Etude des facteurs cellulaires responsables de l'initiation et de la dissémination du virus de l'hépatite C

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THÈSE présentée par :

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soutenue le : **24 JUIN 2014**

pour obtenir le grade de : **Docteur de l'université de Strasbourg**

Discipline/ Spécialité : Sciences du vivant/ Aspects Moléculaires et Cellulaires de la Biologie

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Study of cellular factors responsible for initiation and spread of hepatitis C virus

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« Nous ne sommes savants que de la science présente. »

Montaigne

« La reconnaissance est la mémoire du coeur. »

Hans Christian Andersen

Je tiens à remercier vivement le Pr Thomas Baumert, Directeur de l'unité U1110 pour la confiance qu'il m'a témoignée en acceptant la direction de mes travaux. Je lui suis reconnaissante de m'avoir fait bénéficier tout au long de ce travail de sa grande compétence, de sa rigueur professionnelle, et de son savoir.

Je tiens aussi à remercier le Dr Mirjam Zeisel, ma « co-directrice » qui bien que n'en n'ayant pas le titre fut tout comme. Qu'elle soit assurée de mon attachement et de ma profonde gratitude.

Je suis très honoré de la présence à mon jury de thèse et je tiens à remercier : Le Pr. Philip Meuleman, le Pr. Philippe Roingeard et le Pr. Benoît Jaulhac. Je tiens à les assurer de ma profonde reconnaissance pour l'intérêt qu'ils ont porté à ce travail.

Je remercie toutes les personnes formidables que j'ai rencontrées par le biais de l'unité. Merci pour votre support et vos encouragements et Le climat sympathique dans lequel vous m'avez permis de travailler. Les nombreuses discussions que j'ai pu avoir avec chacun de vous m'ont beaucoup apporté Je pense particulièrement à toutes ces personnes dans l'ombre dont la contribution à mon travail est non négligeable. Un grand merci donc à mes co-équipier autant dans la science que dans les discussions partagées Daniel et Nauman ; à l'équipe de choc d'une efficacité redoutable mais surtout d'une bonne humeur désarmante qui m'a permis de passer de si bons moments Sarah, Laura et Charlotte. Merci à Christine, Sylvie, Joachim, Dan, Tao, Fei, Suc des collègues de valeurs. Un merci spécial à Rajeev pour nos grandes discussions. Merci à celles et ceux qui sont le pilier de ce labo, qui étaient là bien avant moi et le sauront encore bien après Dominique, Sigis, Catherine, Patricia, votre bienveillance et gentillesse m'a beaucoup touchée. Un merci particulier à mes petites fantastiques Alizée et Laetitia, vous avez une place particulière dans ma tête. Et bien sûr un énorme merci à ma petite Isabel, tu es une amie très chère à mon cœur et tu fais partie de mes découvertes réussies au cours de ma thèse, mais tu le sais déjà. Merci aussi à tous ceux que je n'ai pas nommé pour ne pas rentrer dans l'énumération mais qui n'en sont pas moins important à mes yeux.

A mes amis qui m'ont soutenue, qui m'ont accompagnée et prouvé leurs amitié mainte et mainte fois bien que je n'ai pas été très présente, ils se reconnaitront, Epicure disait : « Ce n'est pas tant l'intervention de nos amis qui nous aide mais le fait de savoir que nous pourrons toujours compter sur eux. » alors je leur dit merci. Merci à ma famille, vous êtes une grande partie de ce

que je suis et je vous aime profondément. Papa, maman votre amour inconditionnel et votre présence derrière moi tout au long de ma vie m'ont permis de donner vie à cette thèse un merci n'est pas suffisent mais c'est tout ce que je peux graver sur le papier alors du plus profond de mon cœur et de ce que je suis : MERCI.

Papi, mamie vous n'avez hélas pas pu être là pour voir ce travail achevé, mais la fierté et l'amour que vous m'avez montré le long de votre vie font que je vous dédie cette thèse avec tout mon amour.

ABREVIATIONS

ALAT	Alanine aminotransférase
Alb-uPa	Albumine urokinase-type plasminogen activator
Apo	Apolipoprotéine
ARF	Alternative reading frame
CHC	Carcinome hépatocellulaire
CLDN	Claudine
EGFR	Epidermial growth factor
EL	Extracellular loop
EphA2	Ephrin A2 receptor
GAG	Glycosaminoglycane
GFP	Green fluorescent protein
HCV	Virus de l'hépatite C
HCVcc	particules virales VHC produites en culture cellulaire
HCVpp	Pseudoparticules du VHC
HDL	High density lipoprotein
HIV	Virus de l'immunodéficience humaine
HVR	Région hypervariable
Ig	Immunoglobuline
IRES	Site d'entrée interne du ribosome
JFH1	Japanese fulminant hepatitis
Kb	Kilobase
kDa	Kilodalton
LD	Goutelette lipidique
LDL	Low density lipoprotein
LDL-R	Récepteur aux LDL
LEL	Large extracellular loop
LVP	Lipo viro particule

MLV	Virus de la leucémie murine
Nm	Nanomètre
NS	Non structurale
NPC1L1	Niemann Pick C1 like 1 receptor
NTR	Non traduite
mRNA	microARN
miR-122	microARN 122
OCLN	Occludine
ORF	Open reading frame
RE	Réticulum endoplasmique
GFP	green fluorescent protein
SDC1	Syndecan 1
SCID	severe combined immunodeficiency disorder
SEL	Smal extracellular loop
sE2	Forme soluble de la glycoprotéine E2
siARN	ARN interférent
SP	Signal peptidase
SPP	Signal peptide peptidase
SR-BI	Scavenger receptor class B type I
VDM	Vésicule à double membrane
VLDL	Very low density lipoprotein
VLP	Virus-like particules

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I. INTRODUCTION

En 1970, H.J. Alter, virologiste américain, démontra avec son équipe que la plupart des cas d'hépatite post-transfusionnelles n'étaient pas dus au virus de l'hépatite A ni à celui de l'hépatite B. Ils baptisèrent cette hépatite : hépatite *non A non B*. Ce fut en 1987 que M. Houghton, Q. Choo, et G. Kuo, en collaboration avec D.W.Bradley purent identifier pour la première fois l'organisme responsable de cette *hépatite non A non B*. Et en 1988, l'existence du virus fut confirmée par H.J. Alter qui vérifia sa présence chez un groupe de patients atteints d'*hépatite non A non B*. Mais ce fut au final en avril 1989 que le nouveau nom de virus de l'hépatite C (HCV) fit son apparition pour la première fois avec la publication de deux articles dans *Science* (Choo et al., 1989b)(Kuo et al., 1989).

L'infection par le HCV représente un problème majeur de santé publique. En effet, il est très fréquent qu'après la primo infection le HCV persiste dans l'organisme malgré la présence d'une réponse immunitaire cellulaire et humorale spécifique. L'infection évolue, le plus souvent sans symptôme, vers une hépatite chronique puis vers la cirrhose avec un risque de cancer du foie. Pour ces raisons l'infection par le HCV est une des causes majeures de transplantation hépatique. Par ailleurs, la réinfection du greffon par le HCV se produit chez pratiquement tous les patients après transplantation hépatique et évolue vers une infection persistante, qui conduit à une hépatite chronique et rapidement à une cirrhose chez une grande majorité des transplantés.

D'autre part, les options thérapeutiques restent à ce jour limitées et il n'y a pas de vaccin disponible. Le traitement actuel consiste en une bithérapie par interféron-α pégylé et ribavirine. Ce traitement difficilement toléré avec de forts effets secondaires permet l'élimination du virus dans seulement 50% des cas pour les génotypes les plus difficiles à traiter. L'association d'un antiviral direct (DAA) à ce traitement standard permet d'augmenter le taux de guérison des patients infectés par le génotype 1 à environ 75%. Cependant, les effets secondaires et résistances au traitement restent des enjeux importants. En effet durant ces dernières années de nombreux progrès ont été effectués en vue de l'amélioration des thérapeutiques existantes. Par exemple de nombreuses recherches ont été effectuées afin de cibler les enzymes responsables de la synthèse virale, et de nouveaux inhibiteurs ont pu être testés et approuvés, comme les inhibiteurs de protéases telaprevir et boceprevir, ou sont actuellement à l'essai clinique. Mais l'avènement de ces nouveaux DAA a été accompagné d'une émergence des résistances aux

antiviraux. Ceci appuie l'intérêt de développer de nouveaux traitements se basant sur une combinaison d'antiviraux plutôt que sur le développement d'un traitement en monothérapie.

Le développement de nouveaux traitements contre l'hépatite C a longtemps était freiné par l'absence de moyens d'étude du HCV autant *in vitro* qu'*in vivo.* Ainsi le développement de nouvelles thérapeutiques s'est pas à pas amélioré avec la progression graduelle au long des années de nos modèles d'études du HCV. En effet durant les 20 dernières années de nombreux progrès ont été effectués pour l'étude du HCV. Ceci par exemple avec la mise au point des HCV pseudo particules (HCVpp) permettant l'étude de l'entrée du HCV puis avec le développement des particules virales infectieuses recombinantes dérivées de culture cellulaire (HCVcc) permettant l'étude du cycle viral dans son ensemble. Néanmoins face aux énormes avancées effectuées *in vitro* le développement de modèles d'études *in vivo* reste encore un challenge. La spécificité d'espèce très restreinte (hommes et chimpanzés) et le tropisme cellulaire très spécifique (essentiellement hépatocytaire) du HCV freinent encore le développement de modèle d'étude *in vivo* robustes. En effet, l'étude de l'infection par le HCV sur le chimpanzé est couteuse, compliquée et éthiquement délicate. Et, bien que des modèles murins aient été développés à partir de souris chimères immunodéficientes repopulées avec des cellules hépatocytaires humaines - ce qui a permis de faire de premières expérience sur petit modèle animal- il n'existe pas à ce jour de petit modèle animal immunocompétent permettant d'étudier les antiviraux et l'interaction virus/hôte dans un environnement immunitaire fonctionnel.

Ainsi, une meilleure connaissance des interactions virus-hôte et du cycle réplicatif du virus sont nécessaires afin de développer de nouveaux modèles d'étude ainsi que des thérapeutiques plus efficaces et mieux tolérées. Les travaux de thèse présentés dans ce manuscrit se sont particulièrement intéressés à caractériser le rôle des facteurs de l'hôte dans l'entrée du HCV dans les hépatocytes et à définir les facteurs de l'hôte responsables du tropisme hépatique de ce virus.

1. Epidémiologie et histoire naturelle de l'infection par le virus de l'hépatite C

1. Epidémiologie

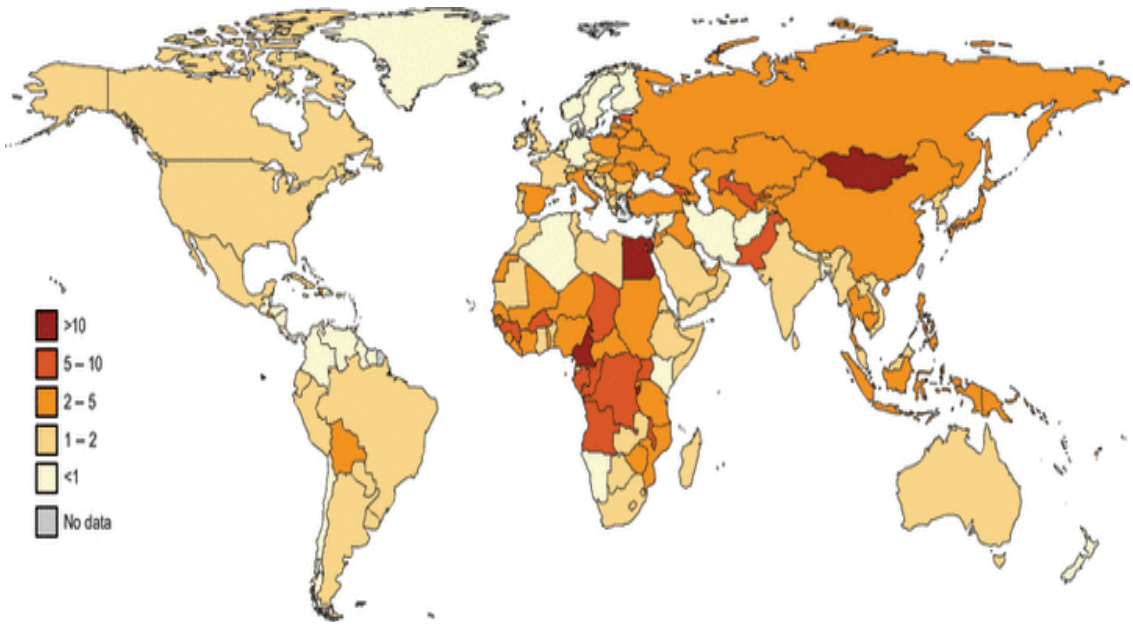


Figure 1 : Evolution épidémiologique du HCV en 2010 : Prévalence globale du HCV en %
Lavanchy Clinical Microbiology and Infection 2011

L'infection par le virus de l'hépatite C (HCV) est un problème majeur de santé publique. En effet, il s'agit de l'une des causes majeures d'hépatite et de cirrhose dans le monde. Avec une prévalence mondiale de 2 à 3 % correspondant à environ 170 millions d'individus infectés chroniquement par le HCV (Figure 1), on estime que 3 à 4 millions de personnes sont nouvellement infectées chaque année. Cette prévalence est variable selon les pays et suit un gradient Nord-Sud : très faible en Europe du Nord (de 0,5% à 2% en fonction des pays), plus élevée en Asie du Sud-Est et en Afrique, atteignant plus de 20% en Egypte. (WHO, 1999) En France, on estime que 550 000 à 600 000 personnes sont porteuses de ce virus, soit 1 à 1,2% de la population. Diverses études récentes permettent d'estimer que seulement 50 à 60% des personnes porteuses du virus se savent atteintes de l'hépatite C. Par ailleurs 10 et 30% des personnes atteintes du virus de l'immunodéficience humaine (HIV) sont également infectées

par le HCV. Cette proportion élevée de personnes coinfectées par le HIV et le HCV est liée au fait que les modes de transmission des deux virus sont en partie communs. (Meffre et al., 2007)

2. Histoire naturelle de l'hépatite C

A. *Transmission du HCV*

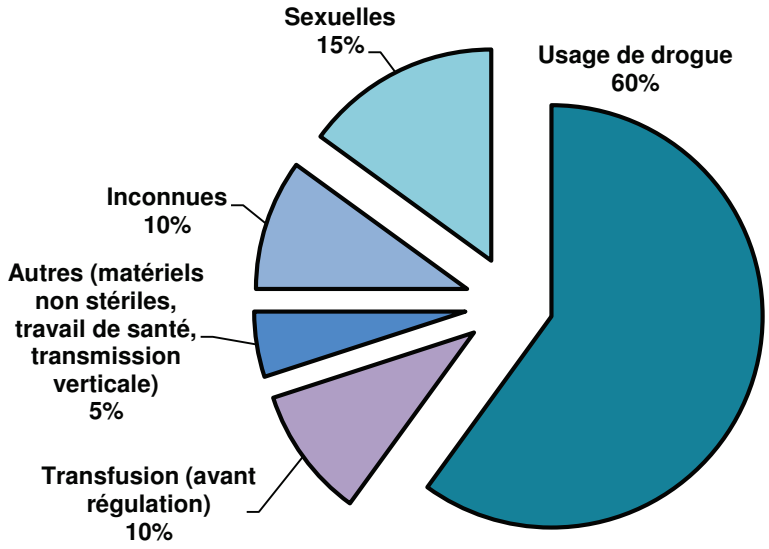


Figure 2 : **sources d'infections du HCV**. Adapté du Schéma ***USA Central Disease Center (CDC) 2007***

Le HCV se transmet principalement par voie sanguine. Dans les pays développés, la plupart des personnes porteuses d'infection chronique par le HCV ont été infectées soit par transfusion de sang ou de produits sanguins non testés avant l'introduction du dépistage systématique des dons du sang en 1991, soit par l'usage de drogues par injection ou par inhalation pratiqué avec du matériel non stérile souvent partagé (Figure 2).

La transmission sexuelle du HCV est considérée comme une éventualité rare (moins de 15 % des cas). De même le risque de transmission verticale materno-fœtal du HCV se produit relativement rarement et uniquement chez les femmes qui sont positives pour l'ARN du HCV au moment de la délivrance, il est de l'ordre de 3-5% et est directement proportionnel à la virémie (quantité de virus circulant).

D'autre part il existe environ 10% des cas d'hépatite C sans qu'aucun facteur de risque ne soit retrouvé; on désigne ces cas par le terme d'« hépatites sporadiques » (CDC 2007).

B. L'hépatite C

L'hépatite C est une maladie progressive : la phase aigüe, en absence de réponse immunitaire adéquate, évolue le plus souvent vers une hépatite chronique.

L'hépatite C aiguë évolue de manière asymptomatique chez la majorité des individus. L'infection aiguë symptomatique implique des symptômes non spécifiques tels que malaise, léthargie, jaunisse et des nausées. On observe aussi dans ce cas une augmentation des enzymes du foie comme l'alanine aminotransférase (ALT) et l'aspartate aminotransférase (AST). Par ailleurs il est intéressant de noter que la majorité des cas de guérisons spontanées seront observés chez les patients atteint d'une infection aigüe symptomatique (Grebely et al., 2010).

Dans de très rare cas, moins de 1% des patients, on peut observer une hépatite fulminante qui conduit généralement à la mort du patient.

Après la primo infection, le HCV va persister chez environ 70% des individus malgré la présence de réponse immunitaire cellulaire et humorale. L'infection chronique par le HCV est définie par la présence de l'ARN du HCV 6 mois après la date estimée de l'infection. On observe alors dans la plupart des infections chroniques l'apparition de lésions d'hépatite chronique et dans certains cas l'apparition d'une fibrose. Il a cependant été observé une élimination spontanée de l'infection chronique par le HCV dans 0,5% -0,74% des cas (Craxì et al., 2008). Mais dans 15-20% des cas l'infection chronique peut amener à des complications graves comme le développement de cirrhose du foie qui peut conduire dans 1-5% des cas à un carcinome hépato cellulaire (CHC). De ce fait, l'infection par le HCV est une des causes majeure de transplantation hépatique.(Chinnadurai et al., 2012)

2. Le HCV et ses modèles d'étude

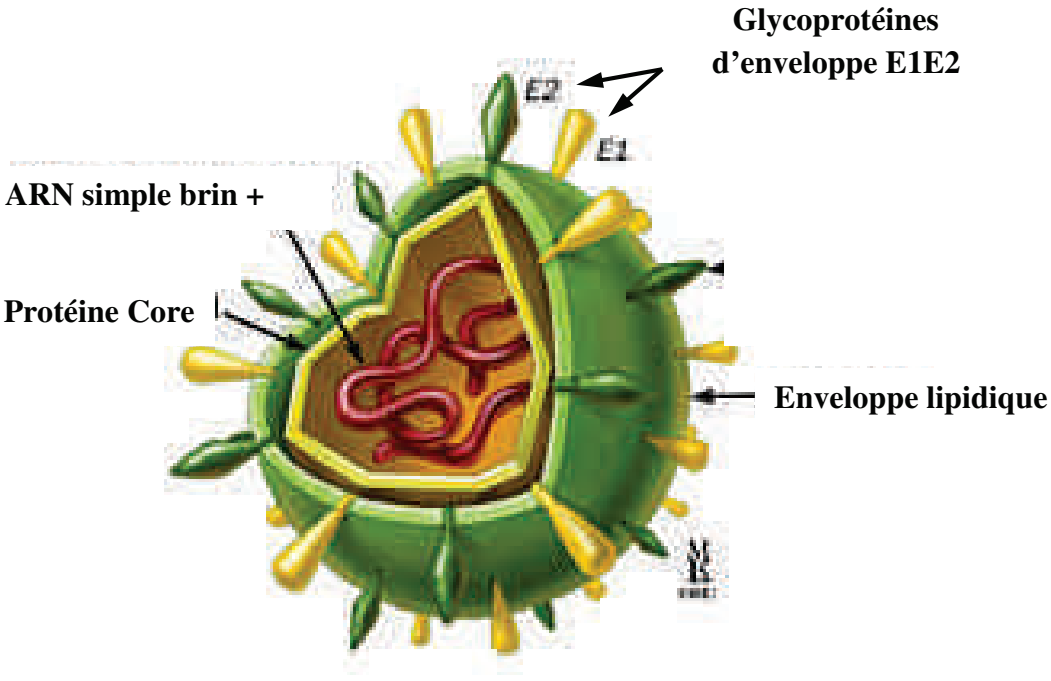


Figure 3 : **Représentation schématique de la morphologie de la particule virale du HCV**. Le HCV est composé d'un brin d'ARN viral contenu dans la nucléocapside composée de la protéine Core et entourée d'une enveloppe lipidique dérivée de la cellule de l'hôte, dans laquelle viennent s'insérer les glycoprotéines d'enveloppe E1E2. Adapté de ***John Hopkins University viral hepatitis reports***

Le HCV est un virus enveloppé d'environ 55 nm de diamètre (Kaito et al., 1994) à ARN simple brin de polarité positive de 9.6 kb (Choo et al., 1989a). Le HCV appartient à la famille des *Flaviviridae*. Cette famille se subdivise en 4 genres : les *Flavivirus* (virus de la fièvre jaune, virus de la dengue), les *Pestivirus* responsables de pathologies animales (virus de la peste porcine, virus de la diarrhée bovine), les *Pegivirus* (GB virus C) et les *Hepacivirus* comprenant le HCV, le GB virus B (GBV-B) et l'Hepacivirus canin (CHV) (Kapoor et al., 2011).

