UNIVERSITÉ DE STRASBOURG

Habilitation à diriger des Recherches

The role of virus-associated signal transduction in viral hepatitis and liver pathogenesis

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1 CONTENTS

2 CURRICULUM VITAE

LUPBERGER, Joachim; born October 03, 1974; married, three children.

2.1 Degrees and diplomas

04/2003 – 04/27/2007	**Degree: Dr. rer. nat. (PhD).** **Humboldt University Berlin, Germany.** Specific field: Biology / Virology. Grade: *magna cum laude* (A).
10/1998 - 01/21/2003	**Diploma: Diplom Ingenieur (FH) (Graduated engineer).** **University of Applied Sciences (Beuth Hochschule Berlin), Germany** Specific field: Biotechnology. Grade: *sehr gut* (A).
1991 – 1993	**Degree: Certified chemical-technical assistant.** **Technical school, Academy of Natural Sciences, Isny, Germany.** Grade: *gut* (B).

2.2 Current position

Since 10/2013	**Inserm UMR1110, University of Strasbourg**, Prof. T.F. Baumert, Strasbourg, France. Function: Staff scientist, CR1 (Inserm). Research focus: Virus host interactions, signaling and liver disease.
04/2007 – 09/2013	**Inserm UMR1110, University of Strasbourg**, Prof. T.F. Baumert, Strasbourg, France. Function: Post-doctoral fellow. Research focus: Virus host interactions, signaling and liver disease. Grants: Funding in framework of the European Associated Laboratory (Inserm UMR1110, University of Strasbourg and the Dept. of Medicine II, University of Freiburg) including a stipend from the Embassy of France in Berlin, Germany.

2.3 Previous laboratories

04/2003 – 12/2006	**University Hospital Freiburg and Robert Koch Institute** Berlin, Pr. E. Hildt, Germany. Function: PhD student.

03/2002 - 11/2002	**Massachusetts Institute of Technology**, Prof. A.J. Sinskey, Cambridge, MA, USA. Function: Diploma student. Grants: DAAD (German Academic Exchange Service).
01/1999 – 03/2003	**Charité, Humboldt University**, Dr. K.A. Kreuzer, Dr. P. le Coutre, Dr. C.A. Schmidt, Berlin, Germany. Function: Research student.
1994 – 1998	**Institute of Lake Research**, Hydrobiology, Dr. H. Güde, Langenargen, Germany. Function: Chemical-technical assistant.

2.4 Committee service

Since 2014	Member of the scientific board CSS7 "Recherches cliniques dans les hépatites virales" of the Agence Agence Nationale de Recherches sur le Sida et les hépatites virales (ANRS).

2.5 Publications

(1802 citations, H-index 16, i10-index 22)

2.5.1 Patents

Lupberger J and Baumert TF, Co-inventors. Combinations of Protein Kinase Inhibitors and Interferons or of Protein Kinase Inhibitors and Direct Acting Antivirals for the Treatment and the Prevention of HCV Infection. PCT/EP2012/066109 (Filing date: August 20, 2012).

Lupberger J and Baumert TF, Co-inventors. Host Cell Kinases as Targets for Antiviral Therapies against HCV Infection. PCT/EP2009/062095 (Filing date: September 26, 2008), US 2011/0229484 A1 (Filing date: September 18, 2009).

2.5.2 Original articles

(2001-2017 accumulated journal impact factor 203.12)

Van Renne N, Roca Suarez AA, Duong FHT, Gondeau C, Calabrese D, Fontaine N, Ababsa A, Bandiera S, Croonenborghs T, Pochet N, De Blasi V, Pessaux P, Piardi T, Sommacale D, Ono A, Chayama K, Fujita M, Nakagawa H, Hoshida Y, Zeisel MB, Heim MH, Baumert TF, **Lupberger J** (2017): miR-135a-5p-mediated downregulation of protein-tyrosine phosphatase delta is a candidate driver of HCV-associated hepatocarcinogenesis. *Gut*, in press. IF=14.92.

Lupberger J, Casanova C, Fischer B, Weiss A, Fofana I, Fontaine N, Fujiwara T, Renaud M, Kopp A, Schuster C, Brino L, Baumert TF, Thoma C (2015): PI4K-beta and MKNK1 are regulators of hepatitis C virus IRES-dependent translation. *Sci Rep.*,5:13344. IF= 5.23.

Mailly L, Xiao F*, **Lupberger J***, Wilson GK, Aubert P, T. DFH, Calabrese D, Leboeuf C, Fofana I, Thumann C, Bandiera S, Lütgehetmann M, Volz T, Davis C, Harris HJ, Mee C, Girardi E, Chane-Woon-

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Lupberger J, Schaedler S, Peiran A, Hildt E (2013): Identification and characterization of a novel bipartite nuclear localization signal in the hepatitis B virus polymerase. *World J. Gastroenterol.*, 19(44):8000-10. IF= 2.37.

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Lupberger J*, Duong FH*, Fofana I, Zona L, Xiao F, Thumann C, Durand SC, Pessaux P, Zeisel MB, Heim MH§, Baumert TF§ (2013): EGFR signaling impairs the antiviral activity of interferon-alpha. *Hepatology*, 58(4):1225-35. *Authors contributed equally, §authors contributed equally. IF= 11.71.

Zona L*, **Lupberger J***, Sidahmed-Adrar N§, Thumann C§, Harris HJ, Barnes A, Florentin J, Tawar RG, Xiao F, Turek M, Durand SC, Duong FH, Heim MH, Cosset FL, Hirsch I, Samuel D, Brino L, Zeisel MB, Le Naour F, McKeating JA, Baumert TF (2013): HRas Signal Transduction Promotes Hepatitis C Virus Cell Entry by Triggering Assembly of the Host Tetraspanin Receptor Complex. *Cell Host Microbe.*, 13(3):302-13. *Authors contributed equally, §authors contributed equally. IF= 12.55.

Lupberger J*, Zeisel MB*, Xiao F, Thumann C, Fofana I, Zona L, Davis C, Mee CJ, Turek M, Gorke S, Royer C, Fischer B, Zahid MN, Lavillette D, Fresquet J Cosset FL, Rothenberg SM, Pietschmann T, Patel AH, Pessaux P, Doffoël M, Raffelsberger W, Poch O, McKeating JA, Brino L and Baumert TF (2011): EGFR and EphA2 are host factors for hepatitis C virus entry and targets for antiviral therapy. *Nat. Med.* 17(5):589-95. *Authors contributed equally. IF= 30.36.

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2.5.3 Reviews and comments

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Zeisel MB, **Lupberger J**, Fofana I, Baumert TF (2012): Host-targeting agents for prevention and treatment of viral hepatitis C - perspectives and challenges. *J. Hepatol.*, 58(2):375-84.

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Turek M, **Lupberger J**, Baumert TF, Zeisel MB (2011): Open Sesame: regulation of hepatitis C virus entry into hepatocytes. *Med Sci (Paris)*. 27(11):929-31.

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Lupberger J, Brino L and Baumert TF (2008): RNAi - A powerful tool to unravel hepatitis C virus-host interactions within the infectious life cycle. *J Hepatol.* 48(3):523-5.

Lupberger J and Hildt E (2007): Hepatitis B virus-induced oncogenesis. *World J Gastroenterol.* 13(1):74-81.

2.5.4 Book chapters

Lupberger J, Van Renne N, Baumert T.F (2015): *Signaling of hepatitis C virus. Signaling pathways in liver diseases*, 3rd ed, Wiley-Blackwell, pp459-468.

2.5.5 Invited lectures

Trinity College, Biochemical Society, Dublin, Ireland (06/04/2017): “HCV-induced signaling and its impact on liver pathogenesis”.

International Center for Infectiology Research (CIRI), Lyon, France (19/02/2015): “EGFR is a co-factor for HCV entry and a regulator of the host antiviral response”.

Paul-Ehrlich Institute (PEI), Langen, Germany (15/10/2014): “EGFR signaling - a regulator of HCV entry and the innate immune response”.

Hôpital Paul-Brousse, Inserm U1004, Villejuif, France (11/06/2013): “EGFR signaling - a regulator of HCV entry and the innate immune response”.

Centre de Recherche en Cancérologie de Marseille (CRCM), France (05/07/2011): “EGFR is a host factor for hepatitis C virus entry and target for antiviral therapy”.

2.5.6 Oral presentations

Van Renne N, Roca Suarez AA, Duong FHT, Gondeau C, Calabrese D, Fontaine N, Pessaux P, Heim MH, Baumert TF and **Lupberger J** (2016): Virus-induced suppression of the tumor suppressor protein-tyrosine phosphatase delta via miR1351-5p promotes a STAT3 transcriptional program in livers of HCV patients. *Annual Meeting of the American Association for the Study of Liver Diseases AASLD*, Boston, MA, USA.

Lupberger J, Croonenborghs T, Jovanovic M, Mertins P, Van Renne N, Bandiera S, Thumann C, Colpitts CC, Kozubek J, Kiani K, Carr SA, Regev A, Zeisel MB, Pochet N, Baumert TF (2016): Spatiotemporal functional genomics uncovers cell circuits mediating persistence and liver disease biology of hepatitis C virus infection. *23rd International Symposium on Hepatitis C Virus and Related Viruses*, Kyoto, Japan.

Van Renne N, Roca Suarez AA, Duong FHT, Gondeau C, Calabrese D, Fontaine N, Pessaux P, Heim MH, Baumert TF and **Lupberger J** (2016): miR-135a-5p-mediated downregulation of protein-tyrosine phosphatase delta is a candidate driver of HCV-associated hepatocarcinogenesis that acts by promoting STAT3 transcriptional activity. *23rd International Symposium on Hepatitis C Virus and Related Viruses*, Kyoto, Japan.

Van Renne N, Duong FHT, Gondeau C, Calabrese D, Fontaine N, Ababsa A, Durand SC, Pessaux P, Heim MH, Baumert TF, **Lupberger J** (2015): The expression of tumor suppressor PTPRD is down-regulated in the liver of patients with HCV infection and in tumor lesions of patients with hepatocellular carcinoma. *46th Annual Meeting of EASL*, Vienna, Austria.

Ababsa A, Duong FHT, Van Renne N, Gondeau C, Heim MH, Baumert TF, **Lupberger J** (2014): Expression of tumor suppressor PTPRD is downregulated in HCV-infected PHH and liver tissue of patients with chronic HCV infection. *15e réunions du réseau national hépatites de l'ANRS*, Paris, France.

Van Renne N, Duong FHT, Gondeau C, Calabrese D, Fontaine N, Ababsa A, Durand SC, Pessaux P, Heim MH, Baumert TF, **Lupberger J** (2015): The expression of tumor suppressor PTPRD is down-regulated in the liver of patients with HCV infection and in tumor lesions of patients with hepatocellular carcinoma. *15e réunions du réseau national hépatites de l'ANRS*, Paris, France.

Ababsa A, Duong FHT, Gondeau C, Calabrese D, Heim MH, Baumert TF, **Lupberger J** (2014): The expression of tumor suppressor PTPRD is downregulated in HCV-infected primary human hepatocytes and liver tissues of HCV patients. *21th International Symposium on Hepatitis C Virus and Related Viruses*, Banff, Canada.

Lupberger J, Zona L, Thumann C, Sid Ahmed-Adrar N, Harris HJ, Zeisel MB, Samuel D, Le Naour F, McKeating JA, BaumertTF (2012): HRas is a host factor for hepatitis C virus entry acting as a switch for CD81- claudin1 co-receptor associations. *9ème Congrès National de la Société Française de Microbiologie*, Lille, France.

Lupberger J, Duong FHT, Fofana I, Zona L, Xiao F, Durand SC, Zeisel MB, Heim MH, Baumert TF (2013): EGFR signaling impairs the antiviral activity of interferon-alpha. *13e réunions du réseau national hépatites de l'ANRS*, Paris,France.

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Lupberger J, Zona L, Thumann C, Harris H, le Naour F, Fischer B, Weiss A, Florentin J, Durand S, Hirsch I, Bachelier P, Brino L, McKeating JA, Baumert TF (2012): Regulation of HCV entry by host cell kinases. *ANRS Basic Research Symposium on HIV and Hepatitis Viruses Replication*, Paris, France.

Lupberger J, Zona L, Thumann C, Fischer B, Weiss A, Florentin J, Duong F, Harris H, Farquhar MJ, Zeisel MB, Nunes JA, Hirsch I, Brino L, McKeating JA, Baumert TF (2011): Identification of EGFR signaling pathways mediating hepatitis C virus entry. *4ème Génomique Fonctionnelle du Foie*, Bordeaux, France.

Lupberger J, Zona L, Thumann C, Fischer B, Weiss A, Florentin J, Duong F, Harris H, Farquhar MJ, Zeisel MB, Nunes JA, Hirsch I, Brino L, McKeating JA, Baumert TF (2011): Identification of EGFR signaling pathways mediating hepatitis C virus entry. *62th Annual Meeting of the American Association for the Study of Liver Diseases AASLD*, San Francisco, CA, USA.

Lupberger J, Zona L, Thumann C, Fischer B, Weiss A, Florentin J, Duong F, Zeisel MB; Brino L, Nunes JA, Hirsch I, McKeating JA, Baumert TF (2011): Identification of EGFR signaling pathways mediating hepatitis C virus entry. *69èmes Journées de l'Association Française pour l'Etude du Foie*, Paris, France.

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Lupberger J (2011): EGFR is a host factor for hepatitis C virus entry and target for antiviral therapy. *46th Annual Meeting of EASL*, Berlin, Germany.

Lupberger J, Zeisel MB, Xiao F, Thumann C, Mee CJ, Davis C, McKeating JA, Fofana I, Soulier E, Zona L, Lavillette D, Cosset FL, Patel A, Doffoël M, Baumert TF (2010): Molecular mechanisms of epidermal growth factor receptor-mediated hepatitis C virus entry. *17th International Symposium on Hepatitis C Virus and Related Viruses*, Yokohama, Japan. (Travel grant).

Lupberger J, Baumert T (2009): Hepatitis C Virus and Hepatocellular Carcinoma. *5ème Journées Scientifiques du Cancéropôle CLARA*, Lyon, France.

Lupberger J, Thumann C, Soulier E, Fischer B, Froidevaux L, Zeisel MB, Gorke S, Fofana I, Royer C, Wolf P, Doffoël P, Raffelsberger W, Poch O, Brino L, Baumert TF (2009): A genome-wide RNAi kinase screen identifies kinase networks as co-factors for hepatitis C virus entry and targets for antiviral therapies. *Alsace BioValley 2009 STEPS Event*, Illkirch, France.

Lupberger J, Thumann C, Soulier E, Fischer B, Froidevaux L, Zeisel MB, Gorke S, Fofana I, Royer C, Wolf P, Raffelsberger W, Poch W, Brino L and Baumert TF (2009): Inhibition of HCV entry and infection by Erlotinib. *60th Annual Meeting of the American Association for the Study of Liver Diseases AASLD*, Boston, MA, USA.

Lupberger J, Thumann C, Soulier S, Zeisel MB, Gorke S, Fofana I, Royer C, Cosset FL, Wolf P, Doffoël M and Baumert TF (2009): Inhibition of HCV entry and infection by Erlotinib. *16th International Symposium on Hepatitis C Virus and Related Viruses*, Nice, France. (Travel grant).

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Lupberger J, Morand AL, Thumann C, Zeisel MB, Raffelsberger W, Cosset FL, Brino L and Baumert TF (2008): Identification of cellular kinases as co-factors for hepatitis C virus entry using a functional high-throughput siRNA screen. *15th International Symposium on Hepatitis C Virus and Related Viruses*, San Antonio, Texas, USA.

Lupberger J, Luckow A, Pairan A and Hildt E (2006): Nuclear import of the hepatitis B virus polymerase is mediated by a bipartite NLS and depends on CKII phosphorylation. *International Meeting on HBV Viruses*, Vancouver, Canada.

Lupberger J, Luckow A and Hildt E (2005): HBV polymerase harbors a bipartite nuclear localization signal, which is subjected to tight regulation. *Fourth workshop of the study group: "Cell biology of Viral Infections" of the German Society for Virology*, Deidesheim, Germany.

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Ligue Nationale Contre le Cancer, France (2007); 30,000 EUR (Principle investigator: TFB).

3 CONTEXT

In order to react to cellular and extracellular stimuli cells have developed a chain of molecular messengers that transmit signals from sensors and receptors to the nucleus where they are translated into genetic response. This signal transduction machinery involves a large variety of enzymes that regulate site-specific protein phosphorylation. Once activated, protein kinases transmit this stimulus within the cell by phosphorylating a specific downstream target, which may

be again another kinase or a transcription factor. As all signals have to be turned off protein phosphatases are feedback regulators of kinase phosphorylation leading to a directed phosphorylation pulse toward the nucleus. This principle represents a fundamental regulatory mechanism that is involved in cellular homeostasis, cellular communication and defense against pathogens and cellular damage. Deregulated signal transduction is associated with specific diseases and cancers.

Many viruses in turn have co-evolved to exploit and manipulate cellular regulatory mechanisms to ensure and promote its life cycle within the cell and to evade the host antiviral defense. This has a profound impact on virus-induced pathogenesis. Many viruses code viral homologues of host molecular switches that persistently activate certain signaling pathways and may ultimately lead to cancer. In the early days of molecular biology many canonical signaling pathways have been discovered by the study of virus infections e.g v-src gene of Rous sarcoma virus is highly homologue to the c-src (for review see [1]).

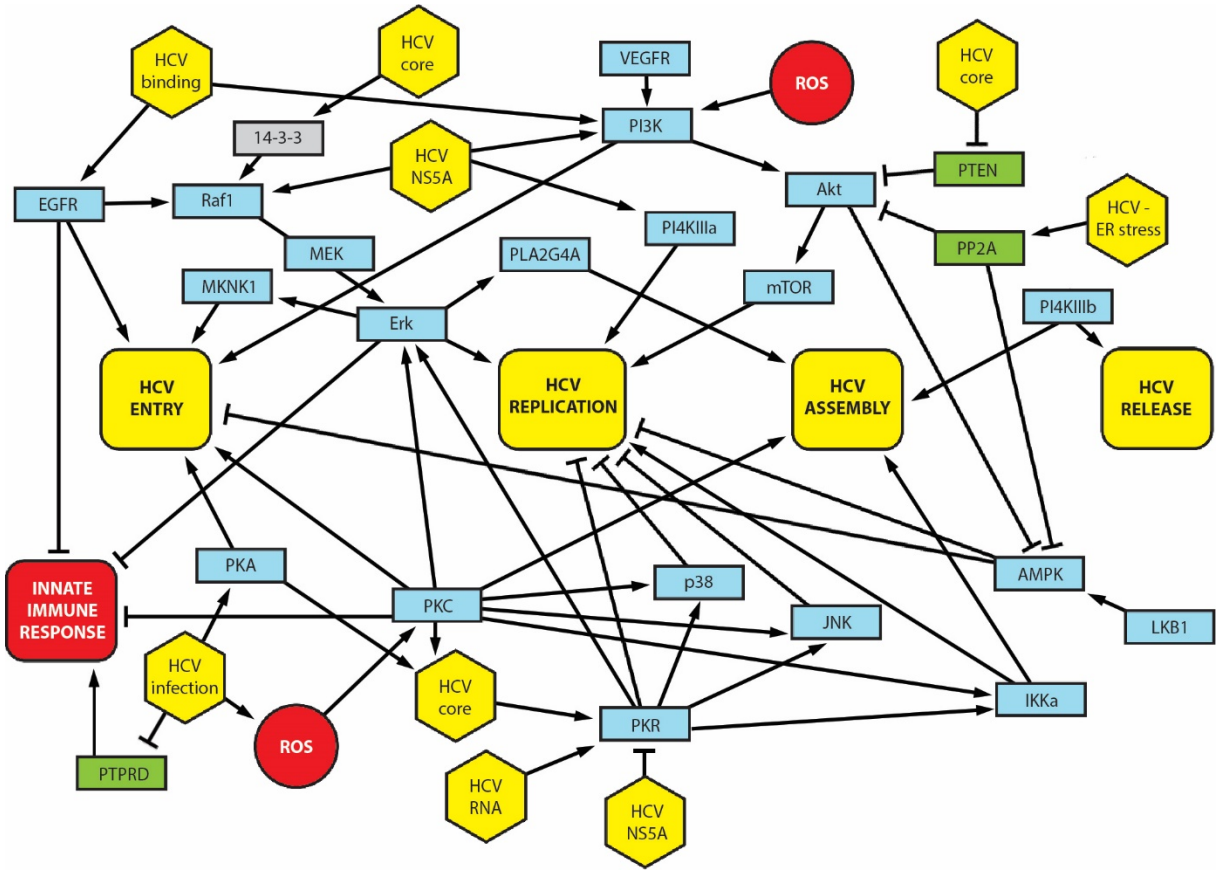

Figure 1 Update of the HCV-host signaling interactome. Yellow=HCV protein, HCV-induced process or aspect of HCV life cycle; grey box= regulatory protein, blue box= host kinase; green box= host protein phosphatase; red square= host antiviral response; red circle= reactive oxygen species. (Image updated from [2]).

Also Hepatitis C virus (HCV) highly depend on the interaction with the host signaling network (figure 1), which has important consequences for the liver pathobiology. I therefore devoted a large portion of my scientific career in the studying of HCV-host interactions and their impact on the development of liver disease and summarized my contributions to this field within this post-doctoral dissertation.

4 Role of cellular signaling for the replication of hepatitis B and C viruses

During my PhD thesis (2003-2007) in the lab of Prof. Eberhard Hildt at the Robert Koch Institute in Berlin and the University of Freiburg, Germany, I studied the role of the MAP kinase signaling pathway on the replication of HBV and HCV. Worldwide, estimated 350 million people are chronically infected with HBV and estimated 170 million individuals are chronically infected with HCV [3]. Viral hepatitis is a major indication for the development of hepatocellular carcinoma (HCC) [4]. Globally 78 % of HCC was attributable to HBV (53 %) or HCV (25 %) infections [5]. It is believed that viral hepatitis contributes to HCC development by direct and indirect perturbation of host regulatory mechanism and genome stability.

For HBV, the integrity of Raf/MAP-kinase signaling is important for HBV gene expression [6], Moreover, HBV induces an anti-proliferative signal via Raf on the cell cycle control [7]. Addressing the need for better cell culture models I revealed that increased cell-cell contacts in three-dimensional cultivation of HBV replicating cells leaded to enhanced MAP kinase signaling and increased HBV secretion of infected cells. My results suggest three dimensional cultivation of infected cells as novel method to produce high titer viral particles and emphasize the important role of MAP kinase signaling for HBV.

For HCV, the viral non-structural protein 5a (NS5A) associates with host signaling components such as Grb2 [8], phosphatidylinositol 3-kinase [9] or p53 [10] and modulates gene expression via the MAP kinase Erk1/2. HCV non-structural protein NS5A is an integral part of the HCV replication complex and plays a crucial role in the biogenesis of the membranous web that is the site of HCV replication [11] and viral assembly at lipid droplets [12]. Using affinity-tagged recombinant NS5A as bait we identified Raf-1 as a novel cellular binding partner to the C-terminal domain of NS5A that leads to increased Raf-1 phosphorylation at serine 338. Our functional data suggest that NS5A-Raf-1 interaction activates Raf-1/MAPK signaling that is required for HCV replication.

Taken together, the results suggest an integral role of host signaling for HBV/HCV biology. Virus-host interactions require and manipulate signaling pathways that are relevant for cancer development and thus may contribute to liver pathogenesis. Interference with these signaling pathways using kinase inhibitors therefore harbor an interesting antiviral potential and may also be interesting for novel chemopreventive strategies targeting liver disease progression in chronically infected patients.

4.1 Associated publications

Lupberger J, Mund A, Kock J, and Hildt E (2006): Cultivation of HepG2.2.15 on micro carrier: Higher HBV production and less subviral particles in comparison to conventional cultivation methods. *J. Hepatol.*, 45(4):547-52. IF= 10.59.

Buerckstuemmer T*, Kriegs M*, **Lupberger J**, Pauli EK, Schmittel S and Hildt E (2006): Raf-1 kinase associates with Hepatitis C virus NS5A and regulates viral replication. *FEBS Lett.*, 580(2):575-80. *Authors contributed equally. IF= 3.52.

4.2 Supervision of students

In frame of these projects I supervised two master student and several internships from the Humboldt University of Berlin and the University of Applied Sciences in Berlin.

5 Functional role of cellular kinases for HCV entry and chronicity

The experience in virus-kinase interactions and general molecular biological methods I acquired during my PhD thesis allowed me to join Prof. Thomas Baumert's lab at the Université de Strasbourg / Inserm UMR 1110 (previously 748) as a postdoc in 2007. The lab of Prof. Baumert provides a strong expertise in HCV-host interactions and state-of-the art HCV infection model systems with access to a P3 containment facility in the unit. UMR 1110 is embedded within an excellent scientific network and has access to platform technologies within Strasbourg. Making use of this environment, I was focusing on the identification and functional characterization of cellular kinases as novel co-factors of the HCV entry process. HCV is a major cause of liver cirrhosis and HCC. Although newly developed direct-acting antivirals targeting HCV proteins leaded to a breakthrough in HCV therapy, high costs and viral resistance remain major challenges [13]. Thus, complementary preventive and therapeutic antiviral strategies are needed. Because HCV entry is required for initiation, dissemination and maintenance of viral infection, it is a promising target for antiviral therapy [14, 15]. The aims of my research in Thomas Baumert's lab were fourfold: (i) to establish and perform a high-throughput siRNA screening assay targeting the human kinome and study the impact of kinase silencing on HCV entry, (ii) to study the molecular mechanism underlying kinase-mediated HCV entry, (iii) to identify and evaluate kinases as novel targets for an antiviral intervention and (iv) to reveal the role of virus-host signaling interaction for the establishment of persistent infection and its consequences for viral pathogenesis.

5.1 RNAi screen identifies 58 kinases with functional impact on HCV entry

At the time when I joined the team of Prof. Baumert several siRNA screens had been published identifying host factors for viral infection like human immunodeficiency virus (HIV) [16] and West Nile Virus [17]. Interestingly, among all screening hits both studies identified about 4-6 % of the human kinome as important co-factors and regulators of viral infection. Since kinases are well established drug targets in cancer therapy [18] I aimed to set-up a high-throughput siRNA screen targeting selectively the human kinome that is able to identify kinases relevant for HCV entry.

In collaboration with the High Throughput Screening platform of the Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC, Illkirch, France) I established a siRNA-based screen silencing 691 human kinases and associated proteins in Huh-7 hepatoma cells. Screening comprised three steps: a primary screen using HCV pseudoparticles (HCVpp) bearing HCV envelope glycoproteins and vesicular stomatitis virus pseudotyped particles (VSVpp) as an unrelated control virus. To validate the relevance of the identified kinases in the complete infectious viral life cycle, identified hits were confirmed in a secondary screen using cell-culture-derived HCV (HCVcc). False positive results due to toxicity were excluded using MTT-based cell viability test. To exclude non-specific off-target effects by siRNA pools, I validated the hits using four individual siRNAs in a third screen.

Using this functional HCVpp entry RNAi assay, I identified 58 human kinases and revealed kinase networks regulating cell morphology including cell polarity, tight junction (TJ) permeability, and cell adhesion, as well as networks of kinases involved in the cell cycle control. Five kinases are targets of clinically licensed protein kinase inhibitors (PKIs). These include ephrin receptor A2 EphA2 (Dasatinib), epidermal growth factor receptor EGFR (Erlotinib), cell division cycle 2 kinase CDC2 (Flavopiridol), cyclin-dependent kinase 4 CDK4 (Flavopiridol) and cyclin-dependent kinase 8 CDK8 (Flavopiridol).

The identification of kinases as HCV entry factors advanced the knowledge of the cellular requirements of HCV entry. The discovery of PKIs as candidate antivirals defines a potential new strategy for preventing and treating HCV infection that resulted in an investigator initiated phase I/IIa clinical trial (NCT01835938) (funding LabEx HEPSYS, Inserm DGOS, Roche), which started in 2013 at the CIC/University of Strasbourg Hospitals. In this trial a team of clinicians and scientists of UMR 1110 and the CIC study the safety and antiviral activity of erlotinib in HCV-infected patients.

5.1.1 Collaborations

Laurent Brino, High-throughput screening platform, IGBMC, Illkirch, France.

Olivier Poch, Bioinformatic platform, IGBMC, Illkirch, France.

5.1.2 Associated patents and publications

Lupberger J and Baumert TF, co-inventors: PCT/EP2009/062095, US 2011/0229484 A1.

Lupberger J*, Zeisel MB*, Xiao F, Thumann C, Fofana I, Zona L, Davis C, Mee CJ, Turek M, Gorke S, Royer C, Fischer B, Zahid MN, Lavillette D, Fresquet J Cosset FL, Rothenberg SM, Pietschmann T, Patel AH, Pessaux P, Doffoël M, Raffelsberger W, Poch O, McKeating JA, Brino L and Baumert TF (2011): EGFR and EphA2 are host factors for hepatitis C virus entry and targets for antiviral therapy. *Nat. Med.* 17(5):589-95. *Authors contributed equally. IF= 30.36 (see ANNEX).

5.2 RTKs mediate HCV entry by promoting co-receptor association and membrane fusion

I aimed to study the molecular mechanism of the identified HCV-kinase interactions and focused on EGFR and EphA2 because they are key components in the identified networks. They are highly expressed in human liver and their kinase function is inhibited by clinically approved PKIs [19-21], allowing us to explore the potential of these molecules as therapeutic targets. In order to address

this objective in an internationally competitive level I was coordinating a team consisting of members of the unit as well as of national and international collaborators (section 5.2.1).

To study the relevance of the identified kinases on the HCV life cycle, I further validated and characterized the functional impact of receptor tyrosine kinases (RTKs) EGFR and EphA2 on HCV entry. We demonstrated that the RTKs do neither modulate HCV receptor expression SR-BI, CD81, CLDN1 and OCLN expression nor do they physically bind to viral glycoproteins. Instead, using a HCV entry kinetic assay we demonstrated that EGFR and EphA2 inhibitors act in a post binding step similar to CD81 antibodies. Therefore, we used a fluorescence resonance energy transfer (FRET)-based assay [22] and demonstrated that specific PKIs or RTK-specific siRNA interfere with CD81-CLDN1 co-receptor interactions, which is an important step for HCV entry. Furthermore, as kinetic assays showed that PKIs inhibit late steps of viral entry we used PKIs and RTK-specific siRNA and demonstrated that RTKs are required for membrane fusion in a viral glycoprotein-dependent cell-cell fusion assay [23].

Our functional experiments with specific ligands, antibodies and kinase inhibitors implicated both ligand-binding and kinase domains of EGFR in promoting HCV entry and cell-cell transmission of HCV via the receptor activity. Given that our functional and mechanistic studies indicate that the expression and activity of EGFR and EphA2 seem to be involved in similar entry steps, it is likely that both RTKs are part of the same entry regulatory pathway indicating that RTK-mediated signaling is required for HCV entry.

5.2.1 Collaborations

Jane McKeating, University of Birmingham, UK.

François-Loïc Cosset, CIRI, ENS, Lyon, France.

5.2.2 Associated publications

Lupberger J*, Zeisel MB*, Xiao F, Thumann C, Fofana I, Zona L, Davis C, Mee CJ, Turek M, Gorke S, Royer C, Fischer B, Zahid MN, Lavillette D, Fresquet J Cosset FL, Rothenberg SM, Pietschmann T, Patel AH, Pessaux P, Doffoël M, Raffelsberger W, Poch O, McKeating JA, Brino L and Baumert TF (2011): EGFR and EphA2 are host factors for hepatitis C virus entry and targets for antiviral therapy. *Nat. Med.* 17(5):589-95. *Authors contributed equally. IF= 30.36 (see ANNEX).

5.3 EGFR-dependent HCV entry is mediated by HRas GTPase signaling

Since we demonstrated that HCV E2 glycoprotein does not engage EGFR or EphA2, RTK activity directly correlated with HCV entry, and both EGFR and EphA2 have very similar mechanisms of action on HCV entry it became evident that signal transduction is a key event in RTK-mediated HCV entry. Therefore, we aimed to identify signaling events in hepatocytes that are required for RTK-dependent entry. We used EGFR as a model since it is a well characterized and clinical relevant RTK with a wide-range of established molecular tools available [24-26].

We demonstrated that upstream members of the MAPK signaling pathway but no other canonical EGFR pathway play a major functional role in HCV entry in hepatocytes. Moreover, we demonstrated that HRas acts as a molecular switch promoting RTK-mediated HCV entry. A key step in HCV entry is the CD81-CLDN1 co-receptor association, which is promoted by EGFR [27]. We analyzed whether tetraspanin-enriched microdomains (TEMs) associating CD81 contain members

of EGFR signaling pathways using proteomic analysis. We demonstrated that activated HRas and HCV entry factors CLDN1 and SR-BI specifically associated with CD81 TEMs and that HRas signaling reduces CD81 mobility by promoting an interaction with other proteins. Furthermore, silencing of HRas expression significantly reduced CD81-CLDN1 association demonstrating a role of HRas in the formation and/or maintenance of the CD81-CLDN1 co-receptor complexes.

Taken together, we conclude that HCV exploits the EGFR/HRas pathway for cellular entry by compartmentalization of host entry factors and receptor trafficking and identify a new mechanism to regulate CD81-dependent pathogen invasion of the liver. HRas is relevant for entry of other viruses including influenza and a potential target for antiviral therapy. Our data demonstrate that viruses exploit HRas signaling for cellular entry by compartmentalization of entry factors and receptor trafficking and identify a new mechanism to regulate CD81-dependent pathogen invasion of the liver.

5.3.1 Collaborations

Jane Mc Keating, University of Birmingham, UK.

François-Loïc Cosset, CIRI, ENS, Lyon, France.

François Le Naour, Inserm U1193, Villejuif, France.

Ivan Hirsch, Centre de Recherche en Cancérologie de Marseille, France.

Markus H. Heim, University of Basel, Switzerland.

Laurent Brino, High-throughput screening platform, IGBMC, Illkirch, France.

Olivier Poch, Bioinformatic platform, IGBMC, Illkirch, France.

5.3.2 Associated publications

Zona L*, **Lupberger J***, Sidahmed-Adrar N§, Thumann C§, Harris HJ, Barnes A, Florentin J, Tawar RG, Xiao F, Turek M, Durand SC, Duong FH, Heim MH, Cosset FL, Hirsch I, Samuel D, Brino L, Zeisel MB, Le Naour F, McKeating JA, Baumert TF (2013): HRas Signal Transduction Promotes Hepatitis C Virus Cell Entry by Triggering Assembly of the Host Tetraspanin Receptor Complex. *Cell Host Microbe.*, 13(3):302-13. *Authors contributed equally, §authors contributed equally. IF= 12.55 (see ANNEX).

5.3.3 Supervision of students

In frame of this project I co-supervised a PhD student (Laetitia Zona) from the Université de Strasbourg together with Thomas Baumert. She graduated in 2014.

5.4 EGFR signaling impairs the antiviral activity of interferon-alpha

Until recently, interferon-alpha (IFN-α) and its pharmacologically modified pegylated derivatives had been part of the standard of care (SOC) of hepatitis C virus (HCV) infection [28] and are also used for the treatment of hepatitis B and D virus infections, albeit with limited efficacy [29, 30]. It has been suggested that the inhibitory effect on HCV replication is mediated through distinct patterns of signal transduction and IFN response gene (IRG)-expression and that viral infections themselves trigger interferon production [31]. The RTK EGFR is a well-established drug target during clinical intervention [32] and EGFR antagonists are relatively well tolerated by patients [21]. As described above,

I demonstrated that EGFR signaling is required for HCV entry and cell-cell transmission and demonstrated that the clinical EGFR inhibitor erlotinib markedly inhibits HCV entry *in vitro* and *in vivo* [27]. Since signaling of RTKs and IFN-α share distinct signal transducers [25, 33] I aimed to investigate whether inhibition of RTK signaling offers an opportunity to improve the therapeutic efficacy of IFN-α and whether RTK signaling is relevant for the antiviral activity of IFN-α.

Combining IFN-α with a small sub-IC_{50} dose of erlotinib, we observed a significant synergistic antiviral effect on HCV infection. In collaboration with the laboratory of Markus H. Heim (University of Basel) we identified a crosstalk of EGFR and IFN-α signaling explaining the marked synergy of IFN-α and erlotinib: (i) erlotinib inhibits STAT3 phosphorylation via SOCS3 shifting the balance of the STAT1:STAT3 seesaw towards an enhanced effect of STAT1; (ii) since STAT3 function is relevant for HCV infection, inhibition of STAT3 phosphorylation by erlotinib may further contribute to the antiviral activity of erlotinib in combination with IFN-α; (iii) inhibition of EGFR function by erlotinib enhances the IFN-α-induced antiviral function of STAT1. The implication of the EGFR-IFN cross-talk is twofold: First, the uncovered interference of signaling is relevant for understanding viral evasion during IFN-α treatment, second, our results open a novel perspective to improve the efficacy of IFN-α-based antiviral therapies.

5.4.1 Collaborations

Markus H. Heim, University of Basel, Switzerland.

5.4.2 Associated patents and publications

Lupberger J and Baumert TF, co-inventors: PCT/EP2009/062095.

Lupberger J*, Duong FH*, Fofana I, Zona L, Xiao F, Thumann C, Durand SC, Pessaux P, Zeisel MB, Heim MH§, Baumert TF§ (2013): EGFR signaling impairs the antiviral activity of interferon-alpha. *Hepatology*, 58(4):1225-35. *Authors contributed equally, §authors contributed equally. IF= 11.71 (see ANNEX).

5.4.3 Supervision of students

In frame of this project I co-supervised a PhD student (Laetitia Zona) from the Université de Strasbourg together with Thomas Baumert. She graduated in 2014.

5.5 CLDN1-specific mAb impairs HCV-induced host cell signaling

Viral resistance against therapy remain a challenge also for the new generation of HCV antivirals [13]. To overcome resistance targeting host factors for virus entry is a concept that potentially increase the genetic barrier of resistance [15]. CLDN1 is an essential host factor of HCV. In particular the tight junction-free CLDN1 -accessible to neutralizing antibodies- is essential for HCV entry. To exploit this functional bottleneck for potential antiviral strategies Inserm UMR1110 developed a CLDN1-specific mouse monoclonal antibody that is able to specifically block the CD81-CDLN1 association [34, 35]. Treatment of a HCV permissive mouse model with the CLDN1-specific monoclonal antibody is able to eradicate chronic HCV infection. The observed efficient and fast viral clearance suggests a mechanism of action that not only block viral entry but also interferes with cellular regulatory processes relevant for the HCV lifecycle. It became very evident during my research at Inserm UMR 1110 that HCV exploit cellular signaling processes to maintain its persistence. HCV infection not only requires EGFR but also stimulates the very pathway. Studying the molecular mechanism of the

monoclonal CLDN1-specific antibody I demonstrated that Erk1/2 phosphorylation is elevated in liver biopsies of patients with chronic HCV infection. While the CLDN1-specific antibody had no impact on EGF-induced Erk1/2 activation the antibody impaired HCV-induced Erk1/2 phosphorylation. The findings suggest a model were CD81-CLDN1 receptor complex induce MAPK signaling through a crosstalk downstream of EGFR. The monoclonal antibody disrupt the receptor complex and impair HCV-induced MAPK signaling that has been shown to be relevant for the HCV life cycle, including viral entry [36, 37]. It is thus very probable that this interference contributes to the ability of the antibody to clear an established infection.

5.5.1 Associated publications

Mailly L, Xiao F*, **Lupberger J***, Wilson GK, Aubert P, T. DFH, Calabrese D, Leboeuf C, Fofana I, Thumann C, Bandiera S, Lütgehetmann M, Volz T, Davis C, Harris HJ, Mee C, Girardi E, Chane-Woon-Ming B, Ericsson M, Fletcher N, Bartenschlager R, Pessaux P, Vercauteren K, Meuleman P, Villa P, Kaderali L, Pfeffer S, Heim MH, Neunlist M, Zeisel MB, Dandri M, McKeating JA, Robinet E, Baumert TF (2015): Clearance of persistent hepatitis C virus infection using a monoclonal antibody specific for tight junction protein claudin-1. *Nat. Biotech.*, 33(5):549-54. *Authors contributed equally. IF= 43.11 (see ANNEX).

