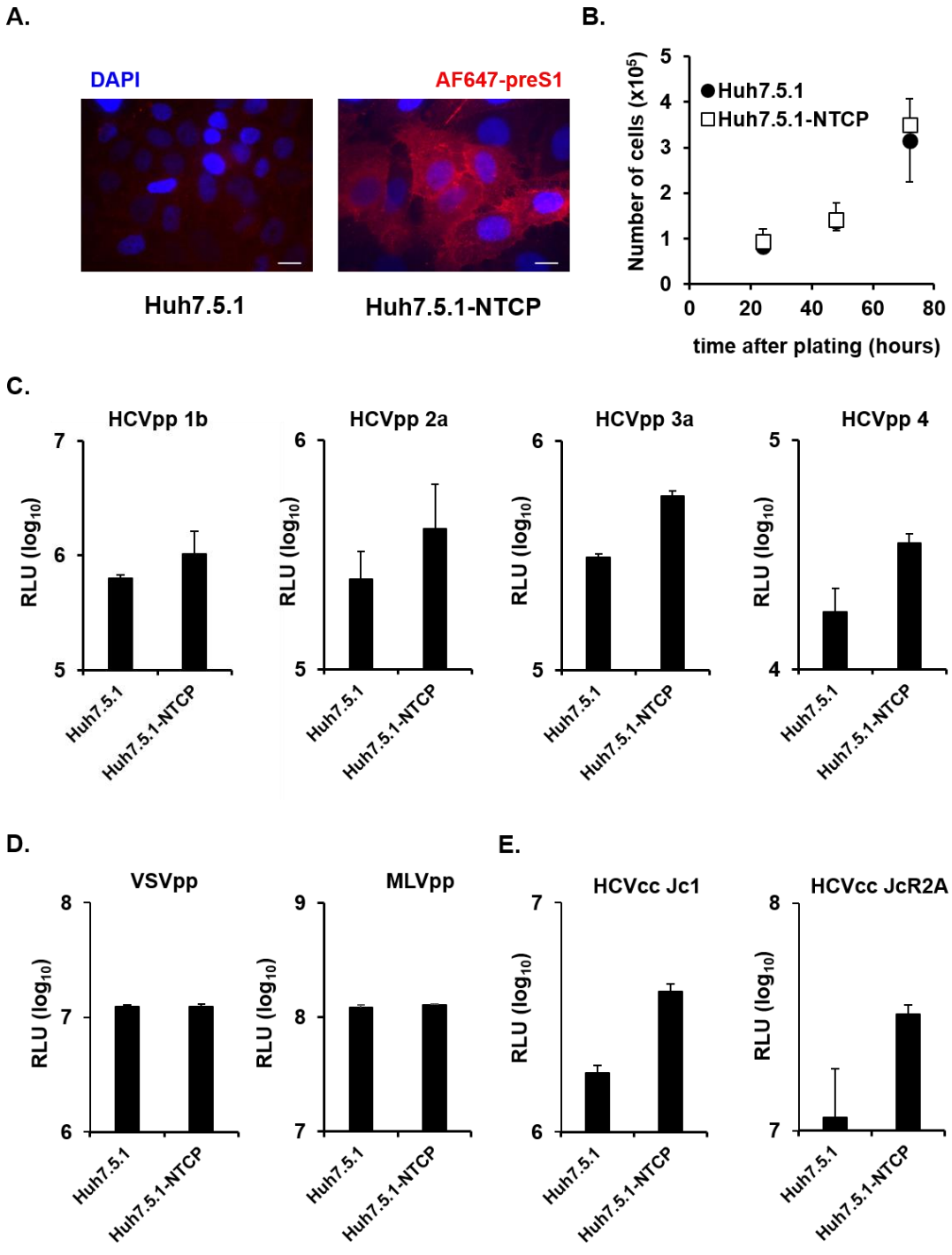

Figure S1. NTCP modulates HCV infection, related to Figure 1. A. Immunodetection of NTCP expression in Huh7.5.1 and Huh7.5.1-NTCP cells. Huh7.5.1 and Huh7.5.1-NTCP cells were treated with AF647-labelled preS1 peptide. One experiment is shown. Scale bar, 10 µm. NTCP protein was visualized in red after AF647-preS1 binding and cell nuclei were stained with DAPI (blue) **B.** Comparable cell growth between Huh7.5.1 and Huh7.5.1-NTCP cells. Huh7.5.1 and Huh7.5.1-NTCP cells were plated and cell growth was assessed every 24 h for 3 days by counting using an automated cell counter. Results are expressed as means ± SD cell counts from three independent experiments performed in triplicate (n=9). **C-E.** HCVpp, VSVpp, MLVpp, and HCVcc infection of NTCP-overexpressing cells. Huh7.5.1 and Huh7.5.1-NTCP cells were infected with HCVpp (genotypes 1b, 2a, 3a and 4, **C**), VSVpp and MLVpp **(D)**, or HCVcc (Luc-Jc1 and JcR2A, **E**). Infection was assessed after 72 h by measuring luciferase activity. Results are expressed as means ± SD log relative light units (RLU) from one representative experiment performed in triplicate (n=3).

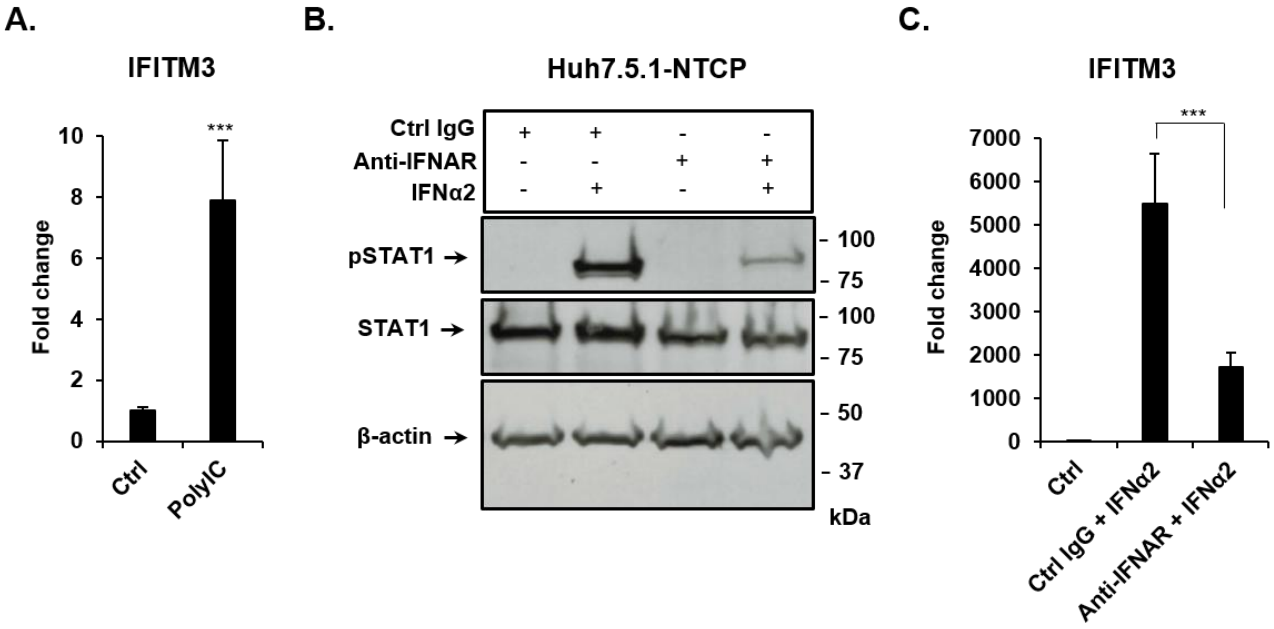

Figure S2. Expression and induction of ISGs in hepatoma cells, related to Figure 5. A. IFITM3 expression is induced by Poly(I:C) in Huh7.5.1-NTCP cells. Huh7.5.1-NTCP cells were reverse-transfected with Poly(I:C) (1 µM/mL) for 24 h. IFITM3 expression was then assessed by qRT-PCR. Results are expressed as means ± SEM relative IFITM3 expression (fold change) compared to IFITM3 expression in non-transfected cells (set at 1) from three independent experiments performed in triplicate (n=9). **B-C.** IFITM3 expression is induced by type I IFN treatment in a STAT1-dependent manner in Huh7.5.1-NTCP cells. Huh7.5.1-NTCP cells were treated with IFNα2 (1,000 UI/mL) in the presence or absence of a specific anti-IFNAR antibody. After 10 minutes of IFNα2 treatment, cells were lysed and STAT1 as well as phospho-STAT1 (pSTAT1) expression was assessed by Western blot (**B**). One experiment is shown. **C**. After 24 h of IFNα2 treatment, cells were lysed and IFITM3 expression was assessed by qRT-PCR. Results are expressed as means ± SD relative IFITM3 expression (fold change) compared to control cells (set at 1) from three independent experiments performed in triplicate (n=9).