Le génome du HCV est constitué d'une région non traduite en 5' (5'NTR), comportant le site d'entrée interne du ribosome (IRES), d'un cadre ouvert de lecture (ORF) codant pour la polyprotéine du HCV, et d'une région non traduite en 3' (3'NTR). Ces régions contiennent les signaux nécessaires à la réplication et à la traduction du génome viral (Niepmann, 2009). Le

génome du HCV code pour une polyprotéine précurseur, d'environ 3010 acides aminés, qui est clivée, de manière co- et post-traductionnelle, par des protéases virales et cellulaires en 10 protéines virales structurales et non structurales. Les protéines structurales comprennent la protéine Core et les glycoprotéines d'enveloppe E1E2 permettant de former la particule virale (Figure 3). Les protéines non structurales comprennent les protéines p7, NS2, NS3, NS4A, NS4B, NS5A et NS5B (Moradpour et al., 2007)(Figure4).

1. Le polymorphisme du HCV

Le HCV se distingue de la pluparts des virus à ARN dans sa capacité à établir une infection chronique et progressive chez la majorité des personnes infectées. Ce mécanisme de persistance virale est complexe et est largement dû à la capacité du virus à échapper à la réponse immunitaire. Cet échappement viral est en grande partie le résultat des variations fréquentes dans les séquences génétiques des glycoprotéines d'enveloppe du HCV qui est la conséquence de l'absence d'activité exonucléasique correctrice de l'ARN polymérase ARN-dépendante. Cette variabilité va conférer aux protéines d'enveloppe une certaine plasticité génétique et va ainsi altérer l'antigénicité du virus. Ainsi, ceci va permettre au HCV un meilleur échappement face à la réponse immunitaire de l'hôte et conclure à un grand polymorphisme du virus. Grâce à des approches phylogénétiques les variants du HCV ont été classés en 6 génotypes (1 à 6) principaux pouvant être subdivisés en de multiples sous-types (1a, 1b, 2a….) (Simmonds et al., 2005). Le HCV chez le patient va être présent sous forme de quasi-espèces virales, c'est-à-dire sous la forme d'un mélange complexe et en équilibre instable de variants viraux génétiquement distincts mais apparentés (Bukh et al., 1995). Le développement de persistance virale et de résistance aux traitements antiviraux dans l'infection par le HCV est la conséquence de la grande capacité d'adaptation génétique du virus (von Hahn et al., 2007).

On observe une répartition géographique de ces différents génotypes : le génotype 1b est le génotype le plus fréquemment observé mondialement parlant tandis que le génotype 1a est plus fréquent en Europe du Nord et aux Etats- Unis d'Amérique. Les génotypes 2a et 2b, représentent 10 à 30 % des génotypes du HCV et sont principalement retrouvés en Italie du Nord et en Asie. Le génotype 3 est principalement observé dans le sous-continent Indien, quant au génotype 4 il

est plus représenté au Moyen-Orient et en Afrique. Les génotypes 5 et 6 sont les génotypes les plus rarement représentés (WHO 2009).

De même que la répartition des génotypes diffère géographiquement, la réponse au traitement sera elle aussi différente en fonction des génotypes. Ainsi les génotypes 1 et 4 sont plus résistants au traitement par l'interféron-α pégylé et la ribavirine, que les génotypes 2 ou 3.Par conséquent le traitement et sa durée sont adaptées en fonction du génotype (Neumann et al., 1998).

2. Les protéines virales

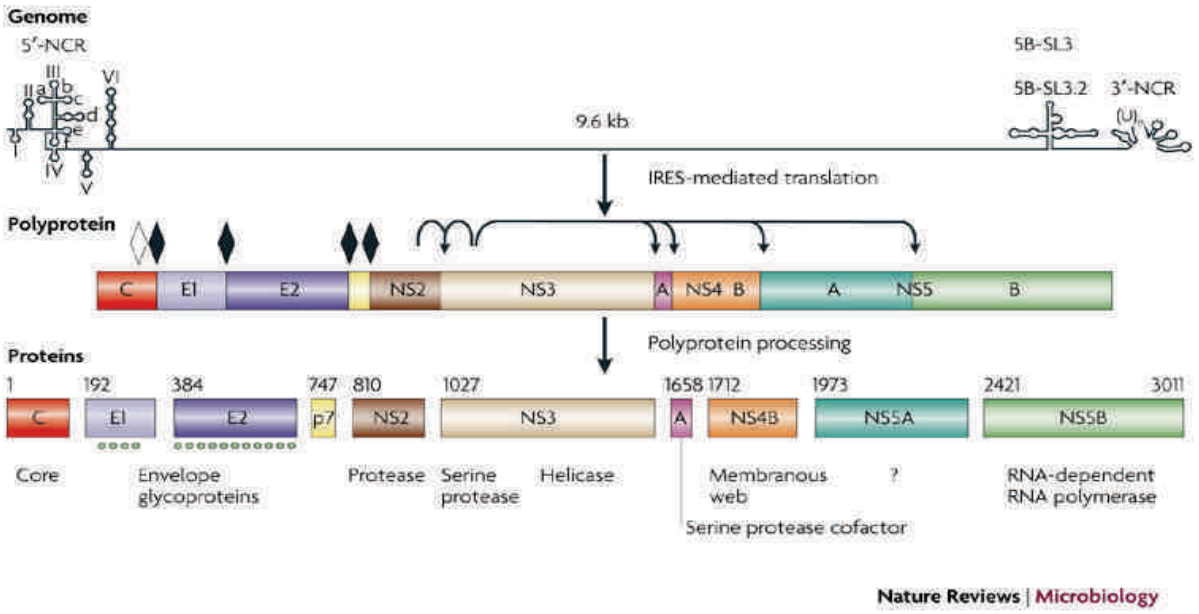


Figure 4 : Représentation schématique de l'ARN et des protéines du HCV. Le schéma ci-dessus représente la simplification des structures secondaires de l'ARN de polarité positive de 9,6 kb dans les les régions 5' et 3'non codantes ainsi que la structure du génome viral. La traduction dépendante de l'IRES conduit à la production d'une polyprotéine précurseur qui sera clivée en protéines structurales et non structurales matures. Les losanges indiquent les sites de clivage du précurseur de la polyprotéine du HCV par la peptidase endoplasmique du réticulum. Les flèches indiquent les sites de clivage par les protéase du HCV NS2-3 et NS3-4A. les points en E1 et E2 indiquent les sites de glycosylation des protéines d'enveloppe. Les différentes étapes de maturation de la polyprotéine se déroulent de manière co-et post-traductionnelle.
Moradpour et al. Nature Reviews Microbiology 2007

A. Les régions non traduites

La région 5'NTR, très conservée, assure la traduction du cadre ouvert de lecture (ORF). L'IRES couvre une région d'environ 340 nucléotides (nt) qui comprend la majeure partie de la région 5'NTR et 24 à 40 nt de la région codant la protéine Core. L'IRES est indispensable à la traduction coiffe-indépendante de l'ARN viral. La région 5'NTR forme une structure tertiaire complexe comprenant 4 domaines distincts, I à IV (Figure 4). Les domaines II, III et IV sont indispensables à l'activité IRES (Fraser and Doudna, 2007). En effet, le domaine III permet la liaison à la sous-unité 40S du ribosome et à eIF2 (facteur d'initiation de la transcription eucaryote) formant un complexe 40S-IRES-eIF3. Le domaine IV contient le codon d'initiation. Il a été suggéré que la région 5'NTR contient à la fois les facteurs déterminants pour la traduction, mais aussi des éléments pour la réplication de l'ARN (Astier-Gin et al., 2005). En effet, il semblerait que la séquence en amont de l'IRES ainsi que certaines séquences dans l'IRES elle-même soient essentielles pour la réplication efficace de l'ARN viral du HCV (Friebe et al., 2001).

La **région 3'NTR** mesure environ 200 à 235 nt, et joue un rôle important dans la réplication virale. Elle est composée de 3 régions distinctes : une région variable, une queue poly (U/UC) et une région hautement conservée de 98 nt, appelée la région X, formant 3 structures tige-boucles (3'SLI, 3'SLII et 3'SLIII) (Figure 4). Cette dernière séquence joue un rôle important dans l'initiation de la synthèse du brin ARN négatif au cours de la réplication.

B. Les protéines virales structurales

La protéine de capside ou protéine C ou protéine Core. C'est une protéine qui se lie à l'ARN pour former la nucléocapside virale. La protéine Core est dans un premier temps clivée par une signal peptidase (SP) endoplasmique conduisant à l'obtention d'une forme immature de 23 kDa puis est à nouveau clivée par une signal peptide peptidase (SPP) en une protéine Core mature de 21 kDa (McLauchlan et al., 2002; Oehler et al., 2012). Elle forme une structure dimérique composée d'hélice alpha et est séparée en deux domaines D1 et D2.
Le domaine hydrophile N-terminal de la protéine Core (D1) est impliqué dans la liaison de l'ARN viral à la région 5'NTR conduisant à l'assemblage de la nucléocapside. Le domaine

hydrophobe C-terminal (D2) quant à lui s'associe aux gouttelettes lipidiques au niveau de la membrane du réticulum endoplasmique (RE) (Boulant et al., 2007, 2008)
L'association de la protéine Core aux gouttelettes lipidiques semble jouer un rôle essentiel dans la pathogenèse et la morphogénèse du HCV et serait impliquée dans la stéatose induite par le virus (Roingeard et al., 2008), processus associé au développement de la fibrose chez les patients infectés chroniquement (Gordon et al., 2000; Westin et al., 2002). Il a aussi été démonté que l'interaction de Core avec NS5A joue un rôle central lors de l'assemblage et de la libération de la particule virale et intervient également lors du désassemblage de la particule virale au cours de l'entrée du virus dans la cellule hôte (Miyamura and Matsuura, 1993; Penin et al., 2004). Ainsi la protéine Core semble cruciale au bon développement du cycle viral.

La protéine F (frameshift) ou ARF (alternative reading frame) ou Core+1 provient d'un cadre de lecture alternatif (ARF) chevauchant la séquence codante de la protéine Core dans le cadre +1. C'est une protéine de 8 à 14kDa qui, après traduction, est localisée dans le RE. Sa durée de vie est très courte, environ 10 minutes, car elle est très vite dégradée par le protéasome (Xu et al., 2003). La détection d'anticorps et de cellules T spécifiques de la protéine F chez des patients infectés par le HCV suggère qu'elle serait exprimée *in vivo* pendant l'infection par le HCV. Sa fonction dans le cycle viral ou la pathogenèse du HCV reste à élucider car elle n'est ni essentielle à la réplication virale ni à la production de particules infectieuses (Wolf et al., 2008).

Les protéines d'enveloppe E1 et E2 sont les constituants essentiels de l'enveloppe, elles jouent un rôle essentiel à différentes étapes du cycle viral du HCV comme les premières étapes de l'entrée virale, la fusion virale avec la membrane de l'endosome et l'assemblage des particules virales. Elles sont la cible des anticorps neutralisants produits au cours de l'infection par le HCV.

Les protéines E1 E2 sont clivées par une signal peptidase. E1 et E2 sont des protéines transmembranaires de type I comprenant chacune un ectodomaine N-terminal (respectivement de 160 et 360aa) et un domaine transmembranaire C-terminal d'environ 30 aa (Cocquerel et al., 2002). Les domaines transmembranaires contribuent fortement aux fonctions d'E1 et E2 comme l'ancrage à la membrane, la rétention au niveau du RE et la formation des hétérodimères non covalents E1E2. Les ectodomaines N-terminaux de ces protéines contiennent

respectivement jusqu'à 6 et 11 sites de glycosylation potentiels qui sont bien conservés (Op De Beeck et al., 2000).

Le rôle de la protéine E1 dans l'infection par le HCV est toujours peu compris. E1 semble être impliquée d'une part dans le processus de fusion nécessaire à l'internalisation du virus dans la cellule hôte (Lavillette, 2007) et d'autre part contribuer à l'association de l'enveloppe virale aux lipoprotéines de l'hôte. Le virus tirerait alors avantage de cette association afin d'échapper à la réponse immunitaire.

La protéine E2 est constituée de 3 domaines (DI, DII et DIII) et de trois régions hypervariables (HVR1, HVR2 et IgVR) (Weiner et al., 1991) aux positions respectives d'acides aminés 1-27, 91-97et 187-197. Le domaine DI contient le déterminant qui permettra l'interaction avec CD81 et se termine par la région HVR1. La région HVR1 est très hétérogène dans sa séquence et est responsable de variations dans le caractère infectieux des particules infectieuses du HCV chez les patients et au sein d'un même patient (quasi-espèces). Grâce à cette forte variabilité, le virus peut facilement échapper au système immunitaire de l'hôte et persister (von Hahn et al., 2007). Cependant de manière intéressante la région HVR1 est très conservée d'un point de vue conformationnel, ce qui corrobore son rôle de région immunodominante et son rôle dans l'attachement du virus à la cellule (Penin et al., 2001). Le domaine DII est constitué de la région HVR2. Le rôle fonctionnel de la région HVR2 est moins bien défini. Cette région semble être essentielle pour le maintien de l'intégrité structurale et de la fonction de l'hétérodimère E1E2 et jouerait un rôle dans la modulation de la liaison d'E2 aux récepteurs (McCaffrey et al., 2011). Le domaine DIII sera quant à lui connecté au domaine DI grâce à la région IgVR qui a la particularité de varier énormément d'un génotype à l'autre (Albecka et al., 2011).

C. Les protéines virales non structurales

Le HCV possède 7 protéines non structurales : **p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B** (Figure 4)**.** Ces protéines non structurales auront chacune un rôle clé au cours du cycle virale et favorisent le bon déroulement du cycle infectieux. La traduction va suivre la libération du génome viral du HCV dans le cytoplasme. La traduction va conduire à l'obtention de la polyprotéine précurseur qui va être redirigée vers le réticulum endoplasmique (RE) où elle subira sa maturation grâce aux **protéases virales NS2 et NS3/NS4A** qui vont permettre le clivage de la polyprotéine. Durant ce processus on observera la formation d'un réseau

membranaire appelé le « complexe de réplication » constituant des vésicules à double membrane contenant les protéines non structurales du HCV, les membranes du RE, l'ARN du HCV et les gouttelettes lipidiques. Il a été démontré que la formation de ces complexes se faisait sous **l'influence de la protéine NS4B associée à la membrane du RE** (Egger et al., 2002; Gosert et al., 2003). La réplication du génome viral aura lieu au niveau de ces complexes grâce à **la protéine NS5B qui est l'ARN polymérase ARN dépendante.** En effet la réplication virale se fait grâce à la synthèse d'un brin complémentaire d'ARN de polarité négative qui utilise le génome viral comme matrice et qui permettra la synthèse de nouveau brin d'ARN génomique de polarité positive. **NS5B** est capable d'initier cette synthèse d'ARN *de novo in vitro* et i*n vivo* (Bartenschlager and Pietschmann, 2005). Mais il a aussi été démontré que **l'activité hélicase de NS3 du complexe NS3/NS4A** participe à la séparation des ARN double brin ou au déroulement des structures secondaires, processus essentiel pour la réplication de l'ARN et l'assemblage des particules virales (Morikawa et al., 2011). Quant aux protéines **p7 et NS5A** elles interviendront lors de l'assemblage des virions nouvellement produits. En effet **la protéine p7** de la famille des viroporines forme un canal ionique (Clarke et al., 2006; Luik et al., 2009) et semble faciliter la production virale. Elle est essentielle à l'assemblage des particules virales et aussi à la libération des virions (Gentzsch et al., 2013; Steinmann et al., 2007). **La protéine NS5A** a été identifié comme élément clé dans l'assemblage des particules virales (Appel et al., 2008). Par ailleurs, il est intéressant de noter qu'il a récemment été démontré que **NS2**, indépendamment de sa fonction protéase, pouvait jouer un rôle crucial dans l'assemblage des particules virales (Jirasko et al., 2010; Phan et al., 2009; Stapleford and Lindenbach, 2011).

3. Les modèles d'études du HCV

Dans des conditions naturelles d'infection, le HCV circule dans le sang des patients infectés en association avec les lipoprotéines de l'hôte ce qui favorise l'interaction virus-cellule hôte et l'échappement aux réponses immunitaires de l'hôte. Afin de pouvoir étudier le cycle viral du virus et afin de développer des traitements performants pour lutter contre l'infection, différents modèles d'étude du HCV ont été développés au cours des dernières décades. De l'utilisation du sérum dérivé des patients infectés sur des cultures cellulaires, système d'étude contraignant et peu efficace pour l'étude du cycle infectieux *in vitro*, à l'utilisation de virus recombinant dérivés des cultures cellulaires (HCVcc), d'énormes progrès ont été effectués. Ces progrès ne sont pas

seulement observés avec les modèles d'étude *in vitro* mais aussi, grâce à notre meilleure compréhension du HCV, avec le développement de nouveaux modèles murins *in vivo.*

A. Les modèles in vitro: des glycoprotéines recombinantes aux HVCcc

Les glycoprotéines solubles recombinantes E1 (sE1) et E2 (sE2) : ces protéines recombinantes sE1 et sE2 sont des formes chimériques solubles des protéines d'enveloppe du HCV. Elles ont la particularité d'avoir le domaine transmembranaire tronqué. sE1 et sE2 ont été utilisées pour étudier les interactions du HCV avec ses cellules hôtes. Il a été démontré que sE2 pouvait se lier spécifiquement aux lignées cellulaires hépatocytaires cancéreuses, suggérant que l'attachement cellulaire du virus à la cellule hôte est médié par E2. Ainsi, l'utilisation de ces modèles a permis d'identifier de nombreux facteurs cellulaires impliqués dans l'entrée du HCV, parmi lesquels les héparanes sulfates (Barth et al., 2003, 2006), la tétraspanine CD81 (Pileri et al., 1998), et le récepteur scavenger BI (SR-BI) (Scarselli et al., 2002). Plus récemment, il a même pu être démontré que l'immunisation de souris et de chimpanzés avec sE2 pouvait induire la production d'anticorps neutralisants (Kachko et al., 2011). Cependant l'utilisation de ces systèmes restreint l'étude de l'infection par le HCV à l'étude de l'attachement de E1 ou E2 seul. Or les glycoprotéines d'enveloppe E1 et E2 sont présentes dans la particule virale sous forme d'un hétérodimère non-covalent. Par conséquent, la protéine sE2 seule se comporte de manière différente (Burlone and Budkowska, 2009) et ne permet pas réellement d'étudier l'étape d'attachement et d'entrée du HCV médiée par les deux glycoprotéines d'enveloppe (Bartosch and Cosset, 2006).

Les HCV-like particules (HCV-LP). C'est une des approches techniques qui fut appliquée à de nombreux autres virus comme le papilloma virus (HPV), l'influenza ou encore le virus de l'hépatite B (HBV) dans le cadre du développement de vaccins (Kushnir et al., 2012). En effet ce sont des particules virales stables possédant d'excellentes propriétés antigéniques. Ces HCV-LP correspondent à des « virus-like particles » (VLP) synthétisés *in vitro* qui s'assemblent de manière autonome. Elles sont produites par l'introduction d'un vecteur codant pour les protéines structurales Core, E1 et E2 du HCV dans des cellules d'insecte ou de mammifères. Ces HCV-LP présentent des propriétés morphologiques et biophysiques proches de celles de virions isolés chez les patient infectés, mais cependant ne se répliquent pas car elles ne possèdent pas le génome viral dans son intégralité. Il a pu être démontré que ces HCV-LP

étaient un outil efficace pour l'étude structurale et fonctionnelle de la protéine Core (Blanchard et al., 2003; Roingeard et al., 2004). Par ailleurs, on observe que ces HCV-LP présentent des propriétés antigéniques semblables à celle du virus natif à la fois dans la liaison aux cellules hôtes et dans la neutralisation, ceci faisant de ces modèles un candidat potentiel pour le développement de vaccin (Baumert et al., 1999). Bien que les caractéristiques des HCV-LP en font un modèle intéressant pour l'étude de l'interaction entre le virus et ses cellules hôtes (Barth et al., 2005; Triyatni et al., 2002), ce modèle d'étude présente néanmoins une grande limitation :d'une part son utilisation nous limite uniquement à l'étude des étapes d'attachement et d'interaction virus-hôte au niveau des récepteurs, et d'autre part l'absence d'un gène rapporteur rend ce modèle contraignant à suivre.