6 HCV infection impairs the expression of the candidate tumor suppressor PTPRD in patients

In 2013 I received a tenure position as staff researcher (CR1) at the French National Institute of Health and Medical Research (Inserm) and continued my work in HCV-host interactions and liver disease at UMR1110. Beside a strong collaborative integration within several projects of the unit I had the chance to acquire own research funding and build an emerging research group within the unit. In 2014 I received a starting grant in frame of the French Excellency Initiative (IdEx, Projets Attractivité) from the Université de Strasbourg that provided resources to hire a technician and to study HCV-associated liver disease development.

In previous projects I demonstrated that HCV exploit and induce host signal transduction to maintain infection and to evade the host innate immune response. Cellular signals transmitted by kinases are tightly regulated by protein phosphatases. Aberrant expression of protein phosphatases are involved in various diseases and syndromes, which are associated with deregulated signaling pathways. However, the global impact of HCV infection on expression pattern of protein phosphatases and their impact on liver disease are largely unknown.

To identify the impact of HCV infection on the regulatory mechanisms of signaling pathways we studied expression patterns of 84 disease-relevant protein phosphatases in liver biopsies of patients with chronic HCV infection. Among the most deregulated phosphatases were several genes involved in processes relevant for the development of liver disease and cancer including the tumor suppressor protein phosphatase delta (PTPRD), which is an established tumor suppressor in a broad spectrum of human cancers. We demonstrated that PTPRD is consistently downregulated by HCV in primary human hepatocytes (PHH) and in liver biopsies. Also PTPRD expression is independent

from the inflammation and fibrosis scores of the studied tissues and the innate immune response in PHH. Moreover, we demonstrated that PTPRD expression is impaired in tumor lesions of paired liver biopsies and that high levels of PTPRD in liver tissue adjacent to HCC correlate with survival and reduced tumor recurrence after surgical resection. Interestingly, the 3'UTR of PTPRD mRNA exhibits two conserved target sites for miR-135a-5p, which we found markedly upregulated in liver biopsies from chronic HCV patients independently from the innate antiviral response in PHH. Moreover, miR-135a-5p expression inversely correlate with the PTPRD mRNA levels in the studied biopsies.

Our results demonstrated impaired PTPRD levels in HCV-infected hepatocytes and HCCs, which potentially represent a hallmark of liver disease progression. PTPRD is a tumor suppressor in various cancers and a STAT3 phosphatase. We previously demonstrated that STAT3 is a co-factor for HCV infection. We show that an elevated STAT3 transcriptional activity is associated to poor prognosis in HCC patients and that an elevated STAT3 transcriptional signature is present in liver biopsies with low PTPRD levels. Taken together, we suggest that HCV maintains STAT3 activity via miR-135a-5p-mediated downregulation of its negative regulator PTPRD, while leaving the liver more prone to malignant transformation.

6.1 Collaborations

Markus H. Heim, University of Basel, Switzerland.

Claire Gondeau, Institut de Médecine Régénératrice et Biothérapie, Université de Montpellier, France.

Tullio Piardi and Daniele Sommacale, Centre Hospitalier Universitaire de Reims, France.

Nathalie Pochet, Broad Institute of MIT and Harvard, Cambridge, MA, USA.

Yujin Hoshida, Icahn School of Medicine at Mount Sinai, New York, NY, USA.

Kazuaki Chayama, Hiroshima University, Japan.

Hidewaki Nakagawa, RIKEN Center for Genomic Medicine, Japan.

6.2 Funding of the project

IdEx (Projets Attractivite), Université de Strasbourg, 2014-2015 (PI: Joachim Lupberger).

6.3 Associated publications

Van Renne N, Roca Suarez AA, Duong FHT, Gondeau C, Calabrese D, Fontaine N, Ababsa A, Bandiera S, Croonenborghs T, Pochet N, De Blasi V, Pessaux P, Piardi T, Sommacale D, Ono A, Chayama K, Fujita M, Nakagawa H, Hoshida Y, Zeisel MB, Heim MH, Baumert TF, **Lupberger J** (2017): miR-135a-5p-mediated downregulation of protein-tyrosine phosphatase delta is a candidate driver of HCV-associated hepatocarcinogenesis. *Gut*, in press. IF=14.92 (see ANNEX).

6.4 Supervision of students

Co-supervision of two PhD students: Nicolaas Van Renne, graduated November 2016 at the Université de Strasbourg and Armando Andres Roca Suarez, started January 2016.

Supervision of two master II students: Amina Ababsa, graduated 2014; Armando Andres Roca Suarez, graduated 2015.

7 Current projects as principle investigator

In this chapter I would like to highlight briefly the current and future directions of my research within Inserm UMR1110. I will focus on two projects that I am coordinating as principle investigator. My long term strategic aim is to consolidate my research group at Inserm UMR1110 and to acquire additional research funding that will allow me to hire staff and PhD students.

7.1 The role of PTPRD as tumor suppressor in the liver

The study of disease-relevant human protein phosphatases in HCV-infected human liver biopsies and primary human hepatocytes identified the candidate tumor suppressor protein-tyrosine phosphatase delta (PTPRD), which is potentially associated to HCV-induced hepatocarcinogenesis (section 6). This was an interesting finding since HCV contribute to HCC-development rather long-term. The downregulation of a tumor suppressor may be partially explained by HCV-induced interference with cellular safety mechanisms and thus may render the cells more susceptible to oncogenic pressure. Using a state-of-the-art PTPRD knockout mouse model, this project will study the impact of PTPRD expression on HCC development and deliver evidence for a role of PTPRD as tumor suppressor in the liver. Understanding the complex signaling network of HCV infection will contribute to reveal general mechanisms that are potentially important of the development of liver disease. Since many signaling processes are well characterized drug targets for clinical intervention we expect that the understanding of these mechanisms will help to define potential new treatments for the prevention and treatment of chronic liver disease including HCC.

Previous studies demonstrate that PTPRD is frequently inactivated and mutated in human cancers [38, 39]. Moreover, PTPRD was downregulated in chemically-induced hepatomas in a rat model [40]. But no direct evidence has been presented demonstrating that a robust PTPRD expression is indeed able to hamper tumor occurrence in the liver. To demonstrate a role of PTPRD as tumor suppressor in the liver we will study chemically-induced HCC development in mice that differentially express PTPRD (figure 2). One intraperitoneal (IP) injection of 0.1 µmol diethylnitrasamine (DEN) / g body weight induces HCC in C57BL/6J mice [41], which is the genetical background of the PTPRD-deficient mouse model described above [42]. These conditions leaded to liver tumors >1mm in 100% males and 78% females C57BL/6J wt mice (PTPRD+/+) after 50 weeks [41].

Table 1 Experimental design – assignment of groups according to PTPRD genotype. +/+ = C57BL/6J, wild type PTPRD expression; +/- = C57BL/6J, heterozygote deletion of PTPRD; -/- = C57BL/6J, homozygote deletion of PTPRD; PBS = phosphate buffered saline; DEN = diethylnitrosamine 0.1 µmol / g body weight.

Group	PTPRD genotype	Amount of male mice	Injection (IP)
1	+/+	12	PBS
2	+/+	12	DEN
3	+/-	12	PBS
4	+/-	12	DEN
5	-/-	12	PBS

6	-/-	12	DEN

To study the impact of PTPRD on the kinetics of DEN-induced hepatocarinogenesis, the PTPRD knockout model has been be transferred to the Institut Clinique de la Souris (ICS) in Strasbourg (frozen sperm). Mice have been generated by *in vitro* fertilization with wild type C57BL/6J, maintained, repopulated and genotyped as described [42]. Male puppies have been separated at day 10 after birth and assigned to six groups of 12 animals according to their PTPRD genotype (table 1). At day 12 of age one half of each group will receive either one single intraperitoneal (IP) injection of 0.1 µmol DEN /g body weight, the other half of the group will receive PBS as negative control.

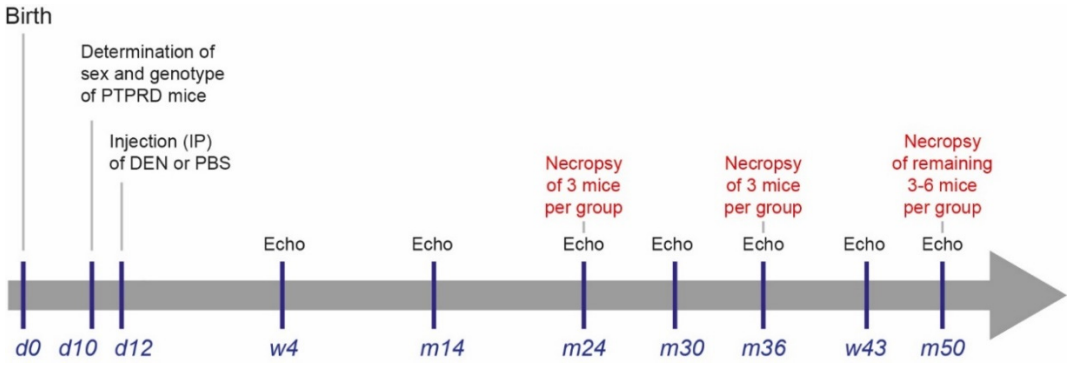

Figure 2 Experimental design for the study of the kinetics of DEN-induced hepatocarcinogenesis in PTPRD-deficient mice. IP=intraperitoneal injection, DEN= 0.1 µmol diethylnitrosamine / g body weight, PBS = control, d= days, m= month, echo = ultrasonic echography of the liver, red = scarify 2 mice from each group for necropsy and preparation of the livers. The experiment will be conducted at the ICS.

As outlined in figure 2, the kinetics of hepatocarcinogenesis in the different groups will be monitored by echography during one year. At week 24, 36, and 50 mice will be sacrificed from each group for necropsy. Two liver samples will be prepared and stored comprising a paraffin block for immunohistochemistry and one cryosection. Samples will be transferred from the ICS to Inserm UMR1110 to analyze of echographic data and liver tissues. These experiments comprise the pathological categorization of liver tumors and immunohistochemistry on liver slices for PTPRD and activity of signaling pathways targeted by PTPRD.

7.1.1 Funding of the project

Appel à projet 2016-1, ANRS, 2016-2019 (PI: Joachim Lupberger).

7.1.2 Supervision of students

Co-supervision of a PhD student: Armando Andres Roca Suarez, started January 2016.

Supervision of a master II student: Oliver Aoun, started January 2017.

7.2 Membrane domains as global platforms for virus entry and antiviral targets

Emerging viruses pose an increasing threat to global socio-economies in the context of progressing globalization and climate change. In the absence of a vaccine, the window of opportunity for a successful treatment of an acute infection is mostly very narrow to prevent complications. Recent

viral outbreaks (i.e. Ebola virus, Zika virus) and endemics (Crimean Congo Hemorrhagic Fever (CCHF) virus, Lassa, Dengue virus) emphasizes the urgent medical need for preventive antiviral strategies to protect high risk individuals including medical staff (Ebola), pregnant women (Zika) or travelers spreading the disease (CCHF, Dengue). Targeting the host provides an interesting perspective for novel antiviral strategies against viral infections because host-targeting agents possess a high genetic barrier to resistance and usually exhibit a pan-genotypic antiviral activity. Using HCV as a model, we previously demonstrated the proof-of-concept that targeting host entry factors using antibodies or small molecule inhibitors efficiently impairs viral entry, infection and spread resulting in cure of infection in state-of-the art animal models with absent toxicity [27, 43, 44]. An additional interesting aspect of targeting the host is that identical host dependency factors may be shared by several pathogens. For example, the tetraspanin CD81 is not only a primary entry receptor for HCV but is also required for entry of *Plasmodium falciparum* and *Listeria monocytogenes* and has been identified as a host factor for influenza infection [45].

Cell entry is a critical step for all viruses to maintain chronic infection and propagation [44, 46] and is therefore an attractive target for cure of viral infection [44, 46, 47]. Although the mechanisms of virus–host interactions during binding and entry are complex, accumulating evidence suggest shared mechanisms of different viruses to penetrate the cell surface [48]. Components of the host plasma membrane are restricted in their lateral movement in order to ensure their functionality at a certain location. Indeed, proteins, lipids and scaffolding proteins on the plasma membrane are organized in major functional domains, including tight junctions, lipid rafts and TEMs. These complexes concentrate co-factors for essential cellular processes including signal transduction and vesicle trafficking and represent potential penetration sites of pathogens including coxsackie virus (tight junctions) [49], SV40 (lipid rafts) [50] and HCV (TEMs) [37]. By definition, all three membrane domains are insoluble to distinct detergents allowing a purification of the complexes from cell lysates. Studying HCV, the consortium recently demonstrated that not only transmembrane proteins but also membrane-associated scaffolding proteins are exploited by the virus to penetrate the host cell [27, 37]. Using CD81 as bait, we pulled down a membrane-associated protein complex and studied the role of the associated proteins in virus entry [37]. Differential mass spec analysis revealed multiple pathogens' receptors associated to CD81. These findings gave rise to a systematic screening of the role of these membrane-associated proteins for entry of several virus families including highly pathogenic representatives for which no or only few treatment options are available. Thus, combining differential proteomics, RNAi, viral pseudoparticles, and infectious live virus, our preliminary results provide proof-of-concept and highlight a subset of proteins associated to TEMs that are important for the entry of multiple viruses including live Ebola virus. In pilot studies using a monoclonal antibody we demonstrate that targeting identified hits exhibit antiviral activity *in vitro*.

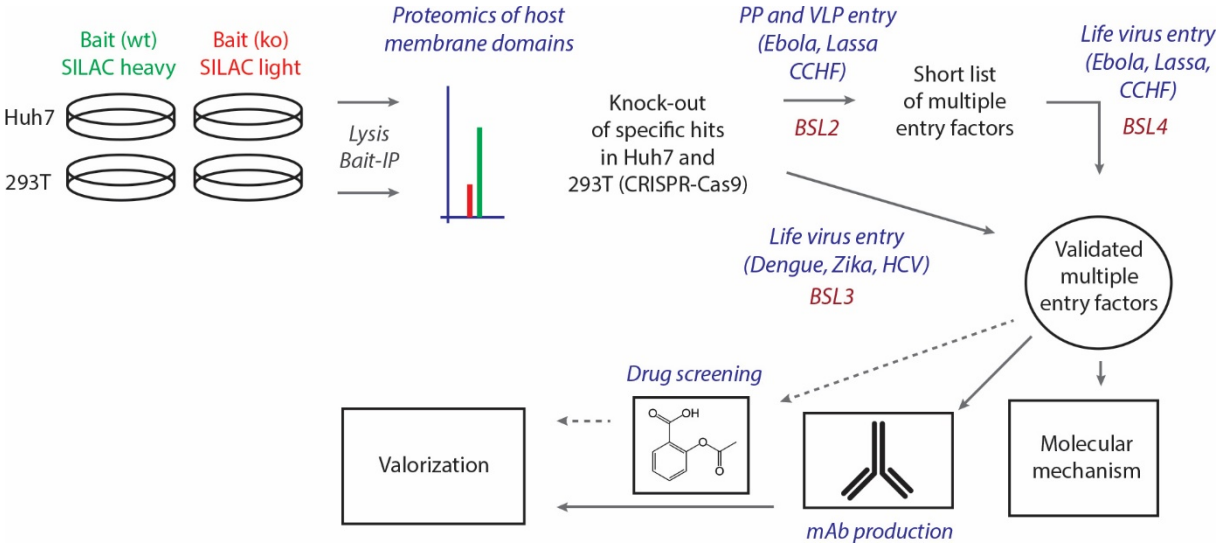

Figure 3 Workflow: Identification of membrane-associated host proteins as targets for broad acting antivirals. PP: pseudoparticles; VLP: virus-like particles; BSL2-4: Biosafety levels 2-4; CCHF: Crimean-Congo hemorrhagic fever; SILAC: Stable isotope labeling by amino acids in cell culture; Bait (ko): engineered cell line with deleted bait protein to control specificity of immunoprecipitations.

We therefore initiated a multidisciplinary approach (figure 3) allowing identify proteins specifically associated to TEMs, lipid rafts or tight junctions that represent bottle-neck limitations for entry of a broad spectrum of clinically relevant viruses. In this regard, I am scientific coordinator of a consortium of three partners aiming (i) to identify protein complexes associated to TEMs, lipid rafts and tight junctions, (ii) to identify specific and shared factors promoting entry of a broad spectrum of viruses, (iii) to uncover the underlying common molecular mechanisms shared by the studied viruses, including the involvement of signal transduction (iv) to identify novel antiviral targets and to generate monoclonal antibodies as specific or broadly acting antivirals.

7.2.1 Project organization

Table 1 Members of the VIRGLOBENT consortium.

Consortium	Name	Affiliation	Task force lead within the consortium
Partner 1 (scientific coordinator)	J. Lupberger	Inserm UMR1110, Strasbourg	Complex labeling and pulldown, screenings, signal transduction
Partner 2	T.F. Baumert	Inserm UMR1110, Strasbourg	Knock-outs, antibody production and validation
Partner 3	F.-L. Cosset	CIRI, ENS, Lyon	Pseudotype particles, viral entry mechanism
Co-partner 3	G. Neveu	CIRI, ENS, Lyon	Proteomic analyses of membrane complexes

7.2.2 Funding of the project

A letter of intent of the project has been submitted to the ANR (AAP-Generique-ANR-2017), decision is pending.

7.2.3 Supervision of students

Supervision of a PhD student that will be hired in frame of this project.

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9 ANNEX

9.1 *Nat. Med.* 17(5):589-95.

nature medicine

EGFR and EphA2 are host factors for hepatitis C virus entry and possible targets for antiviral therapy

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Hepatitis C virus (HCV) is a major cause of liver disease, but therapeutic options are limited and there are no prevention strategies. Viral entry is the first step of infection and requires the cooperative interaction of several host cell factors. Using a functional RNAi kinase screen, we identified epidermal growth factor receptor and ephrin receptor A2 as host cofactors for HCV entry. Blocking receptor kinase activity by approved inhibitors broadly impaired infection by all major HCV genotypes and viral escape variants in cell culture and in a human liver chimeric mouse model *in vivo*. The identified receptor tyrosine kinases (RTKs) mediate HCV entry by regulating CD81–claudin-1 co-receptor associations and viral glycoprotein–dependent membrane fusion. These results identify RTKs as previously unknown HCV entry cofactors and show that tyrosine kinase inhibitors have substantial antiviral activity. Inhibition of RTK function may constitute a new approach for prevention and treatment of HCV infection.

HCV is a major cause of liver cirrhosis and hepatocellular carcinoma. Current antiviral treatment is limited by drug resistance, toxicity and high costs[1]. Although newly developed antiviral substances targeting HCV protein processing have been shown to improve virological response, toxicity and resistance remain major challenges[2]. Thus, new antiviral preventive and therapeutic strategies are urgently needed. Because HCV entry is required for initiation, dissemination and maintenance of viral infection, it is a promising target for antiviral therapy[3,4].

HCV entry is a multistep process involving viral envelope glycoproteins as well as several cellular attachment and entry factors[5]. Attachment of the virus to the target cell is mediated through binding of HCV envelope glycoproteins to glycosaminoglycans[6]. HCV is internalized in a clathrin-dependent endocytic process requiring CD81 (ref. 7), scavenger receptor type B class I (SR-BI)[8], claudin-1 (CLDN1)[9] and occludin (OCLN)[10]. To elucidate the functional role of host cell kinases within the HCV entry process, we performed a functional RNAi screen.

RESULTS

Host cell kinases are host cofactors for HCV entry

Using a siRNA screen, we identified a network of kinases with functional impact on HCV entry (**Supplementary Results**, **Supplementary Tables 1** and **2** and **Supplementary Figs. 1** and **2**). To study the relevance of the identified kinases on the HCV life cycle, we further validated and characterized the functional impact of epidermal growth factor receptor (EGFR), ephrin receptor A2 (EphA2) and cell division cycle 2 kinase (CDC2) (**Supplementary Results** and **Supplementary Fig. 3**) on HCV entry. We focused on EGFR and EphA2 because they are key components in the identified networks (**Supplementary Fig. 2c**), they are highly expressed in human liver (**Supplementary Table 2**) and their kinase function is inhibited by clinically approved protein kinase inhibitors (PKIs)[11–13], allowing us to explore the potential of these molecules as therapeutic targets.

Using individual siRNAs, we first confirmed that silencing of mRNAs reduced EGFR and EphA2 mRNA and protein expression (**Fig. 1a**,**b** and **Supplementary Fig. 4a**,**b**). Infection of siEGFR or

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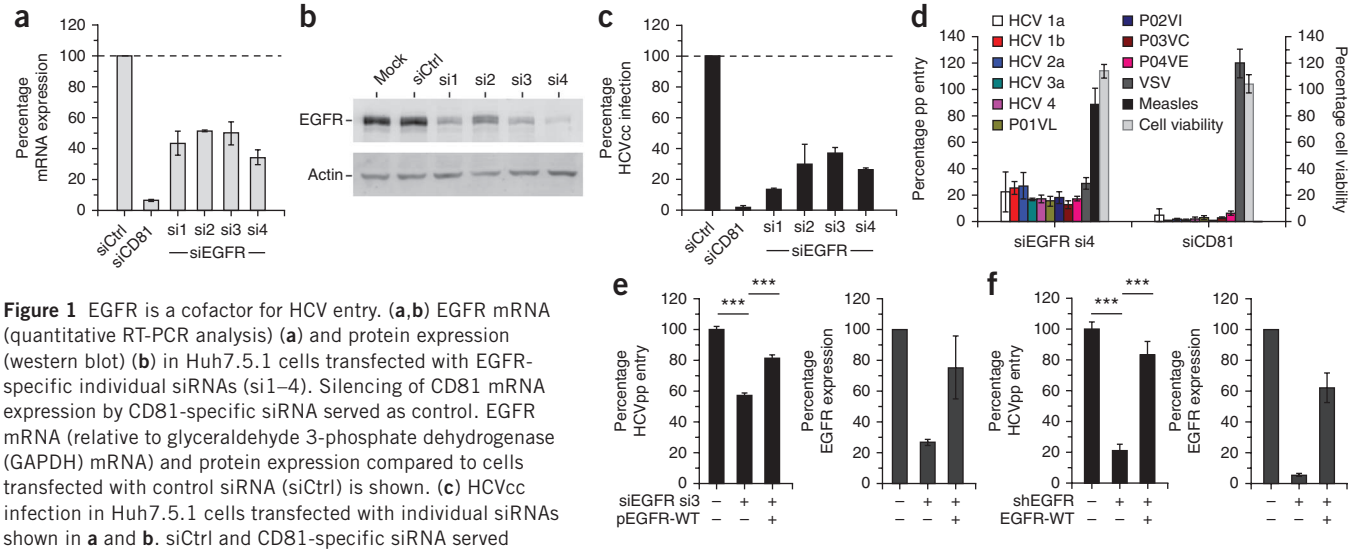

Figure 1 EGFR is a cofactor for HCV entry. (**a**,**b**) EGFR mRNA (quantitative RT-PCR analysis) (**a**) and protein expression (western blot) (**b**) in Huh7.5.1 cells transfected with EGFR-specific individual siRNAs (si1–4). Silencing of CD81 mRNA expression by CD81-specific siRNA served as control. EGFR mRNA (relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA) and protein expression compared to cells transfected with control siRNA (siCtrl) is shown. (**c**) HCVcc infection in Huh7.5.1 cells transfected with individual siRNAs shown in **a** and **b**. siCtrl and CD81-specific siRNA served as internal controls. Data are expressed as percentage HCVcc infection relative to siCtrl-transfected cells (means ± s.d. from three independent experiments in triplicate). (**d**) Entry of HCVpp containing envelope glycoproteins of various isolates[14,39] in Huh7.5.1 cells transfected with si4. Vesicular stomatitis virus (VSV) and measles virus pseudoparticle (pp) entry or cells transfected with CD81-specific siRNA served as controls. Data are expressed as percentage pseudoparticle entry relative to siCtrl-transfected cells (means ± s.d. from three independent experiments in triplicate). (**e**) HCVpp entry and EGFR protein expression in Huh7.5.1 cells concurrently transfected with EGFR-specific individual si3 and a cDNA encoding RNAi-resistant EGFR (pEGFR-WT)[40]. (**f**) HCVpp entry and EGFR protein expression in PHHs concurrently transduced with lentiviruses expressing shEGFR and wild-type EGFR cDNA (EGFR-WT)[40]. Data are expressed as percentage HCVpp entry relative to Ctrl cells or as percentage EGFR expression normalized for β-actin expression (means ± s.d. from four independent experiments in triplicate). ***$P < 0.0005$.

siEphA2-treated cells by cell culture–derived HCV (HCVcc) was markedly reduced, as compared to control siRNA-treated cells indicating that both EGFR and EphA2 are involved in the initiation of a productive infection (**Fig. 1c** and **Supplementary Fig. 4c**). Silencing of kinase expression inhibited the entry of HCV pseudoparticles (HCVpp) derived from major genotypes, including highly diverse HCV strains[14] (**Fig. 1d** and **Supplementary Fig. 4d**).The effects of silencing of endogenous EGFR or EphA2 on HCV infection were rescued by RNAi-resistant ectopic expression of wild-type EGFR or EphA2 (**Fig. 1e**,**f** and **Supplementary Fig. 4e**,**f**), largely excluding the possibility of off-target effects causing the observed phenotype. Furthermore, silencing and rescue experiments using well-characterized lentiviral vectors expressing EGFR-specific shRNA showed a key role for EGFR in HCV entry into primary human hepatocytes (PHHs) (**Fig. 1f**). We then assessed the functional impact of EGFR as a cofactor for HCV entry by expressing human EGFR in mouse hepatoma cell lines engineered to express the four human entry factors CD81, SR-BI, CLDN1 and OCLN (AML12 4R; **Supplementary Fig. 5**). Cell surface expression of human EGFR in AML12 4R cells markedly enhanced the susceptibility of mouse cells to HCVpp entry (**Supplementary Fig. 5**).

RTK kinase function is relevant for HCV entry

We used PKIs to further study the functional relevance of the identified kinases for HCV entry and infection. Erlotinib (an EGFR inhibitor) and dasatinib (an EphA2 inhibitor) impaired HCV entry and infection in a dose-dependent manner without a detectable effect on replication of the corresponding subgenomic replicon (**Fig. 2**, **Supplementary Fig. 6** and **Supplementary Fig. 7**). The half-maximal inhibitory concentration (IC_{50}) values for erlotinib and dasatinib to block HCVpp entry (erlotinib, 0.45 ± 0.09 μM; dasatinib, 0.53 ± 0.02 μM) and HCVcc infection (erlotinib, 0.53 ± 0.08 μM; dasatinib, 0.50 ± 0.30 μM) of human hepatoma Huh7.5.1 cells were comparable (**Fig. 2a** and **Supplementary Fig. 7a**,**b**). These data indicate that inhibiting RTKs by erlotinib and dasatinib has a marked effect on HCV entry.

To evaluate the effects of the inhibitors on HCV entry into cells more closely resembling the HCV target cells *in vivo*, we investigated HCVpp entry into polarized HepG2-CD81 hepatoma cells[15] and PHHs. PKIs markedly and significantly ($P < 0.005$) inhibited HCVpp entry into polarized HepG2-CD81 cells (**Fig. 2c** and **Supplementary Fig. 7d**) and PHHs (**Fig. 2d** and **Supplementary Fig. 7e**). We obtained similar results for infection of PHHs with HCVcc and serum-derived HCV (**Fig. 3** and **Supplementary Fig. 7**), confirming the role of the kinases as auxiliary host cell cofactors in models that more closely mimic *in vivo* infection.

A specific effect of erlotinib on EGFR-mediated HCV entry was further confirmed by the inhibition of HCV entry and infection by other EGFR inhibitors. The EGFR inhibitors gefitinib and lapatinib markedly inhibited HCVpp entry and HCVcc infection in PHHs and Huh7.5.1 cells similarly to erlotinib (**Fig. 2e**,**f**). The specificity of the PKIs in preventing HCV entry was further corroborated by their lack of an effect on murine leukemia virus and measles virus entry (**Fig. 2c** and **Supplementary Fig. 8**). Moreover, PKI treatment of RTK-silenced Huh7.5.1 cells reversed the rescue of HCV entry conferred by expressing EGFR and EphA2 in *trans* (data not shown). Taken together, these results suggest that the RTK kinase function is necessary for efficient HCV entry.

RTK-specific ligands and antibodies modulate HCV entry

We assessed virus entry in the presence of RTK-specific ligands and antibodies. Epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) are well-characterized EGFR ligands whose binding promotes receptor dimerization and subsequent phosphorylation of the intracytoplasmic kinase domain[16]. To confirm the biological activity of EGFR-specific reagents in the target cells of our HCV model systems, we first studied their effect on EGFR phosphorylation. Preincubation of PHHs with EGF markedly increased basal

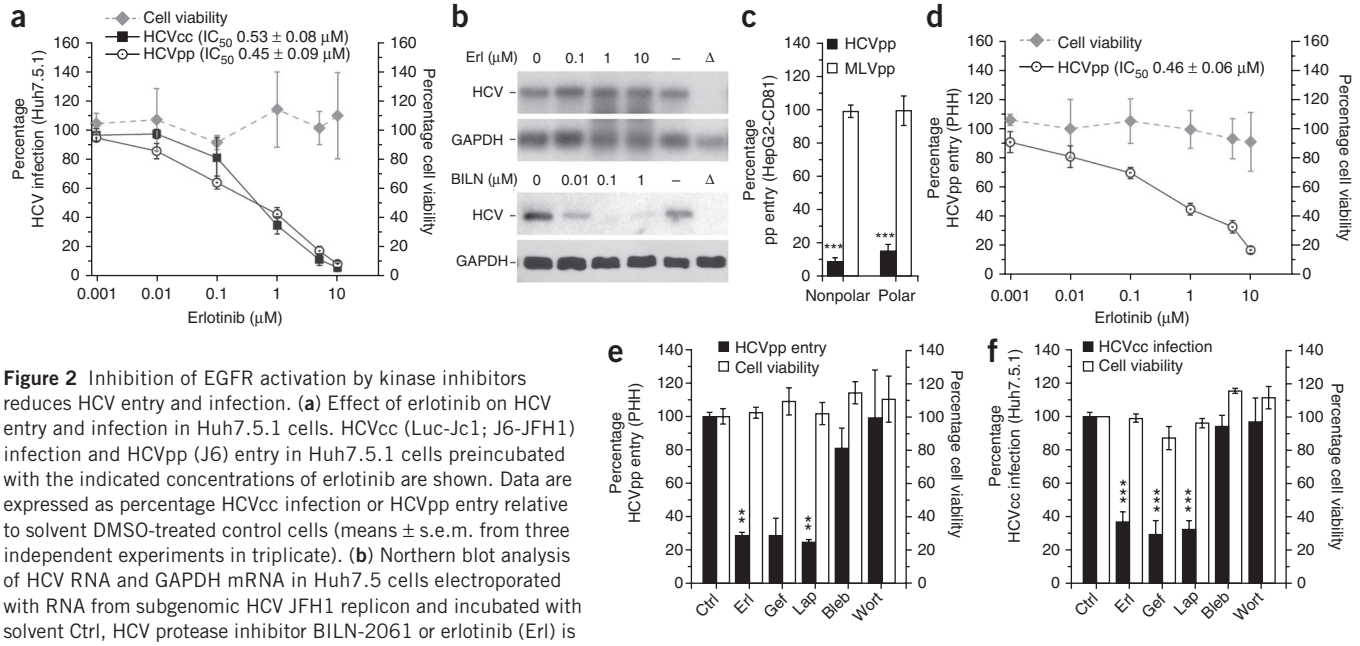

Figure 2 Inhibition of EGFR activation by kinase inhibitors reduces HCV entry and infection. (**a**) Effect of erlotinib on HCV entry and infection in Huh7.5.1 cells. HCVcc (Luc-Jc1; J6-JFH1) infection and HCVpp (J6) entry in Huh7.5.1 cells preincubated with the indicated concentrations of erlotinib are shown. Data are expressed as percentage HCVcc infection or HCVpp entry relative to solvent DMSO-treated control cells (means ± s.e.m. from three independent experiments in triplicate). (**b**) Northern blot analysis of HCV RNA and GAPDH mRNA in Huh7.5 cells electroporated with RNA from subgenomic HCV JFH1 replicon and incubated with solvent Ctrl, HCV protease inhibitor BILN-2061 or erlotinib (Erl) is shown. Analysis of HCV RNA in cells transfected with replication incompetent HCV RNA (GND, Δ) served as negative control. (**c**) Effect of erlotinib on HCVpp and MLVpp entry in HepG2-CD81 cells. The percentage pseudoparticle entry into nonpolarized and polarized HepG2-CD81 cells (generated as previously described[15]) preincubated with erlotinib (10 μM) is shown (means ± s.d. from ten independent experiments). (**d**) Effect of erlotinib on HCVpp entry into PHHs. The percentage HCVpp entry into PHHs preincubated with erlotinib is shown relative to entry into solvent-treated control cells. IC_{50} value is expressed as median ± standard error of the median of three independent experiments performed in triplicate. (**e**,**f**) HCVpp entry into PHHs (**e**) and HCVcc infection of Huh7.5.1 cells (**f**) preincubated with 1 μM erlotinib, gefitinib (Gef), lapatinib (Lap), blebbistatin (Bleb) or wortmannin (Wort) is shown. Cell viability was assessed by MTT assay. Means ± s.d. from three independent experiments in duplicate (**e**) or triplicate (**f**) are shown. $^{**}P < 0.005$; $^{***}P < 0.0005$.

levels of EGFR phosphorylation (**Fig. 3a**). In contrast, EGF had no effect on the phosphorylation of c-mer protooncogene tyrosine kinase (MERTK), an unrelated kinase (**Fig. 3a**). EGF-induced enhancement of basal EGFR phosphorylation was markedly inhibited by erlotinib and an EGFR-specific antibody (**Fig. 3a**), indicating their specific effect on EGFR phosphorylation and activation.

We next examined the role of EGFR ligands on HCV entry. Binding of EGF and TGF-α markedly enhanced entry of HCVpp into

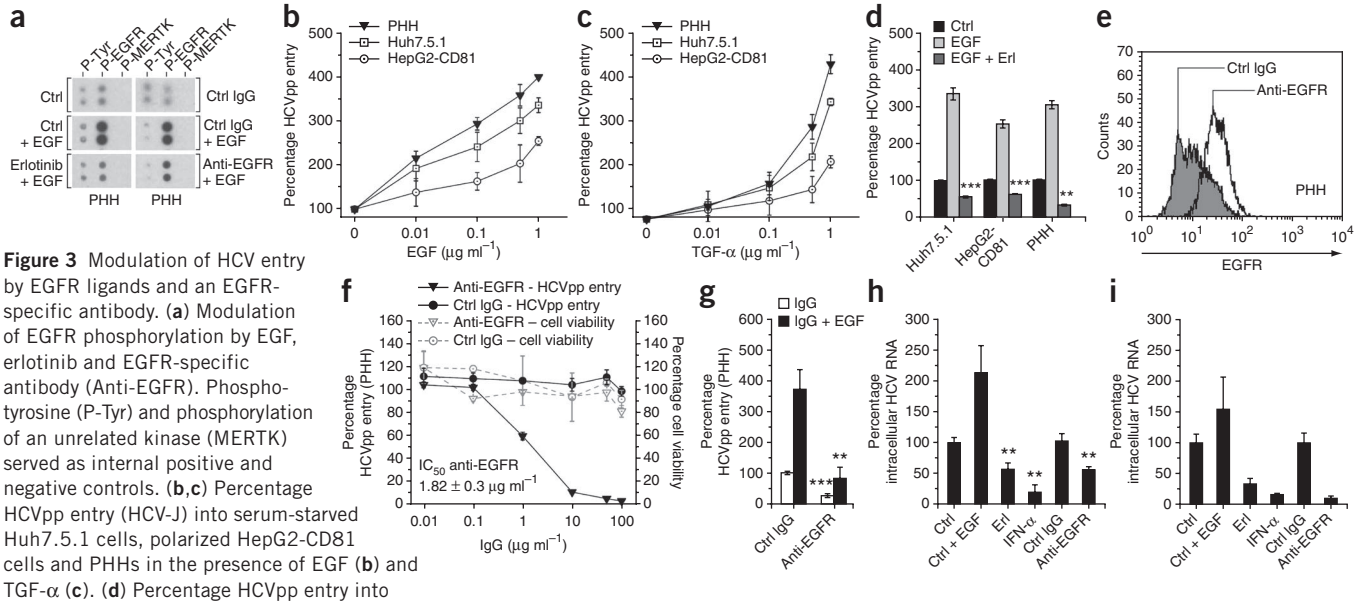

Figure 3 Modulation of HCV entry by EGFR ligands and an EGFR-specific antibody. (**a**) Modulation of EGFR phosphorylation by EGF, erlotinib and EGFR-specific antibody (Anti-EGFR). Phosphotyrosine (P-Tyr) and phosphorylation of an unrelated kinase (MERTK) served as internal positive and negative controls. (**b**,**c**) Percentage HCVpp entry (HCV-J) into serum-starved Huh7.5.1 cells, polarized HepG2-CD81 cells and PHHs in the presence of EGF (**b**) and TGF-α (**c**). (**d**) Percentage HCVpp entry into Huh7.5.1, polarized HepG2-CD81 and PHH incubated with EGF or EGF and erlotinib is shown (means ± s.d. from three independent experiments in triplicate). (**e**) Flow cytometric analysis of nonpermeabilized PHH binding EGFR-specific or control monoclonal antibody (mAb). (**f**) Percentage HCVpp entry into PHHs preincubated with EGFR-specific or control mAb is shown. Viability of cells was assessed by MTT assay. IC_{50} value is expressed as median ± standard error of the median of three independent experiments in triplicate. (**g**) Percentage HCVpp entry into PHHs preincubated with EGF and EGFR-specific mAb. (**h**,**i**) Intracellular HCV RNA levels in PHHs infected with HCVcc (means ± s.d. from three independent experiments in duplicate) (**h**) or serum-derived HCV (one representative experiment) (**i**) as measured by quantitative RT-PCR. $^{**}P < 0.005$; $^{***}P < 0.0005$. Unless otherwise indicated, EGFR-specific and control mAbs: 10 μg ml^{-1}; EGF: 1 μg ml^{-1}; erlotinib: 10 μM.