Supplemental Experimental Procedures

Detection of NTCP protein by Western blot and immunofluoresence. Immunoblots of cell lysates using protein-specific antibodies (a rabbit anti-NTCP polyclonal antibody [Sigma, HPA042727] and a mouse anti-β-actin monoclonal antibody [Sigma, A5441]) were performed in parallel as described (Verrier et al., 2016). Cell lysates were pre-treated with peptide-N-glycosidase (PNGase, NEB) following the manufacturer's instructions. Quantification of protein expression was performed using ImageJ software. For detection of NTCP protein by immunofluorescence, a myristoylated peptide derived from the preS1 domain of the large HBV envelope protein labeled with Alexa Fluor 647 (AF647) fluorophore (Bachem, Switzerland) was used as described (Verrier et al., 2016). Briefly, cells were treated with the AF647-preS1 peptide at 4°C for two hours and then fixed with 4% paraformaldehyde. Nuclei were stained with DAPI. The preS1 peptide specifically binds to NTCP and its primary structure has been described elsewhere (Schieck et al., 2013; Yan et al., 2012). Fluorescent imaging was performed using an Axio Observer Z1 microscope (Carl Zeiss, Germany).

Cell viability assays and quantification of cell proliferation. Cell viability was assessed using PrestoBlue® Cell Viability Reagent (Fisher) as described (Verrier et al., 2016). Cell viability was assessed by measuring absorbance at 560 nm. For quantification of cell proliferation, Huh7.5.1 and Huh7.5.1-NTCP cells were seeded in 24 well plates for three days. Every 24 h, cell growth was assessed by counting cells using a TC20™ Automated Cell Counter (Bio-Rad).

Quantification of gene and protein expression. Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen) or ReliaPrep Kit (Promega). cDNA was reverse-transcribed from total RNA using Maxima reverse transcriptase (Thermo Scientific). qPCR was performed using a CFX96 thermocycler (Bio-Rad) according to the

manufacturer's instructions. Primers and TaqMan® probe for GAPDH, SLC10A1 (NTCP), IFITM2, IFITM3, PARP9, and CXCL10 mRNA detection were obtained from ThermoFisher (TaqMan® Gene Expression Assays). All values were normalized to GAPDH expression. IFITM3 protein expression was assessed by Western blot. Immunoblots of cell lysates using protein–specific antibodies (a rabbit anti-IFITM3 monoclonal antibody [Abcam, ab109429] and a mouse anti-β-actin monoclonal antibody [Sigma, A5441]) were performed as described (Verrier et al., 2016). Quantification of protein expression was performed using ImageJ software.

HCV cell-to-cell transmission assay. The HCV cell-to-cell transmission assay has been described previously (Xiao et al., 2014). Briefly, producer Huh7.5.1 cells were electroporated with HCV Jc1 RNA. GFP-overexpressing Huh7.5 cells (Xiao et al., 2014) were transduced or not with human NTCP-expressing VSVpp (GeneCopoeia). HCV-replicating Huh7.5.1 cells were then co-cultured with uninfected target Huh7.5-GFP or Huh7.5-GFP-NTCP cells in the presence of neutralizing antibody AP33 (25 µg/mL) to inhibit cell-free entry. After co-culture for 24 h, cells were fixed with 1% paraformaldehyde (PFA), stained with an NS5A-specific mouse antibody (0.1 mg/mL) and analyzed by flow cytometry as described (Xiao et al., 2014). Cell-to-cell transmission was defined as percentage HCV infection of Huh7.5-GFP target cells.

siRNAs and shRNA expression plasmids for perturbation studies. One siRNA pool (Qiagen GeneSolution siRNA GS6554) targeting NTCP or one non-targeting siRNA control (Qiagen) were reverse transfected into PHH and Huh7.5.1-NTCP cells with Lipofectamine® RNAiMAX (Invitrogen) as described (Lupberger et al., 2011). Three unique 29mer NTCP-targeting shRNA constructs in lentiviral GFP vector (pGFP-C-shLenti plasmid) expressing shNTCP #1 (ref TL309420A, 5' CCT CAG CAT TGT GAT GAC CAC CTG CTC CA 3'), shNTCP #2 (ref TL309420B, 5' CAT GGA GTT CAG CAA GAT CAA GGC TCA CT 3'), and shNTCP #3 (ref TL309420C, 5' CCA ACT CTG TTC CAC CAT CCT CAA TGT GG 3') were used to transduce cells for 72 h (ORIgene). A non-targeting shRNA sequence (shCTRL - ref TR30021, non-effective scrambled pGFP-lenti) was used as a control. One siRNA pool (SMARTpool ON-TARGETplus, Dharmacon) targeting IFITM3 or one siRNA control (Dharmacon) were reverse transfected into Huh7.5.1-NTCP cells with Lipofectamine® RNAiMAX (Invitrogen) as described (Lupberger et al., 2011).