Les pseudo-particules virales du HCV (HCVpp). Ce modèle de pseudo-particules virales a été utilisé comme système d'étude pour de nombreux virus comme le FLV (Feline leukemia virus) et le HIV (virus de l'immunodéficience humaine) ou bien encore comme outil pour la thérapie génique (Baum et al., 2006). Les HCVpp sont des virus chimères obtenus par incorporation des glycoprotéines E1 et E2, sous leur forme native, à la surface de particules rétrovirales ou lentivirales. Elles sont générées par la transfection des cellules embryonnaires de rein humain (HEK 293T) par trois vecteurs d'expression. Le premier vecteur va coder pour la protéine de capside d'un rétrovirus (virus de la leucémie murine (MLV)) ou un lentivirus (HIV), le deuxième vecteur va coder pour les glycoprotéines d'enveloppe natives E1 et E2 du HCV et le troisième vecteur va coder pour un gène rapporteur codant pour la GFP (Green Fluorescent Protein) ou la luciférase et possède une séquence d'encapsidation. Les HEK 293T une fois transféctées vont sécréter des HCVpp dans leur surnageant, et ce surnageant pourra être utilisé pour infecter des cellules cibles hépatocytaires comme les cellules Huh7 et ses dérivées (cellules cancéreuses hépatocytaires humaines) ou des hépatocytes primaires humains (PHH). L'infection des cellules cibles pourra alors être monitorée par l'observation de l'expression du gène marqueur luciférase ou GFP (Bartosch et al., 2003a) (Figure 5). Les HCVpp représentent le premier vrai succès dans la production d'un système d'étude robuste et flexible à la fois permettant l'étude des fonctions virales dépendantes des glycoprotéines d'enveloppe E1 et E2. Les HCVpp ont la capacité de simuler les premières étapes de l'infection par le HCV : de l'entrée du virus dans la cellule à l'endocytose médiée par les récepteurs de la cellule hôte. Ce modèle a permis de mieux étudier l'entrée du HCV et ainsi d'identifier de nouveaux co-récepteurs du HCV : les protéines de jonction claudine 1 (CLDN1) et occludine (OCLN) (Ploss et al., 2009) et plus récemment les récepteurs à tyrosine kinases tel que

l'epidermal growth factor receptor (EGFR) et l'ephrin receptor A2 (EphA2) (Lupberger et al., 2011) ou bien encore le Niemann-Pick C1-like cholesterol adsorption receptor (NPC1L1) (Sainz et al., 2012). Les HCVpp ont également permis d'étudier les anticorps neutralisants et de caractériser les mécanismes moléculaires du processus de neutralisation *in vitro* et *in vivo* (von Hahn et al., 2007; Pestka et al., 2007). Les HCVpp sont considérées comme l'outil de référence pour étudier les propriétés des glycoprotéines E1E2. Cependant ce modèle possède des limites, en effet, son utilisation ne permet d'étudier que l'étape d'entrée du virus, il ne possède pas de cycle réplicatif. Ainsi les étapes suivantes dans le cycle infectieux du HCV ne peuvent être observées. Par ailleurs le fait que ces particules soient produites dans des lignées non hépatocytaire et qu'elles s'assemblent au niveau de la membrane plasmique du fait de leur enveloppe rétrovirale rend impossible l'association de ces HCVpp avec les lipoprotéines. Ce modèle ne permet donc pas l'étude du rôle des lipoprotéines dans l'entrée (Bartosch et al., 2003a), ni de déterminer l'interaction réelle des virions avec les récepteurs lipidiques tel que SR-BI, le LDL-R ou bien le NPC1L1.

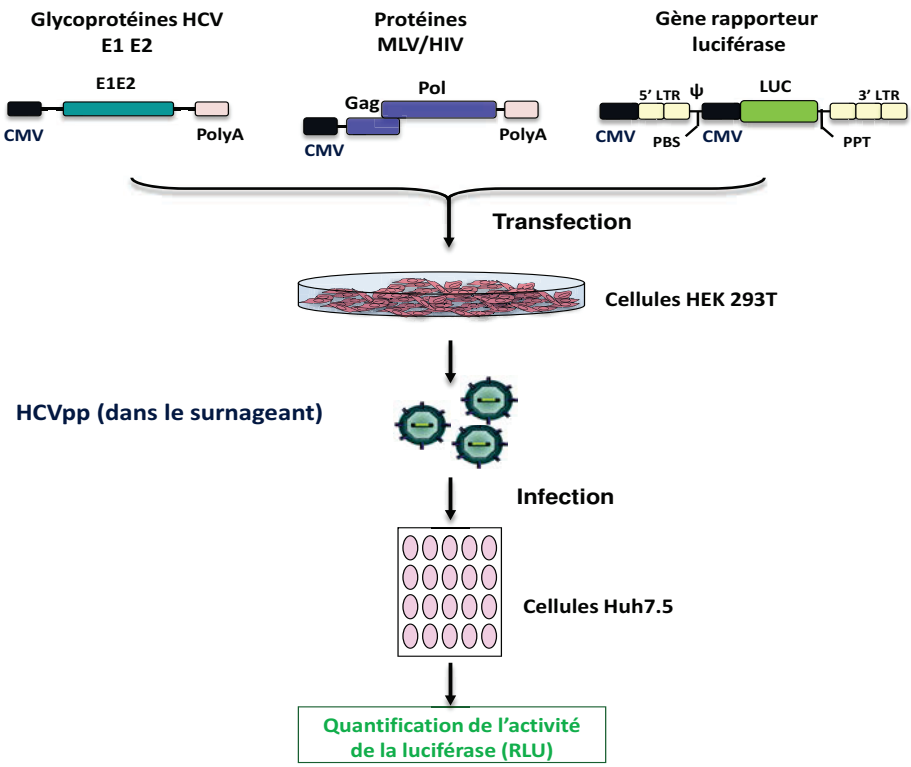


Figure 5 : Représentation schématique de la production des HCVpp : Les cellules 293T sont transfectées avec trois vecteurs d'expression codant : pour les glycoprotéines E1-E2 du HCV ; pour gag et pol permettant la production des protéines de matrice, capside et nucléocapside permettant l'encapsidation de l'ARN et l'assemblage de la particule ; pour un gène rapporteur luciférase et la séquence d'encapsidation. Après transfection de ces plasmides, les HCVpp sont sécrétées dans le surnageant de culture des cellules 293T et recoltées. Elles sont ensuite utilisées pour infecter des cellules Huh7 ou ses dérivées. Les cellules infectées vont intégrer le gène codant la luciférase dans leur ADN génomique et vont exprimer la luciférase. L'infectiosité des HCVpp est quantifiée en monitorant l'activité luciférase résultante el les résultats sont donnés en relative light unit (RLU)

Les réplicons subgénomiques du HCV ont été les premiers modèles à rendre possible l'étude de la réplication virale du HCV (Lohmann et al., 1999). Ce sont des ARN réplicatifs autonomes bicistroniques contenant en 5' l'IRES du HCV qui assure la traduction d'un gène de résistance antibiotique (neomycine), l'IRES du virus de l'encéphalomyocardite (VEMC) permettant la traduction des protéines non-structurales (NS3 à NS5B), le tout entouré par les régions 5' et 3'NTR du HCV. La délétion de la séquence codant pour les protéines structurales du HCV a permis d'y intégrer un marqueur de sélection à la néomycine. Ainsi grâce au marqueur de sélection, seuls les clones résistants à la néomycine répliqueront l'ARN du HCV (Lohmann et al., 1999). Face aux différents modèles permettant l'étude de l'entrée uniquement, le système de réplicon a été d'un grand apport dans l'étude de la réplication du HCV et de certaines interactions virus-hôtes. Ils ont aussi couramment été utilisés pour la validation de drogues antivirales ciblant des enzymes du HCV. Cependant, les réplicons génomiques contenant la séquence complète du virus et, bien que se répliquant efficacement en culture cellulaire sous pression de sélection antibiotique, n'ont pas permis la production de particules virales infectieuses (Pietschmann et al., 2002).

Les particules virales infectieuses recombinantes dérivées de culture cellulaire (HCVcc). Ce modèle a pour origine l'isolement et la description de la séquence entière du génome du HCV de génotype 2a d'un patient japonais soufrant d'une hépatite fulminante C appelée JFH-1 qui a la particularité d'avoir un haut potentiel réplicatif en l'absence de mutation adaptative (Kato et al., 2001). Ce clone JFH-1 a la faculté de produire des particules virales infectieuses recombinantes en cultures cellulaires appelées HCVcc (Lindenbach et al., 2005a; Wakita et al., 2005; Zhong et al., 2005). Ce système est basé sur l'électroporation d'ARN JFH-1 dans les lignées cellulaires hautement permissives qui permettent une forte réplication, les cellules Huh7 et ses dérivées (Figure 6). La production de virus chimérique à partir de JFH-1 combiné à J6 rend possible la production de particules virales hautement infectieuses autorisant la réinfection

des cellules hépatocytaires dans le chimpanzé et la souris humanisée (Lindenbach et al., 2006a; Pietschmann et al., 2006). Ce modèle a permis de reconfirmer les résultats obtenus précédemment sur les facteurs d'entrée et le tropisme cellulaire du HCV (Koutsoudakis et al., 2006a; Lindenbach et al., 2005b; Wakita et al., 2005) ainsi que l'action des anticorps neutralisant sur l'infection (Haberstroh et al., 2008; Zeisel et al., 2007a). Les HCVcc ont aussi la particularité d'avoir permis d'étudier l'association du virus avec les lipoprotéines ce qui jusqu'alors n'était pas possible avec les modèles d'études précédents. (Lindenbach et al., 2006b). Par ailleurs, la construction de virus chimérique inter génotypiques a permis de ne pas se restreindre à l'étude du genotype 2a seulement. (Pietschmann et al., 2006). Ainsi la création de ce modèle a permis une grande avancée dans nos moyens d'études du HCV, car pour la première fois, on possédait un outil permettant le suivi du cycle viral dans son intégralité.

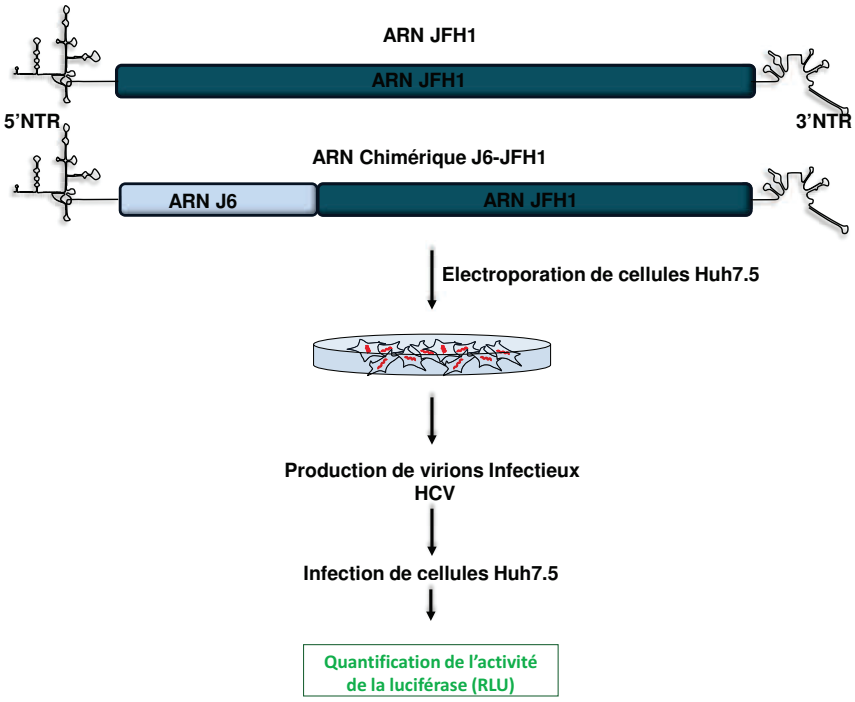


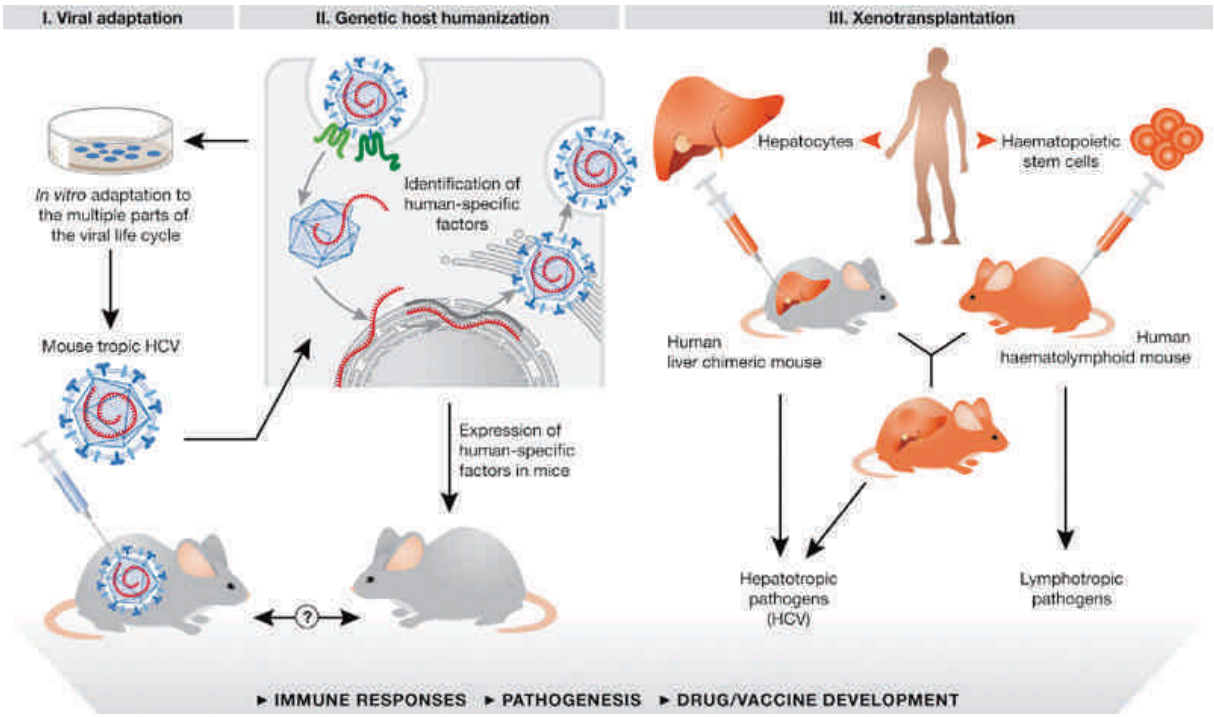
Figure 6 : Représentationschématique de la production des HCVcc. L'ARN du HCV JFH1 ou chimérique JFH1/J6 combiné ou non à un marqueur luciférase est éléctroporé dans les cellules Huh7 et ses dérivées. Une fois éléctroporées, les cellules vont relarguer dans le surnageant des virions infectieux qui pourront par la suite être utilisés pour suivre l'infection par le HCV dans les Huh7 et dérivées en observant l'activité luciférase résultante ou bien en quantifiant l'ARN viral présent par qRT PCR.

B. *Les modèles in vivo : Du chimpanzé aux souris humanisées et aux souris transgéniques*

Les modèles *in vivo* naturels

Les premiers modèles d'étude du HCV *in vivo* ont été **le chimpanzé (*Pan troglodytes*) et le tupaia (*Tupaia belangeri*)**; petits mammifère arboricole vivant dans le Sud-Est asiatique). Ces deux modèles sont des modèles naturellement permissifs aux HCV. Bien que l'évolution de l'infection à HCV ne soit pas tout à fait comparable à celle que l'on observe chez l'homme, leur utilisation a largement contribuée à notre compréhension du déroulement infectieux en phase aigue chronique(Bukh, 2004, 2012). Cependant pour des raisons pratiques, de coût, et en grande partie éthique surtout dans le cadre du chimpanzé (déclaré comme espèce menacée en 1988), ils restent des modèles peu utilisés.

Les modèles *in vivo* adaptés



<u>Figure 7 :</u> schéma récapitulatif des différents modèles d'études murins de l'infection par le HCV et de leurs mécanismes d'obtention. ***Ploss and Rice EMBO Reports 2009***

Afin de dépasser ces limitations, plusieurs modèles murins ont été développés chacun ayant ses propres avantages mais aussi ses propres inconvénients. En effet les hépatocytes murins ne sont pas naturellement permissifs au HCV. Plusieurs stratégies ont été établies afin d'outrepasser cette résistance des souris au HCV.

L'adaptation virale du HCV aux hépatocytes murins : ce système a été utilisé initialement pour d'autres virus comme le virus de l'Ebola (Bray et al., 1998). L'idée de ce modèle d'étude étant que, plutôt que d'adapter la souris à l'infection par le HCV on peut adapter le virus aux hépatocytes murins en forçant le virus à utiliser les facteurs murins CD81 et OCLN (les deux facteurs d'entrée responsables de la spécificité d'espèce) pour entrer dans la cellule (Figure 7). Il a pu ainsi être démontré que, grâce à une approche sélective, on pouvait obtenir avec l'apparition de mutation adaptative sur E1 et E2 des virions HCV ayant un tropisme hépatocellulaire murin (Bitzegeio et al., 2009). Cependant, bien qu'intéressant dans l'idée, ce modèle n'est toujours pas transposé *in vivo* et nécessiterait une étape d'adaptation supplémentaire afin de surmonter l'obstacle de la réplication qui ne s'effectue pas dans les cellules de foie murines.

Les modèles murins génétiquement humanisés : en se basant sur les observations faites au préalable que CD81 et OCLN étaient les facteurs d'entrée humains minimums nécessaires à rendre les cellules murines permissives pour l'entrée du HCV (Ploss et al., 2009), un modèle murin génétiquement humanisé supportant l'entrée du HCV à été généré (Dorner et al., 2011). Les auteurs ont ainsi pu démontrer que suite à l'expression de CD81 et OCLN humains dans des souris immunocompétentes on pouvait observer une entrée efficace du HCV dans les hépatocytes murins *in vivo* en utilisant un système reporter sophistiqué (Figure 7). Cependant suite à l'entrée du HCV dans ces cellules bien que l'on puisse observer la traduction du génome viral (Dorner et al., 2011; McCaffrey, 2002), il ne s'effectue aucune réplication de l'ARN. Ainsi ce modèle à l'avantage de permettre l'étude de l'entrée du HCV *in vivo* dans un système immunocompétent afin d'observer l'efficacité des inhibiteurs d'entrée et des candidats pour certains vaccin par exemple (Giang et al., 2012). Cependant il possède l'inconvénient de ne pas pouvoir étudier le cycle viral dans son ensemble et donc de tester des antiviraux agissant sur des étapes suivant l'entrée du virus, le déroulement du cycle viral étant stoppé à l'étape de la réplication.

Les modèles murins xenotransplantés : les souris Alb uPA-SCID (Mercer et al., 2001) et **FRG** (Bissig et al., 2010)**.** Les souris Alb-uPA-SCID sont des souris transgéniques immunodéficientes qui possèdent un activateur de l'urokinase sous le contrôle d'un promoteur albumine. Ceci va permettre sous l'activation de l'urokinase de détruire les cellules du foie de la souris et on pourra alors transplanter ces souris avec des PHH, permettant la survie des souris et la susceptibilité à l'infection par le HCV. Il a été démontré que l'inoculation de HCVcc ou de sérum de patient HCV positif dans ces souris conduit à une rapide augmentation de la

virémie, et cette virémie est maintenue durant de nombreuses semaines (Lindenbach et al., 2006b; Mercer et al., 2001; Meuleman et al., 2005). Sur le même principe, un autre modèle de souris immunodéficientes a été développé : **les souris Fah-/-Rag2-/-IL2rg-/-** (Bissig et al., 2010)**.** Ce modèle est basé sur le même mécanisme de repopulation du foie des souris par des PHH suite à la mort cellulaire des hépatocyte murins, mais a la particularité de pouvoir réguler la repopulation par administration orale d'un composé le NTCB (2-(2-nitro-4-trifluoro-methylbenzoyl)-1.3-cyclohexanedione). L'utilisation de ces modèles, plus particulièrement des souris Alb-uPA-SCID, a été d'une grande aide dans la confirmation du rôle des anticorps neutralisants et anti-facteurs d'entrée du HCV sous contrôle de l'infection par le HCV (Meuleman et al., 2008, 2012; Vanwolleghem et al., 2007) mais aussi dans les études d'efficacité, de toxicité et de pharmacocinétiques des traitements contre le HCV (Billerbeck et al., 2013). Cependant, bien susceptibles à l'infection par le HCV, ces modèles possèdent les désavantages de nécessiter une intervention chirurgicale complexe sur des souris fragiles dans leur manipulation et de ne pas avoir de système immunitaire fonctionnel. De ce fait, ces modèles ne conviennent ni pour l'étude de l'immunopathogenèse de l'infection par le HCV, ni pour l'étude de vaccins potentiels.

Plus récemment un nouveau modèle murin xenotransplanté a été développé dans l'intention de pouvoir étudier l'infection par le HCV en présence d'un système immunitaire humain (Washburn et al., 2011). Ainsi de nombreux efforts ont été effectués afin de pouvoir combiner les modèles murins au foie humanisé avec des modèles murins possédant un système hémato lymphatique humain. **Ces souris sont générées par co-transplantation d'hépatocytes humains et de cellules souches hématopoïétiques humaines** (Figure 8). Il a été démontré que ces souris sont capables de supporter l'infection par le HCV et qu'une réponse immunitaire cellulaire de type T est générée en retour. Bien qu'il subsiste toujours le désavantage du recours au système chirurgicale difficile, ce modèle à l'énorme avantage de pouvoir permettre l'étude du cycle infectieux du HCV in vivo sous l'influence d'un système immunocompétent. Cependant il est à noter qu'aucune réponse immunitaire humorale n'a pu être mise en évidence dans ce modèle qui se caractérise par ailleurs par l'absence de virémie, le virus ayant été détecté uniquement dans le foie de souris infectées.

3. Le cycle viral du HCV

Le HCV dépend en grande partie des facteurs de l'hôte pour son infection, sa réplication et sa dissémination. Ces interactions virus hôte sont restreintes par un tropisme cellulaire et par une spécificité d'espèce. Durant ces dernières années, de nombreux efforts ont été effectués afin d'identifier et d'élucider les différents facteurs de l'hôte ainsi que les différents mécanismes nécessaires au déroulement du cycle infectieux du HCV (Figure 8). Le développement des différents modèles d'étude du HCV a permis d'établir un certain consensus quant aux facteurs permettant l'interaction virus-hôte aux cours des différentes étapes du cycle virale, comme l'entrée, la réplication, la traduction, l'assemblée, et de définir le tropisme du HCV.