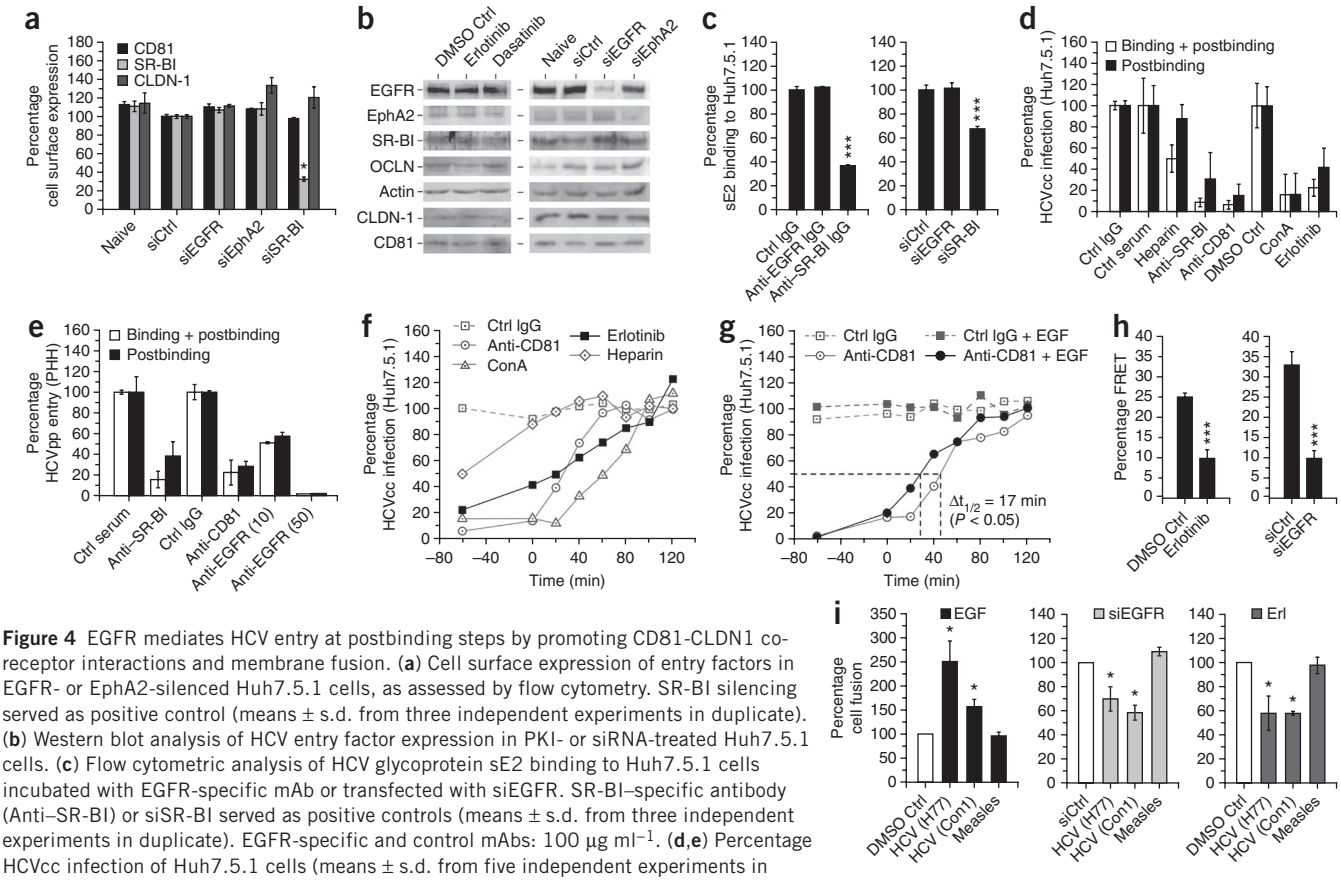

Figure 4 EGFR mediates HCV entry at postbinding steps by promoting CD81-CLDN1 co-receptor interactions and membrane fusion. (**a**) Cell surface expression of entry factors in EGFR- or EphA2-silenced Huh7.5.1 cells, as assessed by flow cytometry. SR-BI silencing served as positive control (means ± s.d. from three independent experiments in duplicate). (**b**) Western blot analysis of HCV entry factor expression in PKI- or siRNA-treated Huh7.5.1 cells. (**c**) Flow cytometric analysis of HCV glycoprotein sE2 binding to Huh7.5.1 cells incubated with EGFR-specific mAb or transfected with siEGFR. SR-BI–specific antibody (Anti–SR-BI) or siSR-BI served as positive controls (means ± s.d. from three independent experiments in duplicate). EGFR-specific and control mAbs: 100 μg ml^{-1}. (**d**,**e**) Percentage HCVcc infection of Huh7.5.1 cells (means ± s.d. from five independent experiments in triplicate) (**d**) and percentage HCVpp entry into PHHs (means ± s.d. from three independent experiments in duplicate) (**e**) after inhibition of binding and postbinding steps by the indicated compounds (EGFR-specific mAb: 10 and 50 μg ml^{-1}). (**f**,**g**) Time course of HCVcc infection of Huh7.5.1 cells after incubation with erlotinib or the indicated compounds (means ± s.d. from five independent experiments in triplicate) (**f**) or EGF at various timepoints during infection (means ± s.d. from three independent experiments in triplicate) (**g**) (**Supplementary Methods**). (**h**) FRET of CD81-CLDN1 co-receptor associations in HepG2-CD81 cells incubated with erlotinib or EGFR-specific siRNA (means ± s.e.m. from ten independent experiments). (**i**) Percentage viral glycoprotein-dependent fusion of 293T with Huh7 cells incubated with EGF, erlotinib or EGFR-specific siRNA, assessed as previously described[25]. Means ± s.d. from three independent experiments in triplicate are shown. *P < 0.05; ***P < 0.0005. Unless otherwise indicated, EGFR-specific and control mAbs: 10 μg ml^{-1}; EGF: 1 μg ml^{-1}; erlotinib: 10 μM.

serum-starved Huh7.5.1 cells, polarized HepG2-CD81 cells and PHHs (**Fig. 3b**,**c**), whereas TGF-β had no effect (data not shown). These data suggest that direct interaction of EGF or TGF-α with the EGFR ligand-binding domain modulates HCV entry. The higher affinity of EGF for EGFR on hepatocytes[17] may explain the differences between EGF and TGF-α in enhancing HCVpp entry. Erlotinib, at doses used in HCV entry inhibition experiments, reversed the enhancing effects of EGF (**Fig. 3d**) and TGF-α (data not shown) on HCV entry. These data confirm that erlotinib inhibits HCV entry by modulating EGFR activity.

We screened a large panel of EGFR-specific antibodies and identified a monoclonal human EGFR-specific antibody that bound PHHs (**Fig. 3e**) and inhibited HCV entry into PHH in a dose-dependent manner (**Fig. 3f**), with an IC_{50} value of 1.82 ± 0.3 μg ml^{-1}. The antibody inhibited EGFR phosphorylation (**Fig. 3a**) and reversed the EGF-induced enhancement of HCV entry (**Fig. 3g**). Ligand-induced enhancement and EGFR-specific antibody–mediated inhibition of HCV entry were also observed for infection of PHHs with HCVcc (**Fig. 3h**) and with serum-derived HCV (**Fig. 3i**). Taken together, these results suggest that the EGFR ligand-binding domain is relevant for HCV entry. Similarly, EphA2 ligands and EphA2-specific antibodies modulated HCV entry, suggesting a functional relevance of the EphA2 ligand-binding domain for HCV entry (**Supplementary Results** and **Supplementary Fig. 9**).

RTKs promote CD81-CLDN1 associations and membrane fusion

To understand the mechanistic role of EGFR and EphA2 in HCV entry, we first investigated whether the RTKs regulate SR-BI, CD81, CLDN1 and OCLN expression. However, silencing RTK expression with specific siRNAs or inhibiting RTK function with PKIs had no significant effect on HCV entry factor expression (**Fig. 4a**,**b**).

Next, we aimed to fine-map the entry steps affected by the RTKs. Viral attachment is the first step of viral entry. To ascertain whether PKI-mediated inhibition of RTK function modulates HCV binding, we used a surrogate model that measures binding of the recombinant soluble form of HCV envelope glycoprotein E2 to Huh7.5.1 cells[18]. RTK-specific antibodies or silencing RTK expression by siRNAs had no significant effect on E2 binding of target cells, whereas preincubation with SR-BI–specific antibodies or silencing SR-BI expression markedly reduced E2 binding (**Fig. 4c** and **Supplementary Fig. 10a**). Furthermore, in contrast to the case with CD81 and SR-BI[19], RTKs did not increase cellular E2 binding when expressed on the cell surface of Chinese hamster ovary cells (data not shown). These data suggest that RTKs do not modulate HCV binding to target cells.

After viral envelope binding, HCV enters its target cell in a multistep temporal process. To identify the time at which the PKIs exert their effects, we used a well-characterized assay allowing us to investigate

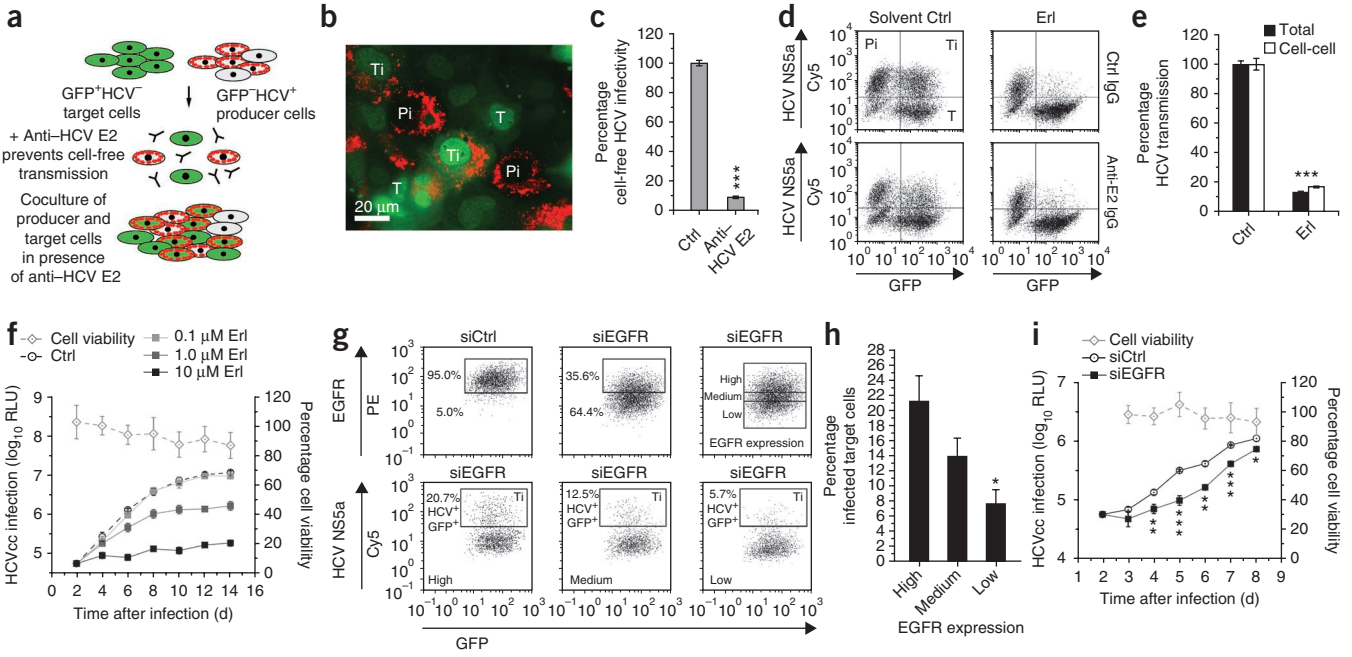

Figure 5 Functional role of EGFR in viral cell-to-cell transmission and spread. (**a**) Experimental setup. HCV producer cells cultured with uninfected target cells[26] were incubated with siEGFR or PKIs. Cell-free HCV transmission was blocked by an E2-neutralizing antibody (Anti–HCV E2, 25 µg ml^{-1})[26]. HCV-infected target cells were quantified by flow cytometry[26]. (**b**) Immunofluorescence analysis of Pi (HCV RNA–electroporated Huh7.5.1 producer cells), T (GFP-expressing Huh7.5 target cells) and Ti (GFP$^+$HCV NS5A$^+$ HCV-infected target cells) cells stained with an HCV non structural protein 5A (NS5A)-specific antibody (red). (**c**) Infectivity of Pi-T cell co-cultivation supernatants (cell-free HCV transmission). (**d**,**e**) Quantification of infected Ti cells during erlotinib (10 µM) treatment in the absence (total transmission) and presence (cell-to-cell transmission) of E2-specific antibody by flow cytometry (means ± s.d. from three independent experiments in duplicate). (**f**) Effect of PKIs on viral spread. Long-term HCVcc infection of Huh7.5.1 cells incubated with erlotinib 48 h after infection at the indicated concentrations. Medium with solvent (Ctrl) or PKI was replenished every second day. Cell viability was assessed by MTT test. Means ± s.d. from three independent experiments in triplicate are shown. RLU, relative light units. (**g**) EGFR expression in target cells with silenced EGFR expression. Cell surface EGFR expression was analyzed by flow cytometry and target cells were divided in three groups displaying high, medium and low EGFR expression. (**h**) HCV infection in GFP-positive target cells expressing EGFR at high, medium and low levels (see **g**) assessed as described above (means ± s.d. from three independent experiments in triplicate). (**i**) Effect of EGFR silencing on viral spread. Long-term analysis of HCVcc infection in Huh7.5.1 cells transfected with EGFR-specific or control siRNA 24 h after infection. Cell viability was assessed by MTT test. Means ± s.d. from three independent experiments in triplicate are shown. *$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$.

whether an inhibitory molecule interferes with viral envelope binding or affects entry steps after binding of the virus to the target cell[19–21]. In contrast to heparin (an inhibitor of HCV binding) but similarly to CD81- and SR-BI–specific antibodies and concanamycin A (an inhibitor of endosomal acidification), PKIs inhibited HCVcc infection when added after virus binding to target cells (**Fig. 4d**). We obtained similar results for HCVpp entry into PHHs after treatment with an EGFR-specific antibody (**Fig. 4e**). These data suggest that the RTKs act at postbinding steps of viral entry.

To further elucidate the entry steps targeted by the RTKs, we performed a kinetic entry assay[19,21] (**Supplementary Fig. 10b**). Notably, the half-maximal times ($t_{1/2}$) for erlotinib ($t_{1/2}$ = 20 min) and dasatinib ($t_{1/2}$ = 26 min) to inhibit HCV entry were similar to the half-maximal time of a CD81-specific antibody ($t_{1/2}$ = 26 min) (**Fig. 4f** and **Supplementary Fig. 10d**). Moreover, similar to concanamycin A, PKIs also had an inhibitory effect when added at late times (60–80 min) after infection (**Fig. 4** and **Supplementary Fig. 10**). We further confirmed the role of EGFR as a postbinding factor by kinetic assays under serum-free conditions. In line with previous reports[22], HCV entry kinetics were delayed under serum-free conditions (**Fig. 4g**). EGF significantly ($P < 0.05$) reduced the time needed for HCVcc to escape the inhibiting effects of a CD81-specific antibody in serum-starved cells from 44 ± 8 min to 27 ± 6 min (mean ± s.d. of three independent experiments), suggesting that EGF markedly and significantly ($P < 0.05$) accelerates the rate of HCV entry (**Fig. 4g**). In summary, these data suggest that EGFR is required for efficient viral entry by modulating early and late steps of postbinding events.

Postbinding steps of HCV entry are mediated by the HCV entry factors SR-BI, CD81, CLDN1 and OCLN. As PKIs inhibited HCV entry at similar timepoints as a CD81-specific antibody, we investigated whether PKIs interfere with CD81-CLDN1 co-receptor interactions using a fluorescence resonance energy transfer (FRET)-based assay[15,23,24]. PKIs significantly ($P < 0.0005$) reduced CD81-CLDN1 FRET in polarized HepG2 cells (**Fig. 4h** and **Supplementary Fig. 10e**). We obtained similar results with RTK-specific siRNAs (**Fig. 4h** and **Supplementary Fig. 10e**), confirming that the observed inhibition is RTK specific and not mediated by off-target effects of the PKIs. These results suggest that EGFR and EphA2 regulate the formation of the CD81-CLDN1 co-receptor complexes that are essential for HCV entry[23] and that erlotinib and dasatinib inhibit HCV entry by interfering with the CD81-CLDN1 co-receptor association.

As kinetic assays showed that PKIs inhibited late steps of viral entry (**Fig. 4f** and **Supplementary Fig. 10d**), we investigated the impact of these kinases in a viral glycoprotein–dependent cell-cell fusion assay[25]. Both PKIs significantly ($P < 0.05$) inhibited membrane fusion of cells expressing glycoproteins derived from genotypes 1a (H77), 1b (Con1) and 2a (J6) (**Fig. 4i** and **Supplementary Fig. 10f**), whereas the EGFR ligand EGF enhanced membrane fusion of cells expressing

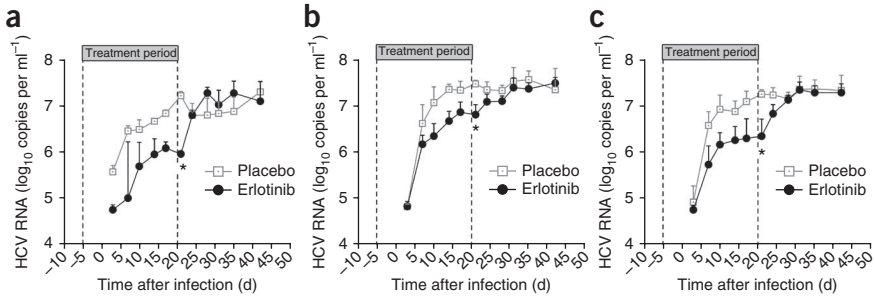

Figure 6 Erlotinib modulates HCV kinetics and inhibits infection *in vivo*. Chimeric uPA-SCID mice repopulated with PHHs[27,28] were treated with erlotinib or placebo during infection with human-derived HCV as indicated by the bar and dashed lines. Serum HCV load was analyzed at the timepoints indicated. Results are shown as median viral load of erlotinib-treated ($n = 4$) or placebo-treated control ($n = 3$) mice, medians ± standard error of the median. (**a**,**b**) Two independent studies (seven mice each) are shown. (**c**) Pooled data of the results shown in **a** and **b** ($n = 14$), medians ± standard error of the median; $^*P < 0.05$.

these HCV envelope glycoproteins (**Fig. 4i**). In contrast, neither erlotinib nor EGF had a marked effect on the membrane fusion of cells expressing measles virus envelope glycoproteins (**Fig. 4i**). We obtained comparable results in EGFR- and EphA2-silenced cells (**Fig. 4i**, data not shown) confirming that the RTKs are involved in viral glycoprotein–dependent membrane fusion.

Impact of RTKs in cell-to-cell transmission and viral spread

To investigate the relevance of RTK-mediated virus-host interactions for cell-to-cell transmission and viral spread, we used a cell-to-cell transmission assay[26] (**Fig. 5a–c**). Erlotinib and dasatinib significantly ($P < 0.0005$) blocked HCV cell-to-cell transmission during short-term coculture experiments (24 h) (**Fig. 5d–f** and **Supplementary Fig. 11a–c**). We also observed a marked inhibition of cell-to-cell transmission when we silenced EGFR and EphA2 with specific siRNAs: infection of GFP-positive target cells directly correlated with RTK cell surface expression (**Fig. 5g,h** and **Supplementary Fig. 11d,e**). Because PKIs inhibited cell-to-cell transmission, we investigated whether erlotinib and dasatinib also impede viral spread in the HCVcc system when added after infection during long-term experiments. Both PKIs inhibited viral spread in a dose-dependent manner for up to 14 d when added 48 h after infection to HCV-infected cells (**Fig. 5f** and **Supplementary Fig. 11c**). Cell viability was not affected by long-term PKI treatment. We also observed a specific decrease in viral spread in cells with silenced RTK expression (**Fig. 5i** and **Supplementary Fig. 11f**). Taken together, these data indicate that PKIs reduce viral spread and suggest a key function of these RTKs in cell-to-cell transmission and dissemination.

Erlotinib inhibits HCV infection *in vivo*

To address the *in vivo* relevance of the identified virus-host interactions, we assessed the effect of erlotinib on HCV infection in the chimeric urokinase plasminogen activator–severe combined immunodeficiency (uPA-SCID) mouse model[27–29]. Erlotinib dosing and administration was performed as described previously for cancer xenograft models[30] and is indicated in **Figure 6**. Erlotinib treatment significantly ($P < 0.05$) delayed the kinetics of HCV infection (**Fig. 6**). The median time to reach steady-state levels of infection increased from 15 d (placebo group) to 30 d (erlotinib group) (median of pooled data from six placebo-treated and eight erlotinib-treated mice). Furthermore, erlotinib treatment decreased steady-state HCV RNA levels by more than 90% (mean of pooled data from six placebo-treated and eight erlotinib-treated mice; $P < 0.05$). After discontinuation of treatment, viral load reached similar levels as in placebo-treated mice (**Fig. 6**). The treatment was well tolerated and did not induce any marked changes in safety parameters such as serum concentrations of alanine transaminase, albumin or body weight (data not shown). Erlotinib plasma concentrations were similar to those described previously in preclinical studies of cancer mouse models[30] (data not shown). Taken together, these data suggest that EGFR acts as a cofactor for HCV entry and dissemination *in vivo* and show that erlotinib has antiviral activity *in vivo*.

DISCUSSION

Using RNAi screening, we uncovered a network of kinases that have a functional impact on HCV entry and identified EGFR and EphA2 as previously unrecognized cofactors for HCV entry. This identification of kinases as HCV entry factors advances knowledge on the molecular mechanisms and cellular requirements of HCV entry, and the discovery of PKIs as candidate antivirals defines a potential new strategy for preventing and treating HCV infection.

EGFR is a RTK that regulates a number of key processes, including cell proliferation, survival, differentiation during development, tissue homeostasis and tumorigenesis[31]. EphA2 mediates cell positioning, cell morphology, polarity and motility[32]. As PKIs had no effect on HepG2 polarization (**Supplementary Fig. 12**), it is unlikely that changes in polarity explain their mode of action. Our results rather highlight a role of these RTKs in the formation of HCV entry factor complexes and membrane fusion. EGF accelerated HCV entry, suggesting that EGFR plays a key part in the HCV entry process, allowing HCV to efficiently enter its target cell. Applying FRET proximity analysis, we found that inhibition of EGFR or EphA2 activity reduced CD81-CLDN1 association. As EGFR activation has been reported to promote CLDN1 redistribution[33,34], and we found that the level of CD81 or CLDN1 cell surface expression was not altered by EGFR silencing (**Fig. 4a**), we hypothesize that EGFR activation modulates intracellular or cell surface trafficking of CLDN1, CD81 or both, which is necessary to form viral envelope-CD81-CLDN1 co-receptor complexes[19,23,24]. The observations that erlotinib inhibits late steps in the kinetic infection assay and in the HCV cell fusion assay suggest a functional role for EGFR in pH-dependent fusion of viral and host cell membranes[25,35].

Our functional experiments with specific ligands, antibodies and kinase inhibitors implicated both ligand-binding and kinase domains of EGFR in promoting HCV entry. EGFR ligands enhanced HCV infection, and an EGFR-specific antibody inhibited HCV infection. This antibody binds between ligand-binding domain III and the autoinhibition (tether) domain IV of the extracellular part of EGFR[36] and prevents EGF- and TGF-α–induced receptor dimerization[37]. Thus, it is likely that receptor dimerization and/or the domain targeted by the antibody are required for HCV entry. Taken together, these findings support a model in which EGFR-ligand binding activates the EGFR kinase function that is required for HCV entry.

We obtained similar results for EphA2, where antibodies specific for the extracellular domain of EphA2 inhibited HCV entry into PHHs and EphA2 surrogate ligands decreased viral entry. Because addition of surrogate ligands only reduced HCV entry to a small extent, it is

conceivable that the effect of EphA2 on HCV entry could be both ligand independent and ligand dependent. This is consistent with other well-characterized EphA2 functions such as cell invasion and migration[38].

Given that our functional and mechanistic studies indicate that the expression and activity of EGFR and EphA2 seem to be involved in similar entry steps, it is likely that both RTKs are part of the same entry regulatory pathway. Because erlotinib and EGF modulated entry of HCVpp but showed minimal effects on the unrelated viruses studied (**Supplementary Fig. 8**), it is likely that the molecular mechanisms that we uncovered are most relevant for HCV entry.

Finally, our results have clinical implications for the prevention and treatment of HCV infection, as they show that licensed PKIs have antiviral activity *in vitro* and *in vivo* and identified a monoclonal RTK-specific antibody that inhibits viral entry. Thus, targeting RTKs as HCV entry factors using small molecules or antibodies may constitute a new approach to prevent and treat HCV infection and address antiviral resistance.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

J.L., M.B.Z. and T.F.B. wrote the manuscript. J.L., M.B.Z., F.X., D.L., F.-L.C., J.A.M., and T.F.B. designed experiments and analyzed data. J.L., M.B.Z., F.X., C.T., I.F., L.Z., C.D., C.J.M., M.T., S.G., C.R., M.N.Z., D.L. and J.F. performed experiments. S.M.R., T.P., A.H.P., P.P. and M.D. contributed essential reagents. W.R. and O.P. performed bioinformatic analyses. J.L., B.F. and L.B. implemented and coordinated the siRNA screen. T.F.B. designed and supervised the project.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Infection of cell lines and primary human hepatocytes with HCVpp, HCVcc and serum-derived HCV. Pseudotyped particles expressing envelope glycoproteins from various HCV strains (**Supplementary Methods**), vesicular stomatitis virus, murine leukemia virus, influenza, measles and endogenous feline leukemia virus (RD114) and HCVcc were generated as previously described[14,41,42,44–46]. Infection of Huh7, Huh7.5.1 cells and PHHs with HCVpp, HCVcc (half-maximal tissue culture infectious dose ($TCID_{50}$) 1×10^3–1×10^4 ml^{-1} for Huh7.5.1 experiments, $TCID_{50}$ 1×10^5–1×10^6 ml^{-1} for PHH experiments) and serum-derived HCV (genotype 1b)[47] was performed as previously described[14,19,21,48]. Polarization of HepG2-CD81, determination of tight junction integrity and cell polarity index were performed, measured and calculated as previously described[15]. Gene silencing was performed 3 d before infection as described for the RNAi screen in the **Supplementary Methods**. Inhibitors, antibodies or ligands were added 1 h before HCVpp or HCVcc infection and during infection unless otherwise stated. Experiments with RTK ligands were conducted with serum-starved cells. Unless otherwise stated, HCV entry and infection was assessed by luciferase reporter gene expression.

Analysis of HCV replication. Electroporation of RNA derived from plasmid pSGR-JFH1 or replication-deficient mutant pSGR-JFH1/GND (Δ)[43] was performed as previously described[42]. Twenty-four hours after electroporation, cells were incubated with inhibitors. Total RNA was isolated and HCV RNA was analyzed by northern blotting as previously described[49].

Rescue of gene silencing. To assess whether silencing of endogenous RTKs could be rescued by expression of RNAi-resistant RTK expression, 4×10^6 Huh7.5.1 cells were co-electroporated with 10 μg siRNA targeting the 3′ untranslated region of the endogenous cellular mRNA (siEGFR si3, siEphA2 si4, HS-CDC2_14) and an RTK-encoding plasmid expressing siRNA-resistant mRNA containing a deletion of the 3′ untranslated region (pEGFR, pEphA2, pCDC2)[40,50,51]. We seeded 2.5×10^4 cells per cm^2 72 h before infection with HCVcc (Luc-Jc1; genotype 2a/2a) or HCVpp (H77; genotype 1a). EGFR rescue in PHHs was performed by co-transduction with lentiviruses expressing shEGFR and/or EGFR[40] 72 h before infection with HCVpp (HCV-J; genotype 1b).

Analysis of EGFR phosphorylation in PHHs and Huh7.5.1 cells. EGFR phosphorylation was assessed in cell lysates with the Human Phospho-RTK Array Kit (R&D Systems), where RTKs are captured by antibodies spotted on a nitrocellulose membrane. Amounts of phospho-RTK were assessed with a horseradish peroxidase–conjugated pan–phospho-tyrosine–specific antibody followed by chemiluminescence detection as described by the manufacturer. Phosphotyrosine (P-Tyr) and phosphorylation of the unrelated c-mer proto-oncogene tyrosine kinase (MERTK) served as internal positive and negative controls. PHHs were incubated in EGF-free William's E medium (Sigma). Huh7.5.1 cells were serum-starved overnight before addition of ligands, inhibitors and antibodies.

Analysis of HCV binding, postbinding and entry kinetics. Analysis of HCV glycoprotein E2 binding to cells and HCV postbinding and entry kinetic assays were performed as previously described[18,19,21] with polyclonal SR-BI-specific[21] or monoclonal EGFR-specific antibodies (10–100 μg ml^{-1}) (Millipore, Roche) or SR-BI[21]– or EphA2-specific serum (produced as described in the **Supplementary Methods** and diluted 1 in 100) and corresponding controls[21] (R&D) (**Supplementary Methods** and **Supplementary Fig. 10**).

Receptor association using fluorescence resonance energy transfer. Homotypic and heterotypic interactions of CD81 and CLDN1 were analyzed as previously described[15,23,24]. The data from ten cells were normalized, and the localized expression was calculated.

Membrane fusion. HCV membrane fusion during viral entry was investigated with a cell-to-cell fusion assay as previously described[25].

Cell-to-cell transmission of HCV. Cell-to-cell transmission of HCV was assessed as previously described[26]. Briefly, producer Huh7.5.1 cells were electroporated with HCV Jc1 RNA and cultured with gene-silenced or naive target Huh7.5-GFP cells in the presence or absence of PKIs (10 μM) (IC Laboratories). An HCV E2–neutralizing antibody[26] (25 μg ml^{-1}) was added to block cell-free transmission[26]. After 24 h of coculture, cells were fixed with paraformaldehyde, stained with an NS5A-specific antibody (0.1 μg ml^{-1}) (Virostat) and analyzed by flow cytometry[26]. Total and cell-to-cell transmission were defined as percentage HCV infection of Huh7.5-GFP$^+$ target cells (Ti) in the absence (total transmission) or presence (cell-to-cell transmission) of an HCV E2–specific antibody.

HCV infection and treatment of chimeric uPA-SCID mice. Chimeric mice repopulated with PHHs[27,28] were infected with serum-derived HCV (genotype 2a, 1×10^4 HCV international units per mouse) via the orbital vein during isofluoran anesthetization (PhoenixBio, Japan). Erlotinib (Roche) administration and dosage (50 mg per kg body weight per day) were performed as previously described in xenograft tumor mouse models[30]. Four mice received 50 mg per kg body weight per day erlotinib and three mice received placebo from day −10 until day 20 of infection in two independent experiments (total 14 mice, two experiments of seven mice each). Serum HCV RNA, alanine transaminase, albumin and erlotinib were monitored as previously described[28,52]. All experimental procedures used to treat live mice in this study had been approved by the Animal Ethics Committee of PhoenixBio in accordance with Japanese legislation.

Toxicity assays. Cytotoxic effects on cells were assessed in triplicate by analyzing the ability to metabolize 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)[53]. Formazan crystals were solubilized 5 h after adding MTT (0.6 mg ml^{-1}) (Sigma) as previously described[53].

Additional methods. Detailed methodology is described in the **Supplementary Methods**.

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Article

HRas Signal Transduction Promotes Hepatitis C Virus Cell Entry by Triggering Assembly of the Host Tetraspanin Receptor Complex

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SUMMARY

Hepatitis C virus (HCV) entry is dependent on coreceptor complex formation between the tetraspanin superfamily member CD81 and the tight junction protein claudin-1 (CLDN1) on the host cell membrane. The receptor tyrosine kinase EGFR acts as a cofactor for HCV entry by promoting CD81-CLDN1 complex formation via unknown mechanisms. We identify the GTPase HRas, activated downstream of EGFR signaling, as a key host signal transducer for EGFR-mediated HCV entry. Proteomic analysis revealed that HRas associates with tetraspanin CD81, CLDN1, and the previously unrecognized HCV entry cofactors integrin β1 and Ras-related protein Rap2B in hepatocyte membranes. HRas signaling is required for lateral membrane diffusion of CD81, which enables tetraspanin receptor complex assembly. HRas was also found to be relevant for entry of other viruses, including influenza. Our data demonstrate that viruses exploit HRas signaling for cellular entry by compartmentalization of entry factors and receptor trafficking.

INTRODUCTION

Viral entry into target cells requires the coordinated interaction of viral and host factors. Cellular kinases play a role in virus uptake (Chakraborty et al., 2012; Mercer and Helenius, 2008; Pelkmans et al., 2005), but the underlying molecular mechanisms and signaling pathways are only poorly understood.

Hepatitis C virus (HCV) is a major cause of liver cirrhosis and hepatocellular carcinoma (HCC) (El-Serag, 2012). Major challenges include the absence of a preventive vaccine and resistance to antiviral treatment in a large fraction of patients (Zeisel et al., 2011). HCV is an enveloped, positive-sense single-stranded RNA virus of the *Flaviviridae* family (Murray and Rice, 2011). Virus entry into hepatocytes is a multistep process that is regulated by receptor tyrosine kinases (RTKs) (Lupberger et al., 2011). Host cell factors for the initiation of infection include heparan sulfate (Barth et al., 2006), CD81 (Pileri et al., 1998), scavenger receptor type B class I (SR-BI) (Scarselli et al., 2002), claudin-1 (CLDN1) (Evans et al., 2007), occludin (OCLN) (Liu et al., 2009; Ploss et al., 2009), and Niemann-Pick C1-like 1 cholesterol absorption receptor (NPC1L1) (Sainz et al., 2012). Virus entry is a promising target for antiviral therapy since host cell receptors exhibit a high genetic barrier to viral resistance (Zeisel et al., 2011).

CD81 belongs to the tetraspanin superfamily of integral transmembrane proteins that have been implicated in a variety of physiological and pathological processes and play a role in pathogen infection (König et al., 2010; Krementsov et al., 2010; Silvie et al., 2003). Tetraspanins are highly organized in microdomains displaying specific and direct interactions with other tetraspanins and molecular partners. Tetraspanins are implicated in membrane protein trafficking, partitioning, and clustering in tetraspanin-enriched microdomains (TEMs) that regulate signaling pathways by membrane compartmentalization (Berditchevski and Odintsova, 2007; Chambrion and Le Naour, 2010).

CD81 has a dynamic nature in HCV entry and its lateral diffusion regulates HCV infection (Harris et al., 2012). The physical interaction of CD81 with CLDN1 in a coreceptor complex is a prerequisite and essential step for HCV entry (Harris et al., 2010; Krieger et al., 2010). We demonstrated that RTKs, like epidermal growth factor receptor (EGFR), act as cofactors for HCV entry by promoting the formation of the CD81-CLDN1 coreceptor complexes, but the molecular mechanism is unknown (Lupberger et al., 2011). Unlike CD81, EGFR does not directly bind HCV E2, and, furthermore, EGFR activity directly correlates with HCV entry (Lupberger et al., 2011). Taken together, this highlights an essential role for RTK signaling in regulating the HCV entry process. Since EGFR supports the uptake of different viruses (Karlas et al., 2010; Lupberger et al., 2011; Pelkmans et al., 2005), it is likely that EGFR signaling plays a role in the entry of other virus families. To uncover the molecular mechanism underlying EGFR-regulated virus entry, we investigated the signaling pathway(s) and cellular transducers mediating HCV entry and investigated their impact on host receptor association and motility.

RESULTS

EGF Predominantly Activates Ras/MAPK Signaling in HCV Permissive Hepatic Cells

To identify the host signaling pathway of RTK-mediated HCV entry, we first studied EGFR signaling in Huh7.5.1 cells—a state-of-the-art permissive cell line for HCV infection, primary human hepatocytes (PHHs), and patient-derived liver biopsies. EGFR activation leads to phosphorylation of tyrosine residues in the intracellular domain that recruit signaling molecules to the plasma membrane that prime subsequent activation of events (Morandell et al., 2008), including mitogen-activated protein kinase (MAPK), phosphoinositide-3 kinase (PI3K), and v-Akt murine thymoma viral oncogene homolog (AKT) pathway activation (Figure 1A). Analysis of signal transduction in Huh7.5.1 cells, PHHs, and liver tissue (liver biopsies 987 and 990) with phosphokinase (Figures 1B and 1C and Figure S1A available online) and phospho-RTK arrays (Figures 1C and S1B) demonstrates that EGFR and the Ras/MAPK pathway are predominantly activated after EGF stimulation in human hepatocytes in vitro and in vivo. In contrast, activation of signaling pathways described in other cell lines and tissues (PI3K/AKT, PLC/PKC, p38/JNK, STAT3/5, Cbl, c-Src/ABL, and FAK) was less relevant in the liver-derived cells, PHHs, or liver tissue (Figures 1B, 1C, and S1) as shown by phosphorylation arrays. To further corroborate the relevance of the Ras/MAPK pathway as the primary signal transducer of EGFR in the liver, we measured the phosphorylation status of extracellular signal-regulated kinases 1 and 2 (ERK1/ERK2) and AKT by dynamic phosphoflow assay following EGFR stimulation (Firaguay and Nunès, 2009) (Figures S1C–S1F) in Huh7.5.1 and PHHs. EGF stimulation of Huh7.5.1 cells and PHHs activates Ras/MAPK signaling, while even prolonged incubation of the cells with EGF failed to activate the PI3K/AKT pathway (Figures S1D–S1F). Similar results were obtained by immunoblot of phosphoproteins in EGF-stimulated Huh7.5.1 or PHH lysates (Figure S1G) and lysates from patient-derived liver tissue (liver biopsies 956, 965, and 968) that had been stimulated with EGF ex vivo (Figure 1D). We noted that EGF activated ERK1/ERK2 at significantly lower doses in Huh7.5.1 cells and PHHs (Figure S1G). In summary, these results demonstrate that EGF predominantly activates the Ras/MAPK pathway in hepatoma cells, PHHs, and liver tissue supporting the key relevance of the Ras/MAPK pathway for EGFR-mediated signaling in the liver.

EGFR Scaffolding Proteins Grb2 and Shc1 Are Relevant for HCV Entry

To identify EGFR-mediated signals important for HCV entry, we performed an unbiased small interfering RNA (siRNA) screen targeting the expression of known EGFR adaptors and associated proteins (Table S1). Among the identified EGFR scaffolding proteins with impact on HCV entry, we identified growth factor receptor-bound protein 2 (Grb2) and Src homology 2 domain-containing transforming protein 1 (Shc1): known activators of Ras GTPases and MAPK pathway (Kolch, 2005) (Figure 1A). Although STAT5b scored as a binding partner with potential functional relevance (Table S1), we observed no evidence for activation of the STAT5 signaling in EGF-treated PHHs or liver tissue (Figures 1 and S1A), and a STAT5b inhibitor had no effect on HCV entry as described below. Thus, we focused on the functional relevance of Grb2 and Shc1 for HCV entry. Silencing of Grb2 or Shc1 expression significantly ($p < 0.01$) decreased HCV pseudoparticle (HCVpp) entry (Figure 2A) and cell-culture-derived HCV (HCVcc) infection (Figure 2B) to similar levels as EGFR silencing, while silencing Grb2-associated binding protein 1 (Gab1) that recruits PI3K (Figure 1A) had no effect on HCV infection (Figures 2A and 2B). Specific Grb2, Shc1, and Gab1 gene silencing was validated by immunoblot (Figure 2C). In contrast, silencing of Grb2 or Shc1 expression had no effect on the entry of murine leukemia virus (MLV) pseudoparticles (Figure 2D), suggesting that the observed inhibitory effect is not related to the pseudoparticle system. Finally, we confirmed the functional relevance of Grb2 and Shc1 in HCVpp infection of polarized HepG2-CD81 cells (Figure 2E). HepG2 cells polarize in vitro and develop bile-canaliculi-like spaces between adjacent cells, thus allowing the study of virus entry in a model system closely related to polarized hepatocytes in the infected liver in vivo (Mee et al., 2009). Taken together, these data show a role for EGFR scaffolding proteins Grb2 and Shc1 in HCV entry and infection.

Inhibition of Ras and Upstream MAPK BRaf Decreases HCV Entry

Since silencing of EGFR scaffolding proteins Grb2 and Shc1 inhibits HCV entry and these proteins activate Ras/MAPK signaling, we investigated whether other members of the MAPK pathway play a role in HCV entry. We thus used a panel of well-characterized small-molecule inhibitors (Figure 1A) of EGFR (erlotinib), rat sarcoma (Ras) (tipifarnib), Raf (sorafenib), BRaf (inhibitor VI), Raf-1 (inhibitor I), mitogen-activated protein kinase 1 and 2 (MEK1/MEK2) (U0126), and ERK1/ERK2 (Fr180204) and studied their effect(s) on HCV entry and infection (Figures 3A–3F). We also ruled out a role of other major EGFR signaling pathways including PI3K/AKT, PLC/PKC, p38/JNK, STAT3/5, and FAK using well-described small-molecule inhibitors (Figure 3G). The biological activity of all used inhibitors was confirmed in functional assays in Huh7.5.1 (Figure S2).