HCV entry factor expression. HCV entry factor expression was assessed by flow cytometry using antibodies targeting CD81 (QV6A8F2C4) (Fofana et al., 2013), SR-BI (QQ4G9A6) (Zahid et al., 2013), CLDN1 (OM7D3B3) (Fofana et al., 2010), OCLN (Invitrogen), or EGFR (Santa Cruz Biotechnologies) (Barth et al., 2008; Lupberger et al., 2011). The antibodies targeting CD81, SR-BI, CLDN1, and EGFR were used without cell permeabilization to allow cell surface protein quantification. For detection of NTCP protein, a myristoylated peptide derived from the preS1 domain of the large HBV envelope protein labeled with Alexa Fluor 647 fluorophore (Bachem, Switzerland) was used (Verrier et al., 2016). Cells were treated with the peptide for 1 h at 37°C and then fixed with 4% PFA. NTCP expression was then quantified by flow cytometry.

Cellular HCV E2 binding. The sE2 binding experiment was performed as described (Krieger et al., 2010; Zahid et al., 2013). Cells were incubated with His-tagged sE2 for 1 h and sE2 binding was quantified by flow cytometry using a RGS-His antibody (Qiagen).

Overexpression of IFITM2 and IFITM3 in Huh7.5.1-NTCP cells. Huh7.5.1-NTCP cells were seeded at 50% confluency 24 h prior to transfection with an empty vector, pCMV-HA-hIFITM2 (Addgene #58398), or pCMV-HA-hIFITM3 (Addgene #58397) (Yount et al., 2010) using FuGENE® HD Transfection Reagent (Promega). Protein expression was assessed by Western blot using a mouse anti-HA tag monoclonal antibody (Abcam ab18181) 72 h after transfection. Cells were then infected with HCVcc (Luc-Jc1) for 3 days and infection was assessed as described.

Stimulation of ISG expression in Huh7.5.1-NTCP cells. For Western blot analysis of STAT1 and pSTAT1 expression, Huh7.5.1-NTCP cells were incubated with a mouse monoclonal antibody targeting the type I IFN receptor (IFNAR) (Millipore MAB1155) or a mouse control IgG (Invitrogen) at 2.5 µg/mL for one hour before treatment with recombinant IFNα2a (BioVision) at 1,000 UI/mL. Following 10 minutes of IFNα2a treatment, cells were lysed and STAT1 and pSTAT1 expression were assessed by Western blot using rabbit specific antibodies (Cell signaling #9172 and #9167, respectively) following manufacturer's instructions. For IFITM3 expression after IFNα2a stimulation, Huh7.5.1-NTCP cells were co-incubated for 24 h with a mouse monoclonal antibody targeting IFNAR or a mouse control IgG at 2.5 µg/mL and IFNα2a at 1,000 UI/mL. IFITM3 gene expression was then assessed by qRT-PCR. For Poly(I:C) stimulation, Poly(I:C) (Sigma) (1µg/mL) was reverse transfected into Huh7.5.1-NTCP cells with Lipofectamine® RNAiMAX (Invitrogen). IFITM3 gene expression was assessed by qRT-PCR 24 h after transfection.

Investigation of the effects of bile acid on viral infection and IFITM3 expression. Huh7.5.1 or Huh7.5.1-NTCP cells were incubated for 72 h with the bile acid sodium taurocholate (25 µM and 100 µM) (Sigma) and/or the preS1 peptide (200 nM) or a scrambled peptide control (200 nM). Total RNA was then extracted, and IFITM3 expression was assessed by qPCR as described above, or cells were lysed and IFITM3 protein expression was assessed as described above. Alternatively, treated cells were infected with HCVcc (Luc-Jc1) for 72 h. PHHs were cultured in serum-free medium (i.e. containing no bile acids) and treated for 72 h with increasing concentrations (100 µM and 500 µM) of sodium taurocholate in the presence of the preS1 peptide or a control peptide (400 nM) and then infected with HCVpp (genotype 1b) for 72 h. Viral entry was assessed by measuring luciferase activity.