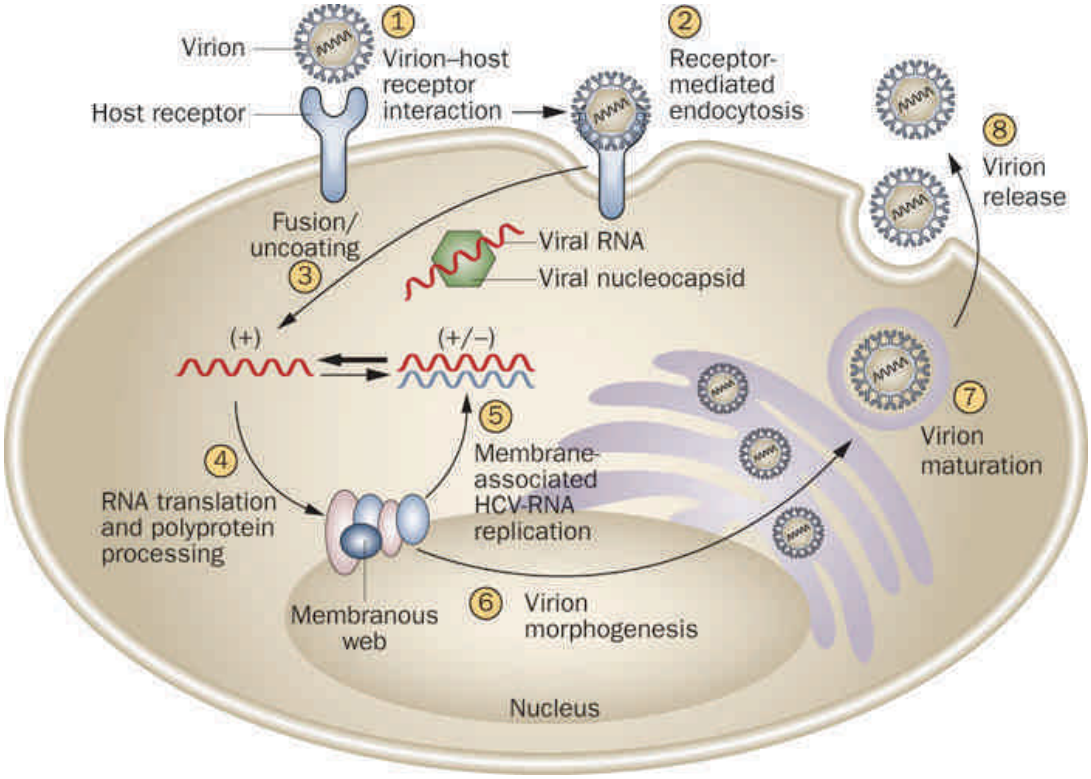


Figure 8: Représentation des différentes étapes du cycle viral du HCV. 1 interaction du HCV avec les facteurs d'entrée de la cellule hôte hépatocytaire. 2 Endocytose du HCV dans la cellule. 3 fusion membranaire et libération du génome viral. 4 Traduction de l'ARN du HCV et maturation de la polyprotéine. 5 Réplication de l'ARN. 6 Assemblage du virus à partir du brin d'ARN+ nouvellement formé. 7 Maturation du virus. 8 Libération du virus

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1. L'entrée du HCV

Première étape du cycle viral infectieux du HCV, l'entrée du virus dans les cellules hôtes implique une série complexe d'interactions qui se déroule de manière séquentielle. On peut diviser l'entrée du HCV en trois grandes étapes : l'attachement du virus à la cellule hôte, l'entrée du virus à proprement parler constituant l'étape de post attachement suivant la liaison du virus à la cellule hôte, puis enfin l'internalisation du virus dans la cellule suivie de la fusion membranaire. L'entrée virale est un processus compliqué qui nécessite d'une part l'engagement des facteurs du HCV comme les glycoprotéines E1 et E2 (Penin et al., 2001) et d'autre part des facteurs de la cellule hôte qui interagissent de manière directe ou indirecte avec les virions. Parmi ces facteurs on retrouve les HSPG (heparan sulfate proteoglycan), le LDL-R (low-density lipoprotein receptor), SR-BI (scavenger receptor class B type I), les récepteurs à tyrosine kinase

EGFR (epidermal growth factor receptor) et EphA2 (ephrin receptor A2), les protéines de jonction serrée CLDN1 (claudin 1) et OCLN (occludin) et, plus récemment découvert le NPC1L1 (Niemann-Pick C1-like 1). Par ailleurs, il a été démontré que l'entrée virale était une étape importante pour le tropisme hépatocellulaire et la spécificité d'espèce (Ploss et al. 2009).

A. *L'attachement du HCV*

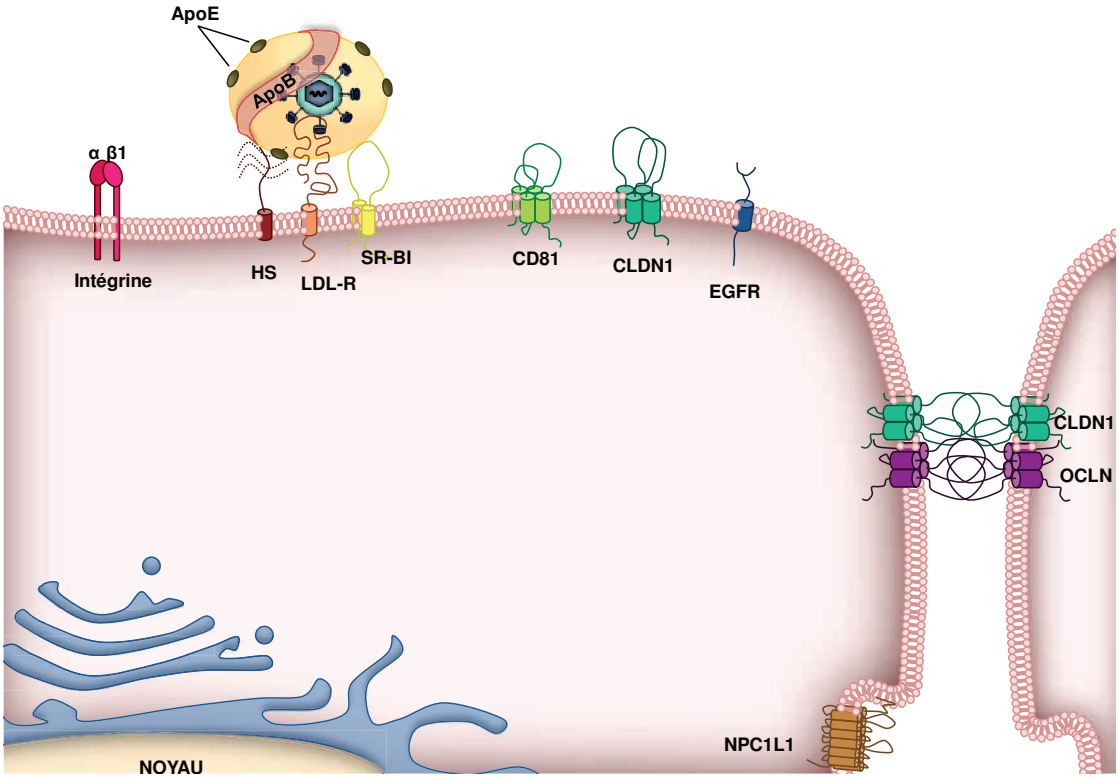

Figure 9 : Attachement du HCV en association avec les lipoprotéines à la surface des hépatocytes

L'étape d'attachement du HCV représente la toute première étape du cycle viral correspondant à la captation du virus à la surface de la cellule hôte (Figure 9).

Les virions du HCV circulent dans le sang sous différentes formes. On peut ainsi retrouver le virus sous forme libre ou sous forme de lipoparticules virales (LVP) qui représentent l'association du virus avec des lipoprotéines comme apoE, apoB, apoC1, apoC2 et apoC3 (Andre et al, 2002). Ainsi il semble qu'à la fois les glycoprotéines d'enveloppe du HCV mais aussi les lipoprotéines contribuent aux premières étapes d'attachement du HCV à la surface de la cellule hôte. Du fait de l'association du HCV avec les lipoprotéines, de nombreuses études ont été effectuées sur l'importance pour l'entrée du HCV des différents facteurs impliqués dans

le métabolisme lipidique comme SR-BI, le LDL-R ou les HSPG. Ces études ont permis d'identifier un certains nombre de facteurs d'entrée, importants pour l'étape d'attachement, qui interagissent soit directement avec les glycoprotéines d'enveloppe, soit via les lipoprotéines des LVP :

Les HSPG appartiennent à la famille des glycosaminoglycanes (GAGs) et sont des polysaccharides linéaires exprimés à la surface des cellules. Ils sont connus pour être utilisés par de nombreux flavivirus (virus de la Dengue (Chen et al. 1997) ; virus de la fièvre jaune) comme moyen de liaison des virus à la membrane cellulaire. Considérés comme le premier site de liaison au HCV, ils représentent des récepteurs abondants mais de faible affinité (Barth et al., 2003). Il a été démontré que l'utilisation d'héparine (un homologue de l'héparane sulfate) ou d'héparinase (enzyme dégradant les HS) empêchait la liaison du HCV à la surface des cellules hépatocytaires (Germi et al. 2002, Koutsoudakis et al. 2006). Il a été montré dans un premier temps que les glycoprotéines E1 et E2 solubles sont capables d'interagir avec les HSPG (Barth et al. 2003; Barth et a. 2006). Cependant des études plus récentes ont permis de démontrer qu'il n'existait pas de liaison entre l'héparine et les hétérodimères E1E2 (Callens et al., 2005), suggérant que la liaison du HCV aux HSPG se ferait de manière indirecte via les lipoprotéines et plus particulièrement apoE (Andreo et al. 2007, Chang et al. 2007). Cette conclusion est appuyée par une récente étude démontrant l'importance de **syndecan-1 (SDC1),** un membre de la famille des HSPG, dans l'attachement du virus (Chi et al. 2013). De plus cette étude démontre que la liaison du HCV à SDC1 se fait par l'intermédiaire de apoE (Chi et al., 2013).

Le LDL-R transporte les lipoprotéines contenant du cholestérol du milieu extracellulaire à l'intérieur des cellules participant ainsi à l'homéostase du cholestérol. Les principaux ligands du LDL-R sont les lipoprotéines de faible densité (LDL) ou de très faible densité (VLDL) qui contiennent des apolipoprotéines telles qu'apoE et apoB (Owen et al., 2009). Par ces caractéristiques de fonctionnement et par le fait que le HCV est retrouvé sous forme de LVP, le LDL-R a naturellement été étudié comme candidat potentiel en tant que facteur d'attachement du HCV. Il a ainsi pu être démontré que des anticorps dirigés contre le LDL-R , apoB ou apoE inhibaient l'endocytose du HCV (Agnello et al., 1999) et plus particulièrement il a pu être démontré que le LDL-R participait aux premières étapes d'entrée du HCV dérivé de sérum dans les PHH (Molina et al., 2007). Cette interaction du HCV avec le LDL-R ne semble pas s'effectuer via E2 (Wunschmann et al., 2000) mais plutôt par l'interaction avec les LDL (Agnello et al., 1999). Cependant, une étude récente pose l'hypothèse que plus que son action sur

l'attachement du HCV, le LDL-R jouerait plutôt un rôle dans la réplication du HCV, ceci en agissant sur la concentration de cholestérol dans les hépatocytes (Albecka et al., 2012). Ainsi la fonction d'internalisation par le LDL-R résulte plutôt en une internalisation non spécifique du HCV conduisant à une infection non productive (Albecka et al., 2012).

Le récepteur SR-BI (aussi appelé CLA-1) est une glycoprotéine de 509 acides aminés avec deux domaines N et C terminaux cytoplasmiques séparés par un large domaine extracellulaire impliqué dans le métabolisme lipidique. SR-BI est connu pour être un récepteur pour un grand nombre de lipoprotéines (HDL, LDL et LDL oxydé) et est responsable de l'incorporation sélective du cholestérol estérifié contenu dans les HDL. Son mécanisme d'action est bidirectionnel : d'une part il permet l'entrée du cholestérol estérifié, et d'autre part il va permettre la régulation de l'efflux du cholestérol cellulaire. SR-BI a une distribution plutôt spécifique, on le retrouve fortement exprimé dans les tissus stéroïdogènes comme les glandes surrénales et les ovaires mais aussi au niveau du foie (Krieger et al. 2001). Grâce à des études de liaison avec sE2 (Scarselli et al. 2002), d'inhibition (Zeisel et al. 2007) et de surexpression de SR-BI (Grove et al. 2008), SR-BI a pu être identifié comme un des récepteurs participant à l'entrée du HCV dans les hépatocytes *in vitro* mais aussi *in vivo* (Meuleman et al. 2012). Par ailleurs, vu sa fonction de liaison aux lipoprotéines, SR-BI interagit probablement avec le virion à travers E2 et/ou les lipoprotéines (Catanese et al. 2007, Dreux et al. 2009). En effet, il est intéressant de noter que SR-BI a une double d'action : SR-BI jouerait un rôle à la fois lors de l'attachement (Catanese et al. 2010) et lors des étapes de post-attachement (Dao Thi et al. 2012; Zahid et al. 2012). L'hypothèse serait que, dans un premier temps lors de l'étape d'attachement, SR-BI interagirait avec le HCV via les lipoprotéines et donc E2 indépendante. Puis, par la suite, lors de l'étape « d'entrée » ou de post attachement, cette interaction deviendrait E2 dépendante (cf chapitre suivant).

Il est intéressant de noter qu'en plus de ces différents facteurs intervenant dans l'attachement du virus, d'autres facteurs interagissant directement avec E2 ont été identifiés, comme **les lectines de type C : les DC-SIGN et L-SIGN** (Gardner et al. 2003). Ces facteurs ne sont pas présents à la surface des hépatocytes mais on les retrouve respectivement à la surface des cellules dendritiques et des cellules endothéliales du foie. Ces protéines semblent potentiellement représenter une voie de transfection permettant la captation du virus via un autre type cellulaire pour ensuite le relocaliser au niveau des hépatocytes où le cycle viral pourra s'effectuer (Ludwig et al., 2004 ; Cormier et al., 2004).

B. L'entrée du HCV

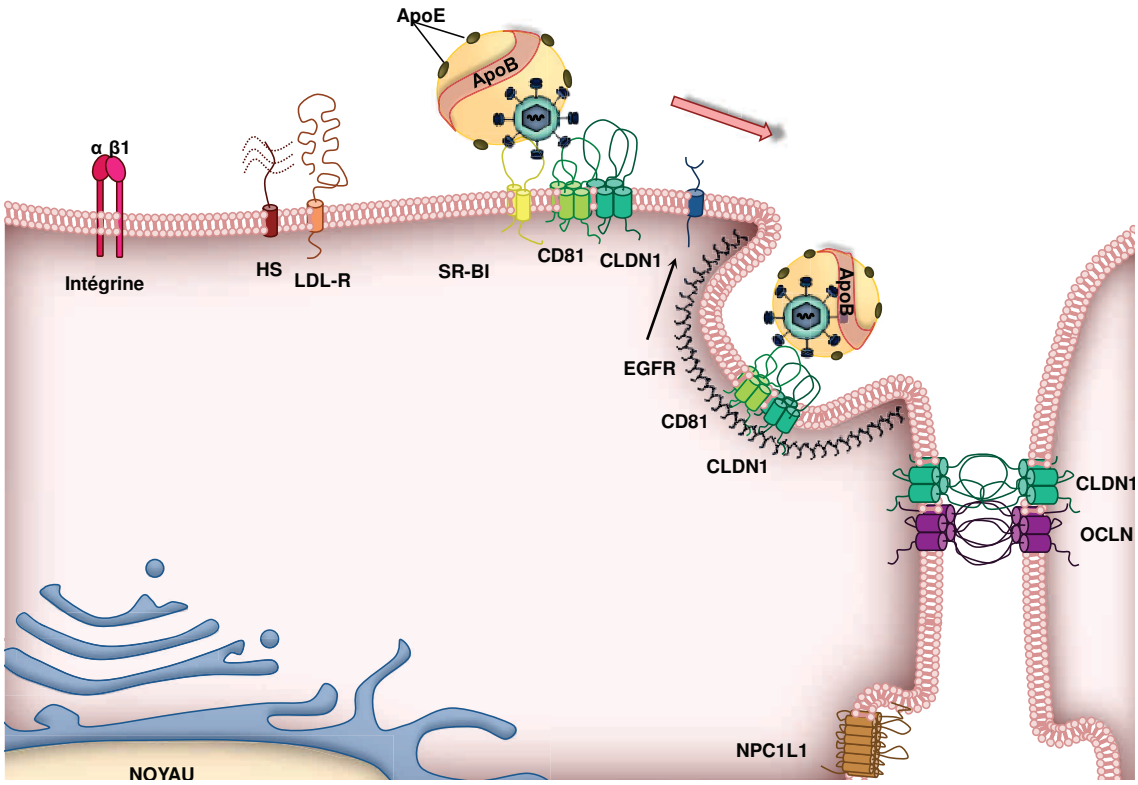

Figure 10 : Post attachement du HCV aux hépatocytes

Une fois le virus lié à la surface de la cellule, s'opère l'étape de post attachement correspondant à l'étape d'entrée du HCV à proprement parler qui conduira à l'endocytose du virus dans les hépatocytes (Figure 10). Cette étape va faire intervenir de nombreux facteurs cellulaires à travers des interactions directes ou indirectes avec le virion et engendrant un réarrangement moléculaire au niveau de la membrane plasmique.

Ainsi on pourra observer le deuxième mode d'action de SR-BI. Correspondant à l'action de SR-BI lors du post-attachement, plusieurs études cinétiques ont permis de démontrer que l'interaction SR-BI/HCV durant cette étape de post attachement était essentielle, l'utilisation d'anticorps ou de petites molécules (ITX-5061) inhibant l'interaction SR-BI/HCV durant cette étape engendrant une très sévère diminution de l'infection (Syder et al., 2011; Zahid et al.,

2012). Il a pu être démontré, grâce à l'utilisation de sE2, que SR-BI pouvait interagir directement avec la glycoprotéine E2 du HCV (Scarselli et al., 2002) et que de manière intéressante cette interaction était spécifique à l'espèce, le SR-BI murin n'étant pas capable de se lier à sE2. Plus particulièrement on a identifié que cette interaction se faisait via la boucle extracellulaire de la région HVR1 de E2, la délétion de HVR1 abolissant l'interaction de sE2 avec SR-BI et conduisant à une diminution de l'entrée du HCV (Bartosch et al., 2003b; Scarselli et al., 2002; Dao Thi et al., 2012a). D'autre part il a été observé grâce à des cinétiques d'infection que SR-BI interviendrait au même moment que CD81 et CLDN1, suggérant une coopération entre les deux récepteurs à une étape faisant suite à l'attachement du virus (Zeisel et al. 2007; Krieger et al. 2010). Cette hypothèse est appuyée par l'étude démontrant que l'augmentation de l'infection des HCVcc médiée par la présence des HDL n'est possible que lorsque CD81 est exprimé (Dreux et al., 2006). Récemment, il a même été identifié une troisième fonction de SR-BI en plus de celle de l'attachement et du post-attachement. SR-BI aurait un troisième mode d'action lors de l'infection qui ne dépend pas du complexe HCV E2/SR-BI, mais qui agit sous l'influence du transfert lipidique en facilitant les différentes étapes d'entrée du virus avec les autres co-facteurs d'entrée du HCV (Dao Thi et al., 2012a). Ces études nous démontrent que SR-BI est un facteur multifonctionnel et que ces différents mode d'action sont essentiels pour l'infection.

CD81 appartenant à la famille des tétraspanine comme les CLDN ou l'intégrine, CD81 est composé de quatre domaines transmembranaires, de deux boucles extracellulaires SEL et LEL (small extracellulaire loop et large extracellulaire loop), d'une petite boucle intracellulaire et de deux extrémités N- et C-terminales intra cytoplasmiques. CD81 a été le premier récepteur d'entrée du HCV identifié (Pileri et al., 1998) comme interagissant avec sE2. De nombreuses études ont pu démontrer l'importance de CD81 dans le processus d'entrée du HCV. L'inhibition de CD81 par des anticorps dirigés contre CD81 ou la réduction de son expression par des siRNA (small interfering RNA) conduit à une forte inhibition de l'entrée autant des HCVpp que des HCVcc dans les cellules Huh7 (Zhang et al., 2004). De plus l'expression ectopique de CD81 dans les cellules HepG2 non permissives au HCV n'exprimant pas CD81 rend ces cellules permissives au HCVpp et HCVcc (Bartosch et al., 2003b; Lavillette et al., 2005), démontrant le rôle crucial de CD81 dans l'infection. Il a été suggéré que CD81 intervenait dans l'étape suivant la liaison du virus à la cellule hôte (l'étape de post attachement) car on a pu démontrer que les anticorps anti CD81 inhibaient l'infection par le HCV uniquement après sa liaison à la

surface de la cellule (Cormier et al., 2004b; Flint et al., 2006; Koutsoudakis et al., 2006), ceci étant confirmé par le fait que la susceptibilité au virus n'est pas seulement dépendante de l'expression de CD81 mais aussi de celle de SR-BI à la surface de la cellule (Kapadia et al., 2007) corroborant leur fonctionnement coopératif (cf chapitre précédent sur SR-BI). En surexprimant différentes combinaisons des quatre co-facteurs CD81, SR-BI, CLDN1 et OCLN murins et humains dans des lignées de hamster ou de souris (cellules CHO et NIH3T3), il a été montré que les récepteurs humains CD81 et OCLN rendent les cellules murines infectables par les HCVpp. Ainsi, en plus de son rôle dans le post attachement CD81 a été identifié comme essentiel à la spécificité d'espèce étant un des deux facteurs minimum humains avec OCLN nécessaire à l'entrée virale dans les cellules hépatocytaires non humaines (Ploss et al., 2009).