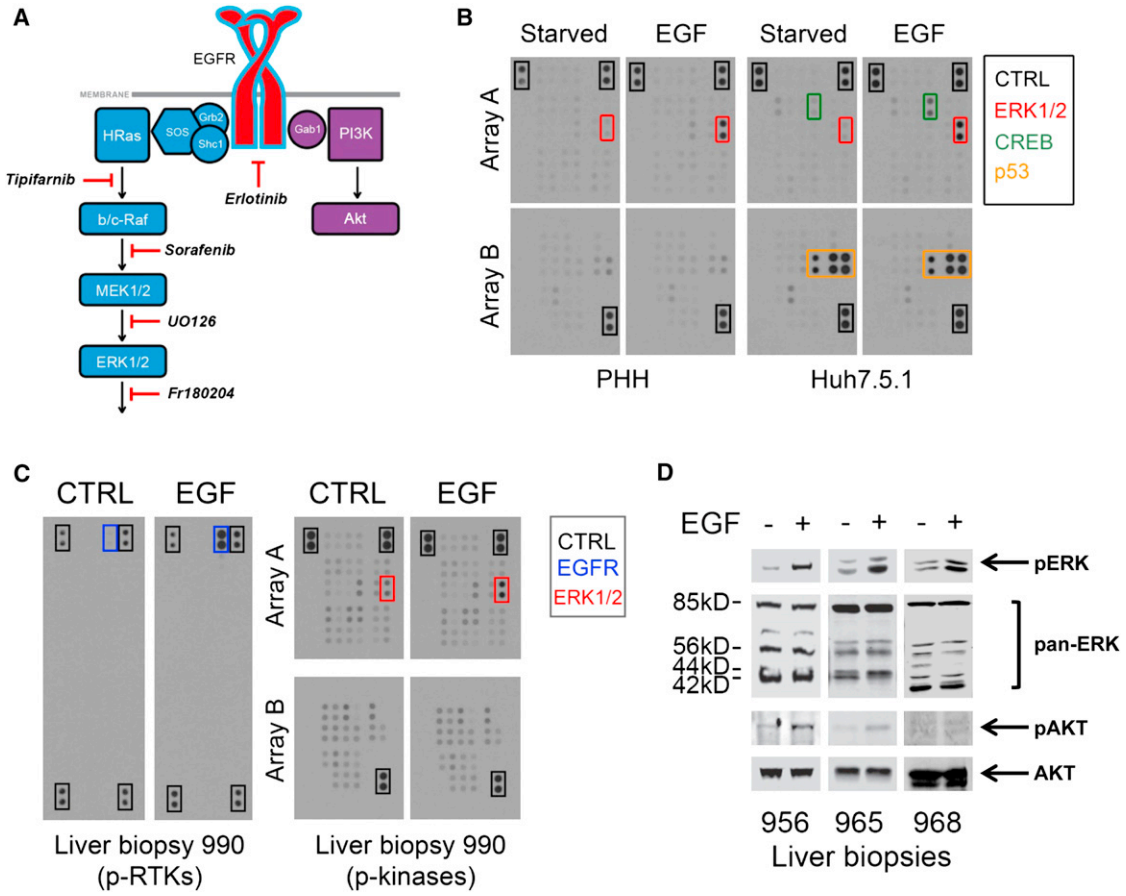

Figure 1. EGFR Signaling Pathways in Human HCV-Permissive Liver Cells, Hepatocytes, and Patient-Derived Liver Tissue

(A) Scheme of the two main canonical EGFR signaling cascades: the MAPK and the PI3K/AKT pathways. Inhibitors targeting members of these pathways are indicated.

(B and C) EGFR-transduced signals in human hepatocytes and liver tissue. (B) Detection of kinase phosphorylation in Huh7.5.1 and PHHs after EGF treatment (1 μg/ml; 15 min) with a human phosphokinase array detecting specific phosphorylation of 46 phosphorylation sites on 32 cellular kinases, which are indicated in Figure S1A. (C) Detection of RTK and kinase phosphorylation in liver tissue of patient biopsy 990 after EGF treatment (1 μg/ml; 15 min ex vivo) with a human phospho-RTK array (detecting specific phosphorylation of 42 different RTKs as indicated in Figure S1B) and a human phosphokinase array.

(D) Analysis of the phosphorylated and total forms of ERK1/ERK2 and AKT with specific antibodies in three different liver biopsies (956, 965, and 968) after 15 min EGF stimulation (1 μg/ml) ex vivo. Total protein (30 μg) was separated by SDS-PAGE and stained for total and phosphorylated forms of ERK and AKT by immunoblot.

See also Figure S1 for more-detailed analyses of EGFR signaling.

All inhibitor concentrations were well tolerated in Huh7.5.1 and PHHs, as shown by MTT assays applied for inhibitors that inhibited HCV entry (Figures 3A and 3B). Since erlotinib and tipifarnib resulted in a robust, donor-independent, and highly significant ($p < 0.0001$) inhibition of HCVpp entry in hepatoma cells and PHHs, we conclude that Ras is a predominant signal transducer required for EGFR-mediated HCV entry. Inhibition of Ras and upstream MAPK Raf, and to some extent MEK1/MEK2, decreased HCVpp and HCVcc infection in Huh7.5.1 cells and PHHs (Figures 3C–3E). Moreover, a different inhibition profile of MLVpp infection of PHHs suggests that the effects are not related to the lentiviral system (Figure 3F). Taken together, our observations demonstrate that the closer the inhibitor's target is toward EGFR in the MAPK signaling cascade (Figure 1A), the more efficiently HCV entry is inhibited. These data suggest that only upstream members of the MAPK signaling pathway, but not other canonical EGFR pathways, play a major functional role in HCV entry.

HRas Is a Transducer of EGFR-Mediated HCV Entry

The classical Ras family comprises three isotypes: Harvey (H)-, Kirsten (K)-, and neuroblastoma (N)-Ras (Boguski and McCormick, 1993). Since Grb2, Shc1 and Raf play a role in EGFR-mediated HCV entry (Figures 2 and 3), we investigated the functional role of the Ras GTPase family in HCV entry using RNA interference (RNAi). HRas, KRas, or NRas expression (Figure S3A) was silenced in Huh7.5.1 with two individual siRNAs per target (Figures 4A–4C). The messenger RNA (mRNA) or protein expression after silencing was studied for all Ras isoforms or HRas, respectively (Figures 4A and 4B). Silencing of HRas expression markedly and significantly ($p < 0.0005$) decreased HCV entry into Huh7.5.1 cells to a comparable level as EGFR

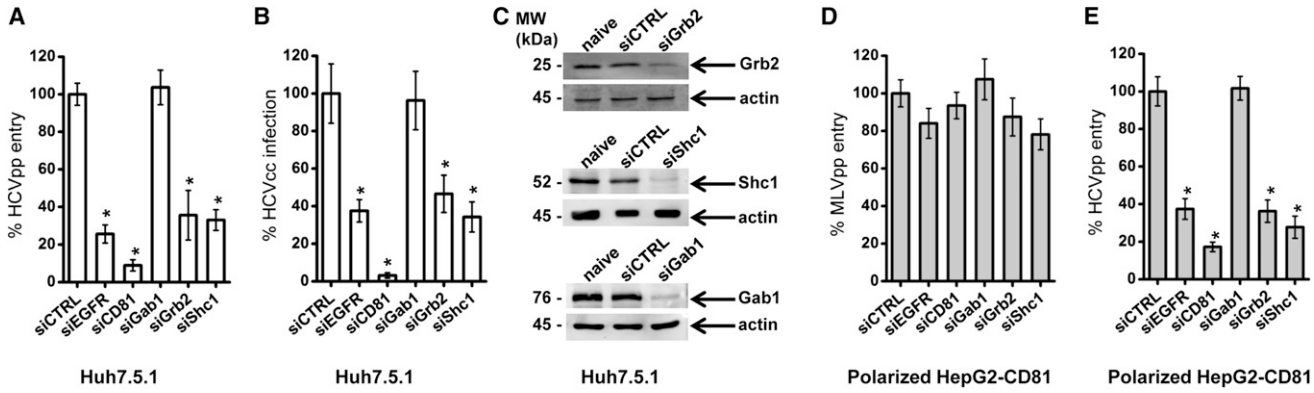

Figure 2. EGFR Adaptors Grb2 and Shc1 Are Relevant for HCV Entry

Silencing of EGFR adaptors Grb2 and Shc1 inhibits HCV entry. Huh7.5.1 (A–C) and polarized HepG2-CD81 (D and E) cells were transfected with individual siRNA directed against Gab1, Grb2, or Shc1 and infected with HCVpp, MLVpp, or HCVcc.

(A, C, and E) Silencing of protein expression was confirmed by immunoblot with specific antibodies targeting Gab1, Grb2, Shc1, or actin (C). HCVpp entry was assessed in Huh7.5.1 (A) and in polarized HepG2-CD81 (E) cells transfected with siRNA. siCTRL, CD81- and EGFR-specific siRNAs served as internal controls. Data are expressed as percentage HCVpp entry relative to siCTRL-transfected cells (means ± SD from three independent experiments in triplicate, n = 9).

(B) HCVcc infection in Huh7.5.1 cells transfected with the same siRNAs. Data are expressed as percentage HCVcc infection relative to siCTRL-transfected cells (means ± SD from three independent experiments in triplicate, n = 9).

(D) MLVpp entry in polarized HepG2-CD81 cells. Data are expressed as percentage MLVpp entry relative to siCTRL-transfected cells (means ± SD from three independent experiments in triplicate, n = 9).

*$p < 0.01$. See also Table S1.

silencing (Figure 4C). In contrast, silencing KRas or NRas expression had no detectable effect on HCV entry (Figure 4C). Isoform specificity of HRas gene silencing was validated by qRT-PCR (Figure S3B). Moreover, HRas silencing reduced EGF-induced ERK1/ERK2 phosphorylation, supporting a role of HRas in EGF-induced MAPK activation in hepatocytes (Figure S3C). These results demonstrate a specific role for the GTPase HRas in the HCV entry process.

Since EGFR mediates entry of viruses other than HCV, we studied the role of HRas on the entry of lentiviral pseudoparticles expressing glycoproteins from avian fowl plague virus influenza A (H7/N1), measles virus, MLV, endogenous feline virus RD114, and vesicular stomatitis virus (VSV) in Huh7.5.1 cells. Silencing HRas expression had a significant ($p < 0.0005$) inhibitory effect on the entry of influenza and measles pseudoparticles (Figure 4D), suggesting that these viruses require similar signaling pathways to enter hepatoma cells as HCV. Although we previously demonstrated that EGFR silencing had no effect on measles virus entry (Lupberger et al., 2011), HRas silencing impacts measles virus entry, suggesting an EGFR-independent role of HRas in this process.

To confirm the HRas dependency of EGFR-mediated HCV entry, we performed inhibition/rescue experiments using the EGFR inhibitor erlotinib and the patient-derived transdominant active V12 mutant of HRas (Beauséjour et al., 2003). Huh7.5.1 or PHHs were transduced to express the HRas V12 mutant and 72 hr later evaluated for their HCVpp permissivity in the presence or absence of 10 μM erlotinib (Figures 4E and 4F). Exogenous HRas expression in Huh7.5.1 and PHHs was confirmed by HRas-specific immunoblot (Figure S3D). The HRas V12 mutant increased HCVpp infection of Huh7.5.1 and PHHs in the absence of erlotinib, demonstrating an involvement of HRas in HCV entry (Figures 4E and 4F). Moreover, HRas V12 mutant rescued the inhibitory effect of erlotinib on HCV entry, confirming that HRas mediates EGFR-dependent HCV entry (Figures 4E and 4F).

Next, we investigated whether HRas mediates EGFR-dependent cell-cell transmission using a well-established cell-cell transmission assay (Lupberger et al., 2011). Reduction of (siHRas.6) or increase of (pHRas V12) HRas expression (Figures 4G and S3E–S3G) in target cells impaired significantly ($p < 0.0005$) or enhanced cell-cell transmission compared to control-transduced cells, respectively. Furthermore, the HRas inhibitor tipifarnib blocked HCV cell-cell transmission (Figures 4G and S3G). These data highlight a previously unrecognized role of HRas during viral spread.

HRas Associates with HCV Cell Entry Factors CD81 and CLDN1

EGFR promotes the association of CD81-CLDN1 coreceptor complexes that are important for HCV entry (Lupberger et al., 2011). To investigate whether EGFR signaling modulates CD81-CLDN1 association, we analyzed whether TEMs contain members of the EGFR signaling pathways using proteomic analysis of CD81 immunoprecipitates. HepG2 and HepG2-CD81 cells were differentially labeled with stable isotope labeling with amino acids (SILAC) (Ong et al., 2002) and lysed with brij97 detergent that is reported to preserve tetraspanin interactions (Le Naour et al., 2006), and HepG2 and HepG2-CD81 lysates were pooled equally according to SILAC protocols (Ong et al., 2002). From this pool, CD81 was pulled down with beads coupled with CD81-specific IgG and coprecipitated protein complexes analyzed by mass spectrometry. Among the CD81 coprecipitated proteins were several integrins (alpha1, alpha6, and beta1) that are well-characterized TEM components. Therefore, we defined the threshold of specificity >2 accordingly to the

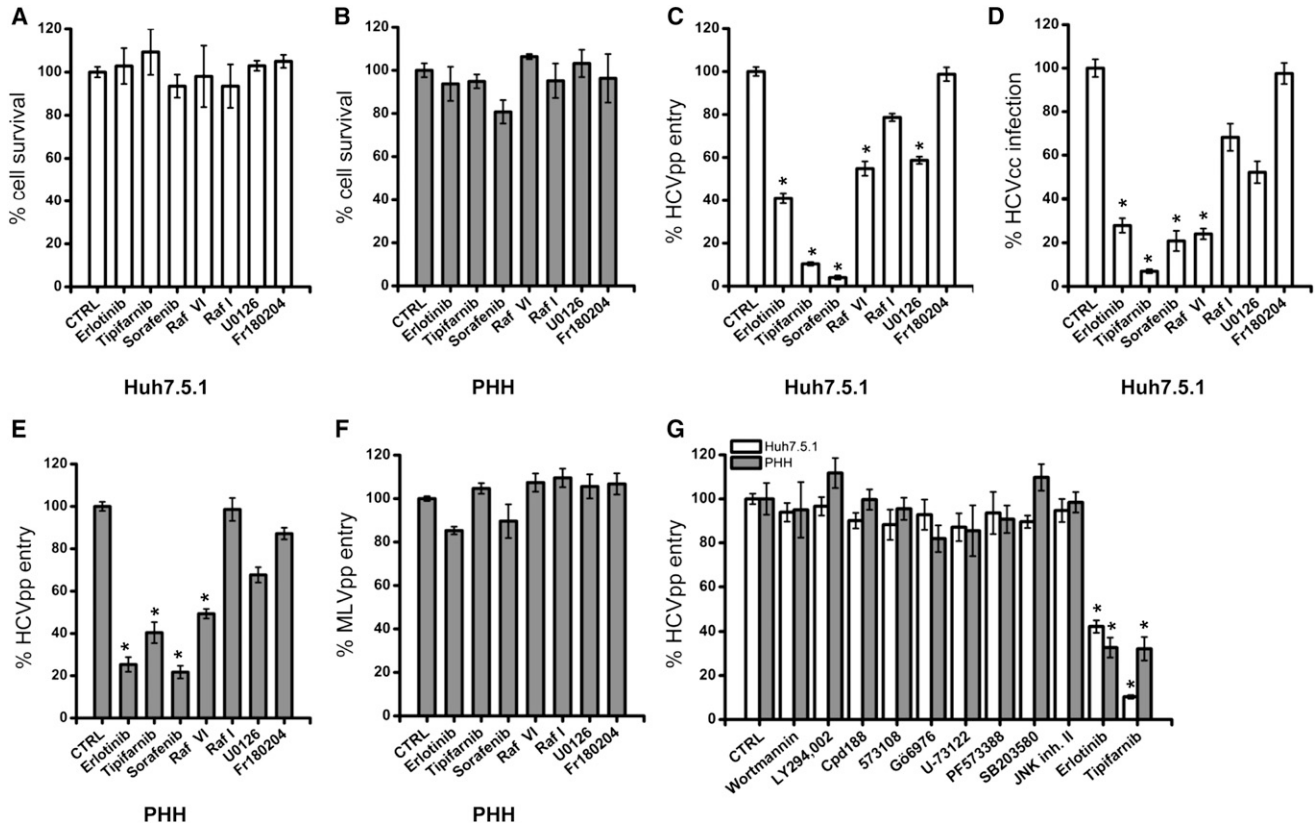

Figure 3. Upstream MAPK Are Relevant for HCV Entry in Hepatoma Cells and Human Hepatocytes

Cell survival (A and B), HCVpp entry (C, E, and G), HCVcc infection (D), or MLVpp entry (F) in Huh7.5.1 cells (A, C, D, and G) or PHHs (B, E, F, and G) incubated with small-molecule inhibitors (10 μM) targeting EGFR (erlotinib), Ras (tipifarnib), Raf (sorafenib,10 μM for pseudoparticles and 1 μM for HCVcc), BRaf (Raf inhibitor VI), Raf-1 (Raf inhibitor I), MEK1/MEK2 (U0126), or ERK1/ERK2 (Fr180204) or the major EGFR pathways, including PI3K (wortmannin and LY294,002), STAT3 (Cpd188), STAT5 (573108), PKC (Gö6976), PLC (U-73122), FAK (PF573288), p38 (SB203580), JNK (JNK inhibitor II), and Ras (tipifarnib). One hour after incubation with inhibitors, HCVpp, MLVpp, or HCVcc was added to the cells in the presence of inhibitors. Cell viability was assessed by MTT assay. EGFR (erlotinib) serves as internal control. Data are expressed as percentage HCVpp or MLVpp entry and HCVcc infection relative to solvent CTRL-treated cells (means ± SEM from four independent experiments in triplicate, n = 12). *p < 0.0001. See also Figure S2.

isotope ratio $^{13}C/^{12}C$ of coprecipitated integrins and accordingly to SILAC standard procedures (Ong et al., 2002). Above this threshold, we identified tetraspanin-associated proteins such as membrane protease ADAM10, several tetraspanins (CD9, CD81, CD151), and known specific interaction partners of CD81, such as EWI-2 and CD9P-1/EWIF, validating this differential proteomic approach to identify CD81-associated proteins. Interestingly, HCV entry factors CLDN1 and SR-BI but not OCLN or NPC1L1 were identified as CD81 TEM components corroborating a close cooperation of CD81, CLDN1, and SR-BI during HCV entry (Krieger et al., 2010). These results were confirmed in a second experiment using SILAC proteomics with inverted isotope labeling (Table 1). Among the 169 components identified in TEMs, we identified HRas as the only member of the canonical EGFR signaling pathways associating with CD81. A physical interaction of HRas with the tetraspanin coreceptor complex was further supported by a partial but robust colocalization of CD81 with HRas at the plasma membrane of Huh7 cells (Figure S4A) as calculated according to Pearson (Rr = 0.25) and Manders (R = 0.659) coefficients and intensity correlation quotient (ICQ = 0.129) (Bolte and Cordelières, 2006; Brown et al., 2010; Manders et al., 1992) (Figure S4). To further study the relevance and robustness of the HRas-CD81 colocalization, we transduced cells to express the HRas V12 mutant (Figure S4B). The colocalization of HRas V12 with CD81 was calculated (Image J software) and significantly ($p < 0.005$) increased as demonstrated by the Pearson (Rr = 0.544 ± 0.047) and Manders (R = 0.825 ± 0.056) coefficients and ICQ (0.387 ± 0.067) as compared to empty vector (Rr = 0.278 ± 0.054; R = 0.820 ± 0.05; ICQ = 0.175 ± 0.022) (Figures S4C and S4D). The distribution of HRas at the plasma membrane was observed as intense punctuated spots and correlated with high CD81 content. Taken together, these data suggest that active HRas physically associates with CD81.

Functional Network Analyses of CD81-Associated Proteins Identified Rap2B and Integrin Beta1 as Cofactors for HCV Entry

To identify members of TEM containing CD81 and CLDN1 with a functional role in HCV entry, we analyzed the 169 identified CD81-associated proteins (Table 1 and data not shown) for known and predicted HRas protein interactions using the

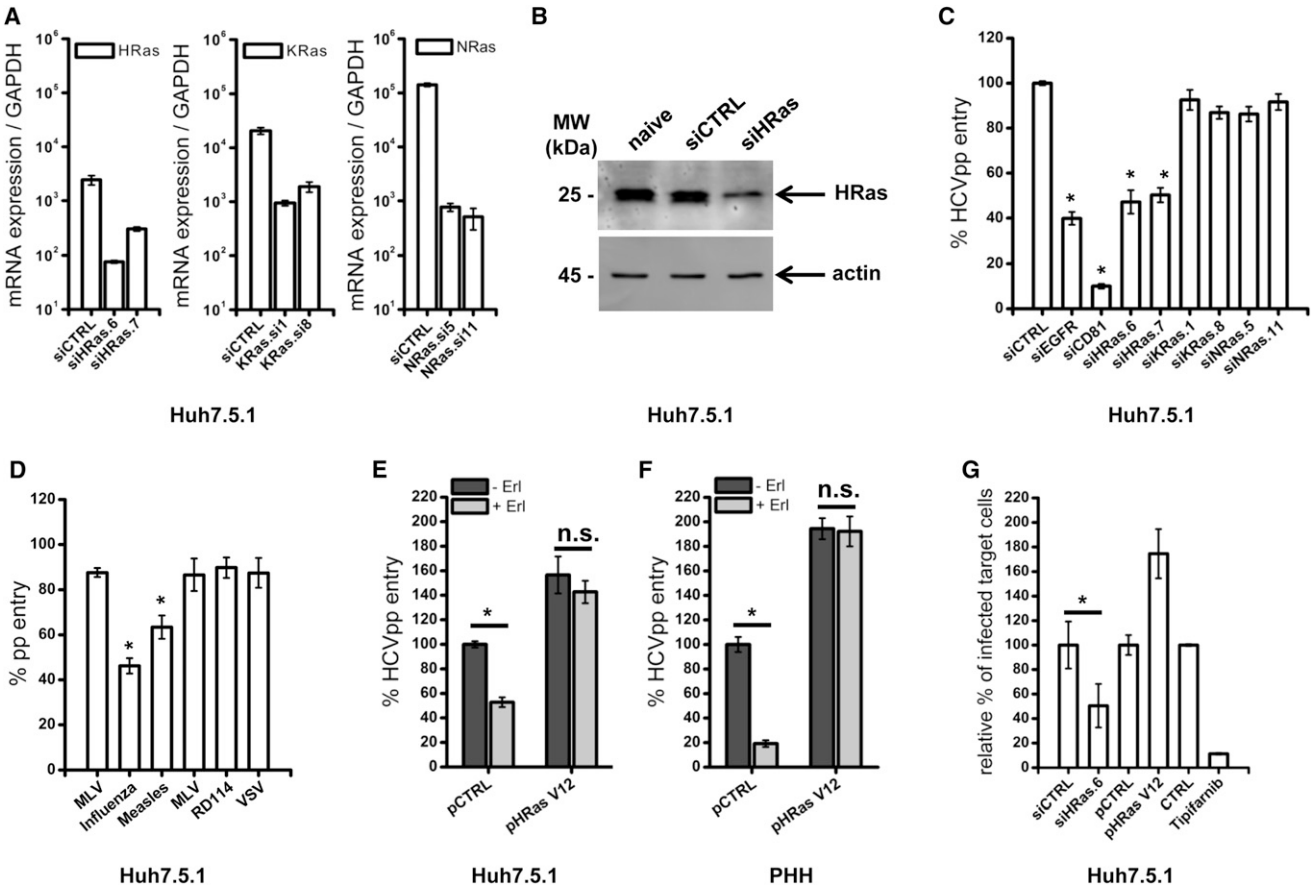

Figure 4. HRas Is a Host Cell Factor for HCV Entry

(A) mRNA expression of HRas, KRas and NRas compared to GAPDH after silencing of each Ras isoform with isoform-specific siRNAs.

(B) Analysis of protein expression by immunoblot with specific antibodies targeting HRas or actin after Ras silencing (siHRas.6).

(C) HCVpp entry in Huh7.5.1 cells transfected with individual siRNAs directed against HRas (si6 and si7), KRas (si1 and si8), and NRas (si5 and si11). siCTRL, CD81, and EGFR-specific siRNAs served as internal controls. Data are expressed as percentage HCVpp entry relative to siCTRL-transfected cells (means ± SEM from four independent experiments in triplicate, n = 12).

(D) Cell entry of pseudoparticles expressing envelope glycoproteins of influenza, measles, MLV, RD114, and VSV in Huh7.5.1 cells transfected with an individual siRNA directed against HRas (si6). Two independent MLVpp preparations were used. siCTRL served as internal control. Data are expressed as percentage pseudoparticle entry relative to siCTRL-transfected cells (means ± SEM from three independent experiments in triplicate, n = 9).

(E and F) Inhibition of HCV entry by erlotinib is rescued by a transdominant active HRas mutant. HCVpp entry in Huh7.5.1 cells (E) and in PHHs (F) transduced with lentiviruses expressing a transdominant active HRas mutant (pHRas V12) and treated with erlotinib (10 μM). For HRas protein expression, see Figures S3C and S3D. Data are expressed as percentage HCVpp entry relative to pCTRL cells (means ± SEM from four independent experiments in triplicate, n = 12).

(G) Functional role of HRas in viral cell-cell transmission. Effect of HRas silencing by siHRas.6, overexpression of HRas V12, or HRas inhibition by tipifarnib (10 μM) on viral spread is shown. Data are expressed as percentage cell-cell transmission relative to respective controls (for RNAi, means ± SD from three independent experiments in triplicate, n = 9; for HRas V12 and tipifarnib, means ± SD from one representative experiment in triplicate, n = 3). SD for CTRL and tipifarnib are 0.77 and 0.68 respectively, and are thus not visible.

*$p < 0.0005$. See also Figure S3.

STRING database (Jensen et al., 2009; Lupberger et al., 2011). STRING represents a metadatabase mapping all known protein-protein interactions onto a common set of genomes and proteins (Jensen et al., 2009). This analysis suggests a potential network of proteins connecting CD81 and HRas (Figure 5A) that includes known HCV entry factors CD81 and CLDN1 and HCV host factor apolipoprotein E (apoE). Functional analysis of members of this network using RNAi/HCVpp studies identified Ras-related protein (Rap2B) and integrin beta1 (ITGB1) as cofactors for HCV entry (Figure 5B). Silencing of ITGB1 and Rap2B expression was confirmed with individual siRNAs (Figures 5C–5E). An ITGB1-specific antibody markedly and significantly ($p < 0.0001$) inhibited HCV infection of Huh7.5.1 cells and PHHs (Figures 5F and 5G), validating the role of ITGB1 for HCV entry. These data suggest a functional network organized by tetraspanins in the plasma membrane consisting of CD81-CLDN1, HRas, Rap2B, and ITGB1.

HRas Is Required for Lateral Diffusion of CD81 Promoting CD81-CLDN1 Associations

Since CD81 plays a role in the lateral diffusion of HCV (Harris et al., 2012), we studied the effect(s) of EGFR/HRas signaling

Table 1. Subset of CD81-Associated Proteins in HepG2-CD81 Cells Identified by SILAC Differential Proteomics, Including HRas Interacting Partners

Protein name	ID	Sequence (%)	MW (kDa)	SILAC Labeling (Ratio H/L)	Inverted SILAC Labeling [Ratio 1 / (H/L)]
CD151	P48509	13.4	28.30	15.86	64.72
EWI-2	Q969P0	47	65.03	13.93	85.67
GNAI3	P08754	22.3	40.53	13.52	41.35
CD9	P21926	7.5	25.41	13.38	231.30
CD9P-1/EWIF	Q9P2B2	36.70	98.55	13.16	6.72
CD81	A6NMH8	30.3	29.81	12.64	111.17
ADAM10	O14672	48.3	84.14	11.45	69.25
GNAI1	P63096	20.1	40.36	11.29	14.11
RAP2B	P61225	32.2	20.50	10.46	22.75
MPZ	Q14902	9.2	27.95	9.05	NaN
APOE	P02649	40.1	36.15	8.13	1.56
CLDN1	A5JSJ9	16.1	22.74	7.83	12.61
CD59	E9PR17	15.4	14.53	7.19	NaN
HRas	P01112	20.1	21.30	5.84	103.91
RALA	P11233	19.4	23.57	5.32	4.73
Integrin alpha6	P23229	28.8	126.63	5.09	15.78
SCAMP3	O14828	9.2	38.29	4.54	NaN
Integrin alpha1	P56199	12.4	130.85	2.98	17.16
Integrin beta1	P05556	27.7	88.41	2.61	6.51
SR-BI	Q59FM4	12.4	64.19	2.56	7.69
LMNA	P02545	19.6	74.14	2.53	0.51
SOD2	P04179	13.1	24.72	NaN	2.91
LGALS1	P09382	17.0	14.72	NaN	2.56

HepG2-CD81 and HepG2 cells were differentially labeled with stable carbon isotopes ^{12}C or ^{13}C (SILAC method). CD81-associated complexes were coprecipitated with CD81, digested, and analyzed by mass spectrometry. The protein ID, its molecular weight, the number of the identified peptides, and total sequence coverage for each identified protein is stated. Specificity threshold of CD81 association from each individual identified protein was defined as a peak volume ratio H/L >2 of the differentially isotope labeled versions of each protein. The results were validated by a second experiment with inverse isotope labeling (inverted). The specificity threshold for the inverted SILAC labeling was 1 / (H/L) > 2. NaN, not a number. See also Figure S4.

on CD81 dynamics by real-time fluorescence recovery after photobleaching (FRAP). Huh7.5.1 cells were transduced to express AcGFP-CD81, and the basal surface was imaged by TIRF microscopy. We observed a significant increase in CD81 diffusion coefficient (CTRL 0.09 $\mu m^2/s$; tipifarnib 0.18 $\mu m^2/s$, $p < 0.05$) in Huh7.5.1 cells treated with the HRas inhibitor tipifarnib (Figure 6A). These data suggest that HRas signaling reduces CD81 mobility by promoting an interaction with other proteins. To investigate whether EGFR acts on CD81-CLDN1 interaction via the putative EGFR/Shc1/HRas/BRaf pathway, we studied whether silencing of Shc1, HRas, or BRaf modulates CD81-CLDN1 coreceptor interaction using a well-established fluorescence resonance energy transfer (FRET)-based assay (Harris et al., 2010). Silencing of Shc1, HRas, or BRaf expression significantly ($p < 0.05$) reduced CD81-CLDN1 FRET in Huh7.5.1 cells (Figures 6B–6D, black bars) while silencing had a minimal effect on CD81-CD81 association (Figures 6B–6D, open bars). These results demonstrate that HRas and BRaf play a role in the formation and/or maintenance of the CD81-CLDN1 coreceptor complexes. We previously reported that although EGFR stimulation increased HCV entry, this process does not require ligand-induced EGFR stimulation since the basal activity of the receptor, even after serum starvation, is sufficient to support HCV entry (Lupberger et al., 2011). Indeed, EGF had no significant effect on CD81-CLDN1 FRET (data not shown), supporting the hypothesis that the steady-state complex of CD81-CLDN1 is dependent on low-level constitutive EGFR signaling.

These data lead us to conclude that HCV exploits the EGFR/HRas pathway to compartmentalize host entry factors and receptor trafficking to regulate CD81-dependent pathogen invasion of the liver.

DISCUSSION

Here, we identified GTPase HRas as key signaling factor in HCV entry. We discovered that HRas associates with CD81-CLDN1, providing a physical link between the EGFR/Shc1/Grb2/HRas signaling pathway and the HCV entry factor complex. Furthermore, we identified the CD81-associated proteins ITGB1 and Rap2B as cofactors for HCV entry. We demonstrate that the identified signaling pathway mediates CD81-CLDN1 coreceptor associations and that HRas signaling regulates CD81 diffusion and confinement in the plasma membrane. Since CD81 lateral diffusion and its association with CLDN1 are essential for HCV entry in vitro (Harris et al., 2012; Harris et al., 2010; Krieger et al., 2010), these findings identify HRas as a trigger of HCV entry.

HCV-CD81 engagement has been suggested to induce MAPK (Brazzoli et al., 2008) and PI3K/AKT (Liu et al., 2012) signaling. Moreover, EGFR function is required for HCV entry (Lupberger et al., 2011) and HCV-CD81 engagement promotes EGFR phosphorylation (Diao et al., 2012). These studies suggest that HCV may use multiple signaling pathways and mechanisms for entry; however, the underlying molecular mechanisms and the relevance of these pathways for HCV entry into human hepatocytes or liver tissue in vivo are unknown. Since transformed hepatoma cells such as Huh7 have deregulated signal transduction pathways, observations might be blurred or confounded by cell-line-specific effects. Here we combined RNAi screening, phosphorylation arrays, and small-molecule inhibitors to study signaling in HCV entry in primary liver cells, concluding a role for EGF priming HRas/MAPK signaling pathway in HCV infection of human hepatocytes and patient-derived liver tissue (Figures 1 and S1).

Interestingly, although in our hands silencing of PI3K regulatory subunit 1 (PI3KR1) expression reduced HCVpp entry (data not shown) as previously shown (Liu et al., 2012), we failed to validate its functional relevance for HCV entry using two different small-molecule PI3K inhibitors (Figures 3G and S2A–S2C).

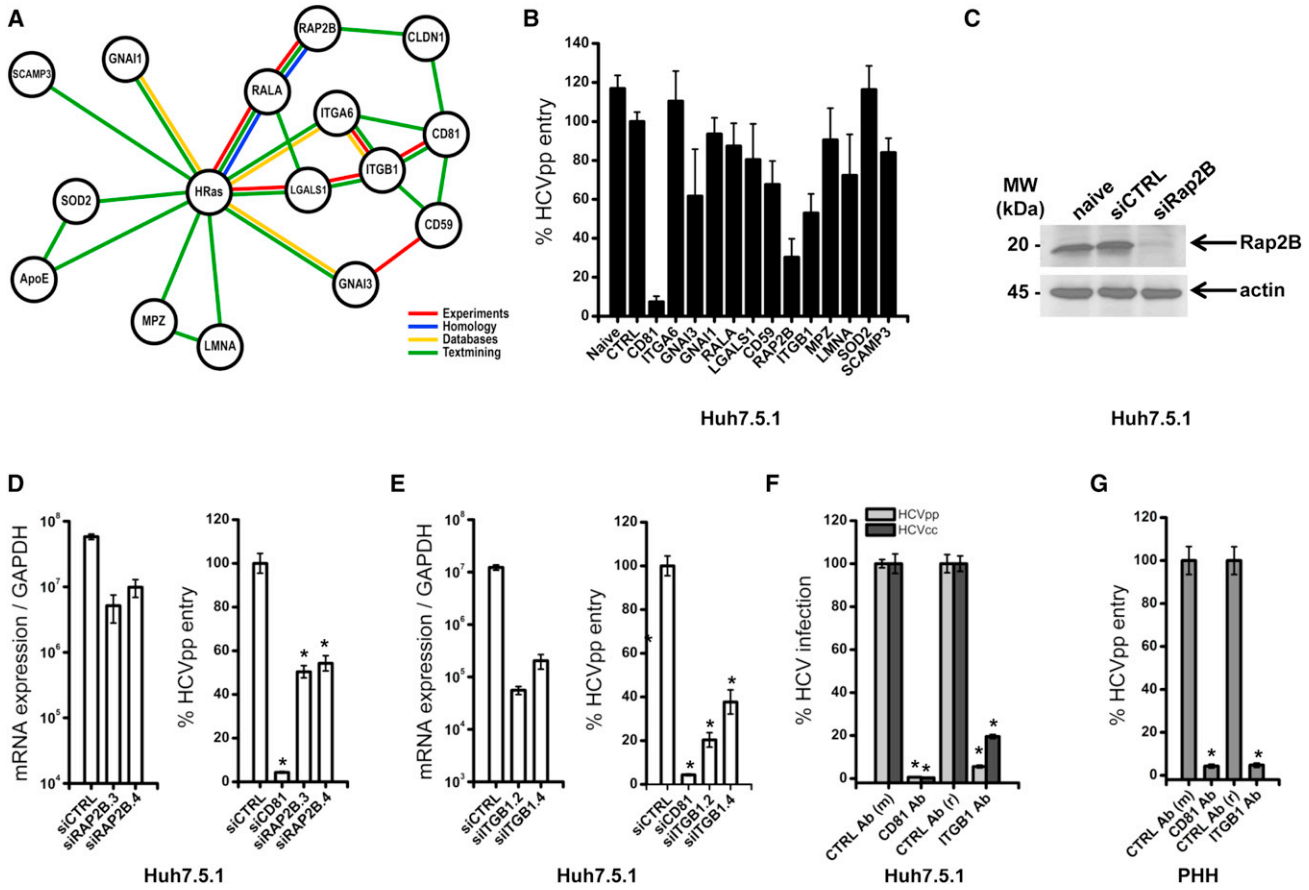

Figure 5. Functional Analysis of HRas-CD81-Associated Proteins Identifies Integrin Beta1 and Rap2B as Previously Undiscovered HCV Entry Factors

(A) Subset of TEM protein association network of the 169 proteins associating with HRas and CD81 identified by STRING analysis (Jensen et al., 2009). Lines connecting proteins show direct (physical) and indirect (functional) associations derived from numerous sources, including experimental repositories (red lines), computational prediction methods (blue lines), databases (yellow lines), and public text collections (green lines).

(B) Functional analysis of protein association network via RNAi. HCVpp entry in Huh7.5.1 cells transfected with pooled siRNA directed against identified members of CD81-associated protein network containing HRas. siCTRL and CD81-specific siRNA served as internal controls. Data are expressed as percentage HCVpp entry relative to siCTRL-transfected cells (means ± SD from one representative experiment, n = 3).

(C) Analysis of protein expression in lysates of Huh7.5.1 with silenced Rap2B expression by immunoblot with specific antibodies targeting Rap2B or actin (Rab2B pool siRNA).

(D and E) mRNA expression of Rap2B (D) and integrin beta1 (ITGB1) (E) compared to GAPDH or HCVpp entry after silencing of each protein with individual siRNAs. Huh7.5.1 cells were silenced with siRAP2B.3 or siRAP2B.4 and siITGB1.2 or siITGB1.4 for 72 hr prior to mRNA expression measurement or to HCVpp infection of Huh7.5.1 cells. siCTRL and CD81-specific siRNA served as internal controls. Data are expressed as mRNA expression of Rap2B or ITGB1 compared to GAPDH (means ± SD from one representative experiment in triplicate, n = 3) or percentage HCVpp entry relative to siCTRL-transfected cells (means ± SEM from three independent experiments in triplicate, n = 9).

(F and G) Effect of a neutralizing ITGB1-specific antibody on HCV entry and infection. Huh7.5.1 cells (F) or PHHs (G) were treated with 25 μg/ml antibodies 1 hr prior and during infection with HCVpp or HCVcc. Irrelevant rabbit and mouse IgGs and a CD81-specific antibody were used as controls. Data are expressed as percentage HCVpp entry or HCVcc infection relative to cells treated with irrelevant IgG (means ± SEM from four experiments in triplicate, n = 12).

*$p < 0.0001$.

Moreover, silencing of Gab1 that binds EGFR and activates PI3K had no significant impact on HCVpp entry (Figure 2), and EGFR signaling through AKT was limited or absent in PHHs or liver tissue in vivo (Figures 1 and S1). Thus, our data suggest that HRas and the upstream MAPK pathway are key signal transducers for EGFR-mediated HCV entry into PHHs and the human liver in vivo and that signal transduction through the PI3K/AKT pathway most likely plays only a minor role.