IFN signal transduction and HCV infection. PHHs were treated for 72 h with 500 µM of sodium taurocholate in the presence of the preS1 peptide or a control peptide (400 nM), in the presence of a mouse monoclonal antibody targeting the type I IFN receptor (IFNAR) (Millipore MAB1155) or a mouse control IgG (Invitrogen) at 2.5 µg/mL and then infected with HCVpp (genotype 1b) for 72 h. Viral entry was assessed by measuring luciferase activity.

Supplemental References

Barth, H., Schnober, E.K., Neumann-Haefelin, C., Thumann, C., Zeisel, M.B., Diepolder, H.M., Hu, Z., Liang, T.J., Blum, H.E., Thimme, R., *et al.* (2008). Scavenger receptor class B is required for hepatitis C virus uptake and cross-presentation by human dendritic cells. J. Virol. *82*, 3466-3479.

Fofana, I., Krieger, S.E., Grunert, F., Glauben, S., Xiao, F., Fafi-Kremer, S., Soulier, E., Royer, C., Thumann, C., Mee, C.J., *et al.* (2010). Monoclonal anti-claudin 1 antibodies prevent hepatitis C virus infection of primary human hepatocytes. Gastroenterology *139*, 953-964.

Fofana, I., Xiao, F., Thumann, C., Turek, M., Zona, L., Tawar, R.G., Grunert, F., Thompson, J., Zeisel, M.B., and Baumert, T.F. (2013). A novel monoclonal anti-CD81 antibody produced by genetic immunization efficiently inhibits Hepatitis C virus cell-cell transmission. PLoS One *8*, e64221.

Krieger, S.E., Zeisel, M.B., Davis, C., Thumann, C., Harris, H.J., Schnober, E.K., Mee, C., Soulier, E., Royer, C., Lambotin, M., *et al.* (2010). Inhibition of hepatitis C virus infection by anti-claudin-1 antibodies is mediated by neutralization of E2-CD81-claudin-1 associations. Hepatology *51*, 1144-1157.

Lupberger, J., Zeisel, M.B., Xiao, F., Thumann, C., Fofana, I., Zona, L., Davis, C., Mee, C.J., Turek, M., Gorke, S., *et al.* (2011). EGFR and EphA2 are host factors for hepatitis C virus entry and possible targets for antiviral therapy. Nat. Med. *17*, 589-595.

Schieck, A., Schulze, A., Gahler, C., Muller, T., Haberkorn, U., Alexandrov, A., Urban, S., and Mier, W. (2013). Hepatitis B virus hepatotropism is mediated by specific receptor recognition in the liver and not restricted to susceptible hosts. Hepatology *58*, 43-53.

Verrier, E.R., Colpitts, C.C., Bach, C., Heydmann, L., Weiss, A., Renaud, M., Durand, S.C., Habersetzer, F., Durantel, D., Abou-Jaoude, G., *et al.* (2016). A targeted functional RNAi screen uncovers Glypican 5 as an entry factor for hepatitis B and D viruses. Hepatology *63*, 35–48.

Xiao, F., Fofana, I., Heydmann, L., Barth, H., Soulier, E., Habersetzer, F., Doffoel, M., Bukh, J., Patel, A.H., Zeisel, M.B., *et al.* (2014). Hepatitis C virus cell-cell transmission and resistance to direct-acting antiviral agents. PLoS Pathog. *10*, e1004128.

Yan, H., Zhong, G., Xu, G., He, W., Jing, Z., Gao, Z., Huang, Y., Qi, Y., Peng, B., Wang, H., *et al.* (2012). Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. Elife *1*, e00049.

Yount, J.S., Moltedo, B., Yang, Y.Y., Charron, G., Moran, T.M., Lopez, C.B., and Hang, H.C. (2010). Palmitoylome profiling reveals S-palmitoylation-dependent antiviral activity of IFITM3. Nat. Chem. Biol. *6*, 610-614.

Zahid, M.N., Turek, M., Xiao, F., Thi, V.L., Guerin, M., Fofana, I., Bachellier, P., Thompson, J., Delang, L., Neyts, J., *et al.* (2013). The postbinding activity of scavenger receptor class B type I mediates initiation of hepatitis C virus infection and viral dissemination. Hepatology *57*, 492-504.