Les protéines de jonction CLDN et OCLN :

La protéine CLDN1 : elle fait partie de la famille des tétraspanines. Elle est composée de quatre domaines transmembranaires, de deux boucles extracellulaires et deux extrémités N- et C-terminales intracellulaires. Elle est exprimée à la surface de tous les tissus épithéliaux, mais on la retrouve en forte abondance au niveau du foie. Avec OCLN, c'est une des protéines essentielles pour la formation des jonctions serrées. Grâce à l'utilisation de screen HCVpp, elle a pu être identifiée comme un facteur important pour l'entrée du HCV (Evans et al., 2007). Son expression dans les cellules non hépatiques 293T non permissives (lignées cellulaires non hépatiques dérivées du rein humain) qui n'expriment pas de CLDN les rend susceptibles au HCVpp. A l'opposé, la suppression de l'expression de CLDN1 dans les Huh7.5 inhibe l'infection par le HCV (Evans et al., 2007). Il est intéressant de noter qu'il n'y a pas d'interaction directe entre les glycoprotéines d'enveloppe du HCV et CLDN1, mais que son rôle est indirect et se joue plutôt lors du post attachement grâce à son interaction avec CD81 (Evans et al., 2007; Harris et al., 2008; Krieger et al., 2010). CLDN1 est un important facteur pour la jonction serrée cependant le complexe de corécepteur CLDN1/CD81 a pu être détecté uniquement au niveau de la membrane basale des cellules hépatocytaires humaines polarisées HepG2, supposant que la CLDN1 qui joue un rôle dans l'étape de post attachement est la CLDN1 qui n'est pas associée aux jonctions serrées (Mee et al., 2009).

Des études ont semblé démontrer que d'autres membres de la famille CLDN auraient un rôle potentiel dans l'entrée du HCV. Ainsi CLDN6 et CLDN9 pourraient être impliquées dans l'entrée du HCV dans la cellule hôte (Zheng et al., 2007) étant donné que la surexpression de

ces deux CLDN dans les 293T entraine une permissivité aux HCVpp (Meertens et al., 2008). Cependant des études plus approfondies sont nécessaires pour confirmer leur réelle implication, l'expression de CLDN6 et CLDN9 n'ayant pas encore été confirmée dans des cellules hépatocytaires humaines.

La protéine OCLN : OCLN est elle aussi une protéine composée de quatre domaines transmembranaires, de deux boucles extracellulaires et deux extrémités N- et C-terminales intracellulaires. Il a été démontré que l'utilisation de siRNA dirigés contre CLDN1 ou OCLN diminuant fortement leur expression conduisait à une inhibition de l'entrée des HCVpp et HCVcc (Liu et al., 2009). Cela démontre qu'OCLN est un autre facteur cellulaire de l'hôte, essentiel pour l'entrée du HCV, probablement à un stade post-attachement (Ploss et al. 2009). Contrairement à CLDN1, des études semblent démontrer que OCLN pourrait interagir avec la glycoprotéine E2. En effet, des analyses microscopiques ont montré qu'OCLN et E2 étaient colocalisées au niveau du RE et que cette association pouvait être confirmée par co-immunoprécipitaion (Benedicto et al., 2008). Une étude a pu démontrer que la suppression de l'expression d'OCLN engendrait une altération de la fusion membranaire glycoprotéine dépendante (Benedicto et al., 2009). L'hypothèse serait qu'OCLN interviendrait entre autre dans les étapes tardives de l'entrée comme lors de l'étape de fusion membranaire. En effet une étude récente s'est focalisée sur l'élucidation du rôle d'OCLN dans l'entrée du HCV. Les auteurs ont pour cela créé des mutants d'OCLN (Flag-OCLN) dont l'activité sur l'entrée virale pouvait être conditionnellement inhibée par l'ajout d'anticorps spécifiques anti-Flag dans des cellules HepG2 polarisées transformées pour exprimées CD81 et miR-122 leur permettant de conduire tout le cycle infectieux. Les auteurs ont pu ainsi confirmer qu'OCLN intervenait dans une étape tardive de l'entrée du HCV et leur étude suggère que cette interaction OCLN/HCV est une interaction directe (Sourisseau et al., 2013). Par ailleurs, comme précisé précédemment (paragraphe CD81), OCLN est aussi un des facteurs responsable de la spécificité d'espèce du HCV (Ploss et al., 2009).

Les récepteurs à tyrosines kinases (RTK) EGFR et EphA2 : il s'agit de protéines transmembranaires avec un grand domaine transmembranaire qui sera le domaine de liaison au ligand et un domaine intracytoplasmique qui contient les sites de phosphorylations responsables de l'activation de la signalisation intracellulaire. EphA2 est connu pour réguler la prolifération cellulaire par l'intermédiaire de l'interaction avec son ligand naturel l'ephrine A1 (Ferluga et

al., 2013). Parmis les ligands de l'EGFR on compte le TGFα (transforming growth factor alfa) et l'EGF (epidermal growth factor). Ce récepteur est connu pour réguler lui aussi la prolifération cellulaire, la survie cellulaire mais il est aussi connu pour participer au processus de tumorogenèse (Schneider and Wolf, 2009). EphA2 et EGFR ont été identifiés comme intervenant dans l'entrée du HCV, grâce à un screen fonctionnel par siRNA et confirmé par l'utilisation d'inhibiteurs de protéine kinase comme dasatinib (inhibiteur d'EphA2) et erlotinib (inhibiteur d'EGFR). Il a pu ainsi être démontré qu'ils étaient deux co-facteurs pour l'entrée et l'infection par le HCV *in vitro* et *in vivo* (Lupberger et al., 2011). Il est cependant intéressant de noter qu'EGFR n'interagit pas directement avec le HCV, mais joue un rôle important au cours des étapes post attachement. EGFR, notamment via l'activation de voies de signalisation intracellulaires, intervient à la fois dans l'association CD81/CLDN1 et lors de la fusion membranaire (Lupberger et al., 2011; Zona et al., 2013).

NPC1L1 est un récepteur impliqué dans le transport du cholestérol. Il possède 13 domaines transmembranaires, avec un grand domaine N terminal extracellulaire et un petit domaine C terminal intracytoplasmique (Wang and Song, 2012). NPC1L1 est localisé au niveau du canalicule biliaire des hépatocytes et est responsable de l'absorption du cholestérol des canalicules vers les hépatocytes. Ce récepteur à récemment été identifié comme intervenant dans l'entrée du HCV (Sainz et al., 2012). En bloquant ou en supprimant l'expression de NPC1L1 à la surface des cellules hépatocytaires, Sainz *et al.* ont pu démontrer une diminution très importante de l'entrée du HCV. De plus avec l'utilisation d'inhibiteurs spécifiques à NPC1L1, comme ezetimibe, ils ont pu démontrer que NPC1L1 intervenait potentiellement lors de l'étape de post attachement du HCV et que les mécanismes d'action de ce récepteur étaient cholestérol dépendant (Sainz et al., 2012). Cependant le rôle exact de NPC1L1 dans l'entrée reste encore à définir.

C. L'internalisation et la fusion membranaire

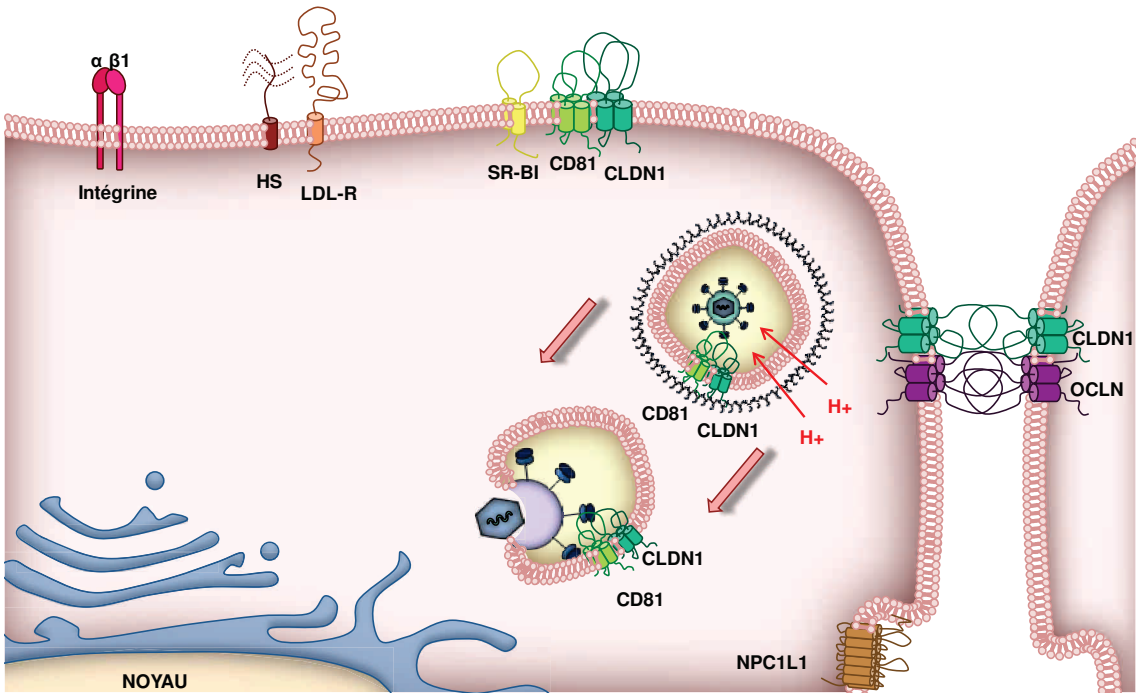

Figure 11: Internalisation clathrine dépendante et fusion membranaire du HCV

L'internalisation du HCV se fait par endocytose dépendante des vésicules à clathrine (Blanchard et al. 2006; Codran et al. 2006). On ignore si tous les récepteurs participant à l'entrée virale sont internalisés en même temps que le HCV. Cependant, il a récemment été démontré que durant ce processus le complexe CD81-CLDN était lui aussi internalisé de manière clathrine dépendante (Farquhar et al 2012). Cela corrobore l'étude précédente démontrant que les particules HCV entraient dans la cellules toujours en association avec le complexe CD81/CLDN1 (Coller et al. 2009) (Figure 11).

Après l'internalisation, on observe l'étape de fusion membranaire : elle se déroule dans l'endosome précoce et correspond à la fusion de l'enveloppe lipidique du virus avec la membrane de l'endosome afin de libérer l'ARN génomique du HCV dans le cytosol (Coller et al. 2009). Ce mécanisme est un processus pH dépendant et va nécessiter une acidification et l'implication à la fois des protéines virales et des protéines de l'hôte (Blanchard et al. 2006; Tscherne et al. 2006). Les glycoprotéines d'enveloppe E1E2 contiennent un domaine de fusion (Lavillette et al., 2007). De plus il a été démontré qu'E2 est nécessaire à la fusion endosome/HCVcc *in vitro* (Haid et al., 2009). De même il a pu être démontré que le complexe CD81/CLDN1 endocyté avec la particule virale jouait lui aussi un rôle dans la fusion

membranaire dépendante des glycoprotéines (Evans et al., 2007). Il semblerait qu'EGFR et EphA2 interviennent eux aussi lors de cette étape de fusion, l'utilisation d'inhibiteurs de protéine kinase engendrant une réduction du processus de fusion (Lupberger et al. 2011).

En résumé, l'entrée et l'internalisation du HCV est un processus multifactoriel très organisé : dans un premier temps le virus va se lier et venir se concentrer à la surface des cellules grâce a différents facteurs comme les HSPG, le LDL-R et SR-BI, cette interaction se fera majoritairement via la lipoprotéine associée au virus. Après la liaison du virus à la surface de la cellule, la particule virale va venir interagir avec différents facteurs cellulaires qui seront responsables de l'entrée virale comme SR-BI, CD81, CLDN1 et OCLN. Il n'y a pas à ce jour de consensus scientifique quant à l'orchestration exacte des évènements qui amènent à l'internalisation du virus. Une hypothèse serait que le HCV dans un premier temps est lié à SR-BI via les lipoprotéines, puis un changement conformationnel pourrait s'observer permettant à HCV de pouvoir interagir avec SR-BI puis avec CD81 via la glycoprotéine E2, les deux récepteurs agissant de manière coopérative (Helle and Dubuisson, 2008). On observe ensuite la formation du complexe de corécepteurs CD81/CLDN1 (Harris et al., 2008) sous l'influence du cofacteur EGFR (Lupberger et al., 2011). Par la suite, le HCV est internalisé par endocytose dépendante de la clathrine et de la dynamine vers les endosomes précoces (Blanchard et al., 2006; Meertens et al., 2006). Puis va s'opérer la fusion membranaire (processus pH dépendant) sous l'influence de certains facteurs comme le complexe CD81/CLDN1, EGFR et OCLN qui va permettre la décapsidation du virus puis la libération de son génome viral dans le cytosol où il pourra subir la traduction.

2. La traduction et la réplication virale

Après la fusion membranaire le génome du HCV sous forme d'ARN simple brin de polarité positive va être libéré dans le cytosol où il va servir directement de patron pour la traduction. **L'initiation de cette traduction** se fera via l'IRES de manière cap indépendante. Il est intéressant de noter que le microRNA-122 (miR-122) plus connu pour son implication dans la réplication (Jopling et al., 2005) a été identifié comme pouvant stimuler la traduction via l'interaction avec la 5'NTR du HCV (Nasheri et al., 2011).

La traduction va conduire à l'obtention d'une polyprotéine précurseur immature qui sera redirigée vers le RE où elle subira sa maturation (cf chapitre : les protéines non structurales). Ce processus se fait de pair avec la formation du « complexe de réplication » où va pouvoir se dérouler la réplication.

Le complexe de réplication : la formation de ce complexe de réplication est importante pour le bon déroulement de la réplication. Ce complexe englobe les vésicules à double membrane (VDM) contenant les protéines non structurales du HCV, les membranes du RE, l'ARN du HCV et les gouttelettes lipidiques. La formation de ce complexe va impliquer un certains nombres de facteurs cellulaires et viraux. Dans un premier temps, sous l'action des protéines non structurales du HCV, la membrane du RE va subir une altération conduisant à la formation de ce qui est appelé un « web membranaire » (Gosert et al. 2003). Bien que les mécanismes engendrant la création de ce web membranaire ne soient pas encore bien définis, il semblerait que la protéine non structurale NS4A soit l'actrice majeure de ce réarrangement membranaire avec la participation secondaire de NS5A, le complexe NS3/4A et NS5B (ARN polymérase ARN dépendante). Ce web membranaire va permettre de former les VDM. Le rôle de ces VDM est encore mal connu mais il semblerait que la synthèse du brin d'ARN négatif (ARN-) qui servira de modèle pour la synthèse des brins d'ARN positifs pour les nouveaux virions a lieu au sein de ces VDM. L'initiation de la synthèse de l'ARN- nécessite la participation de la 3'NTR et NS5B (Binder et al. 2007). Il semblerait qu'un seul exemplaire d'ARN- soit présent par vésicule ce qui va permettre la synthèse de plusieurs nouveaux brins d'ARN+ grâce à NS5B et la fonction hélicase du complexe NS3/4A. Une fois synthétisés ces nouveaux brins d'ARN seront libérés dans le cytoplasme pour venir participer à la formation de nouvelles particules virales HCV.

Les facteurs de l'hôte participant à la synthèse d'ARN : il a pu être démontré qu'un certains nombres de facteurs cellulaires de l'hôte pouvaient participer à la réplication (Georgel et al., 2010). Ainsi par exemple, des études ont démontrés que **les cyclophilines A et B** (CyPA, CyPB) avaient un rôle essentiel lors de la réplication (Watashi et al., 2003). En effet la CyPB interagit avec NS5B et régule sa liaison au brin d'ARN (Watashi et al., 2005) et des études récentes ont démontré que la CyPA possédait elle aussi un rôle critique dans la réplication du HCV (Kaul et al. 2009; Liu et al. 2009). D'ailleurs, l'alisporivir un antiviral ciblant la CyPA, a démontré son efficacité dans la réduction de la charge virale et est actuellement en étude clinique (Gallay and Lin, 2013). Des protéines kinases comme la **phosphatidylinositol 4-kinase III alpha** joueraient également un rôle dans la réplication virale (Reiss PloS Pathogens 2013).

De même, il a été observé que **le microARN-122 (miR-122)** spécifique au foie et représentant près de 70% des miRNA du foie (Chang et al., 2004) régulait la concentration d'ARN viral HCV dans les cellules *in vitro* (Jopling et al., 2005) et *in vivo* (Lanford et al., 2010). De manière surprenante, alors qu'en général la plupart des miRNA ont une action restrictive sur l'expression des gènes en se liant à l'extrémité 3'NTR des ARN messagers (mRNA), miR-122 agit lui en augmentant la production d'ARN viral. Deux sites de liaison à l'ARN du HCV en 5'NTR et 3'NTR ont pu être identifiés et finalement il a pu être démontré que le site crucial pour la réplication de l'ARN viral était le site présent en 5'NTR (Jopling et al., 2005). Assez récemment une étude a pu identifier deux positions nucléotidiques de liaison à miR-122 dans la région 5'NTR qui, quand elles sont mutées, suppriment la réplication de l'ARN du HCV. Les auteurs émettent l'hypothèse que, lorsque que miR-122 se lie à ces deux sites, il se forme un complexe mRNA/cible inhabituel qui permettrait de protéger l'ARN viral de la dégradation par les RNases et/ou de la réponse immunitaire innée (Machlin et al., 2010). Il a également pu être démontré que miR-122 pouvait stabiliser le génome viral sous la dépendance d'Ago2 (Shimakami et al., 2012). Cependant, bien que de nombreuses études démontrent l'importance de miR-122 dans la réplication de l'ARN et dans le tropisme hépatocytaire du HCV, les mécanismes qui permettent cette régulation sont encore mal définis.

3. L'assemblage et la libération de la particule virale

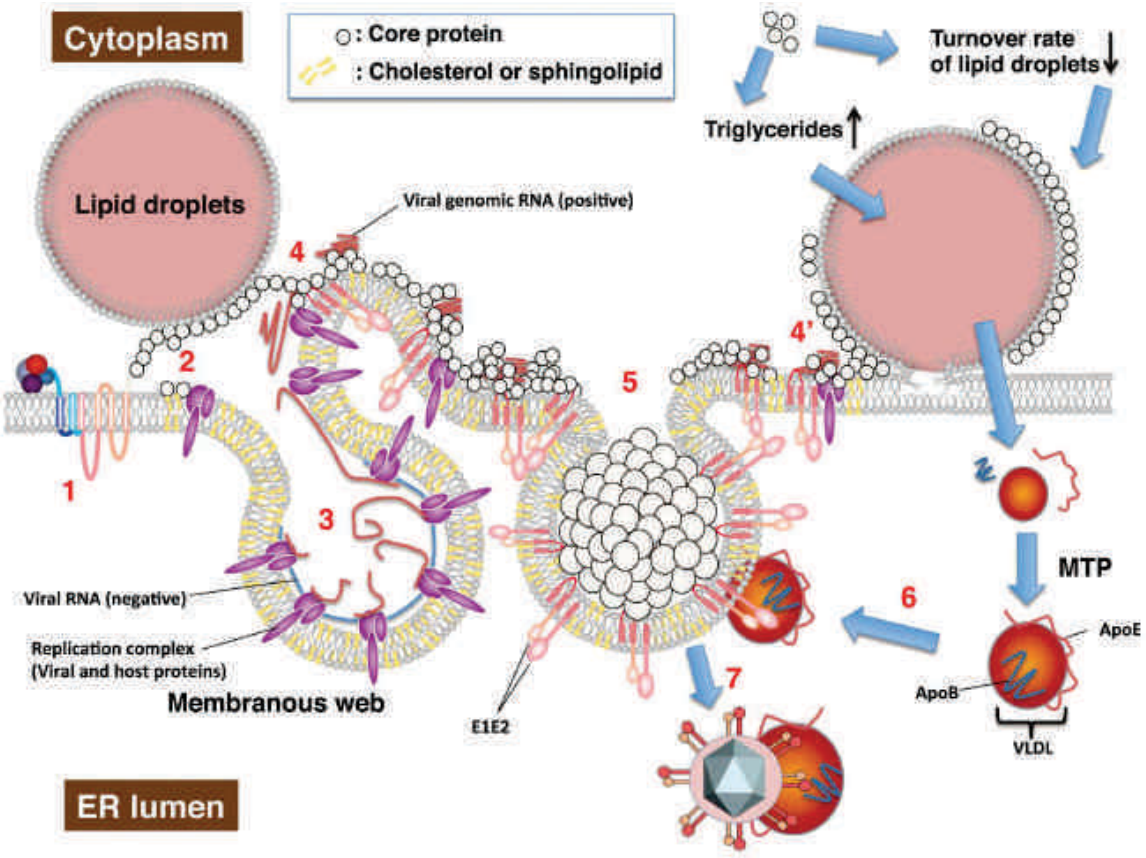

Figure 12: Représentation schématique de la réplication et de l'assemblage du HCV au niveau du RE : 1- L'ARN viral génomique est traduit en une polyprotéine qui va subir sa maturation par clivage. 2- Les protéines non structurales en association avec les facteur de l'hôte vont former le complexe de réplication d'une part . Et d'autre part les protéines Core vont être relocalisées au niveau des gouttelettes lipidiques (LD). 3- Sous l'influence de NS4B on observe la formation du web membranaire et du brin d'ARN-, les brins d'ARN+ étant synthétisés grâce au complexe de réplication. 4- L'ARN+ est empaqueté dans les protéines Core grâce à la coopération des protéines non structurales virales et des facteurs cellulaires qui vont permettre de recruter les protéines Core des LD au RE. 5- La protéine Core englobe l'ARN viral génomique du HCV et forme la nucléocapside qui est associée à la glycoprotéine E1E2. 6- Les VLDL vont venir se lier à la particule virale pour former les LVP. 7- les LVP sont excrétés via la lumière du RE à travers la voie de sécrétion de la cellule.
Moriishi et al. Frontiers in Microbiology 2012

L'assemblage de la particule virale HCV (Figure 12) est un processus finement synchronisé qui nécessite la présence des protéines structurales et non structurales du HCV mais aussi les facteurs cellulaires de l'hôte comme les gouttelettes lipidiques (LD).