Our functional analyses suggest that HRas acts as a molecular switch promoting RTK-mediated HCV entry. Inhibition/rescue experiments highlight that EGFR-mediated HCV entry is dependent on HRas function. Our observation that HRas associates with tetraspanin CD81 supports our biochemical data showing that HRas links RTK signaling to CD81 and promotes CD81-CLDN1 association. This is in line with the recent finding that CD81 internalization via a clathrin- and dynamin-dependent process is independent of the CD81 cytoplasmic domain, suggesting a role for associated partner proteins in regulating CD81 trafficking (Farquhar et al., 2012). Moreover, it has been reported that CD81 engagement activates Rho GTPase family

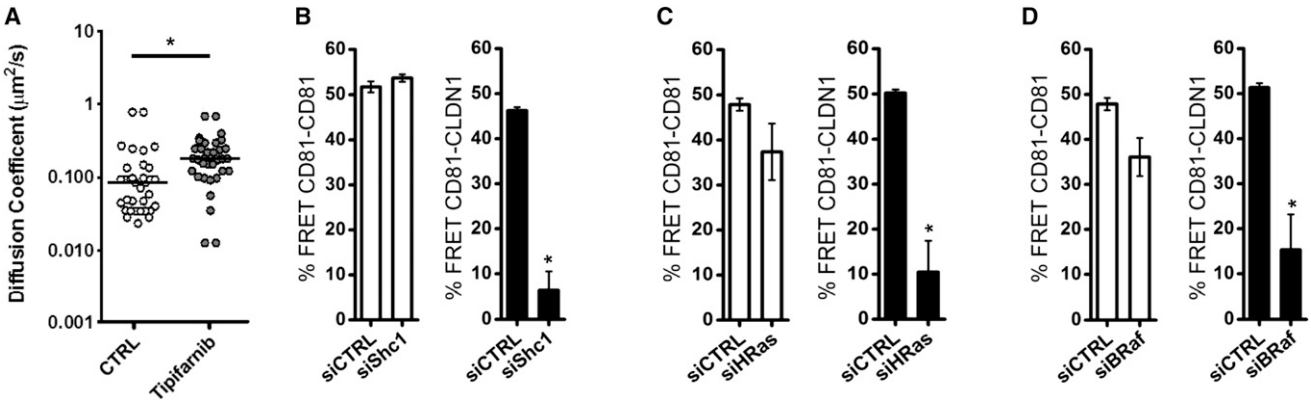

Figure 6. HRas Is Required for Lateral Diffusion of CD81 Promoting CD81-CLDN1 Associations

(A) AcGFP-CD81 diffusion coefficient in Huh7.5.1 cells after 4 hr treatment with DMSO or 10 μM tipifarnib. The median CD81 diffusion coefficient (DMSO, 0.09 $\mu m^2/s$; tipifarnib, 0.18 $\mu m^2/s$) is shown, with each point representing a bleached region of interest and the black line represents the median value.

(B–D) FRET of CD81-CD81 (open bars) and CD81-CLDN1 (black bars) coreceptor associations in Huh7.5.1 cells incubated with siRNA specific for Shc1 (B), HRas (C) or BRaf (D) (means ± SEM from ten independent experiments, n = 10).

*$p < 0.05$.

members leading to actin-dependent relocation of HCV E2-CD81 and activation of Raf/MAPK signaling (Brazzoli et al., 2008). Membrane microdomains, such as TEMs or lipid rafts, play a role in a variety of physiological and pathological processes, for instance as signaling platform (Le Naour et al., 2006). TEMs and lipid rafts differ in their solubility in Triton X-100, as well as in their protein composition, and thus are distinct membrane microdomains (Le Naour et al., 2006). GDP-bound inactive HRas is associated to lipid rafts, whereas GTP-bound active HRas is segregated from lipid rafts to bulk plasma membrane microdomains where it activates signal transduction including the Raf/MAPK pathway (Tian et al., 2007). Thus, it is likely that EGFR-induced signals activate HRas function and that GTP-bound activated HRas leads to rearrangement of tetraspanins, resulting in formation of the essential CD81-CLDN1 entry receptor complex.

Our functional analysis of HRas-CD81-associated proteins demonstrates for the first time a functional role of the GTPase Rap2B and ITGB1 as cofactors for HCV entry. Like HRas, Rap GTPases are known regulators of integrin function. Rap increases integrin avidity by promoting integrin clustering (Kinbara et al., 2003) that may have an important impact on the CD81 TEM formation. ITGB1 is a major TEM component. Integrins are heterodimeric transmembrane proteins composed of an alpha and a beta subunit that couple the extracellular matrix to the F-actin cytoskeleton and signal in a bidirectional manner (Wickström and Fässler, 2011). Conformational changes of integrins elicit signaling events that promote cytoskeletal rearrangement and internalization of many viruses (Stewart and Nemerow, 2007). EGFR can be activated in an ITGB1-dependent manner, and ITGB1 controls EGFR signaling (Morello et al., 2011; Moro et al., 1998), suggesting a crosstalk between ITGB1 and EGFR in HCV entry. Collectively, these findings suggest that HRas acts together with Rap2B and ITGB1 to form a functional complex that may regulate host cell entry receptor mobility, as well as plasma membrane and cytoskeleton organization.

Indeed, the HRas inhibitor tipifarnib promotes CD81 lateral diffusion speed, suggesting an inhibitory role for HRas to regulate CD81 diffusion coefficient at the plasma membrane. We previously reported that hepatoma polarization limits CD81 and HCVpp diffusion coefficient (Harris et al., 2012), concluding that CD81 lateral movement plays an essential role in HCV glycoprotein-dependent particle dynamics that are essential for efficient particle entry.

Our results emphasize that TEMs are active and dynamic areas of the membrane and uncover an important role of GTPases as molecular switches to provide a functional link between TEM-associated tetraspanins and the cytoskeleton, allowing efficient coreceptor complex formation and cellular entry of viruses. Indeed, tetraspanins have been associated with the initiation of infection by various pathogens. Moreover, a recent functional siRNA screen has suggested a potential role for CD81 and HRas for influenza virus entry (Karlas et al., 2010), although their exact function in this process was not investigated. Here, we demonstrate that silencing HRas inhibits the entry of pseudoparticles expressing glycoproteins of influenza A and measles virus but not MLV or VSV. Collectively, these findings highlight a functional relevance for HRas and its role in plasma membrane compartmentalization and receptor trafficking for entry of viruses of other families. Furthermore, our results identify a mechanism to regulate CD81-dependent pathogen invasion of the liver that is HRas dependent.

Finally, our results might have therapeutic implications for the treatment of viral infections. Pharmacological interference with BRaf and HRas might provide an approach for fighting a broad range of viral infections including hepatitis C, influenza, and measles. Indeed, host-targeting agents are an emerging strategy to overcome antimicrobial resistance, a major limitation of direct-acting antivirals or antibiotics (Nathan, 2012). The recent development of safe and efficient clinically licensed small-molecule inhibitors of GTPase and BRaf (Downward, 2003; Maurer et al., 2011; Vanneman and Dranoff, 2012) provides a unique opportunity to develop host-targeting antiviral strategies. In

conclusion, our study has important impact not only for the understanding of viral entry and pathogenesis, but also for the development of preventive and therapeutic antiviral strategies.

EXPERIMENTAL PROCEDURES

Cell Lines and Primary Human Hepatocytes

The sources and culture conditions for 293T, Huh7, Huh7.5.1, HepG2, and HepG2-CD81 cells have been described (Lupberger et al., 2011; Mee et al., 2009). PHHs were isolated and cultured as described (Krieger et al., 2010; Lupberger et al., 2011). Polarization of HepG2-CD81 was induced as described (Mee et al., 2009).

Patient-Derived Liver Biopsies

Liver biopsy tissues were analyzed as described (Dill et al., 2012). Liver tissue was lysed and subjected to immunoblot and phosphorylation array analysis (described below). For ex vivo stimulation, liver tissue was incubated for 15 min with 1 μg/ml EGF. The protocol was approved by the Ethics Committee of the University Hospital of Basel, Switzerland. Written informed consent was obtained from all patients.

Analysis of Cell Signaling with Phosphorylation Arrays

Lysates of cells and liver biopsies were subjected to the proteome Profiler Array human phosphokinase array and human phospho-RTK array (R&D Systems) according to the manufacturer's protocol.

Dynamic Phosphoflow Analyses

Phosphorylated forms of ERK1/ERK2 and AKT were quantified with phospho-specific antibodies in the presence of the phosphatase inhibitor pervanadate, EGF, and kinase inhibitors as described (Firaguay and Nunès, 2009). Cells were fixed, permeabilized, and incubated successively with rabbit antibodies directed against pAKT or pERK1/pERK2, biotinylated anti-rabbit antibodies, and a streptavidin-phycoerythrin solution (Beckman Coulter, Paris, France).

Infection of Cells with Viral Pseudoparticles and Cell-Culture-Derived HCV

Lentiviral pseudoparticles expressing envelope glycoproteins from HCV (strains HCV-J and P01VL), VSV, MLV, measles, RD114, avian fowl plague influenza A (H7N1), and HCVcc (strain Luc-Jc1) were generated as described (Lupberger et al., 2011). Infection of Huh7.5.1, HepG2-CD81 cells, and PHHs with pseudoparticles and HCVcc were performed as described (Krieger et al., 2010; Lupberger et al., 2011). Unless otherwise stated, pseudoparticle entry and HCVcc infection were assessed by measurement of luciferase activity 72 hr after infection as described (Krieger et al., 2010; Lupberger et al., 2011). HCV cell-cell transmission was assayed as described (Lupberger et al., 2011) and is detailed in the Supplemental Experimental Procedures.

Functional RNAi HCV Entry Screens

siRNA screens targeting known EGFR binding partners and CD81-associated proteins were applied in Huh7.5.1 cells as described (Lupberger et al., 2011) with ON-TARGETplus smart pools (pools of four individual siRNAs; Dharmacon). For each target, 5.25 pmol siRNA was reverse transfected in 5,000 Huh7.5.1 cells per well of a 96-well microplate with INTERFERin (Polyplus).

Rescue of EGFR Inhibition with a HRas Transcomplementation Assay

Huh7.5.1 cells (0.66×10^4) or PHHs were seeded as described (Lupberger et al., 2011) 1 day prior to transduction with lentiviruses expressing the trans-dominant active HRas V12 mutant or control (Beauséjour et al., 2003). Seventy-two hours later, cells were infected with HCVpp in the presence of 10 μM erlotinib or DMSO control.

Proteomic Analyses of Tetraspanin Complexes and Microdomains

SILAC was performed as described (Ong et al., 2002). HepG2-CD81 cells and control HepG2 were incubated with either light or heavy isotope labeled amino acids and then lysed with brij97. The two lysates were pooled, and CD81-associated proteins were coimmunoprecipitated as described (André et al., 2006). The proteins were separated by SDS-PAGE and identified by liquid chromatography-mass spectrometry. A peak volume ratio heavy/light >2 was defined as threshold for potential CD81-associated proteins. More details are given in the Supplemental Experimental Procedures.

Imaging Studies

FRAP was performed as described (Harris et al., 2012). Huh7.5.1 cells were transduced with GFP-labeled CD81 (AcGFP-CD81), and CD81 motility was assessed at the membrane of live cells with TIRF microscopy after photobleaching. FRET analyses of homotypic and heterotypic interactions of CD81 and CLDN1 were analyzed in Huh7.5.1 cells as described (Harris et al., 2010). The data from ten cells were normalized, and the localized expression was calculated. Confocal microscopy and staining was performed as described (Chambrion and Le Naour, 2010). Colocalization was calculated according to Pearson and Manders (Bolte and Cordelières, 2006; Manders et al., 1992). More details are given in the Supplemental Experimental Procedures.

Statistical Analysis

All experiments were performed at least three times in triplicate in an independent manner, and results were analyzed with the nonparametric Mann-Whitney test if not indicated otherwise. An F test was performed for analysis of variance (one-way ANOVA) of colocalization studies to compare means of two groups (n = 3) of Pearson's correlation coefficient (Rr) or ICQ. Significant p values are indicated by an asterisk in the individual figure legends.

Additional information on experimental procedures is provided in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2013.02.006.

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Epidermal Growth Factor Receptor Signaling Impairs the Antiviral Activity of Interferon-Alpha

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Interferon-alpha (IFN-α) exhibits its antiviral activity through signal transducer and activator of transcription protein (STAT) signaling and the expression of IFN response genes (IRGs). Viral infection has been shown to result in activation of epidermal growth factor receptor (EGFR)—a host cell entry factor used by several viruses, including hepatitis C virus. However, the effect of EGFR activation for cellular antiviral responses is unknown. Here, we uncover cross-talk between EGFR and IFN-α signaling that has a therapeutic effect on IFN-α-based therapies and functional relevance for viral evasion and IFN resistance. We show that combining IFN-α with the EGFR inhibitor, erlotinib, potentiates the antiviral effect of each compound in a highly synergistic manner. The extent of the synergy correlated with reduced STAT3 phosphorylation in the presence of erlotinib, whereas STAT1 phosphorylation was not affected. Furthermore, reduced STAT3 phosphorylation correlated with enhanced expression of suppressors of cytokine signaling 3 (SOCS3) in the presence of erlotinib and enhanced expression of the IRGs, radical S-adenosyl methionine domain containing 2 and myxovirus resistance protein 1. Moreover, EGFR stimulation reduced STAT1 dimerization, but not phosphorylation, indicating that EGFR cross-talk with IFN signaling acts on the STATs at the level of binding DNA. *Conclusions*: Our results support a model where inhibition of EGFR signaling impairs STAT3 phosphorylation, leading to enhanced IRG expression and antiviral activity. These data uncover a novel role of EGFR signaling in the antiviral activity of IFN-α and open new avenues of improving the efficacy of IFN-α-based antiviral therapies. (HEPATOLOGY 2013;58:1225-1235)

Abbreviations: 3D, three-dimensional; Abs, antibodies; CI, combination index; DMSO, dimethyl sulfoxide; EGFR, epidermal growth factor receptor; EMSA, electrophoretic mobility shift assay; HBV, hepatitis B virus; HCV, hepatitis C virus; HCVcc, cell-culture–derived HCV; HDV, hepatitis D virus; IC_{50}, half-maximal inhibitory concentration; IFN-α, interferon-alpha; IL, interleukin; IRG, IFN response gene; IFNAR, IFN-α receptor; Jak, Janus kinase; mRNA, messenger RNA; Mx1, myxovirus resistance protein 1; PHHs, primary human hepatocytes; P-STAT, phospho-STAT; qPCR, quantitative polymerase chain reaction; RSAD2, radical S-adenosyl methionine domain containing 2; RTK, receptor tyrosine kinase; shRNA, short hairpin RNA; siRNA, small interfering RNA; SOC, standard of care; SOCS, suppressors of cytokine signaling; STAT, signal transducer and activator of transcription protein; $TCID_{50}$, half-maximal tissue culture infectious dose; Tyk2, tyrosine kinase 2.

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Inserm and the University of Strasbourg have filed a patent application on combinations of protein kinase inhibitors and interferons for the treatment and prevention of hepatitis C virus infection.

See Editorial on Page 1200

Interferons (IFNs) are pleiothrophic cytokines produced by the immune system that have a key role in combating viral infections. The IFNs that show the most antiviral properties are the type I and III IFNs alpha (IFN-α) and lambda (IFN-λ).[1,2] It has been suggested that the inhibitory effect on hepatitis C virus (HCV) replication is mediated through distinct patterns of signal transduction resulting in IFN response gene (IRG) expression, and that viral infections themselves trigger IFN production.[1-3] IFN-α leads to recruitment of Janus kinase (Jak) and tyrosine kinase 2 to the IFN-α receptor (IFNAR), causing phosphorylation of the signal transducer and activator of transcription proteins (STATs).[1] Activation of the Jak-STAT pathway by IFN-α involves phosphorylation of STAT1 and STAT2 by Jak, leading to phospho-STAT (P-STAT)1 homodimerization as well as heterotrimerization with P-STAT2 and IFN regulatory factor 9. These complexes migrate to the nucleus, where they bind to *cis*-acting elements in the promoters of IRGs and act as transcriptional activators.[1,4] Hundreds of IRGs are transcribed in response to IFN-α, including genes such as radical S-adenosyl methionine domain containing 2 (RSAD2) and myxovirus resistance protein 1 (Mx1). The gene products of these IRGs facilitate antiviral responses.[2,5-7] IFN-α engagement to the IFNAR also activates STAT3,[8] which is a crucial player in liver regeneration in response to interleukin (IL)−6, promoting cell survival, proliferation, and immune tolerance and counteracting the proinflammatory role of STAT1 in response to IFN-α.[9,10] Additional regulatory mechanisms of STAT signaling involve suppressors of cytokine signaling (SOCS) proteins. SOCS1 and SOCS3 expression is rapidly and transiently induced upon IFN-α exposure to act as negative regulators of the Jak-STAT pathway in a classic feedback loop.[1]

The antiviral activity of IFN-α can be observed in the treatment of hepatitis virus infections, which are the leading causes of liver cirrhosis and hepatocellular carcinoma.[11] IFN-α and its pharmacologically modified derivate, pegylated IFN-α, are part of the standard of care (SOC) of HCV infection[12] and are also used in the treatment of hepatitis B (HBV) and D virus (HDV) infections, albeit with limited efficacy.[13,14] Although the outcome of IFN-α-based treatment of chronic hepatitis C has recently markedly improved by the clinical licensing of protease inhibitors, adverse effects and viral resistance remain important challenges.[12] Furthermore, because the therapeutic options for difficult-to-treat patients remain unsatisfactory and IFN-free regimens have not yet been approved, it is likely that IFN-α will remain a relevant component of HCV treatment for some time to come.[12] Resistance to IFN-α therapy has been explored in great detail.[1,15] In cell-culture models, IFN signaling is impaired by both HCV infection and HCV protein expression.[1,16-18] In patients, a preactivated and refractory hepatic IFN-α-signaling pathway is a hallmark of IFN-α nonresponse.[19]

Epidermal growth factor receptor (EGFR) has been shown to be a host entry factor for several viruses, including HCV.[20-22] Indeed, inhibition of EGFR function using the clinically licensed kinase inhibitor, erlotinib, has been shown to impair HCV infection in a dose-dependent manner.[21,23] Based on these observations, host cell kinases have been suggested as targets for anti-HCV therapy.[24-28] Moreover, because host cell kinases have been shown to be critical in the life cycles of a broad range of other viruses,[20,22,29-31] kinase inhibitors may have utility as broad antiviral interventions. Most recently, data demonstrate that HCV infection not only requires EGFR as a host cell entry factor, but also results in EGFR activation and signaling.[23] Because signaling of receptor tyrosine kinases (RTKs) and IFN-α share distinct signal transducers,[32,33] we aimed to investigate whether (i) inhibition of RTK signaling offers an opportunity to improve the therapeutic efficacy of IFN-α and (ii) RTK signaling is relevant for the antiviral activity of IFN-α.

Materials and Methods

Cell Lines and Primary Human Hepatocytes. The sources and culture conditions for Huh7.5.1 cells have been described.[34] HeLa cells were obtained from American Type Culture Collection (Manassas, VA), and PAP HN5 cells were obtained from the Institut de Génétique et de Biologie Moléculaire et

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Cellulaire (Ilkirch, France). Primary human hepatocytes (PHHs) were isolated and cultured as previously described.[21]

Inhibitors, Ligands, and Antibodies. Erlotinib and sorafenib were obtained from LC Laboratories (Woburn, MA), Cpd188 was obtained from Millipore (Billerica, MA), dimethyl sulfoxide (DMSO) was used at a final concentration of 0.7% for incubation of protein kinase inhibitors and control experiments, IFN-α2b was obtained from Merck (Darmstadt, Germany), IFN-α2a was obtained from Roche (Mannheim, Germany), and human recombinant IL-6 was obtained from PeproTech EC (London, UK). Recombinant EGF and specific antibodies (Abs) targeting β-actin and phospho-EGFR (Tyr1086) were obtained from Sigma-Aldrich (St. Louis, MO). Abs specific for STAT1, STAT3, P-STAT1 (58D6), P-STAT3 (D3A7), and SOCS3 were obtained from Cell Signaling Technology (Danvers, MA). The HCV core Ab (clone C7-50) was obtained from Thermo Fisher Scientific Inc. (Waltham, MA). Horseradish-peroxidase–labeled secondary Abs were obtained from GE Healthcare (Uppsala, Sweden). Secondary Abs IRDye immunoglobulin G (800CW and 680RD) were obtained from LI-COR Biosciences (Lincoln, NE).

Small Interfering RNAs, Short Hairpin RNAs, and Expression Plasmids Used for Functional Studies. siCTRL, siCD81, and siEGFR (Hs-siEGFR_6) have been described.[21] siSTAT3 (Hs-siSTAT3_7) was obtained from Qiagen (Germantown, MD). siSOCS3 (siGenome SMARTpool: 5′-CAG CAU CUC UGU CGG AAG A-3′, 5′-GCG AUG GAA UUA CCU GGA A-3′, 5'-UCA AGA CCU UCA GCU CCA A-3′, and 5′-CCU AUU ACA UCU ACU CCG G-3′) was obtained from Thermo Fisher Scientific. pSIH1-puro-control short hairpin RNA (shRNA) (shCTRL) and shRNA (shSTAT3) were obtained from Frank Sinicrope, M.D. (Mayo Clinic, Rochester, MN) (Addgene plasmids #26597 and #26596).[35] The lentiviral shSOCS3 plasmid was generated by annealing oligonucleotides 3′-GAT CCG GCG GAC CTG GAA TGT GTT TTC AAG AGA AAC ACA TTC CAG GTC CGC CTT TTT G-′5 and 3′-AAT TCA AAA AGG CGG ACC TGG AAT GTG TTT CTC TTG AAA ACA CAT TCC AGG TCC GCC G-5′. The shSOCS3 oligonucleotide was cloned into pSIH1-puro-STAT3 plasmid by replacing the shSTAT3-containing EcoRI/BamHI fragment.

Production and Infection Using Cell-Culture–Derived Recombinant HCV. Recombinant cell-culture–derived HCV (HCVcc) (strain Luc-Jc1) were generated as previously described.[21,34,36] Infection of Huh7.5.1 cells with HCVcc (half-maximal tissue culture infectious dose [$TCID_{50}$]: 10^4 mL^{-1}) was performed as previously described.[21] Unless otherwise stated, HCVcc infection was assessed by luciferase reporter gene expression.

Analysis of Antiviral Activity of Compounds and Combinations on HCV Infection. The *in vitro* antiviral activity of each compound was tested individually or in combination with a second compound using the HCVcc-Huh7.5.1 cell culture model, as previously described.[21,34,36] Huh7.5.1 cells (cultured in 96-well plates) were persistently infected for 5 days with HCVcc (Luc-Jc1) before incubation with IFN-α2a or IFN-α2b and erlotinib or sorafenib for an additional 5 days at 37°C. Viral infection was analyzed by assessing luciferase activity, as previously described.[21]

Mathematical Analysis of Synergy. Synergy was assessed by two independent methods comprising the combination index (CI)[37] and the method of Prichard and Shipman.[38] The CI was calculated as previously described.[37] A CI of <0.9, 0.9-1.1, and >1.1 indicates synergy, additivity, and antagonism, respectively.[37] The method of Prichard and Shipman was applied as previously described.[38] Surface amplitudes $>20\%$ above the zero plane indicate synergistic effects of inhibitor combinations, and surface amplitudes $<20\%$ below the zero plane indicate antagonism.[38] The validity of the assay and methods was confirmed by comparative analyses of combinations showing a nonsynergistic or an antagonistic effect.

Silencing, Inhibition, and Ligand Experiments. Silencing was performed using INTERFERin (Polyplus-transfection SA, Illkirch, France) and DharmaFECT4 (Dharmacon, Inc., Lafayette, CO), as previously described.[21] Experiments with EGFR ligand were conducted on serum-starved cells.

Analyses of Messenger RNA Transcription. Quantitative polymerase chain reaction (qPCR) to analyze IRG transcript levels were performed as previously described.[39]

Analyses of Protein Expression. Western blottings of cell lysates using protein-specific Abs (described above) were performed following GE Healthcare protocols using Hybond-P membranes and visualized using ECF substrate and a Typhoon Trio high-performance fluorescence scanner (GE Healthcare) or using the Odyssey Infrared Imaging System (LI-COR Biosciences).

Electrophoretic Mobility Shift Assay. Electrophoretic mobility shift assay (EMSA) using ^{32}P-labeled m67 oligonucleotide probe has been described.[16]

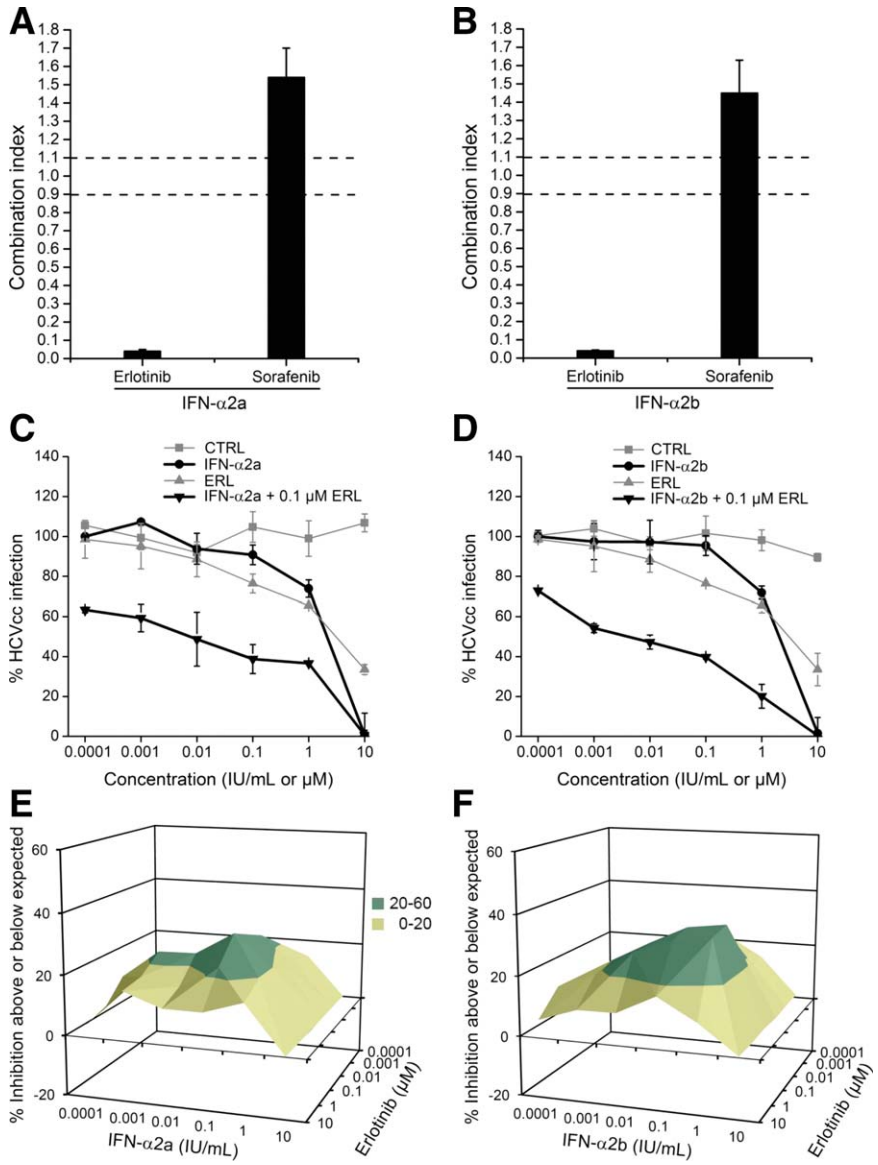

Fig. 1. IFN-α and a clinically licensed EGFR inhibitor inhibit HCV infection in a synergistic manner. Combination of EGFR inhibitor erlotinib with IFN-α potentiates the antiviral effect of IFN-α. Huh7.5.1 cells were infected for 5 days with HCV Luc-Jc1 before treatment with the indicated compounds. Subsequently, cells were incubated for 5 days with increasing concentrations of (A) IFN-α2a or (B) IFN-α2b and 0.1 μM of erlotinib or sorafenib. HCVcc infection was analyzed by luciferase reporter gene expression as previously described.[21,36] (A and B) CI at IC_{50} was calculated as previously described[37] and is indicated in Table 1. Dotted lines at CI values of 0.9 and 1.1 indicate the boundaries of an additive interaction. (C and D) Combination of IFN-α with 0.1 μM of erlotinib resulted in a shift in the IC_{50} value of IFN-α2a and IFN-α2b up to 1,000-fold. Means ± standard deviation from at least three independent experiments performed in triplicates are shown. (E and F) Synergy was confirmed using 3D analysis of the inhibitor-inhibitor combinations according to Prichard and Shipman.[38] Surface amplitudes >20% above the zero plane highlight inhibitor combinations acting synergistically, as highlighted in green. One representative experiment is shown.

Results

Inhibition of EGFR Activity Using Erlotinib Enhances the Antiviral Efficacy of IFN-α in a Highly Synergistic Manner. We previously identified EGFR as a cofactor for HCV entry.[21] We have demonstrated that the clinical EGFR inhibitor, erlotinib, impairs EGFR signaling in human hepatocytes and inhibits HCV infection and viral spread at a half-maximal inhibitory concentration (IC_{50}) of 0.45-0.53 μM.[21] To investigate the relevance of EGFR signaling for the antiviral activity of IFN-α, we incubated Huh7.5.1 cells that had been persistently infected for 5 days with HCVcc (Luc-Jc1) with IFN-α2a or IFN-α2b and a sub-IC_{50} concentration of erlotinib, which exerts only minimal effects on infection with HCVcc. Incubation with an unrelated kinase inhibitor, sorafenib, served as an unrelated control. The CI was calculated as a

Table 1. Synergy of Erlotinib and IFN-α2a or IFN-α2b on Inhibition of HCV Infection

Compound 1	IC_{50} (IU/mL)	Compound 2	IC_{50} (IU/mL) for Combination	CI
IFN-α2a	2.1 ± 0.3	Erlotinib	0.007 ± 0.002	0.04 ± 0.010
IFN-α2b	2.0 ± 0.4	Erlotinib	0.002 ± 0.003	0.04 ± 0.005

Five days postinfection with HCVcc (Luc-Jc1), Huh7.5.1 cells were incubated with serial concentrations of IFN-α2a or IFN-α2b and 0.1 μM of erlotinib or control reagent for 5 days at 37°C. HCVcc infection was analyzed by luciferase reporter gene expression.[21] CI was calculated as previously described.[37] A CI less than 0.9, between 0.9 and 1.1, and more than 1.1 indicates synergy, additivity, and antagonism, respectively. Means ± standard deviation from at least three independent experiments performed in triplicate are shown. IC_{50} of erlotinib: 0.5 ± 0.06 μM.

relative measure of synergy, as described previously.[37] We observed an enhanced and synergistic antiviral effect on HCVcc infection of IFN-α2a and IFN-α2b when combined with 0.1 μM of erlotinib (CIs of 0.04 ± 0.01 and 0.04 ± 0.005; Fig. 1A,B). In contrast, an antagonistic effect was observed when IFN-α2a and IFN-α2b were combined with sorafenib (CIs of 1.54 ± 0.16 and 1.45 ± 0.18, respectively; Fig. 1A,B). These data demonstrate that the observed synergy is specific for erlotinib. The addition of a very low dose of erlotinib (0.1 μM) to IFN-α2a or IFN-α2b decreased their IC_{50} values up to 1,000-fold (from 2.1 ± 0.3 to 0.007 ± 0.002 IU/mL for IFN-α2a and from 2.0 ± 0.4 to 0.002 ± 0.003 IU/mL for IFN-α2b; Fig. 1C,D; Table 1). Furthermore, three-dimensional (3D) modeling, according to the method of Prichard and Shipman, was used to validate synergy.[38] Surface amplitudes >20% above the zero plane confirmed synergistic effects of the indicated combination of IFN-α with erlotinib (Fig. 1E,F).

Taken together, these data suggest that the EGFR inhibitor, erlotinib, enhances the antiviral activity of IFN-α in a highly synergistic manner when added postinfection. The finding that very low doses of erlotinib (0.1 μM) with absent or minimal effects on HCV in preinfection[21] or postinfection experiments (Fig. 1C,D) efficiently potentiated the antiviral effect of IFN in postinfection experiments indicates that EGFR activity has a functional role for the antiviral activity of IFN-α that is independent from its role in HCV entry. Interestingly, we observed that, at high concentrations, erlotinib also inhibited HCV in persistently infected cells (Fig. 1C,D). Because we have previously shown that erlotinib monotherapy does not have a robust effect on HCV replication in replicon models,[21] we conclude that this effect is probably the result of the inhibition of HCV cell-cell transmission, which is required for maintenance of persistent infection.[21]

IFN-α Signal Transducer STAT3 Is a Host Factor for HCV Infection. To investigate the molecular mechanism of the synergistic effects of erlotinib and IFN-α, we investigated the role of IFN signal transducers in HCV infection using functional silencing of gene expression by STAT-specific siRNAs. STAT3 is an important regulator in the STAT1-mediated IFN-α

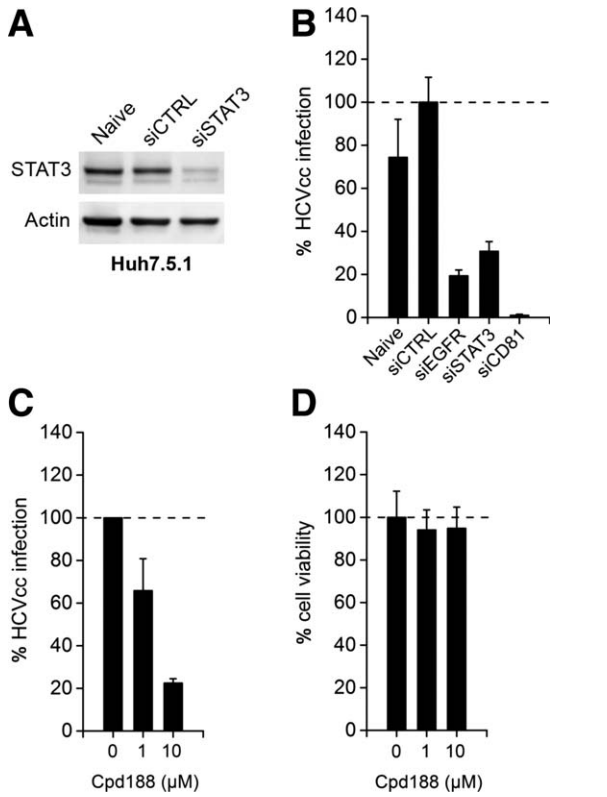

Fig. 2. STAT3 is relevant for HCV infection. Inhibition of STAT3 expression and function decreases HCV infection. (A) Silencing of STAT3 protein expression in Huh7.5.1 cells transfected with STAT3-specific siRNA (siSTAT3) or control siRNA (siCTRL). Immunoblottings of 30 μg of total proteins in cell lysates using a STAT3-specific Ab (upper panel) or a β-actin Ab (lower panel) are shown. (B) Inhibition of HCVcc (Luc-Jc1) infection in Huh7.5.1 cells with silenced STAT3 expression. HCV infection was assessed by luciferase reporter gene expression. Cells with silenced expression of EGFR (siEGFR) or CD81 (siCD81) and cells transfected with a control siRNA (siCTRL) serve as positive and negative controls, respectively. Data are expressed as percentage HCVcc infection relative to siCTRL-transfected cells. Means ± standard deviation from three independent experiments in triplicate are shown. (C) STAT3-specific inhibitor Cpd188[42] impairs HCVcc infection in Huh7.5.1 cells. Cells were preincubated for 1 hour with Cdp188 (1 and 10 μM) dissolved in 0.7% DMSO or 0.7% DMSO alone (control). Subsequently, cells were incubated with HCVcc Luc-Jc1 in the presence of the inhibitor and infection was assessed by luciferase reporter gene expression. Data are expressed as percentage HCVcc infection relative to cells treated with drug solvent 0.7% DMSO. Means ± standard deviation of three experiments in triplicate are shown. (D) Viability of cells treated with Cpd188 shown in panel (D), as measured by methyl thiazol tetrazolium assay.[21] Means ± standard deviation of three experiments in triplicate are shown.

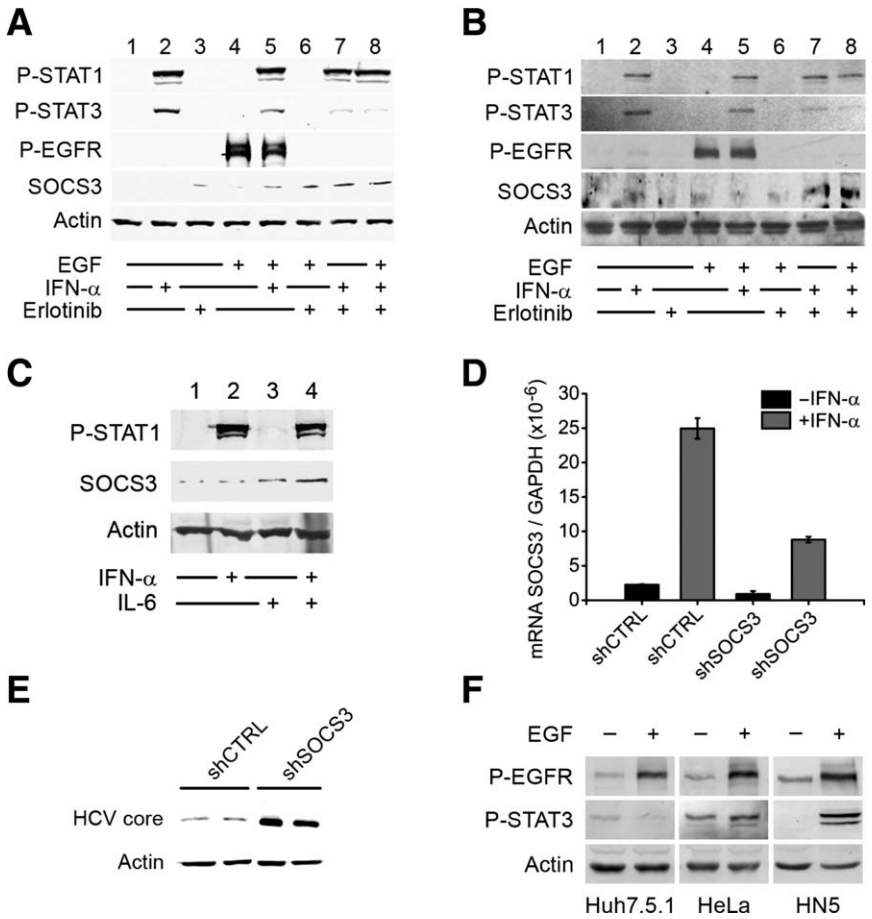

Fig. 3. Erlotinib inhibits IFN-α-induced STAT3 phosphorylation. Inhibition of EGFR function using erlotinib triggers SOCS3 expression that inhibits STAT3 phosphorylation. (A) Twelve-hour serum-starved Huh7.5.1 cells and (B) PHHs were incubated before cell lysis for 15 minutes with 1 μg/mL of EGF, 15 minutes with 1,000 IU/mL of IFN-α, 1 hour with 10 μM of erlotinib, or combinations of compounds as indicated. Cell-culture medium contained 0.7% DMSO. STAT1 phosphorylation at Tyr701 (P-STAT1), STAT3 phosphorylation at Tyr705 (P-STAT3), EGFR phosphorylation at Tyr1086 (P-EGFR), expression of SOCS3, and actin were measured by western blotting. (C) IL-6-induced SOCS3 expression does not influence IFN-α-mediated STAT1 phosphorylation in Huh7.5.1 cells. SOCS3 expression was induced by incubation of Huh7.5.1 cells for 2 hours with 10 ng/mL of IL-6 before induction of STAT signaling for 15 minutes with 1,000 IU/mL of IFN-α. SOCS3 expression and STAT1 phosphorylation at Tyr701 (P-STAT1) were measured by western blotting. (D) Transduction of shSOCS3 inhibited IFN-α-mediated increase of SOCS3 mRNA expression. Huh7.5.1 cells were transduced with lentiviruses coding irrelevant shRNA (shCTRL) or targeting SOCS3 mRNA (shSOCS3) before 1-hour incubation with 1,000 IU/mL of IFN-α or PBS (CTRL). Expression of SOCS3 mRNA is shown relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. (E) Silencing of SOCS3 expression stimulated HCV replication. Huh7.5.1 cells were transduced with lentiviruses coding shCTRL or shSOCS3 before infection with HCVcc (Luc-Jc1). After 3 days of infection, HCV core protein expression was measured from cell lysates as a measure for HCV replication. (F) EGFR activation and STAT3 phosphorylation in hepatic and nonhepatic cell lines. Human cell lines Huh7.5.1 (hepatoma), HeLa (cervix carcinoma), and HN5 (head neck cancer) were serum starved for 12 hours and then incubated for 15 minutes with 1 μg/mL of EGF before cell lysis. EGFR phosphorylation at Tyr1086 (P-EGFR), STAT3 phosphorylation at Tyr705 (P-STAT3), and actin were measured by western blotting using specific Abs. Western blottings were analyzed using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE) (A and C) and chemoluminescence exposure to ECL X-ray films (GE Healthcare Uppsala, Sweden) (B).

response[40] and has been described as an interaction partner of EGFR in proliferative cells.[41] The effect of STAT3 on HCV infection was studied using RNAi and a pharmacological inhibitor in HCV-permissive Huh7.5.1 cells. Silencing of STAT3 expression in Huh7.5.1 cells (Fig. 2A) decreased infection of HCVcc (Fig. 2B). Pharmacological inhibition of STAT3 function using Cpd188[42] dose dependently impaired HCVcc infection (Fig. 2C). Analysis of cell viability revealed no detectable toxicity, excluding antiviral effects that were the result of unspecific adverse effects (Fig. 2D). Collectively, these data suggest that STAT3 expression and STAT3 function are relevant for HCV infection and that STAT3 is a host factor for HCV infection.

Inhibition of EGFR Activity Reduces IFN-α-Induced STAT3 Activity by Induction of SOCS3 Expression. Because the EGFR interaction partner, STAT3, plays a role in HCV infection (Fig. 2), we studied the effect of combinations of IFN-α, EGFR

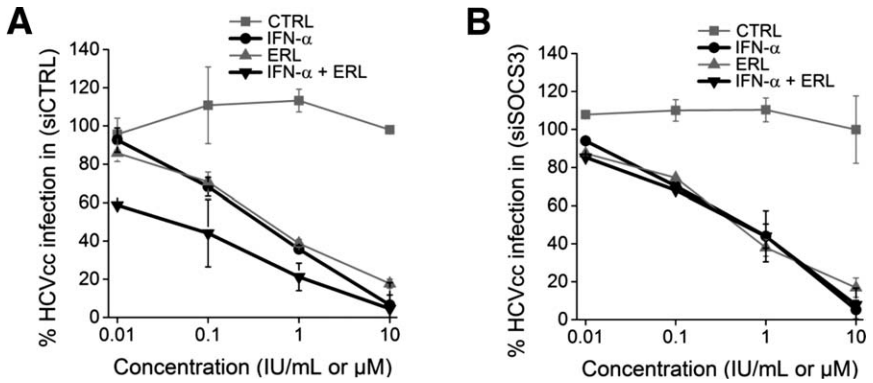

Fig. 4. SOCS3 silencing impairs the synergistic antiviral effect of IFN-α and erlotinib. Huh7.5.1 cells were transfected with (A) irrelevant siRNA (siCTRL) or (B) siRNA targeting SOCS3 (siSOCS3). After 3 days, cells transfected with siRNAs were preincubated for 1 hour with serial concentrations of IFN-α2a and erlotinib (ERL) before incubation for 72 hours with HCVcc (Luc-Jc1) in the presence of both compounds. HCVcc infection was analyzed by luciferase reporter gene expression, as previously described.[21,36] Means ± standard deviation of one representative experiment performed in triplicate are shown.

inhibitor erlotinib, and EGFR ligand EGF on STAT1 and STAT3 activity using phosphoimmunoblotting. Figure 3A,B confirmed the biological activity of EGF (P-EGFR, lane 4), erlotinib (P-EGFR, lane 6), and IFN-α (P-STAT1, lane 2) in Huh7.5.1 and PHHs. Incubation of Huh7.5.1 cells or PHHs with IFN-α enhanced the phosphorylation of STAT1 and STAT3 (Fig. 3A,B, lane 2). Although modulation of EGFR activity by EGF or erlotinib had no effect on IFN-α-induced STAT1 phosphorylation, IFN-α-induced STAT3 phosphorylation decreased in the presence of erlotinib (Fig. 3A,B, lanes 7 and 8). Moreover, the expression of SOCS3, a suppressor of STAT3 activity, was induced in the presence of erlotinib (Fig. 3A,B, lanes 6-8). To study whether SOCS3 also modulates STAT1 function in hepatic cells, we analyzed the effect of SOCS3 on IFN-α-mediated STAT1 phosphorylation in Huh7.5.1 cells by inducing SOCS3 expression with IL-6 before stimulation with IFN-α. Because SOCS3 up-regulation did not impair STAT1 phosphorylation in Huh7.5.1 cells (Fig. 3C), we conclude that SOCS3 expression does not have a major role for STAT1 function in hepatic cells.

The relevance of SOCS3 in HCV infection was confirmed by lentiviral transduction of SOCS3-specific shRNA in Huh7.5.1 cells. Silencing of SOCS3 messenger RNA (mRNA) expression by shSOCS3 in the absence or presence of IFN-α (1,000 IU/mL) was confirmed by qPCR (Fig. 3D). Silencing of SOCS3 expression increased HCV core protein production in Huh7.5.1 cells infected with HCVcc, which is a measure for HCV replication (Fig. 3E). This observation is in line with our observations that (1) STAT3 function is required for HCV infection (Fig. 2) and that (2) erlotinib-induced enhancement of IFN-α-dependent SOCS3 expression leads to reduced STAT3 phosphorylation (Fig. 3A,B). Interestingly, in contrast to results obtained in nonhepatic human cell lines HeLa and HN5 (Fig. 3F), EGF did not induce STAT3 phosphorylation in Huh7.5.1 cells and PHHs (Fig. 3A,B). These data indicate that, in hepatic cells, EGF does not result in STAT3 activation in the absence of IFN-α.

The role of SOCS3 for the synergistic action of erlotinib on the antiviral activity of IFN was confirmed by functional experiments in cells with silenced SOCS3 expression. Although erlotinib enhanced the antiviral activity of IFN-α (IC_{50} (IFN-α) = 0.4 IU/mL; IC_{50} (IFN-α + 0.1 μM of erlotinib) = 0.04 IU/mL) in a highly synergistic manner in Huh7.5.1 cells transfected with irrelevant siRNA not affecting SOCS3 expression (CI = 0.2; Fig. 4A), erlotinib acted only in an additive manner (IC_{50} (IFN-α) = 0.6 IU/mL; IC_{50} (IFN-α + 0.1 μM of erlotinib) = 0.6 IU/mL) in cells with silenced SOCS3 expression (CI = 1.1; Fig. 4B). These data clearly confirm a key role of SOCS3 for the observed synergy of erlotinib on the antiviral activity of IFN-α.

Taken together, these data indicate that IFN-α-dependent SOCS3 expression is enhanced by erlotinib. Enhanced SOCS3 expression results in repression of STAT3 activation with subsequent synergistic enhancement of the antiviral activity of IFN-α.

Inhibition of EGFR Activity Enhances STAT1-Mediated Antiviral Response. Because STAT1 and STAT3 have opposing roles in many aspects of the IFN response,[10] we next studied whether elevated SOCS3 levels resulting from inhibited EGFR activity contribute to an enhanced antiviral response by STAT1 activation. Phosphorylated STATs form dimers

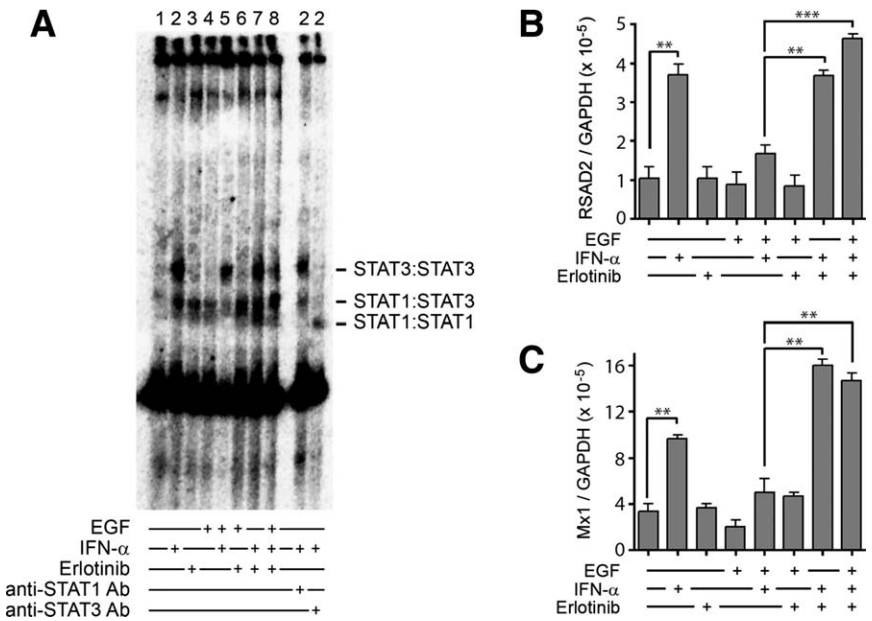

Fig. 5. Erlotinib enhances STAT1 homodimerization and expression of IFN-α response genes RSAD2 and Mx1. (A) EMSA measuring IFN-α-induced STAT1:STAT1, STAT1:STAT3, and STAT3:STAT3 dimerization. Before cell lysis, 12-hour serum-starved Huh7.5.1 cells were incubated for 30 minutes with 1 μg/mL of EGF, 30 minutes with 1,000 IU/mL of IFN-α, 1 hour with 10 μM of erlotinib, or combinations of compounds as indicated. Cell-culture medium contained 0.7% DMSO. EMSA was performed using the STAT1/STAT3-specific oligonucleotide probe, m67.[16] Sample 2 (IFN-α treated) was used in lanes 9 and 10 for supershift with specific STAT1 or STAT3 Abs to confirm STAT1 and STAT3 bands on the gel. STAT1:STAT1 and STAT1:STAT3 bands disappeared (supershift) in the presence of STAT1-specific Ab (lane 9). STAT3:STAT3 and STAT3:STAT1 disappeared in the presence of STAT3-specific Ab (lane 10). (B and C) Quantification of RSAD2 and Mx1 mRNAs using qPCR. Serum-starved Huh7.5.1 cells were incubated for 4 hours with 1 μg/mL of EGF, 4 hours with 1,000 IU/mL of IFN-α, 1 hour with 10 μM of erlotinib, or combinations of compounds as indicated. Cell-culture medium contained 0.7% DMSO. mRNAs were quantified from cell lysates relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA using qPCR. $^{**}P < 0.005$; $^{***}P < 0.0005$.

or trimers that translocate to the nucleus, where they bind to DNA and direct specific transcriptional initiation.[4] P-STAT1 homodimerization, in the presence of IFN-α, EGF, erlotinib, or combinations of the three compounds, was quantified using EMSA. Therefore, a ^{32}P-labeled oligonucleotide probe (m67) derived from the promoter of the c-fos gene[16] was used, which specifically binds to P-STAT1 and P-STAT3 dimers. The presence of EGF reduced P-STAT1 homodimerization (Fig. 5A, lanes 2 and 5) correlated with reduced transcription levels of the IRGs, Mx1 and RSAD2 (Fig. 5B,C). IFN-α-induced Mx1 and RSAD2 expression play an important role during viral infections.[2,5-7] This suggests a negative regulatory role of EGFR for STAT1 function (Fig. 5A, lanes 2 and 5). In contrast, inhibition of EGFR activity by erlotinib increased STAT1 homodimerization (Fig. 5A, lanes 5 and 8) and removed the inhibitory effect of EGF on IFN-α-induced STAT1 function, leading to significantly ($P < 0.05$) increased Mx1 and RSAD2 transcription levels (Fig. 5B,C, bars 5 and 8). These data confirm the regulatory role of EGFR on STAT1 function and suggest that inhibition of EGFR function by erlotinib enhances the IFN-α-induced antiviral function of STAT1. Moreover, these data demonstrate that the regulation of IFN-α-induced gene expression by EGFR occur at the DNA-binding level of STAT1 (Fig. 5), because STAT1 phosphorylation is not affected by EGF treatment (Fig. 3A,B, lanes 2 and 5).

Discussion

Our results uncover a previously undiscovered regulatory role of EGFR for STAT3 activity that counteracts the proinflammatory and antiproliferative function of STAT1. In the cell, STAT1 and STAT3 activity delicately balance IFN-α signaling.[10,40] Cross-talk influences that balance, which may favor the opposing roles of STAT1 or STAT3. For example, it has been shown that EGF activates STAT3 transcriptional activity.[43] Moreover, Wang et al. have recently demonstrated that STAT3 knockout enhances type I IFN-mediated antiviral activity.[44] Although STAT3 has been described as a binding factor to EGFR in highly proliferating cells,[45] we demonstrate that STAT3 activity is not directly mediated by EGFR engagement in HCV-permissive cells, because, in hepatic cells, EGFR stimulation by EGF in the absence of IFN-α is not sufficient

to mediate STAT3 phosphorylation (Fig. 3A,B,F). Rather, our data demonstrate that inhibition of EGFR function in hepatocytes unleashes SOCS3 expression that inhibits IFN-α-induced STAT3 activity. This hypothesis is supported by previous observations showing that EGFR overexpression down-regulates SOCS3 expression in mouse embryonic fibroblasts,[46] consistent with a negative regulatory role of EGFR for SOCS3 expression by acting as a repressor. Interestingly, serum starvation of cells (Fig. 3A,B, lanes 1-3) did not release the repression of IFN-α-induced SOCS3 expression. Because EGF did not further enhance IFN-α-mediated STAT3 phosphorylation, we conclude that, in PHHs and Huh7.5.1 cells, ligand-independent EGFR basal activity (indicated by basal EGFR phosphorylation in serum-starved Huh7.5.1 cells and PHHs, as shown previously[21]; Fig. 3F) is sufficient to maintain SOCS3 repression. This repressive effect by basal EGFR activity is not further enhanced by more-pronounced EGFR activation through EGF (Fig. 3A,B).

Moreover, we observed that EGF inhibited STAT1 homodimerization that correlated with reduced ISG expression, whereas STAT3 homodimerization was unchanged (Fig. 5). Taken together, these observations appear to point toward a regulatory role of EGFR signaling on STAT1/STAT3 in human hepatocytes, which is different from nonhepatic cells. Indeed, in contrast to nonhepatic human cell lines HeLa and HN5 (Fig. 3F), EGF did not enhance STAT3 phosphorylation in Huh7.5.1 cells and PHHs (Fig. 3A,B,F). Although further experiments need to be performed to better understand these findings, it is conceivable that this observation is relevant for the special role of EGFR during liver regeneration[9,47] and HCV infection.[23] EGFR activation may inhibit the proapoptotic role of STAT1 signaling on a DNA-binding level, resulting in promotion of cell proliferation and viral spread.

Collectively, identification of the cross-talk of EGFR and IFN-α explains the marked synergy of IFN-α and erlotinib through the following findings: (1) Synergy at low doses of erlotinib (0.1 μM) with absent effects on HCV entry and infection in monotherapy (Fig. 1C,D) indicates that EGFR activity has a functional role for the antiviral activity of IFN-α that is independent from its role in HCV entry; (2) SOCS3 expression is required for synergy on antiviral activity (Fig. 4); (3) inhibition of STAT3 phosphorylation by erlotinib through SOCS3 shifts the balance of STAT1/STAT3 activity toward an enhanced antiviral effect of STAT1 that is reflected by enhanced IRG transcription levels (Fig. 5); (4) STAT3 function is relevant for HCV infection (Fig. 2), and inhibition of STAT3 phosphorylation by erlotinib (Fig. 3) may further contribute to the antiviral activity of erlotinib in combination with IFN-α; and (5) inhibition of EGFR function by erlotinib enhances the IFN-α-induced antiviral function of STAT1 (Fig. 5).

There are two implications of EGFR-IFN cross-talk. First, the interference of signaling explains viral evasion during IFN-α treatment. Because HCV infection has been shown to result in EGFR activation,[23] virus-induced EGFR signaling and cross-talk may contribute to viral evasion and resistance to IFN-α. Furthermore, EGFR expression is up-regulated in response to treatment, presumably as a homeostatic cellular response to antiproliferative stimuli by IFN-α.[48] Because EGFR is a cofactor for HCV entry, and EGFR expression correlates directly with enhanced viral entry and propagation,[21] elevated EGFR levels in IFN-α-treated patients might be further exploited by the virus to counteract the antiviral effect of IFN-α. Collectively, these findings suggest an important role for EGFR signaling in viral evasion and IFN-α resistance. Second, our results open a novel possibility of improving the efficacy of IFN-α-based therapies. Although the clinical licensing of protease inhibitors has improved the outcome of IFN-α-based therapy for HCV, adverse effects, drug-drug interactions, and a low genetic barrier to resistance are important limitations.[49] These limitations result in unsatisfactory treatment options in patients with advanced liver disease, liver transplantation, comorbidity, and human immunodeficiency virus coinfection.[50] Although promising IFN-α-sparing regimens are in clinical development, early clinical trials demonstrate that nonresponders to previous IFN-based therapies appear only partially to clear viral infection.[51] Thus, it is likely that a clinically relevant subset of patients will require IFN-α in the future, despite the anticipated approval of IFN-free regimens.

Our mechanistic data suggest that a clinical combination therapy of IFN-α and erlotinib may result in unique benefits to address the limitations of IFN-α. First, as shown here, erlotinib synergistically enhances the antiviral activity of IFN-α by interfering with IFN-α signaling. Enhanced efficacy may contribute to break the tolerance to IFN-α in patients with slow virological response or primary nonresponders.[19] Second, erlotinib may decrease IFN resistance by overcoming a potential proviral effect of enhanced EGFR expression induced by IFN.[48] Third, erlotinib may increase the genetic barrier to viral resistance to therapy by inhibiting a host cell HCV entry factor.[21,23] In contrast to the very

high genetic variability of the virus, the genetic variability of host factors, such as EGFR, is low.[26,28]

It is important to note that the combination of high doses of erlotinib and IFN-α did not result in detectable toxicity in PHHs (data not shown). In clinical use, the safety of erlotinib in cancer patients is comparable to HCV protease inhibitors.[52-54] An investigator-initiated phase I clinical trial is in preparation at Strasbourg University Hospital (Strasbourg, France) to specifically evaluate the safety, antiviral activity, and resistance profile of erlotinib in HCV-infected patients.

Because the IFN-α-sensitizing effect of SOCS3/STAT3 and STAT1 is independent of the specific host-targeting activity of erlotinib on HCV entry, it is possible that the synergistic action of erlotinib also potentiates the antiviral activity of IFN-α against other viruses, such as HBV or HDV. Collectively, our data reveal a novel concept and strategy to enhance the antiviral efficacy of IFN-α and decrease resistance of IFN-α-based therapies.

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Clearance of persistent hepatitis C virus infection in humanized mice using a claudin-1-targeting monoclonal antibody

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Hepatitis C virus (HCV) infection is a leading cause of liver cirrhosis and cancer[1]. Cell entry of HCV[2] and other pathogens[3–5] is mediated by tight junction (TJ) proteins, but successful therapeutic targeting of TJ proteins has not been reported yet. Using a human liver–chimeric mouse model[6], we show that a monoclonal antibody specific for the TJ protein claudin-1 (ref. 7) eliminates chronic HCV infection without detectable toxicity. This antibody inhibits HCV entry, cell-cell transmission and virus-induced signaling events. Antibody treatment reduces the number of HCV-infected hepatocytes *in vivo*, highlighting the need for *de novo* infection by means of host entry factors to maintain chronic infection. In summary, we demonstrate that an antibody targeting a virus receptor can cure chronic viral infection and uncover TJ proteins as targets for antiviral therapy.

The TJ protein claudin-1 (CLDN1) mediates hepatitis C virus (HCV) entry into host cells[2]. TJs are also implicated in the entry of other pathogens including dengue virus[5], adenovirus[3], coxsackievirus[3] and shigella[4]. However, the role of TJ proteins in viral pathogenesis and as antiviral targets is unknown. To investigate this we used a recently developed inhibitory CLDN1-specific monoclonal antibody (mAb)[7] and the human chimeric uPA-SCID (urokinase-type plasminogen activator-transgenic severe combined immunodeficiency) mouse model[6]. Owing to discontinuation of research in chimpanzees for ethical reasons, this mouse model is the only available model that supports robust long-term chronic HCV infection. Although these mice lack a functional immune system, precluding the study of immune-mediated events, they have substantially contributed to our understanding of viral pathogenesis and the development of antivirals[8–11].

First, we analyzed CLDN1 expression in the chimeric liver using a commercially available mAb that recognizes human and mouse CLDN1 (**Supplementary Fig. 1**). Confocal imaging demonstrated that the majority of CLDN1 on human hepatocytes co-localized with apical marker CD10, demonstrating the formation of bile canalicular structures (**Fig. 1a**). A minor pool of protein was detected at the basolateral membrane as identified with cytokeratin-8 staining[12] (**Fig. 1a** and data not shown). Comparative staining of normal human liver tissue demonstrated comparable subcellular localization (**Fig. 1a**). Transmission electron microscopic (TEM) analysis confirmed that hepatocytes in the chimeric mouse liver form TJs that were structurally indistinguishable from those in human liver tissue (**Supplementary Fig. 2a**,**b**). The similar localization of hepatocellular CLDN1 and hepatocyte architecture suggests that the uPA-SCID chimeric mouse is a relevant model to evaluate CLDN1 as a therapeutic target.

Next, we characterized the subcellular localization of CLDN1 recognized by the inhibitory CLDN1-specific mAb (OM-7D3-B3) when administered intraperitoneally *in vivo*. TEM and immunogold labeling of the CLDN1 mAb showed reactivity with basolateral and

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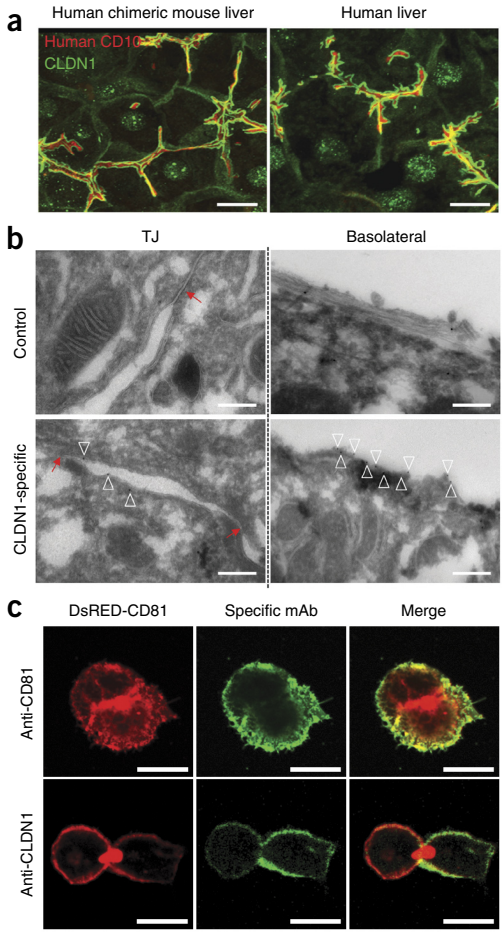

Figure 1 Human CLDN1 expression and tight junction ultrastructure in the livers of human chimeric mice. (**a**) Human CLDN1 expression in noninfected chimeric livers of uPA-SCID mice (left panel) as well as noninfected human livers (right panel) assessed by confocal microscopy. Representative 3D composite images show human CLDN1 (green) co-stained with apical membrane marker human CD10 (red). Scale bars, 20 µm. (**b**) Binding of CLDN1-specific or control mAb to hepatocyte ultrastructures assessed by transmission electron microscopy analyses and immunogold labeling of tissue sections of chimeric human mouse livers from mAb-treated mice. Left panels, TJ area; right panels, basolateral membranes of hepatocytes. Red arrows indicate TJ, empty triangles indicate immunogold staining. Scale bars, 500 nm. (**c**) Confocal microscopy of polarized HepG2-CD81 HCV-permissive cells stained with CLDN1-specific antibody. HepG2 DsRED-CD81 cells were stained live with CD81-specific (upper panels) or CLDN1-specific (lower panels) antibodies. CLDN1-specific staining is predominantly limited to the basolateral membrane of polarized cells. Scale bars, 20 µm.

sinusoidal hepatocyte membranes *in vivo* (**Fig. 1b**), whereas we failed to detect staining of TJs (**Fig. 1b**). Basolateral membrane staining was confirmed using live-cell imaging of polarized hepatoma HepG2 cells (**Fig. 1c**)[13]. Collectively, these data suggest that the CLDN1-specific mAb predominantly binds to nonjunctional pools of CLDN1 on the hepatocyte basolateral membrane *in vivo*.

To investigate the antiviral effect of this CLDN1-specific mAb, we administered to chimeric uPA-SCID mice CLDN1-specific ($n = 5$) or control isotype-matched irrelevant mAb ($n = 4$) 1 day before inoculation, and 1 and 5 days after infection with a primary HCV genotype 1b serum. The CLDN1-specific mAb provided complete and sustained protection from infection as evidenced by undetectable HCV RNA in the sera for up to 6 weeks after infection (**Fig. 2a** and **Supplementary Table 1**). In contrast, all control-mAb–treated mice became infected (**Fig. 2a**). Similar results were observed following infection with an HCV of the difficult-to-treat genotype 4 (isolate ED43 (ref. 14)), **Fig. 2b** and **Supplementary Table 1**), demonstrating a significant level of anti-viral activity ($P < 0.0002$, pooled data from experiments of both genotypes, Mann-Whitney test). Pharmacokinetic studies showed that CLDN1-specific mAb levels declined rapidly and were undetectable 2 weeks after administration (**Fig. 2f,g**). Collectively, these data show that short-term administration of a CLDN1-specific mAb prevents *de novo* HCV infection and suggests that it might be used to prevent HCV re-infection during liver transplantation.

To investigate the effect of the CLDN1-specific mAb on chronic HCV infection, we used mice persistently infected with the cell culture–derived HCV (HCVcc) strain Jc1 (genotype 2a/2a chimera)[15], a JFH1-derived[16] prototype strain. Persistently infected mice (24–50 days after infection) were given four doses total (one in each week of the study period) of CLDN1-specific ($n = 5$) or control mAbs ($n = 4$). All CLDN1-specific mAb–treated mice had undetectable HCV RNA levels in the blood after 2 to 4 injections (**Fig. 2c** and **Supplementary Table 1**) and, except for one case, animals remained free of virus until the end of the study period. The relapsed mouse showed a low antibody serum concentration during the early treatment phase (**Fig. 2h**). However, low levels of mAbs did not always correlate with relapse (**Fig. 2i,j**). Sequencing and functional analysis of viral envelope glycoproteins from antibody-treated mice in HCV pseudoparticles (HCVpp) and HCVcc (**Supplementary Fig. 3a–e**) suggested that the relapse was unlikely to be explained by the presence of resistant variants that escaped the CLDN1-specific mAb (**Supplementary Fig. 3c,d**) or to CLDN1-independent cell entry (**Supplementary Fig. 3e**). Notably, human hepatocytes engrafted into uPA-SCID mice expressed lower CLDN6 levels than Huh7.5.1 cells (**Supplementary Fig. 4a–d**). The mAb concentration peaked during the treatment period and declined rapidly following discontinuation of mAb injection (**Fig. 2h**). Similar results were observed in four additional HCV Jc1-infected and CLDN1-specific mAb–treated mice with follow-up of 3 weeks (data not shown).

CLDN1 mAb also induced viral clearance from mice chronically infected with HCVcc VL-JFH1 (genotype 1b/2a chimera) ($n = 5$), a well-characterized strain[17] encoding the structural proteins from a highly infectious HCV neutralization escape variant isolated from a patient undergoing liver transplantation (**Fig. 2d** and **Supplementary Table 1**). One mouse showing a partial viral response (**Fig. 2d** and **Supplementary Table 1**) was characterized by undetectable mAb concentration after the second injection (**Fig. 2i**). The therapeutic effect was robust (HCV RNA decline > 2 logs), sustained (until the end of the study period corresponding to 11 weeks) and significant ($P < 0.0007$, pooled data for experiments of both genotypes, Mann-Whitney test). Furthermore, a similar effect was observed in mice chronically infected with HCV genotype 2a serum (**Fig. 2e** and **Supplementary Table 1**). Mice infected with primary isolates of HCV genotype 4 ($n = 4$) also showed a marked and time-dependent decrease in HCV RNA levels (**Supplementary Fig. 5a**) with no viral rebound and stable function of the engrafted human hepatocytes as shown by serum albumin levels (**Supplementary Fig. 5b**). In summary, these data show that a CLDN1-specific mAb can clear chronic HCV infection *in vivo* and exhibits antiviral activity against different viral genotypes. These findings suggest CLDN1 as a therapeutic target in a clinically relevant animal model.

To assess antibody safety, we analyzed the histopathology of chimeric uPA-SCID mouse livers. Human hepatocyte-specific staining

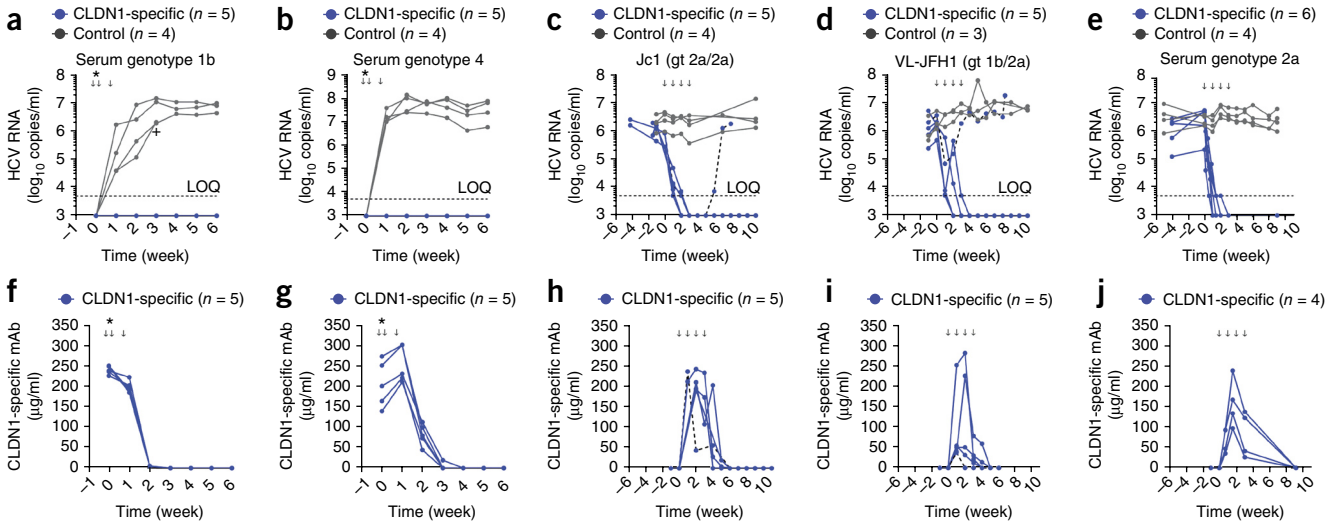

Figure 2 Prevention and clearance of chronic HCV infection using a CLDN1-specific mAb *in vivo*. (**a**,**b**) Prevention studies. Chimeric uPA-SCID mice received 500 µg CLDN1-specific ($n = 5$) or control mAb ($n = 4$) on days −1, 1 and 5 (arrows) of inoculation (asterisk) with genotype 1b (**a**) or genotype 4 (**b**) HCV-containing serum. One infected mouse injected with control mAb died on day 26 (**a**, cross). (**c**–**e**) Treatment studies. Chimeric uPA-SCID mice were chronically infected with HCVcc Jc1 (ref. 15) (genotype 2a/2a) (**c**), HCVcc VL-JFH1 (ref. 17) (genotype 1b/2a) (**d**) or serum of genotype 2a (**e**). Twenty-four to 50 days following inoculation, the animals received 500 µg control (**c**,**d**,**e**; $n = 4$, 3 and 4, respectively) or CLDN1-specific mAb (**c**,**d**,**e**; $n = 5$, 5 and 6, respectively) each week (arrows) for 4 weeks. Two CLDN1-specific mAb-treated mice were euthanized following viral clearance for FISH studies (**e**). (**f**–**j**) Serum concentration of CLDN1-specific mAb from (**a**–**e**, respectively) was determined by ELISA. Serum viral load (**a**–**e**) was quantified by the clinically licensed Abbott RealTime HCV assay. The horizontal dashed line indicates the limit of quantification (LOQ). (**c**,**d**,**h**,**i**) The viral load and antibody concentration of the mice exhibiting a relapse is indicated by a dashed line. Values of individual animals are shown.

demonstrated similar repopulation and structure of human hepatocytes in HCV Jc1-infected mice treated with the control or CLDN1-specific mAb (**Supplementary Fig. 6a**). TEM analysis of chimeric-infected livers of treated mice showed no detectable alteration in hepatocyte morphology or TJ ultrastructure (data not shown). Human albumin, transaminases (ALT, AST) and total bilirubin levels remained stable following antibody administration and were similar in control and CLDN1-specific mAb–treated mice at all time points tested (**Supplementary Fig. 6b**–**e**).

To assess the functional integrity of CLDN1–mAb–treated human hepatocytes in mice, we challenged the mice with HCV of a different strain and genotype. CLDN1-specific mAb–treated animals previously protected from HCV infection (**Fig. 2a**) supported viral infection following mAb elimination (**Supplementary Fig. 6f**). These functional data corroborate the presence of fully viable and functional hepatocytes following anti-CLDN1 mAb treatment and exclude adverse effects on hepatocyte function *in vivo*.

To address potential side effects of the CLDN1-specific mAb in other organs and tissues, we assessed antibody binding to human and murine CLDN1 (**Supplementary Fig. 7a**–**g**). The antibody-bound primary mouse hepatocytes and 293T cells expressing murine CLDN1 with an apparent K_d of 83.6 ± 14.1 nM compared to the apparent K_d for human CLDN1 expressed in 293T cells (17.05 ± 1.14 nM) (**Supplementary Fig. 7g**). Moreover, the latter was in a similar range as the apparent K_d of CLDN1-specific mAb binding to human hepatocytes[7]. Next we measured *in vivo* biodistribution in BALB/c mice and observed enrichment in skin, kidneys, lungs, intestines and liver (**Supplementary Fig. 8a**,**b**). Toxicity studies in BALB/c mice including clinical (data not shown), biochemical and hematological parameters as well as histopathological analyses did not reveal any toxicity (**Supplementary Fig. 9** and **Supplementary Tables 2** and **3**). Because TJs play a key role in intestinal paracellular permeability, we investigated the effect of the CLDN1-specific mAb on intestinal barrier function *in vivo*. The CLDN1-specific mAb had no effect on intestinal paracellular permeability (**Supplementary Fig. 8c**) or total intestinal transit time (**Supplementary Fig. 8d**), in contrast to irradiation, which was used as a positive control for intestinal injury. Additional *ex vivo* studies showed that the CLDN1-specific antibody had no detectable effects on paracellular permeability (**Supplementary Fig. 8e**) or tissue conductance in the small intestine or colon (**Supplementary Fig. 8f**). Moreover, the mAb had no effect on the transepithelial electrical resistance of intestinal Caco-2 cells (**Supplementary Fig. 8g**), which express abundant CLDN1 levels (**Supplementary Fig. 8h**), or on TJ function in hepatoma HepG2 cells[18]. Whereas CLDN1 knockdown significantly reduced TJ integrity ($P < 0.001$, *t*-test) in polarized HepG2 cells (**Supplementary Fig. 10a**,**b**), the CLDN1-specific mAb had no impact (**Supplementary Fig. 10c**). Furthermore, flow cytometry experiments measuring CLDN1 cell surface expression suggested that the CLDN1-specific mAb did not promote cellular CLDN1 internalization (**Supplementary Fig. 10d**). Finally, a detailed analysis of cytokine mRNA expression in antibody-treated mice did not reveal evidence for antibody-induced inflammatory changes in the mouse jejunum (data not shown). Collectively, these data exclude major toxicity or side effects induced by the CLDN1-specific mAb in mice. Because the rat antibody did not interact with the mouse Fc receptor, immune-mediated adverse effects, such as antibody-dependent cell-mediated cytotoxicity (ADCC), cannot be assessed in this model. Humanization of the antibody using an IgG4 backbone—a well-established strategy to minimize ADCC[19]—is underway for its clinical development.