Un certain nombre de facteurs viraux sont impliqués dans l'assemblage viral. D'une part il faut considérer les protéines structurales qui permettront la formation de la particule englobant l'ARN viral : la protéine Core qui sera redirigée vers le RE après la SP (Signal peptidase) et par la suite à la surface des LD du cytosol après un nouveau clivage par la SPP (Signal peptide peptidase) (McLauchlan, J. et al. 2002). Les glycoprotéines E1E2, quant à elles, seront retenues au niveau du RE sous forme d'hétérodimères. D'autre part, on a les protéines non stucturales

comme la protéine p7 (Jirasko et al .2010), NS2 (Jirasko et al., 2008), NS5A et plus particulièrement le DIII de NS5A (Masaki, T. et al., 2008). Plus récemment il a même pu être démontré que NS4B et le complexe NS3/NS4A pouvaient influencer la production *de novo* de particules virales (Jones et al. 2009 ; Jirasko et al. 2010).

De nombreux facteurs cellulaires sont eux aussi impliqués dans l'assemblage. La plupart sont impliqués dans la voie de synthèse des VLDL afin de former la LVP. Il a pu être montré que l'utilisation d'inhibiteur de la microsomal triglyceride transfer protein (MTP), enzyme clé impliquée dans la production des VLDL, engendrait une diminution de la production des virus, démontrant le rôle crucial des VLDL pour la formation de nouveau virion HCV (Gastaminza et al., 2008). Les facteurs identifiés comme les plus cruciaux sont apoE, apoB et les LD. ApoE est une apolipoprotéine connue pour être présente sur les LVP infectieuses et pour participer à l'entrée du HCV (cf chapitre l'entrée du HCV), mais il a aussi été démontré que c'était un facteur crucial pour l'assemblage de la particule virale grâce à son association avec NS5A (Chang et al. 2007 ; Benga et al. 2010). Le rôle d'apoB dans l'infection et l'assemblée reste encore controversé (Bartenschlager et al., 2011; Jiang and Luo, 2009). Les LD semblent avoir un rôle clé dans l'assemblage par leur association avec les protéines Core. Il a pu être démontré que si l'on bloque le clivage de Core par le SPP empêchant ainsi son recrutement à la surface des LD, on bloque la production de virions (Targett-Adams et al. 2008). Plus récemment un autre facteur a été identifié comme facteur essentiel à la formation des particules HCV : la diacylglycerol acyltransferase-1 (DGAT1) (Herker, et al. 2010)(Camus JBC 2013). Cette enzyme connue pour être impliquée dans la biogénèse des LD se lie à la protéine Core pour la localiser au niveau des LD et donc au niveau des sites d'assemblage. Lorsque DGAT1 est inhibée on observe une diminution marquée de la production de particules virales HCV argumentant l'hypothèse que l'assemblage nécessite la formation des LD de manière DGAT1 dépendante. (Herker et al., 2010).

Bien que le détail des évènements qui conduisent à l'assemblage des particules HCV ne soit toujours pas entièrement élucidé et reste sujet à controverse, l'hypothèse serait que les brins d'ARN+ qui ne sont pas utilisés pour la synthèse des protéines virales vont être encapsidés par la protéine Core pour former de nouveau virions. Ce processus prend place quand l'ARN viral va venir interagir avec les protéines Core présentes à la surface des LD pour former la nucléocapside (Roingeard and Hourioux, 2008). Les mécanismes qui permettent cette encapsidation ne sont toujours pas compris. Une fois la nucléocapside formée, on observera la maturation des particules virales qui consiste en l'enveloppement et l'incorporation de lipides

ainsi que des glycoprotéines E1E2 à la nucléocapside. Ce processus se fait conjointement à la voie des VLDL. Comment l'incorporation de E1E2 se fait, ou comment les apolipoprotéines sont associées aux particules virales infectieuses matures, reste cependant encore à être défini.

Les LVP ainsi formées se retrouvent dans la lumière du RE où elles pourront être excrétées de la cellule grâce à la voie sécrétoire : transport à travers le RE, puis à travers l'appareil de Golgi et enfin exocytose.

4. La transmission virale cellule à cellule

L'initiation de l'infection se fait par les virions libres présents dans le sang qui entrent dans les hépatocytes (voies décrites dans les chapitres précédents décrivant l'entrée virale) que l'on peut appeler « infection libre ». Toutefois la dissémination virale, afin d'établir une infection chronique, pourra se faire via plusieurs routes d'infection, dont une qui va se faire directement d'hépatocytes à hépatocytes adjacents, qu'on appelle « infection cellule à cellule » (Timpe et al., 2008). Bien qu'utilisant de nombreux facteurs cellulaires identiques à ceux utilisés pour « l'infection libre », l'infection cellule à cellule semble plus efficace, et est résistante à la majorité des anticorps neutralisants. Elle représente ainsi un moyen potentiel d'échappement à la réponse immunitaire humorale afin d'établir une infection chronique (Brimacombe et al., 2011)

L'infection cellule à cellule comme citée précédemment possède un certain nombre de similarité de mécanismes avec l'infection libre. Par exemple elle requiert un grand nombre de facteurs cellulaires qui sont communs avec l'infection libre comme SR-BI, CD81, CLDN1, OCLN, EGFR, EphA2 et probablement NPC1L1 (Brimacombe et al., 2011; Lupberger et al., 2011; Sainz et al., 2012b; Schwarz et al., 2009; Timpe et al., 2008; Zahid et al., 2012). Il est cependant intéressant de noter que l'on observe toutefois certaines différences : une infection cellule à cellule CD81 indépendante a pu être identifiée dans certaines études (Jones et al., 2010; Timpe et al., 2007; Witteveldt et al., 2009). Ces informations nous démontrent qu'une meilleure connaissance de ce mécanisme d'infection est essentielle à la compréhension de l'établissement de la chronicité du HCV et pourrait conduire au développement de thérapies qui cibleraient à la fois l'initiation et la dissémination de l'infection.

II. OBJECTIFS

L'objectif principal de mon travail de thèse a été d'étudier les facteurs cellulaires responsables de l'initiation et de la dissémination du HCV afin de mieux comprendre les mécanismes impliqués dans l'infection et le tropisme cellulaire de ce virus.

Dans la première partie de ma thèse, nous nous sommes intéressés au rôle et à la fonction de SR-BI et CD81 dans le processus d'entrée et de dissémination du HCV afin d'explorer leur potentiel en tant que cible antivirale. Ainsi, au cours de mon travail de thèse, j'ai pu démontrer que la fonction de SR-BI suivant l'étape de liaison du virus à la surface de la cellule hôte joue un rôle majeur dans l'initiation de l'infection et la dissémination virale du HCV, notamment par son rôle dans la transmission cellule à cellule. D'autre part, j'ai démontré l'importance de CD81 dans ce processus.

Puis, dans la seconde partie de ma thèse, notre objectif a été de déterminer quels étaient les facteurs cellulaires nécessaires à l'infection par le HCV et d'élucider les facteurs restreignant l'infection par le HCV aux hépatocytes humains afin d'avoir une meilleure compréhension de ces processus qui permettrait à l'avenir de développer de nouvelles stratégies préventives et/ou des thérapeutiques efficaces. J'ai pu démontrer que l'expression et/ou la surexpression de facteurs d'entrée du HCV, tels SR-BI, CD81, CLDN1 et OCLN ainsi que de facteurs cruciaux à la réplication, tel le miR-122, et à l'assemblage, tel apoE, permettait la reconstitution du cycle viral en entier dans des cellules non hépatiques.

Ces résultats permettent d'approfondir la connaissance des mécanismes moléculaires et cellulaires d'initiation, de dissémination, mais aussi de tropisme cellulaire qui sous tendent l'infection par le HCV.

III. RESULTATS ET DISCUSSION

PARTIE I : Le rôle de SR-BI et CD81 dans les processus d'entrée et de dissémination du HCV

1. Introduction

Dans la première partie de ma thèse, je me suis intéressée au rôle et à la fonction de SR-BI et CD81 dans le processus d'entrée et de dissémination du HCV afin d'explorer leur potentiel en tant que cible antivirale. Les résultats sont présentés ci-dessous sous forme d'une publication originale (Zahid, Turek et al. Hepatology 2013) et de figures issues d'une publication originale présentée en annexe (Fofana et al. 2013; Annexe 1). Ces résultats sont ensuite discutés dans leur ensemble.

2. Résultats

Publication n°1: The post-binding activity of scavenger receptor BI mediates initiation of hepatitis C virus infection and viral dissemination

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* Ces auteurs ont contribué de manière équivalente
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Hepatology 2013

SR-BI fut originellement proposé en tant que récepteur du HCV car il se liait à la glycoprotéine E2 du HCV (Scarselli et al., 2002). Depuis, de nombreuses études ont démontré que les mécanismes moléculaires sous tendant l'association HCV/SR-BI étaient complexes et impliquaient une interaction orchestrée entre le HCV, SR-BI et les HDL afin de permettre une entrée virale et une infection efficace (Dreux and Cosset, 2007).

Pour étudier ces mécanismes, le laboratoire avait au préalable généré des anticorps polyclonaux anti SR-BI et avait démontré que SR-BI était requis lors d'une étape d'entrée proche de l'interaction du virus avec CD81 et CLDN1 (Krieger et al., 2010; Zeisel et al., 2007b).

Au cours de ma thèse, j'ai pu étudier plus en détail le rôle clé de SR-BI grâce à la production d'anticorps monoclonaux de rat et de souris anti-SR-BI qui, contrairement aux précédents anticorps monoclonaux anti-SR-BI existants (Catanese et al., 2010), n'inhibent pas la liaison des protéines recombinantes E2 du HCV ni des particules virales HCVcc à SR-BI (Figure 2A de la publication n°1) mais inhibent l'infection par le HCV, principalement durant les étapes suivant la liaison du virus à la surface de la cellule hôte. J'ai pu démontrer que ces anticorps anti-SR-BI sont capables d'inhiber l'entrée des HCVpp portant les glycoprotéines d'enveloppe des principaux génotypes du HCV (Figures 5 et S3 de la publication n°1). De plus, la combinaison d'anticorps anti-protéine d'enveloppe du HCV avec ces anticorps anti-SR-BI conduit à un effet synergique sur l'inhibition à la fois de l'entrée et de l'infection par le HCV sans pour autant interférer directement avec la liaison E2-CD81. Nous avons également pu démontrer que ces anticorps monoclonaux anti-SR-BI n'interfèrent pas avec la liaison des HDL à SR-BI, mais influencent la fonction de transfert lipidique de SR-BI. De plus, j'ai pu démontrer que ces anticorps monoclonaux anti-SR-BI sont de puissants inhibiteurs de la transmission cellule à cellule ainsi que de la dissémination virale du HCV (Figure 3 et S2 de la publication n°1). L'utilisation de cellules exprimant SR-BI humain, capables d'interagir avec sE2, ou murin, incapables d'interagir avec sE2, m'a par ailleurs permis de confirmer que la fonction post attachement de SR-BI était impliquée dans ce processus (Figure 2B de la publication n°1). Finalement, j'ai utilisé des mutants de SR-BI (Dreux et al., 2009; Dao Thi et al., 2012b) pour commencer à caractériser les épitopes des anticorps anti-SR-BI. J'ai pu montrer que les acides aminés 402, 418, 420 et 424 de SR-BI pourraient faire partie des épitopes des anticorps anti-SR-BI inhibant sa fonction post attachement ou que la mutation de ces acides aminés induit des changements conformationnels dans ces épitopes (Figure S5 de la publication n°1).

Par conséquent, ces anticorps représentent un outil intéressant pour l'étude des mécanismes d'action de SR-BI lors de l'entrée et la dissémination virale ainsi que pour le développement d'inhibiteurs d'entrée ciblant l'interaction SR-BI/HCV.

HEPATOLOGY
Official Journal of the American Association for the Study of Liver Diseases

The Postbinding Activity of Scavenger Receptor Class B Type I Mediates Initiation of Hepatitis C Virus Infection and Viral Dissemination

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Scavenger receptor class B type I (SR-BI) is a high-density lipoprotein (HDL) receptor highly expressed in the liver and modulating HDL metabolism. Hepatitis C virus (HCV) is able to directly interact with SR-BI and requires this receptor to efficiently enter into hepatocytes to establish productive infection. A complex interplay between lipoproteins, SR-BI and HCV envelope glycoproteins has been reported to take place during this process. SR-BI has been demonstrated to act during binding and postbinding steps of HCV entry. Although the SR-BI determinants involved in HCV binding have been partially characterized, the postbinding function of SR-BI remains largely unknown. To uncover the mechanistic role of SR-BI in viral initiation and dissemination, we generated a novel class of anti–SR-BI monoclonal antibodies that interfere with postbinding steps during the HCV entry process without interfering with HCV particle binding to the target cell surface. Using the novel class of antibodies and cell lines expressing murine and human SR-BI, we demonstrate that the postbinding function of SR-BI is of key impact for both initiation of HCV infection and viral dissemination. Interestingly, this postbinding function of SR-BI appears to be unrelated to HDL interaction but to be directly linked to its lipid transfer function. *Conclusion:* Taken together, our results uncover a crucial role of the SR-BI postbinding function for initiation and maintenance of viral HCV infection that does not require receptor-E2/HDL interactions. The dissection of the molecular mechanisms of SR-BI–mediated HCV entry opens a novel perspective for the design of entry inhibitors interfering specifically with the proviral function of SR-BI. (HEPATOLOGY 2013;57:492-504)

Hepatitis C virus (HCV) is a major cause of liver cirrhosis and hepatocellular carcinoma. Preventive modalities are nonexistent, and the current antiviral treatment is limited by resistance, toxicity, and high cost.[1] Viral entry is required for initiation, spread, and maintenance of infection, and thus is a promising target for antiviral therapy. HCV binding and entry into hepatocytes is a complex process involving the viral envelope glycoproteins E1 and E2, as well as several host factors,

Abbreviations: CE, cholesteryl ester; GFP, green fluorescent protein; HCV, hepatitis C virus; HCVcc, cell culture-derived HCV; HCVpp, HCV pseudoparticle; HDL, high-density lipoprotein; hSR-BI, human SR-BI; IC_{50}, 50% inhibitory concentration; IgG, immunoglobulin G; mAbs, monoclonal antibodies; mSR-BI, mouse SR-BI; sE2, soluble E2; SR-BI, scavenger receptor class B type I; VSV-Gpp, vesicular stomatitis virus glycoprotein pseudoparticle.

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Received February 3, 2012; accepted September 22, 2012.

Supported by the European Union (ERC-2008-AdG-233130-HEPCENT, INTERREG-IV-Rhin Supérieur-FEDER-Hepato-Regio-Net 2009), Laboratoire d'Excellence HEPSYS (Investissement d'Avenir; ANR-10-LAB-28), ANR-05-CEXC-008, ANRS (2008/354, 2009/183, 2011/132, 2012/239), Pro Inno II (KA0690901UL8), Région Alsace, INSERM, University of Strasbourg, and Aldevron Freiburg. The group in Leuven was funded by a grant from the Fund for Scientific Research (G.0728.09N) and KULeuven GOA 10/014. M. N. Z. was supported by a Higher Education Commission fellowship.

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including highly sulfated heparan sulfate, CD81, the low-density lipoprotein receptor, scavenger receptor class B type I (SR-BI), claudin-1, occludin, and receptor tyrosine kinases.[2,3]

Human SR-BI is a glycoprotein that is highly expressed in tissues with a high cholesterol need for steroidogenesis and the liver.[4] SR-BI is a multifunctional molecule well known to modulate high-density lipoprotein (HDL) metabolism. SR-BI binds a variety of lipoproteins and mediates selective uptake of HDL cholesterol ester (CE) as well as bidirectional free cholesterol transport at the cell membrane. Genetic SR-BI variants have been associated with HDL levels in humans, and a recent study uncovered a functional mutation in SR-BI impairing SR-BI function and affecting cholesterol homeostasis.[5] SR-BI also interacts with different pathogens, including HCV,[6-8] and mediates their entry and uptake into host cells. SR-BI is relevant for HCV infection *in vivo*, and its potential as an antiviral target has been reported.[9]

SR-BI directly binds HCV E2,[6,8] but virus-associzated lipoproteins also contribute to host cell binding and uptake.[10,11] Moreover, physiological SR-BI ligands modulate HCV infection.[12-14] This suggests the existence of a complex interplay between lipoproteins, SR-BI, and HCV envelope glycoproteins for HCV entry. SR-BI has also been demonstrated to mediate postbinding events during HCV entry.[15-17] HCV–SR-BI interaction during postbinding steps occurs at similar time points as the HCV utilization of CD81 and claudin-1, suggesting that HCV entry may be mediated through the formation of coreceptor complexes.[15,18,19] These data suggest that SR-BI plays a multifunctional role during HCV entry at both binding and postbinding steps.[15,20] This is corroborated by the fact that murine SR-BI does not bind E2,[20,21] although it is capable of promoting HCV entry.[20,22]

To elucidate the mechanistic function of SR-BI in the HCV entry process and to explore its potential as an antiviral target, we generated a novel class of monoclonal antibodies directed against human SR-BI that inhibit HCV entry during postbinding steps without preventing E2 binding to target cells.

Material and Methods

Additional details are provided in the Supporting Information.

Cells. HEK293T, Chinese hamster ovary, Buffalo rat liver, Huh7, Huh7.5-GFP, and Huh7.5.1 cells were cultured as described.[18,23-25] Primary human hepatocytes were isolated as described.[18] Chinese hamster ovary and Buffalo rat liver cells expressing SR-BI were produced as described.[11,15,23]

Antibodies. Polyclonal[15] and monoclonal antibodies (mAbs) directed against the extracellular loop of SR-BI were raised by genetic immunization of Wistar rats and Balb/c mice as described[15] according to proprietary technology (Aldevron GmbH, Freiburg, Germany). Anti–SR-BI mAbs were purified using protein G affinity columns and selected via flow cytometry for their ability to bind to human SR-BI.[15] To determine the affinity of the anti–SR-BI mAbs for human SR-BI, Huh7.5.1 cells were incubated with increasing concentrations of mAbs, and binding was assessed using flow cytometry. Kd values were determined as half-saturating concentrations of the mAbs.[26,27] Antibodies will be provided on request using a material transfer agreement (MTA). Anti-CD81 (JS-81), anti–SR-BI (CLA-1), and phycoerythrin-conjugated anti-mouse antibodies were from BD Biosciences. Anti-His and fluorescein isothiocyanate–conjugated anti-His antibodies were obtained from Qiagen, rabbit anti-actin (AA20-30) antibodies were obtained from Sigma-Aldrich, and mouse anti-NS5A antibodies were obtained from Virostat. Anti-E1 (IGH520, IGH526, Innogenetics), anti-E2 (IGH461, Innogenetics; AP33, Genentech; CBH23, a kind gift from S.K.H. Foung), and patient-derived anti-HCV immunoglobulin G (IgG) have been described.[16,25,27]

Cell Culture–Derived HCV and Pseudoparticle Production and Infection. Luciferase reporter cell culture-derived HCV (HCVcc), HCV pseudoparticles (HCVpp), murine leukemia virus pseudoparticles, and vesicular stomatitis virus glycoprotein pseudoparticles (VSV-Gpp), infection and kinetic experiments have

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DOI 10.1002/hep.26097

Potential conflict of interest: INSERM and the University of Strasbourg have filed a patent application on scavenger receptor class B type I–specific monoclonal antibodies and inhibition of hepatitis C virus infection. Dr. Pietschmann consults for Janssen and Biotest. Dr. Thompson received grants from and holds intellectual property rights with Aldevron. Dr. Grunert received grants from Aldevron.

Additional Supporting Information may be found in the online version of this article.

been described.[15,18,25,27,28] Unless otherwise stated, HCVcc experiments were performed using Luc-Jc1, and infection was analyzed in cell lysates via quantification of luciferase activity.[29] For combination experiments, each antibody was tested individually or in combination with a second antibody. Huh7.5.1 cells were preincubated with anti–SR-BI or control mAb for 1 hour and then incubated for 4 hours at 37°C with HCVcc (Luc-Jc1) or HCVpp (P02VJ) (preincubated for 1 hour with or without anti-envelope antibodies). Synergy was assessed using the combination index and the method of Prichard and Shipman.[30-32] Cell viability was assessed using a MTT test.[2]

Cellular Binding of Envelope Glycoprotein E2. Recombinant His-tagged soluble E2 (sE2) was produced as described.[23] Huh7.5.1 cells were preincubated with control or anti–SR-BI serum (1:50), anti–SR-BI or control mAbs (20 μg/mL) for 1 hour at room temperature, and then incubated with sE2 for 1 hour at room temperature. Binding of sE2 was revealed using flow cytometry as described.[18,23]

HCVcc Binding. Huh7.5.1 cells were preincubated with heparin (100 μg/mL), control or anti–SR-BI serum (1:50), or anti–SR-BI or control mAbs (20 μg/mL) for 1 hour prior to incubation with HCVcc at 4°C as described.[18] Nonbound HCVcc were removed by washing of cells with phosphate-buffered saline, and cell bound HCV RNA was then quantified by reverse-transcription polymerase chain reaction.[18]

HCV Cell-to-Cell Transmission. HCV cell-to-cell transmission was assessed as described.[2,24] Producer Huh7.5.1 cells were electroporated with Jc1 RNA[33] and cultured with naïve target Huh7.5-GFP cells in the presence or absence of anti–SR-BI or control mAbs. An HCV E2-neutralizing antibody (AP33, 25 μg/mL) was added to block cell-free transmission.[24] After 24 hours of coculture, cells were fixed with paraformaldehyde, stained with an NS5A-specific antibody (Virostat), and analyzed via flow cytometry.[2,24]

Immunofluorescence of Viral Dissemination. Cell spread was assessed by visualizing Jc1-infected Huh7.5.1 cells by immunoflorescence using anti-NS5A (Virostat) and anti-E2 (CBH23) antibodies as described.[2]

HDL Binding. HDL was labeled using Amersham Cy5 Mono-Reactive Dye Pack (GE Healthcare). Unbound Cy5 was removed by applying labeled HDL on illustra MicroSpin G-25 Columns (GE Healthcare). Blocking of Cy5-HDL binding with indicated reagents was performed for 1 hour at room temperature prior to Cy5-HDL binding for 1 hour at 4°C on 10^6 target cells.

Lipid Transfer Assays. Selective HDL-CE uptake and lipid efflux assays were performed as described.[23,34] HDL-CE uptake was assessed in the presence or absence of anti–SR-BI mAbs (20 μg/mL) and ^{3}H-CE-labeled HDL (60 μg protein) for 5 hours at 37°C. Selective uptake was calculated from the known specific radioactivity of radiolabeled HDL-CE and is denoted in μg HDL-CE/μg cell protein. For lipid efflux assay, Huh7 cells were labeled with ^{3}H-cholesterol (1 μCi/mL) and incubated at 37°C for 48 hours as described.[23,35] Cells were incubated with anti–SR-BI mAbs (20 μg/mL) for 1 hours prior to incubation with unlabeled HDL for 4 hours. Fractional cholesterol efflux was calculated as the amount of label obtained in the medium divided by the total in each well (radioactivity in the medium + radioactivity in the cells) regained after lipid extraction from cells.

Statistical Analysis. Unless otherwise stated, data are presented as the means ± SD of three independent experiments. Statistical analyses were performed using a Student *t* test and/or Mann-Whitney test; $P < 0.01$ was considered statistically significant.

Results

Production of SR-BI–Specific Monoclonal Antibodies Interfering with the Postbinding Steps of Viral Entry. To further explore the role of HCV–SR-BI interaction during HCV infection, we generated five rat and three mouse monoclonal antibodies (mAbs) directed against the human SR-BI (hSR-BI) ectodomain (Table 1). These antibodies bound to endogenous SR-BI on human hepatoma Huh7.5.1 cells and primary human hepatocytes but did not bind to mouse SR-BI (mSR-BI) expressed on rat BRL cells (Fig. 1A,B and Supporting Fig. 1). Three rat mAbs (QQ-4A3-A1, QQ-2A10-A5, and QQ-4G9-A6) and one mouse mAb (NK-8H5-E3) significantly ($P < 0.01$) inhibited HCVcc infection in a dose-dependent manner with 50% inhibitory concentrations (IC_{50}) between 0.2 and 8 μg/mL (Fig. 1C,D and Table 1). The apparent K_d ($K_d$app) corresponding to the half-saturating concentrations for binding to Huh7.5.1 cells ranged from 0.5 to 7.4 nM, demonstrating that these antibodies recognize SR-BI with high affinity (Table 1). It is noteworthy that there seems to be a correlation between the antibody affinity and inhibitory capacity, with the low affinity antibodies unable to block HCV infection. We next aimed to characterize the viral entry steps targeted by these anti–SR-BI mAbs. We first assessed their ability to interfere with viral binding. To reflect the complex interaction between HCV and hSR-BI during viral binding, we

Table 1. mAbs Directed Against hSR-BI

mAb	Isotype	Kd_{app} Huh7.5.1 (nM)	IC_{50} HCVcc (μg/mL)	Inhibition of HDL-CE Uptake (Mean % ± SD)	Inhibition of Cholesterol Efflux (Mean % ± SD)
QQ-4A3-A1	Rat IgG2b	1.0	0.7	44.18 ± 1.42	40.97 ± 0.92
QQ-2A10-A5	Rat IgG2b	0.5	0.2	47.64 ± 1.2	40 ± 1.01
QQ-4G9-A6	Rat IgG2b	0.5	1.0	44.64 ± 1.57	39.02 ± 1.14
PS-6A7-C4	Rat IgG2b	NA	NA	10.24 ± 1.52	−2.52 ± 1.25
PS-7B11-E3	Rat IgG2b	NA	NA	11.73 ± 2.1	5.04 ± 0.83
NK-8H5-E3	Mouse IgG2b	7.4	8.0	56.28 ± 0.8	44.74 ± 0.55
NK-6B10-E6	Mouse IgG1	NA	NA	1.28 ± 1.69	18.41 ± 0.81
NK-6G8-B5	Mouse IgG1	NA	NA	5.64 ± 1.04	13.08 ± 0.77

Isotype, binding affinity to Huh7.5.1 cells (Kd_{app}), and inhibition of HCVcc infection (IC_{50}) and lipid transfer of anti–SR-BI mAbs are shown. Huh7.5.1 cells were incubated with increasing concentrations of mAbs, and Kd values were determined as half-saturating concentrations of the mAbs. IC_{50} was determined after incubation of Huh7.5.1 cells with serial dilutions of anti–SR-BI mAbs for 1 hour at room temperature before infection with HCVcc. The results represent the mean of three independent experiments performed in triplicate. Lipid uptake and efflux were assessed in Huh7 cells as described in Materials and Methods in the presence of anti–SR-BI mAbs (20 μg/mL). The results are expressed as % inhibition of lipid transfer relative to cells incubated in the absence of antibody and represent the mean ± SD of three independent experiments.

Abbreviation: NA, not applicable.

studied the effect of anti–SR-BI mAbs on HCVcc binding to Huh7.5.1 cells at 4°C. Incubation of Huh7.5.1 cells with anti–SR-BI mAbs before and during HCVcc binding did not inhibit virus particle binding (Fig. 2A). Similar results were obtained using sE2 as a surrogate model for HCV (Supporting Results and Supporting Fig. 1). These data suggest that, in contrast to described anti–SR-BI mAbs,[20] these novel anti–SR-BI mAbs do not inhibit HCV binding but interfere with HCV entry during postbinding steps. Next, to characterize potential postbinding steps targeted by these anti–SR-BI mAbs, we assessed HCVcc entry kinetics into Huh7.5.1 cells in the presence of anti–SR-BI mAbs inhibiting HCV infection (QQ-4A3-A1, QQ-2A10-A5, QQ-4G9-A6, and NK-8H5-E3) added at different time points during or after viral binding (Fig. 2B). This assay was performed side-by-side with an anti-CD81 mAb inhibiting HCV postbinding[15,18,29] and proteinase K[36] to remove HCV from the cell surface. HCVcc binding to Huh7.5.1 cells was performed for 1 hour at 4°C in the presence or absence of compounds. Subsequently, unbound virus was washed away, cells were shifted to 37°C to allow HCVcc entry, and compounds were added every 20 minutes for up to 120 minutes after viral binding. These kinetic experiments indicate that anti–SR-BI mAbs inhibited HCVcc infection when added immediately after viral binding as well as 20-30 minutes after initiation of viral entry (Fig. 2C), demonstrating that QQ-4A3-A1, QQ-2A10-A5, QQ-4G9-A6, and NK-8H5-E3 indeed target postbinding steps of the HCV entry process. This time frame is comparable to the kinetics of resistance of internalized virus to proteinase K (Fig. 2C), indicating that these postbinding steps precede completion of virus internalization. Taken together, these data indicate that a postbinding function of SR-BI is essential for initiation of HCV infection. In contrast to previous anti–SR-BI mAbs inhibiting HCV binding[20] as well as polyclonal anti–SR-BI antibodies and small molecules interfering with both viral binding and postbinding,[15,17,23] these antibodies are the first molecules exclusively targeting the postbinding function of SR-BI and thus represent a unique tool to more thoroughly assess the relevance of this function for HCV infection.

A Postbinding Function of SR-BI Is Essential for Cell-to-Cell Transmission and Viral Spread. HCV disseminates via direct cell-to-cell transmission.[24,37] To assess the role of SR-BI postbinding function in viral dissemination, we first investigated the ability of anti–SR-BI mAbs to interfere with neutralizing antibody-resistant viral spread by studying direct HCV cell-to-cell transmission in the presence of QQ-2A10-A5 and QQ-4G9-A6. Viral "producer" cells containing replicating HCV Jc1 (Pi) are cocultured with green fluorescent protein (GFP)-expressing "target" cells (T) in the presence of E2-neutralizing mAb (AP33, 25 μg/mL) to prevent cell-free HCV transmission.[24] AP33 reduces cell-free transmission by >90%, and infectivity of producer cell supernatants is minimal at the time of coculture; viral transmission thus occurs predominantly via cell-to-cell transmission in this assay.[2,24] HCV cell-to-cell transmission is assessed by quantifying HCV-infected, GFP-positive target cells (Ti) by flow cytometry.[2,24] Both anti–SR-BI mAbs (10 μg/mL) efficiently blocked HCV cell-to-cell transmission (Fig. 3A and Supporting Fig. 2A,B), indicating that these antibodies may prevent viral spread *in vitro*. Because these anti–SR-BI mAbs do not block HCV–SR-BI binding (Fig. 2A) but inhibit HCV entry during postbinding

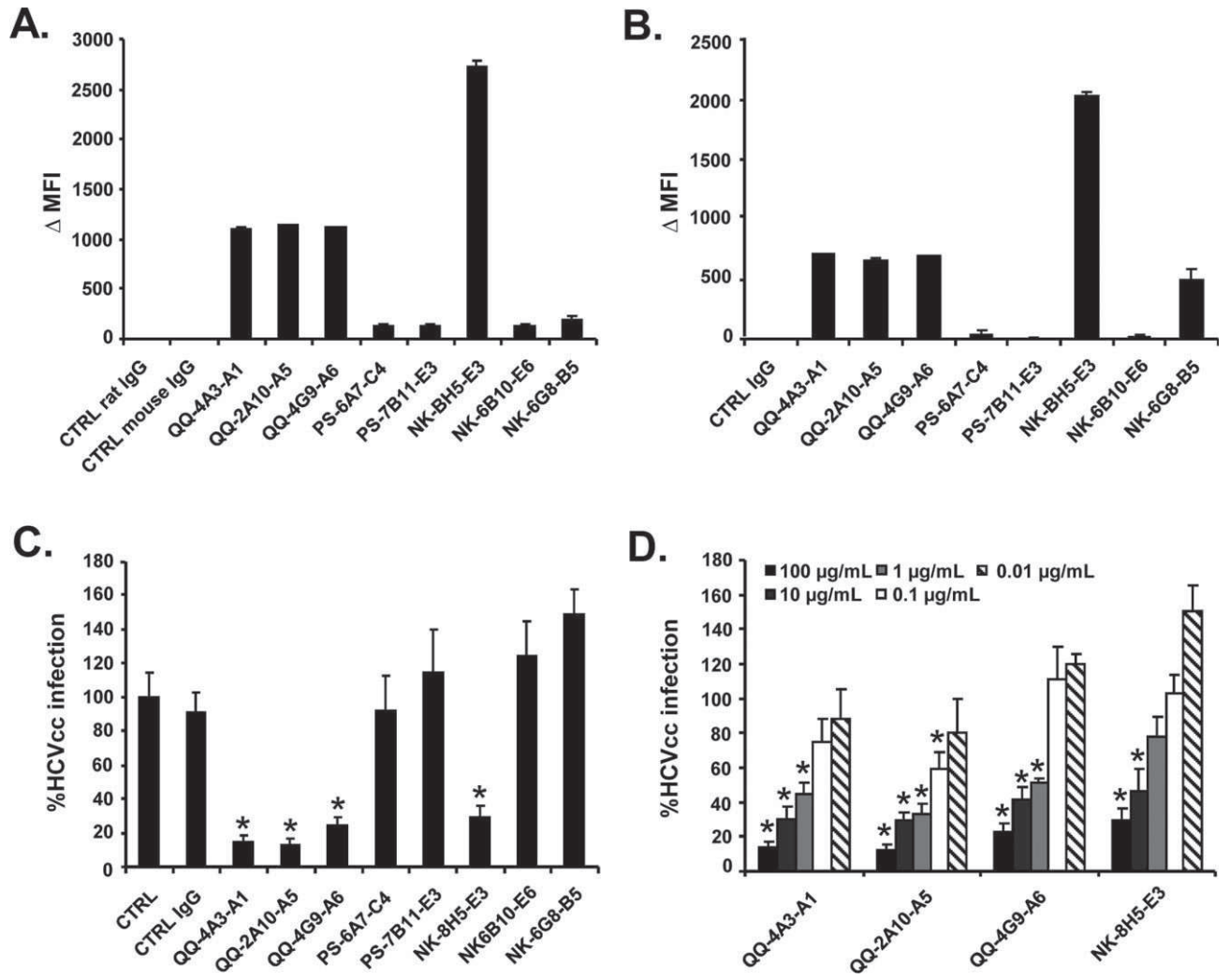

Fig. 1. Binding of monoclonal anti–SR-BI antibodies to human hepatocytes and inhibition of HCV infection. (A) Huh7.5.1 cells and (B) primary human hepatocytes were incubated with anti–SR-BI mAbs, and antibody binding was assessed using flow cytometry. Results are expressed as the net mean fluorescence intensity (ΔMFI) of a representative experiment. (C) Inhibition of HCVcc infection by anti–SR-BI mAbs. Huh7.5.1 cells were preincubated for 1 hour at 37°C with anti–SR-BI or control mAbs (100 μg/mL) before infection with HCVcc (Luc-Jc1) for 4 hours at 37°C. HCV infection was assessed by luciferase activity in lysates of infected Huh7.5.1 cells 72 hours postinfection. Results are expressed as the mean ± SD % HCVcc infectivity in the absence of antibody of three independent experiments. (D) Dose-dependent inhibition of HCVcc infection by anti–SR-BI mAbs. Huh7.5.1 cells were preincubated for 1 hour at 37°C with anti–SR-BI or control mAbs at the indicated concentrations before infection with HCVcc (Luc-Jc1) for 4 hours at 37°C. HCV infection was assessed by luciferase activity in lysates of infected Huh7.5.1 cells 72 hours postinfection. Results are expressed as the mean ± SD % HCVcc infectivity in the absence of antibody of three independent experiments performed in triplicate. *$P < 0.01$.

steps (Fig. 2C), these data suggest that an SR-BI postbinding function plays an important role during HCV cell-to-cell transmission. To ascertain the importance of the SR-BI postbinding function in this process, we performed additional cell-to-cell transmission assays using mSR-BI, which in contrast to hSR-BI is unable to bind E2. Cells lacking SR-BI and robustly replicating HCV, which would be an ideal model cell to study cell-to-cell transmission by mSR-BI in the absence of hSR-BI, have not been described. However, hSR-BI has been reported to be a limiting factor for HCV spread in Huh7-derived cells, as overexpression of hSR-BI increases cell-to-cell transmission.[37] We thus used Huh7.5 cells or Huh7.5 cells overexpressing either mSR-BI or hSR-BI as target cells. Cell-to-cell transmission was enhanced in Huh7.5 cells overexpressing either hSR-BI (2.04 ± 0.03 fold) or mSR-BI (1.92 ± 0.19 fold) compared with parental cells (Fig. 3B). These data indicate that E2–SR-BI binding is not essential for viral dissemination and confirm the crucial role of SR-BI postbinding function in this process. Furthermore, to assess whether anti–SR-BI mAbs prevent viral dissemination in already HCV-infected cell cultures when added postinfection, we performed a long-term analysis of HCVcc infection by culturing Luc-Jc1–infected Huh7.5.1 cells in the presence or absence of control or anti-SR-BI mAbs QQ-4G9-A6 and NK-8H5-E3 as previously described.[2] When added 48

hours after infection and maintained in cell culture medium throughout the experiment, these anti–SR-BI mAbs efficiently inhibited HCV spread over 2 weeks in a dose-dependent manner without affecting cell viability (Fig. 3C,D and Supporting Fig. 2C,D). We also assessed Jc1 spread in Huh7.5.1 cells via immunostaining of infected cells as described.[2] While 74.5 ± 2.3% and 70.0 ± 3.2% of cells incubated with control rat or mouse mAbs stained positive for NS5A and E2, respectively, incubation with QQ-4G9-A6 and NK-8H5-E3 markedly reduced the number of NS5A-positive (14.2 ± 3.4%) and E2-positive (16.7 ± 2.6%) cells (Fig. 3E,F). Taken together, these data indicate that a postbinding function of SR-BI is required for HCV cell-to-cell transmission and spread.

SR-BI Determinants Relevant for HCV Postbinding Steps May Be Linked to the Lipid Transfer Function of the Entry Factor. The SR-BI ectodomain has been demonstrated to be important for both HDL binding and CE uptake, but the determinants involved in these processes have not yet been defined. To assess whether anti–SR-BI mAbs inhibiting HCV postbinding steps affect HDL binding to SR-BI, we studied Cy5-labeled HDL binding to hSR-BI in the presence or absence of anti–SR-BI mAbs. In contrast to polyclonal anti–SR-BI serum, which inhibited Cy5-labeled HDL binding, none of the anti–SR-BI mAbs markedly interfered with HDL–SR-BI binding at concentrations inhibiting HCV infection by up to 90% (Fig. 4A, statistically not significant). Furthermore, we investigated the effect of these mAbs on CE uptake and cholesterol efflux. Whereas PS-6A7-C4, PS-7B11-E3, NK-6B10-E6, and NK-6G8-B5 had no effect on lipid transfer, QQ-4A3-A1, QQ-2A10-A5, QQ-4G9-A6, and NK-8H5-E3 partially reduced both CE uptake and cholesterol efflux at concentrations inhibiting HCV infection by up to 90% (Fig. 4B,C). These data indicate that the anti–SR-BI mAbs inhibiting HCVcc infection also partially inhibit SR-BI–mediated lipid transfer (Table 1). Taken together, these data suggest that SR-BI determinants involved in HCV postbinding events do not mediate HDL binding but may contribute to lipid transfer, in line with the reported

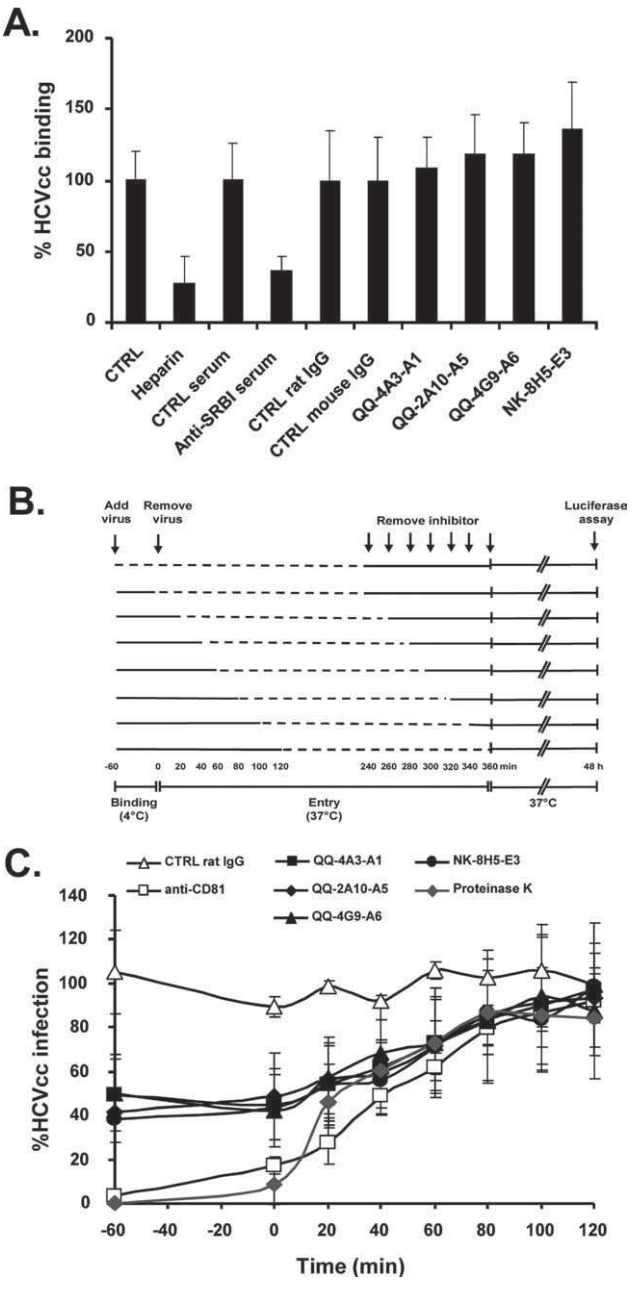

Fig. 2. Monoclonal anti–SR-BI antibodies do not interfere with HCV binding to SR-BI but inhibit HCV entry at postbinding steps. (A) To assess the effect of anti–SR-BI mAbs on viral binding, Huh7.5.1 cells were preincubated with heparin (100 μg/mL), anti–SR-BI or control (CTRL) serum (1:50), or anti–SR-BI or control (CTRL IgG) mAbs (20 μg/mL) for 1 hour prior to incubation with HCVcc (Jc1) at 4°C in the presence of compounds. Nonbound HCVcc were removed by washing of cells with phosphate-buffered saline, and HCVcc binding was then quantified by reverse-transcription polymerase chain reaction of cell-bound HCV RNA. Results are expressed as the mean ± SD of one representative experiment performed in quintuplicate. (B) Schematic drawing of the experimental setup. To discriminate between virus binding and postbinding events, HCVcc (Luc-Jc1) binding to Huh7.5.1 cells was performed in the presence or absence of anti-CD81 (5 μg/mL), anti–SR-BI (20 μg/mL) or control mAbs (20 μg/mL), or proteinase K (50 μg/mL) for 1 hour at 4°C, before cells were washed and incubated for 4 hours at 37°C with compounds added at different time points during infection. Compounds were then removed and cells were cultured for an additional 48 hours. Dashed lines indicate the time intervals where compounds were present. (C) HCV entry kinetics. Time course of HCVcc infection of Huh7.5.1 cells following addition of the indicated antibodies at different time points during infection is shown. HCV infection was assessed by luciferase activity in lysates of infected Huh7.5.1 cells 48 hours postinfection. Results are expressed as the mean % HCVcc infectivity in the absence of antibody of three independent experiments performed in triplicate. *$P < 0.01$.