To explore the antiviral mechanism of the CLDN1-specific mAb, we performed both *in vitro* and *in vivo* studies. Using protein association studies and a Förster resonance energy transfer (FRET)-based assay, we found that the CLDN1-specific mAb interfered with CD81-CLDN1 co-receptor complex formation (**Supplementary Fig. 11a**)—a key step required for HCV entry[20]. Co-receptor

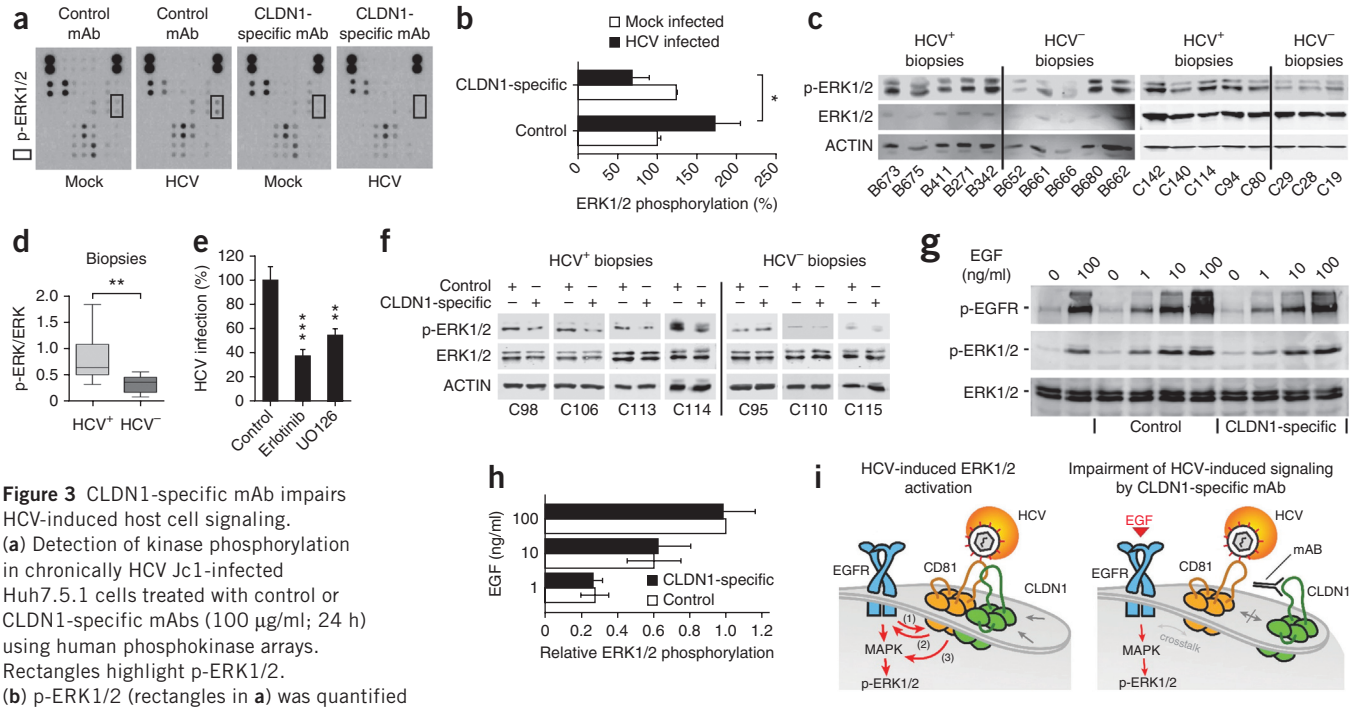

Figure 3 CLDN1-specific mAb impairs HCV-induced host cell signaling. (**a**) Detection of kinase phosphorylation in chronically HCV Jc1-infected Huh7.5.1 cells treated with control or CLDN1-specific mAbs (100 µg/ml; 24 h) using human phosphokinase arrays. Rectangles highlight p-ERK1/2. (**b**) p-ERK1/2 (rectangles in **a**) was quantified using ImageJ software. Results are shown as mean ± s.e.m. of integrated dot blot densities from two independent experiments performed in duplicate. (**c**) Western blot analysis of p-ERK1/2, total ERK1/2 and ACTIN expression in liver tissue of patients (representative full-length blots are presented in **Supplementary Fig. 13a**). (**d**) Quantification of signal intensities of p-ERK1/2 in **c** normalized to total ERK1/2 expression. The quantification of p-ERK1/2 (normalized to ACTIN expression) relative to total ERK1/2 expression (normalized to ACTIN expression) is shown. Results are shown as the interquartiles (box) with the min. and max. values (whiskers). The line within the box indicates the median value. (**e**) Inhibition of the MAPK pathway inhibits persistent HCV infection in Huh7.5.1 cells. Luc-Jc1-infected Huh7.5.1 cells were treated 3 days after inoculation with erlotinib or UO126 (each at 10 µM) for 3 days. HCV replication was assessed by luciferase assay. Results are presented as mean ± s.e.m. of six independent experiments performed in triplicate. (**f**) CLDN1-specific mAb reduces ERK1/2 phosphorylation in liver tissue from patients with chronic HCV infection. Fresh liver biopsies were divided in two equal pieces and maintained in DMEM medium supplemented with 10% fetal calf serum and 100 µg/ml control mAb or CLDN1-specific mAb for 4 h, respectively. Expression of p-ERK1/2, total ERK1/2 and ACTIN was determined by western blot analysis. (Representative full-length blots are presented in **Supplementary Fig. 13b**.) (**g**) p-EGFR, p-ERK1/2 and total ERK1/2 of serum starved Huh7.5.1 cells treated with antibodies and EGF was quantified by western blot analysis. (Full-length blots are presented in **Supplementary Fig. 13c**). (**h**) EGF-induced ERK phosphorylation in the presence of CLDN1-specific or control mAb. p-ERK1/2 and total ERK1/2 western blot signals described in **g** were quantified using a Typhoon Trio laser scanner and ImageQuant Software. Values are expressed as relative ratio of p-ERK1/2 to total ERK1/2 densities (two independent experiments ± s.e.m.). (**i**) Model of HCV-induced signal transduction during cell entry (left panel) and its impairment by CLDN1-specific mAb (right panel). Left panel: (1) HCV entry is dependent on EGFR signaling, which promotes CD81-CLDN1 co-receptor interactions[9,24]; at the same time, viral binding to the target cell induces (2) EGFR phosphorylation and signaling through CD81-EGFR interactions[23] and (3) MAPK signaling by cross-talk[22]. Right panel: proposed model that the CLDN1-specific mAb impairs virus-induced MAPK/ERK1/2 signaling by interfering with CD81/CLDN1-MAPK cross-talk without impairing direct EGF-induced ERK1/2 phosphorylation. $*P < 0.05$, $**P < 0.01$, $***P < 0.0001$ (Student's *t*-test (**b**,**e**), Mann-Whitney test (**d**)).

perturbation most likely occurs at the hepatocyte basolateral membrane as shown by immunofluorescence studies of polarized HepG2 cells or immunogold affinity labeling of hepatocytes (**Fig. 1b**,**c**). Furthermore, we demonstrate that the CLDN1-specific mAb significantly inhibits cell-free and cell-cell routes ($P < 0.01$, *t*-test) of transmission (**Supplementary Fig. 11b–e**). Of note, inhibition of cell-cell transmission led to a marked decrease of virus spread in hepatoma cells when the CLDN1-specific mAb was added 48 h after infection (**Supplementary Fig. 11e**). The ability of the antibody to inhibit cell-free and cell-cell infection may explain, at least partially, the antiviral effects of the CLDN1-specific mAb to cure persistent HCV infection *in vivo*. As this mAb impaired neither HCV replication nor assembly and release (**Supplementary Fig. 11f,g**), we conclude that the mAb acts predominantly by interfering with HCV entry.

Because claudins can modulate signaling pathways[21], we investigated whether the antibody modulated the microRNA (miRNA) and kinome profiles of persistent HCV-infected Huh7.5.1 cells. Consistent with previous observations, our deep sequencing studies confirmed that HCV infection modulates miRNA profiles in Huh7.5.1 cells. However, the CLDN1-specific mAb had no detectable effect on miRNA expression (**Supplementary Fig. 12**).

HCV binding to hepatocellular CD81 and associated receptors activates EGFR and mitogen-activated protein kinase (MAPK) signaling[22,23]. HCV Jc1 infection increased ERK1/2 phosphorylation, and this was reversed by the CLDN1-specific mAb (**Fig. 3a**,**b**). The significance of HCV-induced MAPK signaling was supported by analyzing liver biopsies from HCV-infected patients. Liver biopsies from HCV-infected patients showed a significant ($P < 0.01$, Mann-Whitney test) 2.45-fold increase in p-ERK1/2 levels over that observed in the control (**Fig. 3c**,**d**, **Supplementary Fig. 13a** and **Supplementary Table 4**). We observed an antiviral effect of small-molecule inhibitors targeting MAPK signaling (**Fig. 3e**) at nontoxic concentrations[9,24] (**Supplementary Table 5**). To confirm the effect of the CLDN1-specific mAb on HCV-induced signaling in liver tissue from HCV-infected patients (**Supplementary Table 4**), freshly isolated liver biopsies were treated *ex vivo* with CLDN1-specific or control mAb, and we observed a reduction of ERK1/2 phosphorylation compared to control-treated biopsies (**Fig. 3f** and **Supplementary Fig. 13b**).

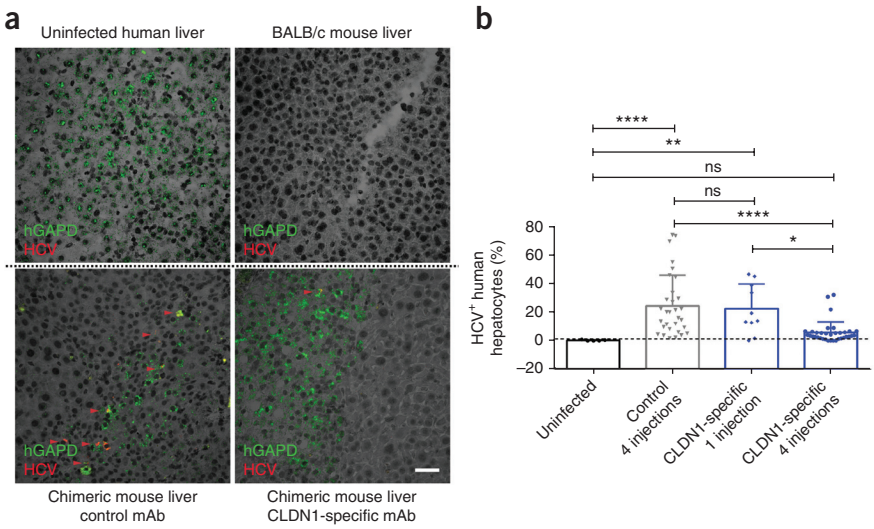

Figure 4 CLDN1-specific mAb leads to elimination of HCV-infected cells from the livers of human chimeric mice in a dose- and time-dependent manner. (**a**) Detection of human hepatocytes and HCV RNA with probes specific for human GAPDH mRNA (green) or HCV Jc1 RNA (red) using FISH. HCV Jc1 RNA probe specifically stained HCV-infected human hepatocytes but not uninfected human liver (left top panel) or BALB/c mouse liver (right top panel). FISH analyses were done in livers of control mAb (left bottom panel) or CLDN1-specific mAb (right bottom panel)-treated chimeric uPA-SCID mice. Red arrowheads indicate HCV+ cells. Scale bar, 50 µm. (**b**) Quantification of the percentage of infected cells in the liver of uninfected chimeric mice (black circles) or HCV Jc1-infected mice treated either four times with control mAb (gray triangles), or once (blue diamonds) or four times (blue circles) with CLDN1-specific mAb. Livers were collected 1 week after the last antibody injection. The quantification was performed after confocal microscopy acquisition. ns, not significant. $*P < 0.05$, $**P < 0.01$, $****P < 0.0001$ (Kruskal-Wallis test).

These studies show that HCV infection activated MAPK signaling and CLDN1-specific mAb partially reversed this virus-dependent signaling event and did so without inhibiting ERK activation by the physiological EGFR ligand EGF (**Fig. 3g**,**h** and **Supplementary Fig. 13c**). These data suggest a model in which the anti-CLDN1 mAb impairs HCV-induced cross-activation of EGFR and ERK by interfering with CD81-CLDN1 association (**Fig. 3i**). Because MAPK signaling has been shown to be relevant for the HCV life cycle, including viral entry[24,25], it is likely that this interference contributes to the ability of the antibody to clear an established infection.

Finally, we performed mechanistic studies in chimeric mice to study the consequences of the observed mechanistic findings for viral clearance *in vivo*. Using fluorescence *in situ* hybridization (FISH) with HCV- and human GAPDH-RNA-specific probes (**Fig. 4a**), we demonstrate that the CLDN1-specific mAb results in a dose- and time-dependent decrease in the number of HCV-infected cells leading to their final elimination (**Fig. 4b**). The decline of HCV-infected hepatocytes paralleled the decline in peripheral HCV RNA levels, showing a monophasic decline (**Fig. 4b** and **Supplementary Fig. 14**). Notably, the baseline frequency of HCV-infected hepatocytes (**Fig. 4b**) in the chimeric mouse liver were comparable to those reported in chronically infected human liver[26].

Assuming an inhibition of entry (**Supplementary Fig. 11**) and a negligible effect on viral replication or assembly (**Supplementary Fig. 11f**,**g**), mathematic modeling predicts a time-dependent elimination of virus-infected hepatocytes with concomitant lack of *de novo* infection. Applying this model, we estimated the half-life of infected cells to be ~1.3 days for HCV genotype 2a and 5.4 days for HCV genotype 4, which is in the range of hepatocyte half-lives reported in patients[27]. Indeed, HCV has been reported to promote apoptosis[28] (**Supplementary Fig. 15**). During serum HCV RNA decline, virus-infected hepatocytes are constantly replaced by noninfected hepatocytes resulting in viral control and clearance. This model is supported by both FISH analyses of liver tissue sections (**Fig. 4**) and cell culture studies where the CLDN1-specific mAb eliminates HCV-infected cells (**Supplementary Fig. 15c**,**d**). The different half-lives of HCV-infected cells and the magnitude of virus-induced cell death may explain the varying magnitude of the decline in viral load for diverse HCV strains (**Supplementary Figs. 5a** and **14**)[27].

Collectively, we demonstrate that a host-targeting entry inhibitor clears chronic viral infection (**Fig. 2c–e**). Our results show that viral entry and spread are required for persistent infection *in vivo* and that CLDN1 is a valid target to prevent and treat chronic HCV infection. The interaction of the mAb with nonjunctional pools of CLDN1 (**Fig. 1b**,**c**) that complex with the viral co-receptor CD81 (**Supplementary Fig. 11a**) may explain the non detectable toxicity (**Supplementary Figs. 6a–f**, **8c–g** and **9** and **Supplementary Tables 2** and **3**).

In summary, therapeutic administration of a CLDN1-specific mAb demonstrates activity against several HCV genotypes (**Fig. 2** and **Supplementary Figs. 5** and **11b**) without detectable toxicity (**Supplementary Figs. 6**,**8**,**9** and **Supplementary Tables 2** and **3**). The absence of detectable viral resistance (**Supplementary Fig. 3**) suggests that CLDN1 is an antiviral target with a high genetic barrier for resistance. Although strain-dependent use of CLDN1 and CLDN6 has been described in hepatoma cells[29], our data suggest that CLDN1 is essential for HCV infection *in vivo* and low expression levels of CLDN6 and CLDN9 in human hepatocytes[29,30] (**Supplementary Fig. 4**) appear to preclude a relevant entry function of these molecules *in vivo* in the human chimeric mouse model.

In contrast to clinically licensed direct-acting antivirals, such as HCV protease, NS5A or polymerase inhibitors the CLDN1-specific mAb eliminates viral infection in monotherapy in the state-of-the-art animal model (**Fig. 2** and **Supplementary Fig. 5a**) without detectable resistance (**Supplementary Fig. 3**). These features are likely due to its unique mechanism of action targeting a host factor (**Supplementary Fig. 11a**) that is essential for viral persistence *in vivo* as well as for modulating signaling of virus-infected cells (**Fig. 3**), likely contributing to clearance. Our data suggest that antibodies or small molecules targeting CLDN1 are promising candidates to prevent liver graft infection and to eliminate chronic HCV infection. Finally, similar antimicrobial strategies may be designed for a broad range of pathogens that utilize TJ proteins as host factors including dengue virus, coxsackievirus, adenovirus and shigella[3–5].

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

T.F.B. initiated and supervised the study. T.F.B., E.R., J.A.M., M.N., M.B.Z., M.H.H., R.B., S.P., P.M., P.V. and J.L. designed experiments and analyzed data. L.M., P.A., K.V. and E.R. performed *in vivo* experiments and analyzed data. L.M., F.X., J.L., S.B., G.K.W., P.A., F.H.T.D., D.C., C.L., M.E., I.F., C.D., H.J.H., C.J.M., C.T., E.G., B.C.-W.-M., N.F., M.B.Z. and L.K. performed *ex vivo* and *in vitro* experiments and analyzed data. R.B., P.P. and P.M. provided key reagents. M.D., M.L. and T.V. produced chimeric uPA-SCID mice. L.M., J.L., S.B., M.B.Z., J.A.M., E.R. and T.F.B. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Research experiments on live vertebrates. *In vivo* experiments were performed in the Inserm Unit 1110 animal facility according to local laws and ethical committee approval (CREMEAS, project numbers AL/02/19/08/12 and AL/01/18/08/12).

Human subjects. Human material including liver biopsies and liver tissue from patients undergoing surgical resection was obtained with informed consent from all patients. The respective protocols were approved by the Ethics Committee of the University Hospital of Basel, Switzerland (EKBB 13 December 2004), University of Strasbourg Hospitals, France (CPP 10-17), and the University of Birmingham, UK (LREC, Birmingham, West Midlands, project 04/Q2708/40).

Antibodies. The human CLDN1-specific (OM-7D3-B3, IgG2b, rat[7]), CLDN6-specific (WU-9E1-G2, IgG2b, rat[30]), CLDN9-specific (YD-4E9-A2, IgG2b, rat[30]) mAbs have been described previously and were produced by Aldevron Freiburg. HCV E2-specific AP33 (mouse)[17], E1-specific IGH433 (human)[7], E2-specific CHB-23 (human)[17] mAbs and patient-derived HCV-specific IgG[7] have been described. Control mAb (rat IgG2b clone LTF-2, Bio X Cell), human CLDN1-specific (Life Technologies, rabbit; R&D, rat polyclonal), human CD10-specific (clone 56C6, Vector Laboratories, mouse), human CD81-specific (clone 2s131, mouse), human CK18-specific (clone VP-C414, Vector Laboratories, mouse), Alexa-fluor 488–conjugated IgG rabbit-specific, Alexa-fluor 488–conjugated IgG mouse-specific, Alexa-fluor 488–conjugated IgG rat-specific and Alexa-fluor 594–conjugated IgG mouse-specific Abs (Invitrogen, goat) were used for fluorescent imaging as described[31]. ERK1/2 (rabbit, Cell Signaling), pERK1/2 (Thr202, Tyr204, mouse, clone E10, Cell Signaling) and beta-actin (clone AC-15, mouse, Sigma)-specific antibodies and alkaline–phosphatase (AP)–labeled (goat, GE Healthcare) as well as IRDye 800CW-conjugated goat anti-rabbit and IRDye 680-conjugated goat anti-mouse (Li-Cor Biosciences) secondary antibodies were used for immunoblot analyses. Rabbit anti-rat bridging antibody (Cappel #55704) was used for immunogold labeling.

Cell lines. Huh7.5.1, Huh7.5-GFP, Huh7.5 shCD81.1, 293T, HepG2 DsRed-CD81 and Caco-2 cells were cultured as described[9,32–35].

Imaging of CLDN1 in human and chimeric livers. Chimeric mouse and human liver samples were formalin fixed and paraffin embedded. Human liver tissue was obtained from patients with HCV undergoing liver transplantations or from normal livers taken from surplus donor tissue used for reduced size transplantation. Informed consent from each patient was obtained with regional ethics committee approval (project number 04/Q2708/40). Sections were subjected to low-temperature antigen-retrieval pretreatment, followed by fluorescent detection of CLDN1, CD10 or CK18, as previously described[31]. Labeled sections were visualized using laser scanning confocal microscopy (Zeiss LSM 780, 100 x Plan Apochromat 1.4NA oil immersion objective). Background and autofluorescence of tissue samples were corrected throughout and 3D composite images were generated using the Zeiss Zen analysis software.

TEM and immunogold labeling of CLDN1 *in vivo*. Human liver–chimeric mice were injected with 500 µg control or CLDN1-specific mAb and euthanized 5 h later. Mice were perfused with PBS for 3 min through portal vein infusion followed by 5 min perfusion with 4% electron microscopy (EM)-grade paraformaldehyde (PFA). Livers were harvested, minced in 3-mm pieces and fixed in 4% PFA at room temperature for 24 h. Subsequently liver pieces were placed in PBS and kept at 4 °C before processing for EM. Prior to freezing in liquid nitrogen the tissue were infiltrated with 2.3 M sucrose in PBS containing 0.2 M glycine for 15 min. Frozen samples were sectioned at −120 °C, the sections were transferred to formvar-carbon-coated copper grids. Grids were floated on PBS at room temperature (RT) until the immunogold labeling was carried out at RT on a piece of parafilm using control or CLDN1-specific mAb, rabbit anti-rat bridging antibody and 10 nm (for CLDN1-specific mAb) or 15 nm (for isotype control mAb) protein-A gold. Contrasting and embedding of the labeled grids was carried out on ice in 0.3% uranyl acetate in 2% methyl cellulose for 10 min. Grids were examined in a JEOL 1200EX TEM and images were recorded with an AMT 2k CCD camera.

CLDN1 staining on polarized HepG2 cells. HepG2 DsRED-CD81 cells were grown on 13-mm diameter borosilicate glass coverslips at 4×10^4 cells/coverslip (Fisher Scientific, UK). Laser scanning confocal microscopy (LSCM) was performed on a Zeiss Meta Head Confocal Microscope with a 63× water immersion objective. For live cell receptor staining of CD81 and CLDN1 in HepG2 DsRED-CD81 cells, cells were washed twice with PBS before blocking in PBS-BSA-0.01% sodium azide (Sigma, UK) for 15 min. Cells were incubated with the following primary antibodies: anti-CD81 (1 µg/ml) and anti-CLDN1 (1:200) antibodies in PBS-BSA-azide for 30 min at 37 °C. Cells were washed 3× in PBS before fixing with 3.6% PFA for 30 min. Cells were washed 3× with PBS before addition of a goat anti-mouse secondary antibody (Alexa 488, Invitrogen) at a 1:1,000 dilution in PBS-BSA-azide or a goat anti-rat secondary antibody (Alexa 488, Invitrogen) for 30 min at 37 °C. Cells were then washed with PBS before mounting and visualization.

Human liver-chimeric mice. Human liver–chimeric mice were produced by transplanting cryopreserved human hepatocytes into the spleen of 3-week-old homozygous urokinase-type plasminogen activator-transgenic severe combined immunodeficiency-beige (uPA-SCID-bg) mice[6,36] of both sexes. Human hepatocytes were purchased from Becton Dickinson or isolated as described[7,9].

***In vivo* experimentation.** For prevention studies, chimeric uPA-SCID mice were intraperitoneally injected with 500 µg CLDN1-specific or control mAb at day −1 before and days 1 and 5 after inoculation of a genotype 1b or 4 HCV-infected serum. HCV infection was performed as previously described[6]. For treatment studies, chimeric uPA-SCID mice were chronically infected with HCVcc of prototype strain Jc1 (ref. 15), a genotype 1b/2a chimeric VL-JFH1 (ref. 17) or genotype 2a and 4 serum. Chronically infected mice received 500 µg CLDN1-specific or control mAb once a week for 4 weeks, except for genotype 4 where mice received 500 µg twice a week for 3 weeks followed by 200 µg twice a week up to the end of the study period. Blood was harvested by retro-orbital puncture under general anesthesia. Mice were randomly assigned to the different experimental groups by a blinded technician. Experiments were performed in the Inserm Unit 1110 animal facility according to local laws and ethical committee approval (AL/02/19/08/12 and AL/01/18/08/12).

Viral load. Viral load was quantified by a blinded technician using Abbott RealTime HCV assay (Abbott). The linear range of the assay is 12 IU/ml to 10^8 IU/ml, the limit of quantification (LOQ) of HCV RNA is 12 IU/ml. Given a mouse serum dilution of 1:100 in PBS, LOQ is 1,200 UI/ml, i.e., 5,160 copies/ml.

Analysis of CLDN1-specific mAb serum concentrations. Serum antibody concentrations were quantified using a rat IgG2b antibody-specific enzyme-linked immunosorbent assay (ELISA) (ref. E110-111, Bethyl Laboratories, Euromedex) following the recommendations of the supplier.

Liver biopsies. Eligible patients were identified by a systematic review of patient charts at the Hepatology outpatient clinic of the University Hospital of Basel, Switzerland. The protocol was approved by the Ethics Committee of the University Hospital of Basel, Switzerland. Written informed consent was obtained from all patients. Histopathological grading and staging of the HCV liver biopsies according to the Metavir classification system was performed at the Pathology Institute of the University Hospital Basel and are summarized in **Supplementary Table 3**. All patients that donated liver tissue were male between 27 and 61 years old and female between 39 and 62 years old (**Supplementary Table 4**). Liver biopsy tissues from patients were analyzed as described[37]. In brief, liver tissue was lysed in 100 mM NaCl, 50 mM Tris pH 7.5, 1 mM EDTA, 0.1% Triton X-100, 10 mM NaF, 1 mM PMSF, and 1 mM sodium ortho-vanadate during mechanical homogenization followed by 20 min incubation on ice. Lysates were cleared by centrifugation at 4 °C and 21,000*g* for 5 min before immunoblot analysis of 5 µg protein per sample.

For *ex vivo* stimulation, fresh liver biopsies were divided in two equal pieces and incubated for 4 h at 37 °C in DMEM medium supplemented with 10% FCS and 100 µg/ml control or CLDN1-specific mAb, respectively.

Analysis of protein expression and phosphorylation. Immunoblots of cell lysates using ERK1/2-specific antibodies were performed using Hybond–P membranes and visualized using ECF substrate (GE Healthcare), according to the manufacturer's protocol. Fluorescence emission was detected using Typhoon Trio high-performance fluorescence scanner (GE Healthcare) and quantified using Image Quant TL software (GE Healthcare). Phospho-array analysis was performed using Proteome Profiler Human Phospho-kinase Array (R&D Systems) as described by the manufacturer. For imaging, blots were incubated with ECL (GE Healthcare) and exposed to ECL Hyperfilm (GE Healthcare). Phospho-kinase array results were quantified by integrating the dot blot densities using ImageJ software (NIH). Immunoblots from biopsies were analyzed as described[24].

Inhibition of HCV infection by protein kinase inhibitors. Huh7.5.1. cells were plated in 96-well plates (2×10^3 cells/well) and infected 24 h later for 3 days with HCVcc Luc-Jc1. Medium was then removed and replaced with medium containing 10 µM erlotinib (LC Laboratories) or UO126 (Calbiochem) and final concentration of 1% DMSO. Control medium contains only 1% DMSO. Three days later, HCV replication was quantified by luciferase activity measurement.

EGF-induced MAPK activation. Huh7.5.1 cells were serum starved for 4 h before 1 h incubation with 100 µg/ml control or CLDN1-specific mAb and 15-min incubation with increasing concentrations (1, 10 and 100 ng/ml) of EGF. p-EGFR, p-ERK1/2 and total ERK were detected by western blot analysis. Quantification was performed using a Typhoon Trio laser scanner and ImageQuant Software (GE Healthcare).

FISH analyzes of human chimeric livers. Liver samples from mice were collected during necropsy, immediately embedded in optimal cutting temperature compound (OCT) and frozen in liquid nitrogen chilled 2-methylbutane. Tissues were then stored at −80 °C until use. Sections (10 µm) were cut at cryostat (Leica), fixed overnight in 4% formaldehyde at 4 °C and hybridized, as previously described[26], with the following modifications. Briefly, tissue sections were pretreated by boiling (90–95 °C) in pretreatment solution (Affymetrix – Panomics) for 1 min, followed by a protease QF (Affymetrix – Panomics) digestion for 10 min at 40 °C. Hybridization was performed using probe sets against JFH-1 HCV RNA (target region 4513-6253) and against human GAPDH mRNA (VA6-12786-06, Affymetrix-Panomics). Pre-amplification, amplification and detection were performed according to provider's protocol. Images were acquired with a LSCM (LSM710, Carl Zeiss Microscopy) and Zen2 software, using the same settings for all the tissues analyzed. Five to seven random fields were selected from each section, based only on the presence of human GAPDH signal to ensure the presence of human hepatocytes. Images were then acquired also including the channel for the HCV detection. Image analysis was performed using ImageJ and CellProfiler software, with a customized pipeline. Total number of cells, number of human hepatocytes (HH), number of HCV^+ HH and HCV signal average intensity (and s.d.) were evaluated.

Statistical analyses. For *in vivo* and *ex vivo* data, the one-way ANOVA followed by Tukey's *post-hoc* test or the Kruskal-Wallis followed by Dunn's *post hoc* test were used after determination of distribution by the Shapiro-Wilk normality test. The Wilcoxon rank test as well as the two-tailed Mann-Whitney test were also used. The *in vitro* data are presented as the mean ± s.d. except where mean ± s.e.m. is indicated, and were analyzed by the unpaired Student's *t*-test or the two-tailed Mann-Whitney test as indicated. $P \leq 0.05$ was considered significant. Statistical analyzes were performed with GraphPad Prism 6 software.

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ORIGINAL ARTICLE

miR-135a-5p-mediated downregulation of protein tyrosine phosphatase receptor delta is a candidate driver of HCV-associated hepatocarcinogenesis

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ABSTRACT

Background and aims HCV infection is a leading risk factor of hepatocellular carcinoma (HCC). However, even after viral clearance, HCC risk remains elevated. HCV perturbs host cell signalling to maintain infection, and derailed signalling circuitry is a key driver of carcinogenesis. Since protein phosphatases are regulators of signalling events, we aimed to identify phosphatases that respond to HCV infection with relevance for hepatocarcinogenesis.

Methods We assessed mRNA and microRNA (miRNA) expression profiles in primary human hepatocytes, liver biopsies and resections of patients with HCC, and analysed microarray and RNA-seq data from paired liver biopsies of patients with HCC. We revealed changes in transcriptional networks through gene set enrichment analysis and correlated phosphatase expression levels to patient survival and tumour recurrence.

Results We demonstrate that tumour suppressor protein tyrosine phosphatase receptor delta (PTPRD) is impaired by HCV infection in vivo and in HCC lesions of paired liver biopsies independent from tissue inflammation or fibrosis. In liver tissue adjacent to tumour, high PTPRD levels are associated with a dampened transcriptional activity of STAT3, an increase of patient survival from HCC and reduced tumour recurrence after surgical resection. We identified miR-135a-5p as a mechanistic regulator of hepatic PTPRD expression in patients with HCV.

Conclusions We previously demonstrated that STAT3 is required for HCV infection. We conclude that HCV promotes a STAT3 transcriptional programme in the liver of patients by suppressing its regulator PTPRD via upregulation of miR-135a-5p. Our results show the existence of a perturbed PTPRD–STAT3 axis potentially driving malignant progression of HCV-associated liver disease.

Significance of this study

What is already known on this subject?

- Chronic HCV infection is a leading cause of hepatocellular carcinoma (HCC); HCC risk remains elevated even after viral clearance.
- The mechanisms contributing to HCC development are poorly understood.
- HCV requires host cell signalling including the STAT3 pathway to maintain infection.
- Protein tyrosine phosphatase receptor delta (PTPRD) suppresses tumour by controlling STAT3 activity.

What are the new findings?

- PTPRD expression is impaired in hepatocytes of HCV-infected liver tissues and in HCC lesions.
- PTPRD expression in liver tissue of patients with HCC is associated with STAT3 transcriptional activity, patient survival and tumour recurrence after surgical resection.
- STAT3-mediated transcriptional programme is enriched in HCV-infected livers.
- miR-135a-5p is strongly upregulated in livers of patients with HCV and is a regulator of PTPRD mRNA.

How might it impact on clinical practice in the foreseeable future?

- HCV infection promotes the activity of its cofactor STAT3 by suppressing its negative regulator PTPRD via miR-135a-5p. Our model suggests the existence of a perturbed PTPRD–STAT3 axis driving malignant progression of liver disease. This finding is of further clinical relevance since it provides a target for urgently needed HCC chemoprevention.

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INTRODUCTION

More than 150 million people worldwide are infected by HCV,[1] which is a leading cause of liver cirrhosis and hepatocellular carcinoma (HCC).[2] The arrival of highly effective new therapies consisting of direct-acting antivirals can cure the vast majority of patients,[3] but those with advanced liver disease remain at risk for developing HCC even after viral clearance.[4] Moreover, the exact mechanisms responsible for this increased

susceptibility remain only partly understood. Traditionally, carcinogenesis has been attributed to a multistep accumulation of genetic damage resulting in gain of function by proto-oncogenes and inactivation of tumour suppressor genes.[5] Only a minority of these driver genes is frequently involved in cell transformation, while the majority is only occasionally affected.[6] In other words, a myriad of combinations in genetic and epigenetic alterations can lead to cancer, and as a consequence, every tumour displays a heterogeneous molecular profile, further clouding understanding of cancer development. In chronic hepatotropic virus infections such as hepatitis C and B, the picture is even more complicated as both viral and non-viral factors are the drivers of hepatocarcinogenesis.[7 8] Nonetheless, the complexity of malignant transformation can be reduced to a handful of logical underlying principles, of which derailed signalling circuitry is a key component.[9] We have previously demonstrated that cellular receptor tyrosine kinase (RTK) signalling is involved in regulating HCV entry into hepatocytes and that the virus binding to the hepatocytes triggers RTK activation.[10 11 12] This indicates that chronic HCV infection modulates host signalling patterns that may contribute to the development of virus-induced liver disease. Since these signalling processes are tightly regulated by protein phosphatases and aberrant phosphatase expression is involved in various syndromes and diseases,[13] we screened for disease-relevant phosphatase expression in liver biopsies of chronic patients with HCV.

MATERIALS AND METHODS

Liver tissue, cells and virus

Human needle liver biopsies and liver tissue from patients undergoing surgical resection were collected at the Gastroenterology and Hepatology outpatient clinic of the Basel University Hospital, Switzerland, the Centre Hospitalier Universitaire de Reims, France and the Hôpitaux Universitaires de Strasbourg, France. Protocols for patient tissue collection were reviewed and approved by the ethics committees of the respective university hospitals. Written informed consent was obtained from all patients. Eligible patients were identified by a systematic review of patient charts at the hepatology centres of the university hospitals of Basel, Reims and Strasbourg. Histopathological grading and staging of the HCV liver biopsies, according to the METAVIR classification system, were performed at the pathology institutes of the respective university hospitals. All the patients that donated liver tissue are summarised in online supplementary table S1. Liver biopsy tissues were analysed as described.[14] Mouse tissue was obtained from C57BL/6J mice. Tissues were lysed and subjected to quantitative PCR (qPCR) and immunoblot analysis (described below). Primary human hepatocytes (PHH) were isolated and cultured as previously described.[11] Huh7.5.1, HEK293T cells and cell culture derived HCV (HCVcc) strains Luc-Jc1 and Jc1E2^{FLAG} have been described.[11 12 15] Jc1E2^{FLAG} was affinity-purified as described.[15] Huh7.5.1 were infected with Jc1E2^{FLAG} as described.[16]

Patient cohorts

Protein tyrosine phosphatase receptor delta (PTPRD) expression was correlated with corresponding clinical data in three independent patient cohorts from Icahn School of Medicine at Mount Sinai, New York, USA (National Centre for Biotechnology Information Gene Expression Omnibus GSE10140) (cohort A),[17] from the Hiroshima University Hospital, Hiroshima, Japan (European Genome-phenome Archive, https://ega.crg.eu, accession number, EGAD00001001880) (cohort B)[18] and from the University Hospitals of Strasbourg and Reims, France (cohort C).

Antibodies and western blotting

P-STAT1 mAb (58D6) was obtained from Cell Signaling Technology, β-Actin mAb (AC-15) was obtained from Invitrogen and PTPRD pAb (C-18) from Santa Cruz Biotechnology. Western blots were performed using Hybond-P membranes (GE Healthcare), visualised using ECF substrate (GE Healthcare) and quantified with a fluorescence scanner (Typhoon Trio, GE Healthcare).

Analysis of mRNA and miRNA expression

mRNA expression of 84 disease-relevant protein phosphatases was assessed in six liver biopsies from patients with chronic HCV infection and in six non-infected biopsies using qPCR (Human Protein Phosphatases RT^2 Profiler PCR Array, Qiagen). Total RNA from Huh7.5.1 cells, PHHs and liver tissue was extracted using RNeasy Mini Kit (Qiagen) or Trizol (Life Technologies). Gene expression in the total RNA extracts was assessed using two-step qPCR. The reverse transcription on total RNA extract was made using Maxima reverse transcriptase (Thermo Scientific). qPCR for detecting PTPRD, radical S-adenosyl methionine domain containing 2 (RSAD2), ubiquitin-specific peptidase 18 (USP18) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed with RT^2 qPCR Primer Assays (Qiagen) using Real-Time PCR ABI Prism 7500 (Thermo Scientific) or Corbett Rotor Gene 6000 (Qiagen). All qPCRs were performed following manufacturers' instructions. Specificities of phosphatase PCR products were validated by melting curve analysis and PCR product sequencing. Differential gene expression of patient biopsies was calculated after $2^{-\Delta CT}$ transformation into individual data points. The transcriptome of Jc1E2^{FLAG}-infected Huh7.5.1 cells was measured by RNA-seq at the Broad Institute as previously described.[19] All microRNA (miRNA) indicated in this study are human (hsa-miR) if not indicated differently. miRNA expression was measured using miScript (Qiagen) with forward DNA primers derived from the miRNA sequence implemented in Sanger miRBase database (V.21.0) and referenced with a MIMAT accession number (see online supplementary table S2). miRNA target sites were predicted using miRSystem.[20] Synthetic miRNAs (mimics) of miR-135a-5p and the non-targeting negative control miRNA cel-miR-67 (miR-CTRL) were obtained from GE Healthcare.

Luciferase reporter assay

The 3′-untranslated region (3′UTR) of PTPRD was amplified with 5′-TTT CTC GAG CTT TGA CCA CTA TGC AAC GTA G-3′ and 5′-TTT CTC GAG CTG TCC TCG CCG TTT TCT AA-3′, and subcloned in psiCheck-2 (Promega) using *Xho*I. As specificity control, the seed sequence AGCCAT of two miR-135a-5p target sites on the 3′UTR of PTPRD was replaced by AAAAAA using site-directed mutagenesis. An amount of 150 ng luciferase reporter plasmid was cotransfected with 10 nmol/L miRNA in HEK293T cells using Lipofectamine 2000 (Thermo Fisher Scientific). Activity of *Renilla* and firefly luciferase was assessed 48 hours post transfection using Dual-Luciferase Reporter Assay (Promega) and a Mithras LB940 plate reader (Berthold Technologies).

FISH analysis

Human needle liver biopsies were collected, immediately embedded in optimal cutting temperature compound and frozen

in liquid nitrogen chilled 2-methylbutane. Tissues were then stored at −80°C until use. Sections (10 μm) were cut at cryostat (Leica) and mounted onto Superfrost Plus Gold glass slides (K5800AMNZ72, Thermo Fisher Scientific), fixed overnight in 4% formaldehyde at 4°C and hybridised, as previously described,[21] with the following modifications: tissue sections were pretreated by boiling (90°C–95°C) in pretreatment solution (Panomics, Affymetrix) for 1 min, followed by a protease digestion for 10 min at 40°C. Hybridisation was performed using probe sets against patient-specific HCV RNA sequence (type 1) and against human PTPRD mRNA (NM_002839, target region 4754-5835). Preamplification, amplification and detection were performed according to provider's protocol. Images were acquired with a laser scanning confocal microscope (LSM710, Carl Zeiss Microscopy) and Zen2 software, using the same settings for all the tissues analysed. Five random fields were acquired from each section. Image analysis was performed using ImageJ and CellProfiler software, with a customised pipeline. Total number of cells, frequency of HCV-positive and PTPRD-positive cells, and signal intensity were then evaluated and exported for statistical analysis (two-way analysis of variance (ANOVA), $p \leq 0.05$).

Bioinformatics of gene expression database

Paired gene expression of 62 formalin-fixed paraffin-embedded tumour and adjacent liver tissues was explored from cohort A[17]). Downregulation of PTPRD expression in tumour tissue was analysed *in silico* for 46 patients with chronic HCV. Values of p were calculated with Wilcoxon signed-rank test (two-tailed). Probability of survival and tumour recurrence were evaluated by the log-rank test available on the GenePattern Survival Analysis module (http://www.broad.mit.edu/cancer/software/genepattern).[22] Gene Set Enrichment Analysis (GSEA) was performed as described.[23 24] The normalised enrichment score (NES) is a measure expressing to what extent the members of a gene set shift towards the top or bottom in the gene list ranked on differences in expression between two phenotypes. A false discovery rate (FDR) smaller than 0.25 is generally considered an appropriate cut-off. The GSE10143 database comprised 78 of the 87 response genes designated as the Hallmark_IL6 JAK STAT3 signalling gene set.[25]

RESULTS

Chronic HCV infection impairs PTPRD expression in vivo

To study the impact of chronic HCV infection on phosphatase expression in patient liver tissue, we extracted RNA from six biopsies of patients with chronic hepatitis C (CHC) and six non-infected biopsies (see online supplementary table S1), and quantified expression of 84 disease-relevant protein phosphatases using qPCR. We identified 24 phosphatases that were significantly ($p<0.01$, U-test) deregulated in HCV-infected tissue compared with non-infected biopsies (figure 1A). Interestingly, among the phosphatases with most deregulated expression levels in HCV biopsies, we observed an enrichment of candidates with potential relevance for the development of HCC. More particularly, some of these phosphatases act as tumour suppressors in various cancers including PTPRD. PTPRD is frequently inactivated and mutated in human cancers[26 27] including HCC.[28] In order to validate HCV-induced PTPRD downregulation, we measured mRNA expression in a second sample series of 24 liver biopsies of patients with chronic HCV and validated that PTPRD mRNA expression is significantly ($p=0.0003$, U-test) downregulated compared with 11 additional non-viral control biopsies (figure 1B).

PTPRD expression in patients with HCV is independent from fibrosis or inflammation

Since biological circuits in liver tissues from patients with CHC reflect a complex interaction of different cell types integrating an inflammatory immune response, we first compared phosphatase expression levels with METAVIR grading and staging data (see online supplementary table S1). No significant relationship between METAVIR score and PTPRD expression was observed in 30 liver biopsies (figure 1C, D), suggesting that PTPRD expression is independent from the degree of inflammation and fibrosis of the studied biopsies and rather susceptible to a more direct virus-induced mechanism within infected hepatocytes.

PTPRD expression is impaired in HCV-infected hepatocytes

To identify the cell type displaying impaired PTPRD expression levels upon HCV infection, we applied an established protocol for multiplex fluorescent in situ hybridisation (FISH) analysis of liver biopsies at single cell resolution.[21] This strategy enabled us to distinguish HCV-infected cells from uninfected cells in liver biopsies. Simultaneous hybridisation with specific probe set for PTPRD mRNA demonstrated that PTPRD was significantly and specifically ($p<0.05$, two-way ANOVA) impaired in infected cells compared with uninfected cells in three biopsies from different patients infected with HCV genotype 3a (see figure 1E, online supplementary table S1 and figure S1). Differential PTPRD expression was detected in cells with hepatocyte morphology. The analysed fields did not show an abnormal level of infiltrating non-parenchymal cells, and the level of PTPRD expression in those cells was marginal compared with hepatocytes (data not shown). This indicates that HCV impairs PTPRD expression directly in hepatocytes. To validate this observation, we measured PTPRD expression levels in isolated PHH infected in vitro with HCV. We observed that PHH robustly express PTPRD both at the mRNA and the protein level, providing a competent model system to study PTPRD. Two days after isolation, PHH were infected for 5 days with HCVcc strain JFH1, after which cells were lysed, and both mRNA and protein were isolated. With this established protocol, up to 10%–30% of hepatocytes can be infected by HCVcc (strain JFH1) as shown[29] (see online supplementary figure S2) using a reporter red fluorescence protein–nuclear localisation sequence–interferon-β promoter stimulator (RFP-NLS-IPS)[30] HCV-infected PHH from different donors showed a significant decrease ($p=0.015$, U-test) in PTPRD mRNA expression (figure 2A), which also translated into a decreased PTPRD protein level as detected by immunoblot ($p=0.016$, U-test) (figure 2B, C). To exclude that PTPRD downregulation was caused by an antiviral response mounted by immunocompetent PHH, we treated PHH for 5 days with interferon-α (IFN-α) (figure 2D). No change in PTPRD protein levels was observed by western blot for different concentrations of IFN-α, while STAT1 phosphorylation was properly induced. In parallel, interferon response genes *USP18* and *RSAD2* displayed an increase in mRNA transcription after IFN-α stimulus, while PTPRD remained at baseline levels (figure 2E). These data confirm that PTPRD downregulation is independent from the innate immune response.

miR-135a-5p is a mechanistic regulator of PTPRD expression in HCV-infected hepatocytes

The miRNA machinery is a regulator of gene expression and is exploited by HCV to maintain its replication.[31 32] We applied multiple bioinformatical algorithms[20] scanning for human miRNAs potentially targeting PTPRD mRNA. Combining

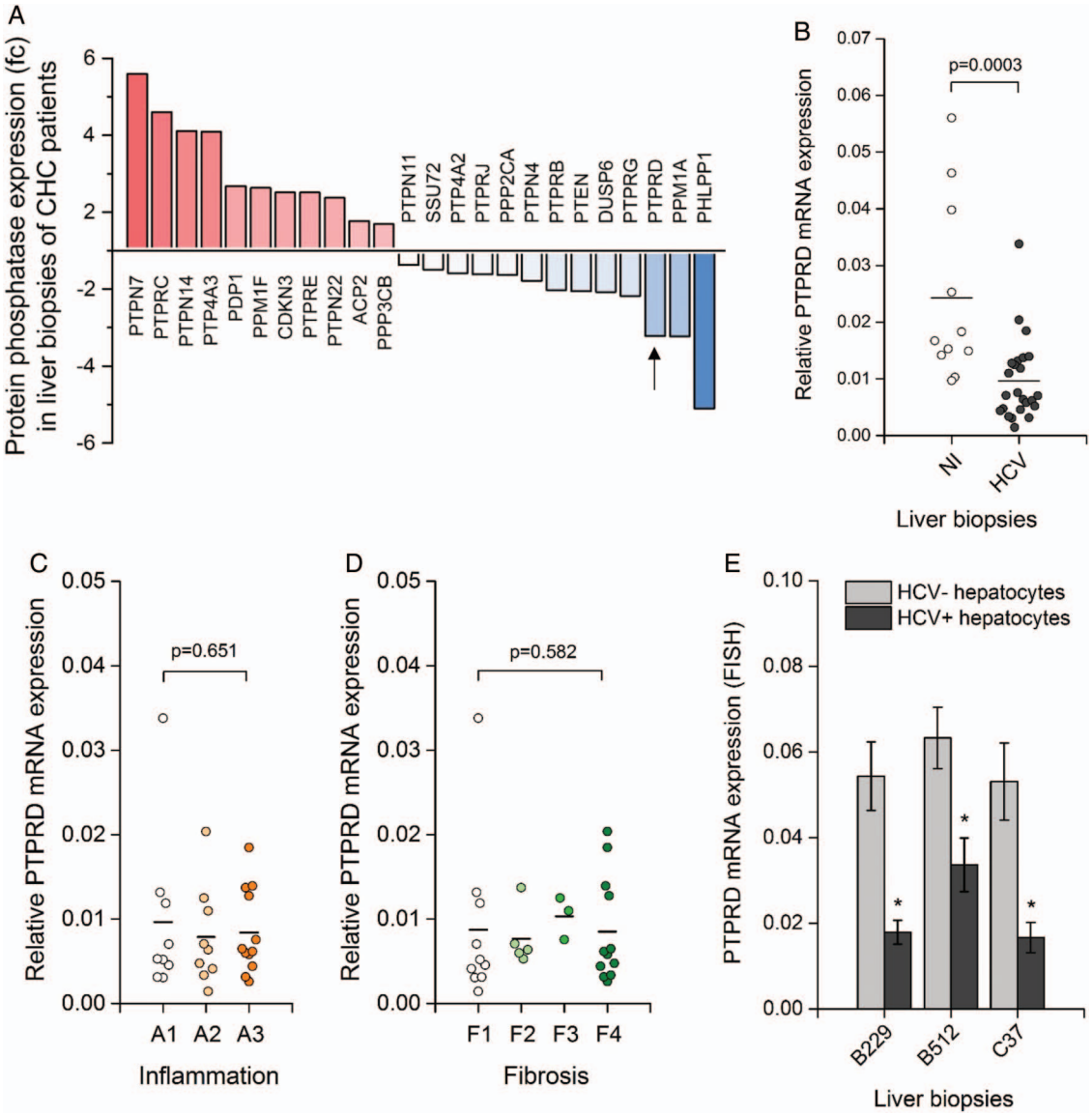

Figure 1 Protein tyrosine phosphatase receptor delta (PTPRD) expression is significantly impaired in livers of patients with chronic hepatitis C (CHC). Expression of 84 protein phosphatases associated with diseases and syndromes was quantified in needle stick biopsies using qPCR and in situ hybridisation of RNA probes. (A) mRNA expression of 11 phosphatases was significantly ($p<0.01$, U-test) increased (red), and expression of 13 phosphatases was significantly ($p<0.01$, U-test) decreased. Data are expressed as fold change (fc) phosphatase expression of non-HCV (n=6) and HCV-infected biopsies (n=6) relative to the average expression levels of five housekeeping genes (*actin, B2M, GAPDH, HPRT1, RPLP0*). Arrow indicates tumour suppressor PTPRD. (B) Expression of PTPRD is significantly impaired in liver biopsies of patients with chronic HCV ($p=0.0003$, U-test). Validation of HCV-induced downregulation of PTPRD compared with non-infected biopsies (NI) observed in (A) in additional liver biopsies from patients infected with HCV (see online supplementary table S1). Data are expressed as PTPRD mRNA expression relative to GAPDH and visualised as individual data points and median (line). (C and D) Expression of PTPRD is independent from fibrosis and inflammation in liver tissues. PTPRD expression was correlated with METAVIR score of the HCV-infected liver biopsies, but no significant correlation ($p>0.5$, U-test) between METAVIR grading (C) or staging (D) of the studied biopsies in (A) and (B) (see online supplementary table S1) was observed. Data are expressed as PTPRD mRNA expression relative to GAPDH and visualised as individual data points and median (line). (E) PTPRD expression is impaired in HCV-infected hepatocytes in liver biopsies. Fluorescent in situ hybridisation (FISH) analysis of liver biopsies infected with HCV genotype 3 by simultaneous hybridisation with HCV-specific and PTPRD-specific probe sets. PTPRD labelling was assessed in five random fields from each section. Data are expressed as mean of PTPRD fluorescence intensities in HCV-positive and HCV-negative cells per patient (±SD, $p<0.05$, two-way analysis of variance).

computational miRNA prediction with observations in HCV-infected Huh7.5.1 cells, we defined a panel of eight miRNAs with two essential characteristics: they potentially target PTPRD and they are upregulated by HCVcc in Huh7.5.1 cells (see online supplementary table S2). To validate the relevance of these eight miRNAs in a real-life setting, we screened for their expression in liver biopsies of patients infected with HCV (figure 3A). This uncovered a striking twofold upregulation of miR-135a-5p in liver specimens of patients with HCV ($p=0.0006$, U-test) and establishes it as a possible PTPRD regulator (figure 3B). Moreover, similar to PTPRD, miR-135a-5p expression is independent from the host antiviral response, as shown by a kinetics study measuring miR-135a-5p in IFN-α stimulated PHH. miR-135a-5p expression remained level ($p=0.5$, U-test), while the interferon response gene *RSAD2* was significantly ($p=0.02$, U-test) upregulated (figure 3C). This suggests that miR-135a-5p does not take part in the innate immune response, but is rather relevant to the HCV life-cycle itself. To

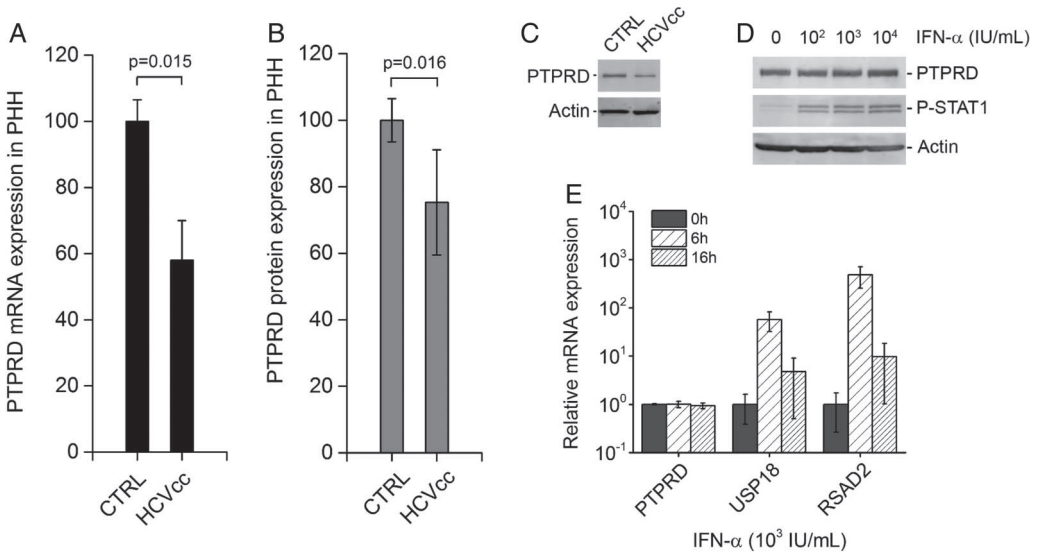

Figure 2 Protein tyrosine phosphatase receptor delta (PTPRD) is significantly impaired by HCV in hepatocytes independent from the innate immune response. (A) PTPRD mRNA expression is significantly impaired in primary human hepatocytes (PHH) after 5 days of infection with HCVcc (strain JFH1). Data are expressed as mean PTPRD expression relative to GAPDH±SEM (p=0.015, U-test; six independent infections of PHH from three donors). (B) PTPRD protein expression is significantly impaired by HCV in PHH. PTPRD and actin expression were assessed by western blotting 5 days after infection of PHH with HCVcc (strain JFH1) compared with non-infected PHH (NI). Band intensities were quantified using Image Quant and are expressed as mean PTPRD expression relative to actin±SEM (p=0.016, U-test; five independent infections of PHH from two different donors). (C) A representative western blot quantified in (B) is shown. (D) PTPRD protein expression is independent from innate immune response. Host antiviral response was simulated by incubation of PHH with interferon-α (IFN-α). Cell culture medium with IFN-α was replaced every day. Cells were lysed after 5 days of incubation, and PTPRD, phospho-STAT1 (p-STAT1) and actin were measured by western blotting. (E) PTPRD is not an IFN-response gene. PHH were incubated for 10 min with 10^3 IU/mL IFN-α, and mRNA expression of PTPRD and the known interferon-response genes *USP18* and *RSAD2* was assessed by RT-qPCR relative to GAPDH 6 and 16 hours post IFN stimulation. CTRL, control.

prove that miR-135a-5p targets PTPRD mRNA, we subcloned the 3′UTR of PTPRD mRNA in the *Renilla* luciferase expression cassette (Luc-3′UTR) of a bicistronic *Renilla*/firefly luciferase reporter construct (psiCheck-2, Promega). The 3′UTR of PTPRD harbours two predicted highly conserved miR-135a-5p target sites (see online supplementary table S2). Cotransfection of a miR-135a-5p mimic with the Luc-3′UTR reporter significantly ($p=3.29\times10^{-4}$, U-test) impaired normalised luciferase activity compared with empty vector, while the repression of luciferase expression was lost when the Luc-ΔmiR-135a-5p reporter with mutated miR-135a-5p binding sites was used (figure 3D). This demonstrates that miR-135a-5p is able to silence PTPRD expression, which is also reflected in vivo where a significant (p=0.04, r=−0.03, one-tailed Spearman's correlation test) inverse correlation of PTPRD mRNA and miR-135a-5p levels could be discerned in liver biopsies studied in figure 3B. Remarkably, high levels of miR-135a-5p always corresponded with low PTPRD expression, while low amounts of miR-135a-5p give PTPRD expression more leeway to vary. Interestingly, some of the studied liver biopsies did neither exhibit high PTPRD mRNA nor miR-135a-5p levels (figure 3E), suggesting the presence of an additional more general regulatory mechanism of PTPRD expression that is independent from HCV. Taken together, these data establish miR-135a-5p as a potent HCV-driven regulatory element for PTPRD expression.

STAT3 transcriptional activity is induced in HCV-infected Huh7.5.1 cells in vitro and associated with attenuated PTPRD expression in vivo

PTPRD is a signal transducer and activator of transcription 3 (STAT3) phosphatase,[27] and therefore dampened PTPRD expression should promote the interleukin 6-Janus Kinase–STAT3 (IL6-JAK–STAT3) signalling axis. STAT3 signalling is essential for liver regeneration[33] and an integral part of the regulation of the host interferon response.[10] Moreover, STAT3 is a cofactor for HCV infection, suggesting an accumulation of STAT3 activity in host cells during HCV infection.[10] Indeed, a temporal GSEA of HCVcc-infected Huh7.5.1 transcriptomes revealed an accumulation of a STAT3 transcriptional signature (Hallmark_IL6 STAT3 signalling gene set[25]) over the first 7 days of infection (figure 4A). However, PTPRD is not expressed in hepatoma cells and cannot be rescued in our hands (data not shown), precluding the in vitro study of PTPRD on the induction of the IL6–JAK–STAT3 signalling cascade. Therefore, we analysed a publicly accessible gene expression database of 82 adjacent non-tumour liver tissue specimens of patients with HCC (cohort A).[17] Those patients with the lowest 20 percentile of hepatic PTPRD expression in adjacent tissue showed a strong and marked enrichment (NES=1.75, FDR=0.009) of the STAT3 transcriptional programme compared with patients with the highest 20 percentile (see figure 4B, C and online supplementary figure S3), which is testimony of a PTPRD-mediated STAT3 deactivation in vivo. For 62 of these 82 patients, HCV infection status was available. We could thus confirm PTPRD expression downregulation in 46 HCV(+) biopsies compared with 16 HCV(−) biopsies (figure 4D) and show the enhanced STAT3 activity in HCV(+) through GSEA (NES=1.96, FDR=0.001) (see figure 4E and online supplementary figure S4). Taken together, these findings demonstrate that transcriptional activity of the oncogene STAT3 is clearly linked to PTPRD in the livers of patients. Because STAT3 activity is associated with HCCs with poor prognosis,[34 35 36] our findings suggest that HCV infection accumulates STAT3 signalling via suppression of its negative regulator PTPRD and as such may contribute to the exacerbation of chronic liver disease.

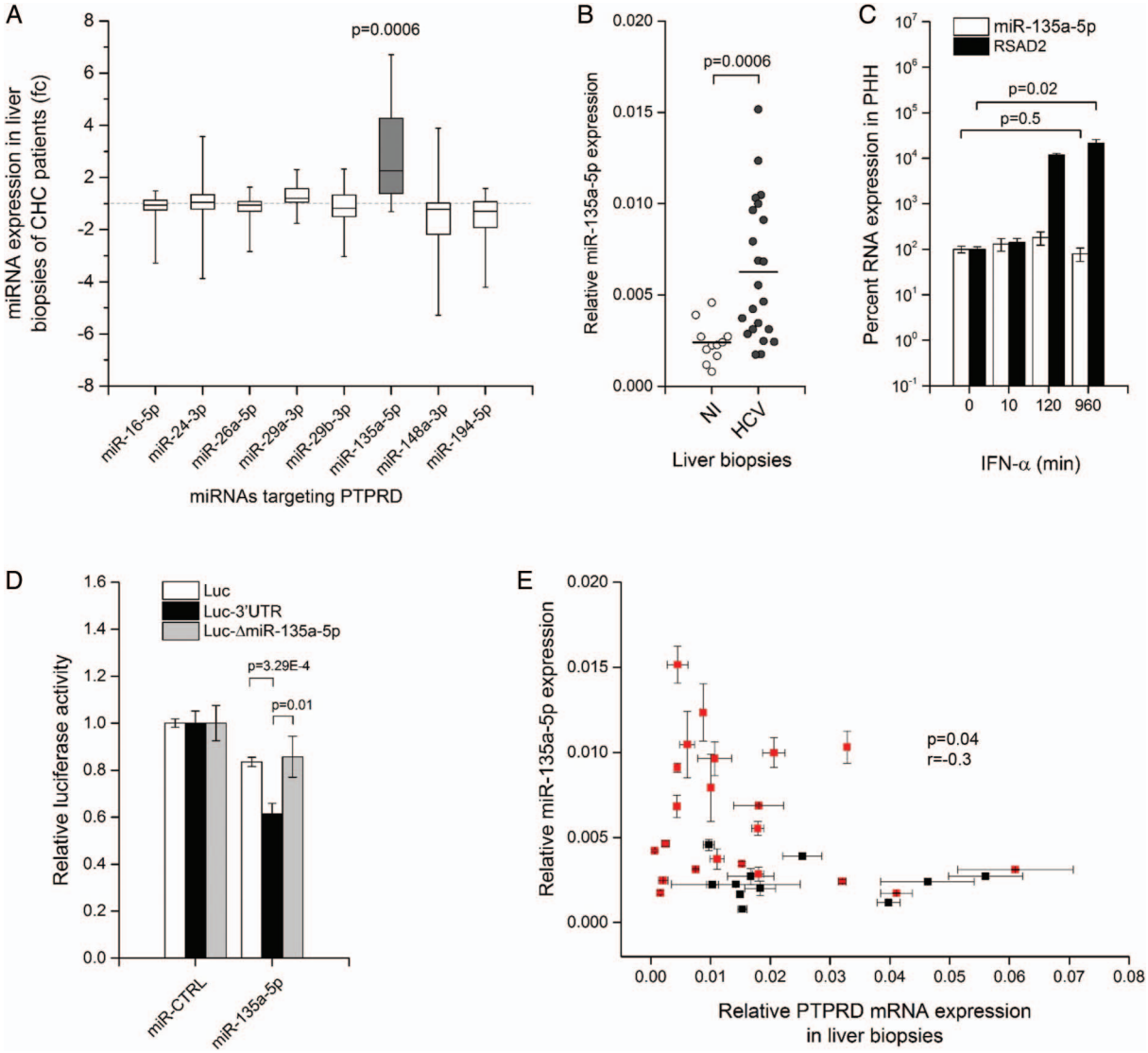

Figure 3 HCV-induced expression of miR-135a-5p mediates protein tyrosine phosphatase receptor delta (PTPRD) silencing. (A) miRNA expression screening of potential regulators of PTPRD mRNA in livers of patients with chronic hepatitis C (CHC). Predicted miRNAs potentially regulating the PTPRD mRNA and that are upregulated by HCV in Huh7.5.1 cells (see online supplementary table S2) were screened in liver biopsies using qPCR. Data are expressed as fold change (fc) miRNA expression relative to SNORD61 and visualised as box-and-whisker plot (box=50% of biopsies, line=median, whiskers=minimal and maximal values) centred to the median expression levels of the miRNA in the corresponding non-infected (NI) liver biopsies. Grey bar highlights miR-135a-5p that is upregulated more than twofold in patients with HCV. (B) miR-135a-5p expression is significantly (p=0.0006, U-test) upregulated in livers of patients with HCV (dark grey circles) compared with NI tissues (open circles). (C) miR-135a-5p is not induced by the antiviral response to HCV infection. Isolated primary human hepatocytes (PHH) were treated over 16 hours with 10^3 IU/mL interferon-α (IFN-α) prior to qPCR analysis of miR-135a-5p and the interferon-stimulated gene *RSAD2*. While interferon-response gene *RSAD2* is significantly (p=0.02, U-test) induced by IFN-α, miR-135a-5p remains level (p=0.5, U-test). (D) miR-135a-5p induces silencing of PTPRD expression. 3′UTR of PTPRD harbouring two miR-135a-5p target sites was subcloned in the *Renilla* luciferase expression cassette of a bicistronic *Renilla*/firefly luciferase reporter plasmid (Luc-3′UTR). As a control (Luc-ΔmiR-135a-5p), the seed sequence AGCCAT in Luc-3′UTR was replaced by AAAAAA. Cotransfection of Luc-3′UTR but not Luc-ΔmiR-135a-5p with a miR-135a-5p mimic significantly ($p=3.29\times10^{-4}$, U-test) impairs *Renilla* luciferase expression in HEK293T cells compared with empty vector (Luc). A minor unspecific effect of miR-135a-5p on empty vector (Luc) was observed. Data are expressed as mean *Renilla* luciferase activity±SEM normalised to firefly luciferase (five independent experiments in triplicate) relative to cotransfection of the vectors with non-targeting miRNA derived from *Caenorhabditis elegans* (miR-CTRL). (E) PTPRD mRNA levels significantly and inversely correlate (p=0.04, r=−0.03, one-tailed Spearman's correlation test) with miR-135a-5p levels in liver biopsies. PTPRD mRNA and miR-135a-5p expression in NI and HCV-infected liver biopsies analysed in (B) were compared. All liver biopsies with high miR-135a-5p levels exhibited low PTPRD mRNA expression and *vice versa*. Liver biopsies from patients infected with HCV are highlighted in red.

PTPRD expression is impaired in HCC and associated with survival of patients with HCC and decreased tumour recurrence after surgical resection

We observed that PTPRD mRNA is only marginally expressed in hepatoma cell lines such as Huh7.5.1. Moreover, it does not translate into detectable protein levels as assessed by western blot in a panel of hepatic cell lines tested including HepG2, Huh7, Huh7.5.1, differentiated HepaRG and non-differentiated HepaRG (data not shown), which suggests that impaired PTPRD is a hallmark of cell transformation. To ascertain whether PTPRD is also downregulated in HCC, we assessed PTPRD protein expression in six paired biopsies of patients

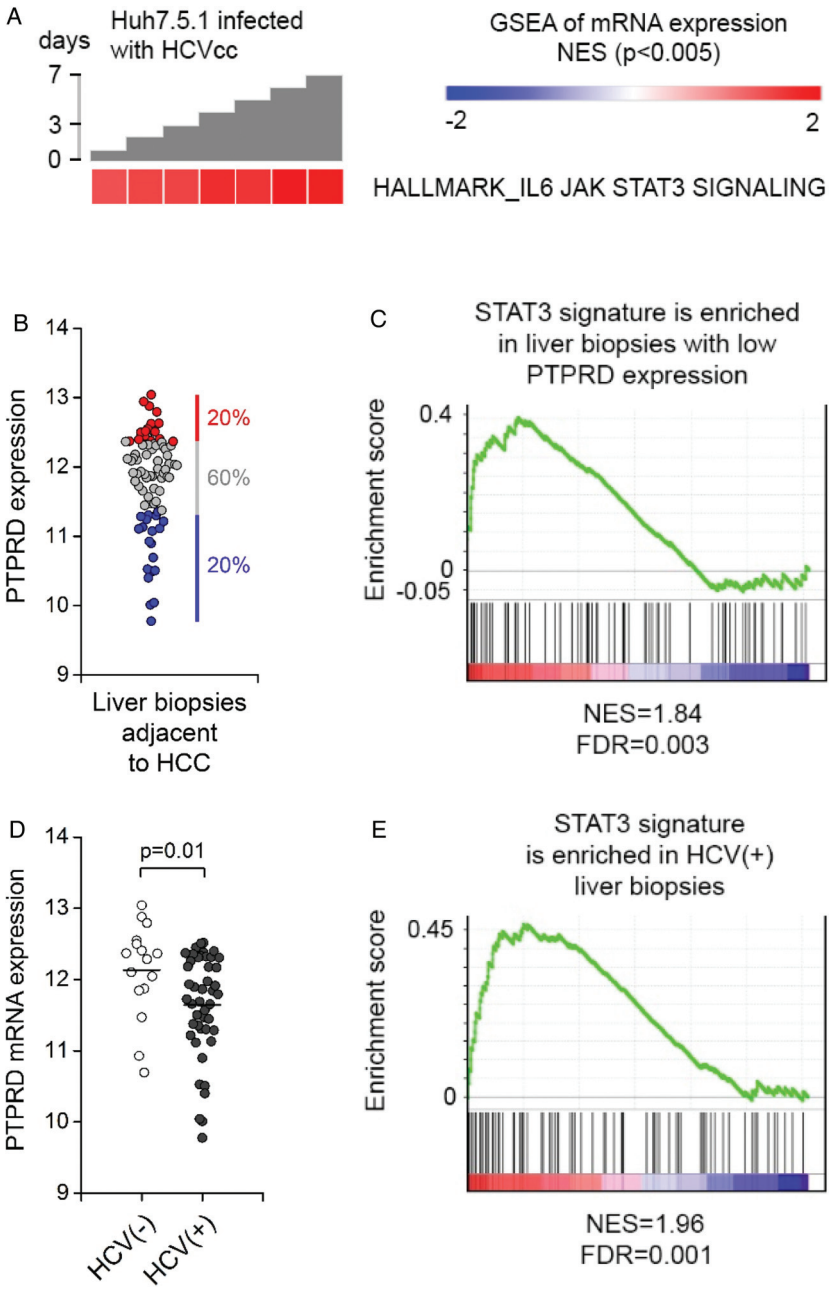

Figure 4 Dampened protein tyrosine phosphatase receptor delta (PTPRD) expression in liver biopsies is associated with an accumulated STAT3 transcriptional programme. (A) HCV infection induces STAT3 transcriptional activity. Huh7.5.1 cells were subjected to infection with affinity-purified HCVcc (Jc1E2^{FLAG}) for up to 7 days. Control cells were mock-infected using FLAG peptide. Cell transcriptome was profiled every day by RNA-seq as previously described,[19] and the modulation of the Hallmark_IL6 STAT3 signalling gene set was assessed by Gene Set Enrichment Analysis (GSEA) in HCV-infected cells as compared with controls. Significant (p<0.005) normalised enrichment scores (NES) of the gene set are indicated as red (positive enrichment) for each day of infection (grey stairs). (B) Patients were classified according to PTPRD expression in tissue adjacent to hepatocellular carcinoma (HCC) into the highest 20 percentile (red), lowest 20 percentile (blue) and intermediate (grey). GSEA of these 82 adjacent liver biopsies (cohort A)[17] revealed (C) a significant enrichment (NES=1.75, false discovery rate (FDR)=0.001) of the Hallmark_IL6 STAT3 signalling gene set (STAT3 signature) in biopsies with 20 percentile lowest PTPRD expression (B, blue). (D) In the adjacent tissues described in (B), PTPRD expression is significantly (p=0.01, U-Test) impaired in the HCV-infected (HCV(+), grey circles, n=46) versus confirmed HCV-negative (HCV(−), empty circles, n=16) biopsies and (E) associated with a significant enrichment (NES=1.96, FDR=0.001) of the STAT3 signature. Gene expression patterns of the leading edge genes of the STAT3 signatures in liver tissues are provided as online supplementary figures S3 and S4.

infected and uninfected with HCC (see online supplementary table S1). In four out of six donors, PTPRD expression was downregulated or even completely absent in tumour lesions compared with paired adjacent tissue samples (figure 5A). To verify whether this downregulation was also specifically present in HCV-infected patients with HCC, we analysed PTPRD expression in paired tumour and adjacent liver tissue specimens.[17] In this data set of patients undergoing surgical resection of liver tumours, information for 46 patients with HCV-associated HCCs could be retrieved. In 30 out of 46 paired biopsies was PTPRD expression downregulated in tumour lesions compared with adjacent non-tumoural tissue (figure 5B). This 30/46 ratio echoes the western blot results of paired HCC biopsies (figure 5A), and the observed downregulation in tumour tissue was statistically significant (p=0.01, two-sided Wilcoxon signed-rank test) (figure 5B). Next, we took advantage of the long-term clinical follow-up information provided in the database. Strikingly, when looking at all patients with HCC (n=82), those ranking in the highest 20 percentile levels of PTPRD expression in adjacent tissue (figure 4B) had a higher long-term survival rate (p=0.048, log-rank test) (figure 5C) and had less chance of recurrent liver cancer (p=0.02, log-rank test) (figure 5D), irrespective of cancer aetiology. We confirmed the same trend of prognostic association in two additional independent cohorts with shorter patient follow-up (see online supplementary figures S5 and S6).

DISCUSSION

HCC is the second largest cause of death from cancer worldwide and poses an increasing burden on global health.[37] Chronic HCV infection is a main cause of HCC, and even though recent pharmacological breakthroughs can efficiently eradicate HCV infection, the risk of developing HCC in patients after sustained virological response remains elevated. Moreover, given the epidemiological history of the virus, HCV-associated

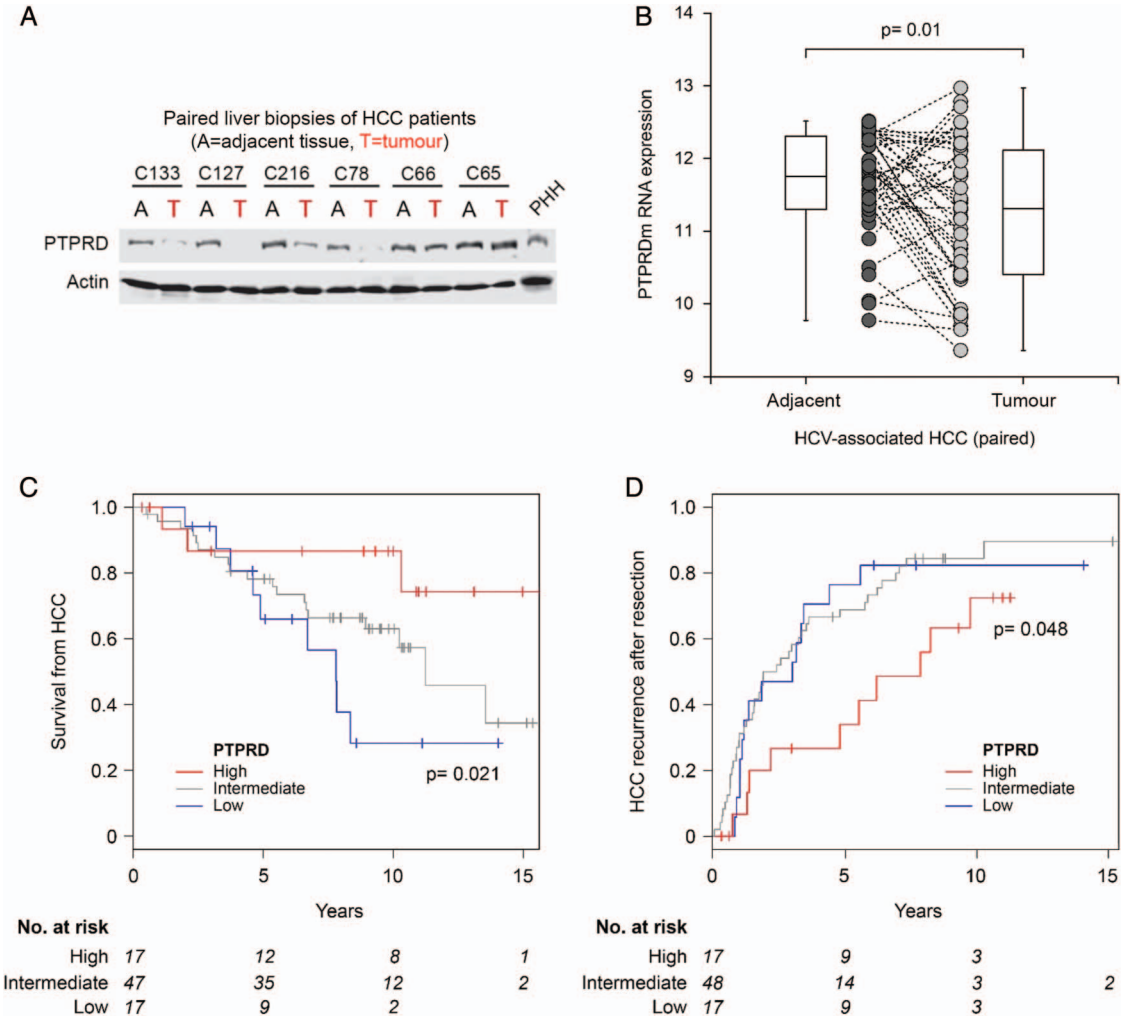

Figure 5 Dampened protein tyrosine phosphatase receptor delta (PTPRD) expression in liver biopsies is associated with a decreased patient survival and increased tumour recurrence after surgical resection. (A) PTPRD is impaired in tumour lesions of patients with hepatocellular carcinoma (HCC). Expression of PTPRD and actin was assessed in HCC lesions (*T*), and the corresponding paired adjacent tissue (*A*) of six patients with HCC by western blotting using specific antibodies. Patient codes are indicated. A random primary human hepatocytes (PHH) lysate served as positive control. (B) PTPRD is significantly (p=0.01, two-sided Wilcoxon signed-rank test) impaired in HCV-associated HCCs. The gene expression database of cohort A was analysed for PTPRD expression in paired liver biopsies from 46 patients infected with HCV.[17] Pairs of tumour lesions (dark grey) with the corresponding adjacent non-tumour tissue (light grey) are connected by a dashed line. Same data are summarised side by side as box-and-whisker plot (box=50% of biopsies, line=median, whiskers=minimal and maximal values) in the same panel. (C) Patients from cohort A with high PTPRD expression in adjacent non-tumour tissues (red) exhibit a significantly (p=0.021, log-rank test) higher survival rate from HCC and (D) a significantly (p=0.048, log-rank test) decreased tumour recurrence after surgical resection of the tumours. Product-limit estimation of PTPRD expression in adjacent non-tumour tissue of 82 patients compared with disease progression data from patients. Patient survival from HCC (C) and tumour recurrence after surgical resection (D) were compared between biopsies with highest PTPRD expression (figure 4B) (top 20 percentile; red) and biopsies exhibiting low PTPRD expression (lowest 20 percentile; blue) using Kaplan-Meier estimator. Number of patients at risk (No. at risk) is indicated.

complications are set to peak over the next decade, and novel preventive strategies are urgently needed. By studying the impact of HCV infection on protein phosphatase expression patterns in liver biopsies, we aimed to identify drivers for HCV-associated disease development. Here, we show that tumour suppressor PTPRD is consistently downregulated upon HCV infection, both in vivo, in chronically infected patient biopsies, and in vitro in infected PHH. PTPRD is a well-established tumour suppressor[27] whose chromosomal locus on 9p23–24.1 is regularly subject to genetic deletion or epigenetic inactivation in a broad spectrum of human malignancies including neuroblastoma, glioblastoma,[27] lung cancer, cutaneous squamous cell carcinoma,[38] laryngeal squamous cell carcinoma,[39] melanoma[40] and also HCC.[28] In addition, PTPRD copy number loss associates with a poor prognosis in breast cancer, colon cancer[41] and gastric adenocarcinoma.[42] Here, we demonstrate for the first time that PTPRD protein expression is downregulated in patients chronically infected with HCV. Moreover, we show that this downregulation is even more pronounced in tumour lesions of paired liver biopsies compared with adjacent non-tumour tissue of non-infected patients. Taken together, the evidence suggests that a gradual loss of PTPRD expression is a common event in liver disease progression, and it highlights the role of PTPRD as a potential suppressor of hepatocarcinogenesis, irrespective of aetiology.

In this study, we identified a HCV-specific regulatory mechanism that silences PTPRD expression in vivo. We demonstrate that PTPRD mRNA is targeted by miR-135a-5p, a miRNA that

is expressed in the liver (see online supplementary figure S7) and that was described to be markedly elevated in HCC lesions from patients with poor prognosis.[43 44] We demonstrate that miR-135a-5p expression is also significantly elevated in liver biopsies of patients with HCV (figure 3A, B). Interestingly, miR-135a has been recently suggested as a cofactor for HCV replication by interacting with the 5′UTR of the viral genome.[45] Our data demonstrate HCV-induced upregulation of miR-135a-5p expression is a cause of PTPRD silencing (figure 3D) and that high miR-135a-5p levels in liver tissues significantly ($p=0.04$, one-tailed Spearman's correlation test) correlate with low PTPRD levels and *vice versa*. The existence of a population of samples where both miR-135a-5p and PTPRD are weakly expressed (figure 3E) suggests the existence of additional regulatory mechanisms of PTPRD expression that may as well involve HCV-dependent or independent epigenetic modification of the PTPRD promoter or histones. These findings support the view that miR-135a-5p controls PTPRD in HCV-infected livers, potentially together with additional so far unidentified mechanisms for PTPRD mitigation.

Interestingly, the transcription factor STAT3 is a confirmed target for PTPRD and a key player during liver regeneration. Loss of PTPRD function leads to aberrant STAT3 phosphorylation in glioblastoma.[27 46] Here we demonstrate a significant association of PTPRD levels with STAT3 transcriptional activity in the livers of patients (figure 4). We have previously demonstrated that STAT3 is an indispensable host factor for HCV infection and that the viral infection is promoted by STAT3 activation.[10] Thus, it is conceivable that HCV downregulates PTPRD in order to benefit from a STAT3-driven transcriptional programme. Since STAT3 activity also plays a role in liver disease progression and HCC development,[34 35 36 47] this may contribute to the protumourigenic environment during chronic HCV infection. In other words, our model suggests the existence of a perturbed PTPRD–STAT3 axis driving malignant progression of liver disease. We believe that although not associated to other hallmarks of HCC development like inflammation and fibrosis (figure 1), a perturbed PTPRD–STAT3 axis adds additional oncogenic pressure to the liver as STAT3 is associated to HCCs with poor prognosis.[34 35 36] This finding may also be of further clinical relevance since it provides a target for HCC chemoprevention. Indeed, therapeutic intervention on signalling events constitutes a new chemopreventive strategy, as proof of concept has been demonstrated using the clinical epidermal growth factor receptor inhibitor erlotinib to attenuate liver fibrosis and the development of HCC in an animal model.[48] Given that PTPRD expression is suppressed by chronic HCV infection and associated with HCC and patient survival, our data suggest a PTPRD-centred signalling network as a potential target for novel chemopreventive strategies for HCV-induced HCC.

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