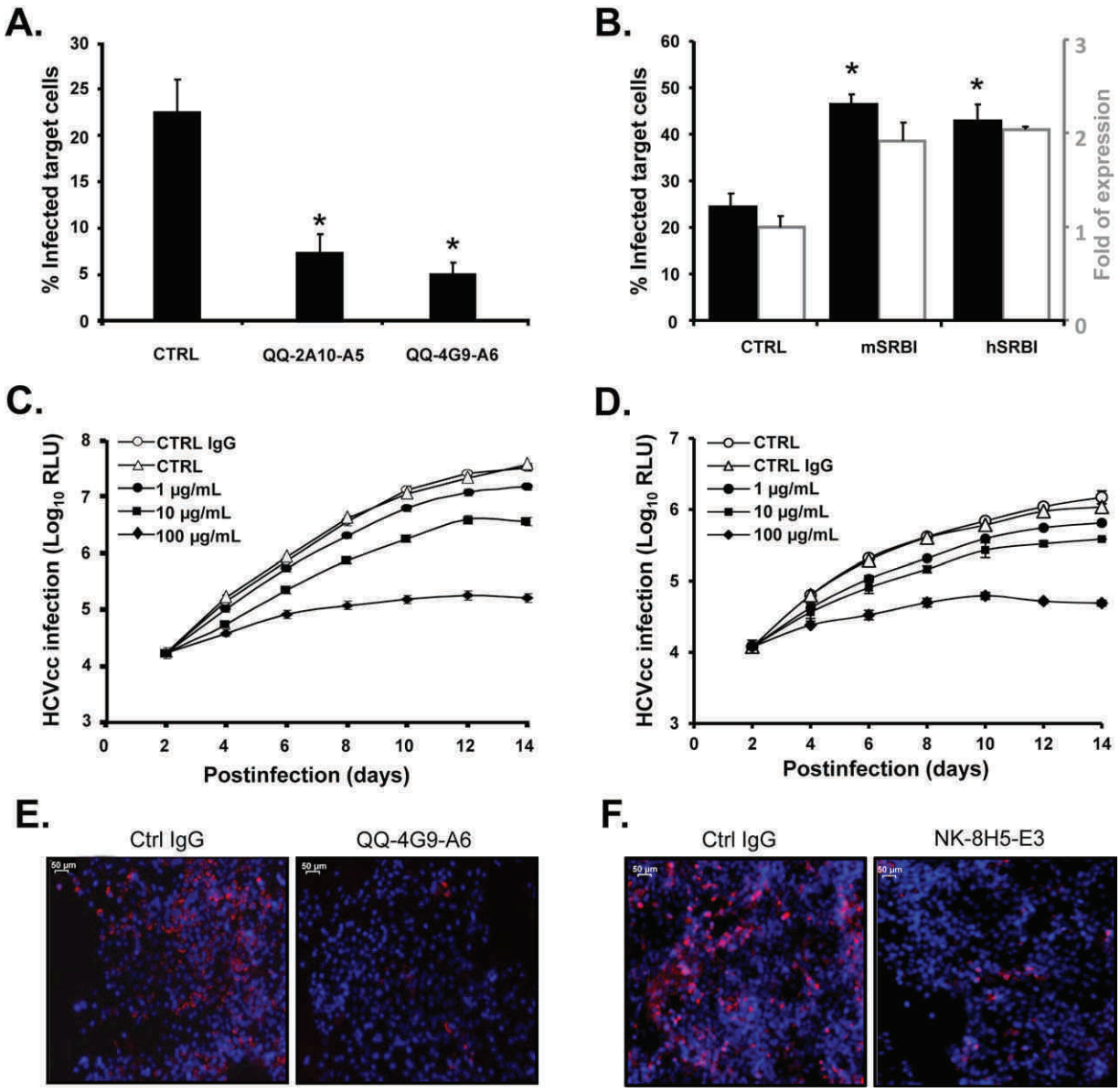

Fig. 3. The SR-BI postbinding function is relevant for HCV cell-to-cell transmission and viral spread. (A) Quantification of HCV-infected target cells (Ti) after cocultivation with HCV (Jc1) producer cells (Pi) during incubation with control or anti–SR-BI mAbs (10 μg/mL) in the presence of E2-neutralizing antibody AP33 (25 μg/mL) via flow cytometry. Data are expressed as % infected target cells and represent the mean $\pm$ SD of three independent experiments. (B) Quantification of HCV cell-to-cell transmission in parental target cells compared with target cells overexpressing mSR-BI or hSR-BI. Data are expressed as the mean $\pm$ SD from three different experiments. (C,D) Long-term analysis of HCVcc (Luc-Jc1) infection in the presence or absence of control (10 μg/mL) or anti-SR-BI mAbs (C) QQ-4G9-A6 or (D) NK-8H5-E3 at the indicated concentrations. Antibodies were added 48 hours after HCVcc infection and control medium or medium containing antibodies were replenished every 4 days. Luciferase activity was determined in cell lysates every 2 days. Data are expressed as $\log_{10}$ RLU and represent the mean $\pm$ SD of one representative out of three different experiments performed in duplicate. (E,F) Cell spread in the presence or absence of anti-SR-BI mAbs. Antibodies were added 48 hours after HCVcc (Jc1) infection and control medium or medium containing antibodies were replenished every 4 days. HCV-infected cells were visualized 7 days postinfection via immunofluorescence using (E) anti-NS5A or (F) anti-E2 (CBH23) antibodies. The percentage of infected cells was calculated as the number of infected cells relative to the total number of cells as assessed by 4',6-diamidino-2-phenylindole staining of the nuclei. *$P < 0.01$.

link between the SR-BI lipid transfer function and HCV infection.[11,12,23]

Synergy Between Antibodies Targeting SR-BI Postbinding Function and Neutralizing Antibodies on Inhibition of HCV Infection. To assess the clinical relevance of blocking SR-BI postbinding function to inhibit HCV infection, we determined the effect of anti–SR-BI mAbs on entry into Huh7.5.1 cells of HCVcc and HCVpp of major genotypes and highly infectious HCV strains selected during liver

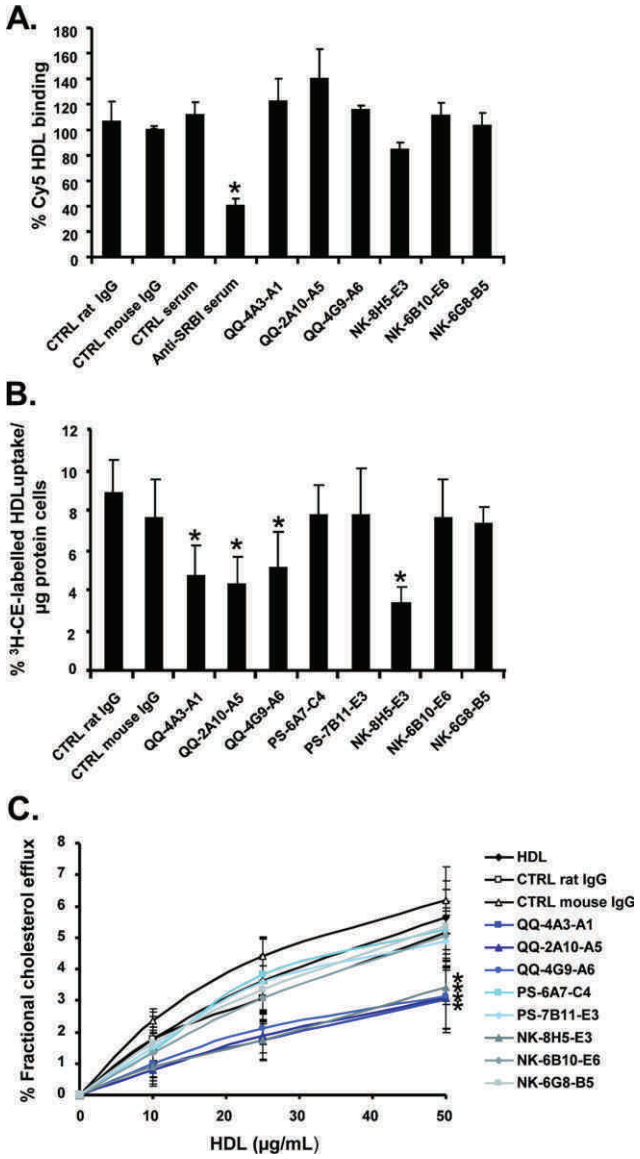

Fig. 4. Anti–SR-BI mAbs do not interfere with HDL binding but partially inhibit lipid transfer. (A) HDL binding to BRL3-hSR-BI cells. BRL3-hSR-BI cells were incubated in the presence or absence of anti–SR-BI mAbs (20 μg/mL) or polyclonal serum (1:50) or respective controls, prior to Cy5-HDL binding for 1 hour at 4°C. Bound Cy5-HDL was quantified using flow cytometry. Results represent the mean ± SD of two different experiments performed in duplicate. (B) Lipid uptake by Huh7 cells. Huh7 cells were incubated with a mixture of anti–SR-BI mAbs (20 μg/mL) and ^{3}H-CE–labeled HDL for 5 hours before incubation with unlabeled HDL for 30 minutes. Selective uptake was calculated from the known specific radioactivity of radiolabeled HDL-CE and is denoted in μg HDL-CE/μg cell protein. Results represent the mean ± SD of three different experiments performed in sextuplicate. (C) Cholesterol efflux from Huh7 cells. Huh7 cells were first incubated with 3H-cholesterol for 48 hours and then with BSA (0.5%) for 24 hours. Subsequently, cells were first incubated with anti–SR-BI mAbs (20 μg/mL) for 1 hour and then with unlabeled HDL for 4 hours. Fractional cholesterol efflux was calculated as the amount of the label obtained in the medium divided by the total label in each well regained after lipid extraction from cells. Results represent the mean ± SD of three different experiments performed in triplicate. *$P < 0.01$.

transplantation (P02VJ). All anti–SR-BI mAbs inhibiting HCVcc genotype 2a (Jc1) infection (QQ-4A3-A1, QQ-2A10-A5, QQ-4G9-A6 and NK-8H5-E3) also inhibited infection of HCVcc and HCVpp of all major genotypes ($P < 0.01$), whereas VSV-Gpp entry was unaffected (Fig. 5 and Supporting Fig. 3). Moreover, entry of patient-derived HCVpp P02VJ into both Huh7.5.1 cells and primary human hepatocytes was also efficiently inhibited by these anti–SR-BI mAbs (Supporting Fig. 7 and data not shown). Given that combinations of drugs targeting both viral and host factors represents a promising future approach to prevent and treat HCV infection, we next determined whether the combination of anti–SR-BI mAbs NK-8H5-E3 or QQ-2A10-A5 and anti-HCV envelope antibodies results in an additive or synergistic effect on inhibiting HCV infection. Thereto, each antibody was tested individually or in combination with a second antibody in a checkerboard format, and synergy was assessed using the combination index and the method of Prichard and Shipman[30-32]. Combination of anti–SR-BI and anti-HCV envelope antibodies resulted in a synergistic effect on inhibition of HCVpp P02VJ entry and HCVcc infection as reflected by a combination index of 0.06-0.67 (Supporting Fig. 7), and synergy of low doses was confirmed using the method of Prichard and Shipman (Fig. 6). These combinations reduced the IC_{50} of anti–SR-BI mAbs by up to 100-fold (Supporting Fig. 7). The marked observed synergy may be explained by the fact that the envelope- and SR-BI–specific antibodies target highly complementary steps during HCV entry. Taken together, these data indicate that interfering with SR-BI postbinding function may hold promise for the design of novel antiviral strategies targeting HCV entry factors.

Discussion

We generated novel anti–SR-BI mAbs specifically inhibiting HCV entry during postbinding steps that enabled us for the first time, using endogenous SR-BI, to explore and validate the hypothesis that SR-BI has a multifunctional role during HCV entry and to elucidate the functional role of SR-BI postbinding activity for HCV infection. Our data demonstrate that the HCV postbinding function of hSR-BI can indeed be dissociated from its E2-binding function. Moreover, we demonstrate that the postbinding activity of SR-BI is of key relevance for cell-free HCV infection as well as cell-to-cell transmission.

SR-BI mediates uptake of HDL-CE in a two-step process including HDL binding and subsequent

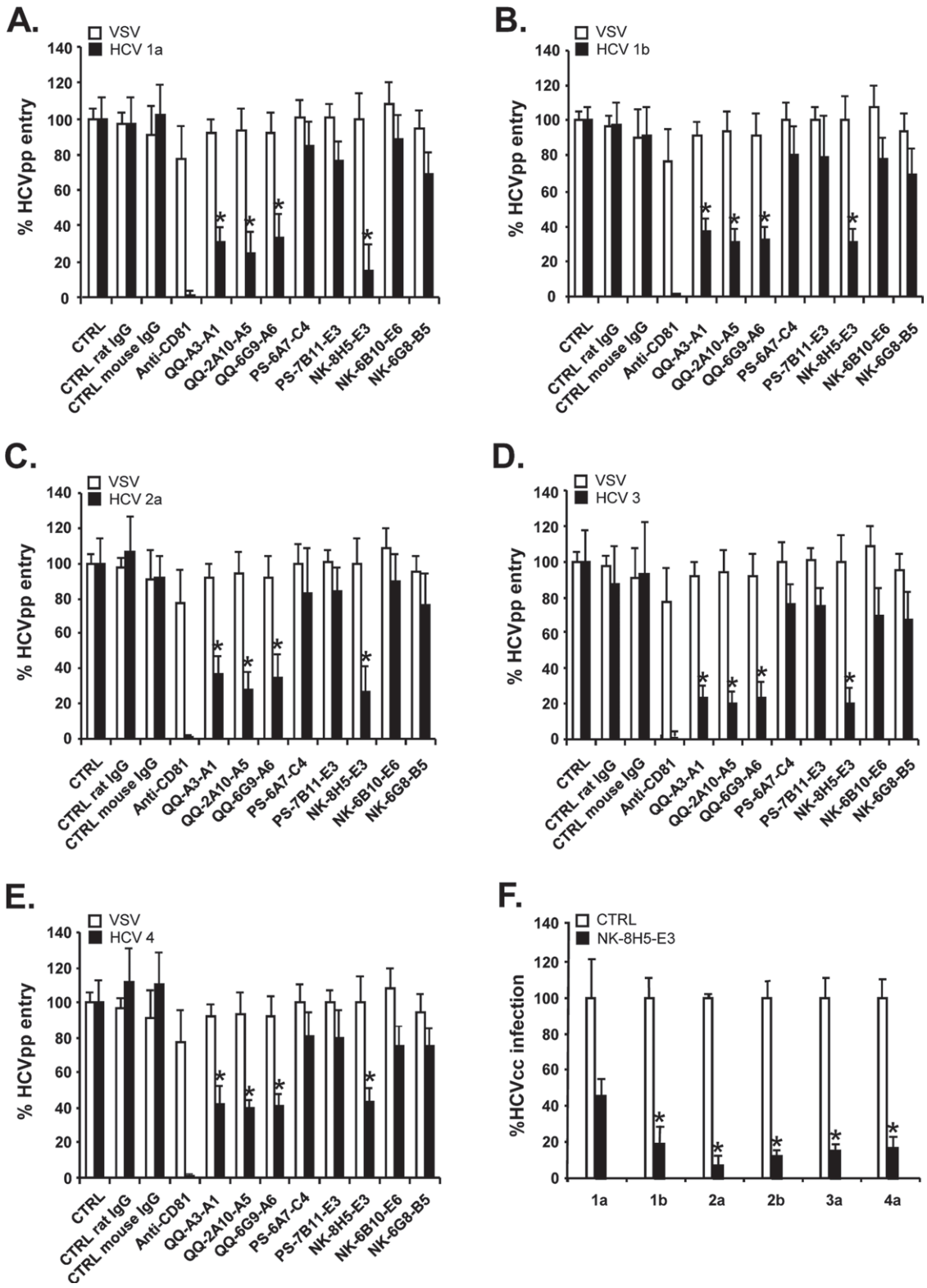

Fig. 5. Genotype-independent inhibition of HCVpp and HCVcc infection by monoclonal anti–SR-BI antibodies. (A-E) Inhibition of entry into Huh7.5.1 cells of HCVpp bearing envelope glycoproteins from genotypes 1-4. Huh7.5.1 cells were preincubated with control (CTRL IgG) or anti–SR-BI mAbs (50 μg/mL) for 1 hour at 37°C before infection with HCVpp bearing envelope glycoproteins of strains H77 (1a), HCV-J (1b), JFH1 (2a), UKN3A1.28 (3a), or UKN4.21.16 (4) and VSV-Gpp. The results represent the mean ± SD from three experiments performed in triplicate. (F) Inhibition of infection of Huh7.5.1 cells with HCVcc bearing envelope glycoproteins from genotypes 1-4. Huh7.5.1 cells were preincubated with anti–SR-BI mAb (NK-8H5-E3, 50 μg/mL) for 1 hour at 37°C before infection with HCVcc. HCVpp and HCVcc infection was analyzed by luciferase reporter gene expression. Results are expressed as % HCVpp entry or HCVcc infection and represent (A-E) the mean ± SD from three independent experiments performed in triplicate and (F) the mean ± SEM from three independent experiments performed at least in triplicate. *$P < 0.01$.

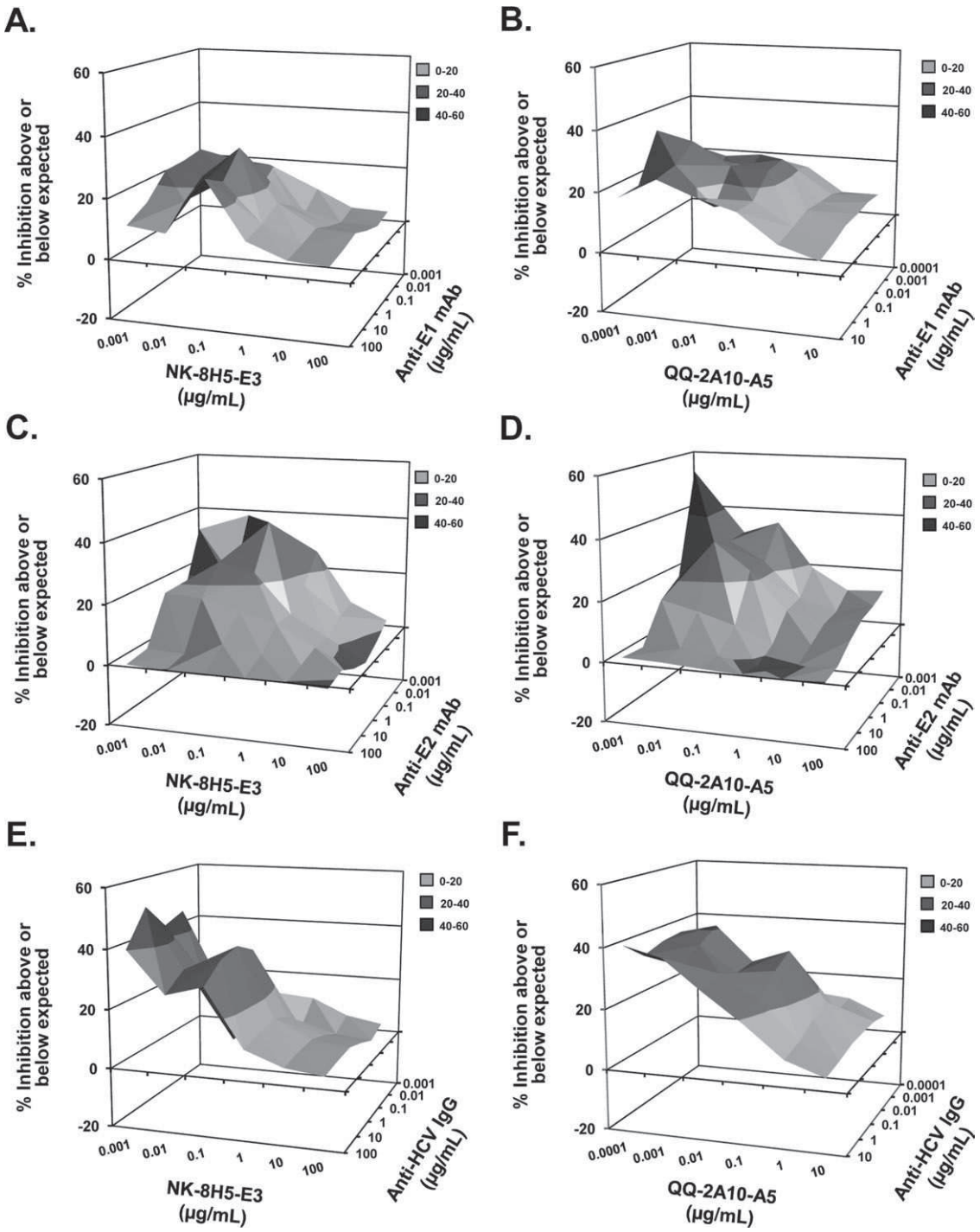

Fig. 6. Synergy between anti–SR-BI and neutralizing antibodies in inhibiting HCV infection. HCVcc (Luc-Jc1) were preincubated with increasing concentrations of (A,B) anti-E1 (IGH520) or (C,D) anti-E2 (AP33) mAbs or (E,F) purified heterologous anti-HCV IgG obtained from an unrelated chronically infected subject or isotype control IgGs for 1 hour at 37°C and added to Huh7.5.1 cells preincubated with increasing concentrations of control or anti–SR-BI mAbs (A,C,E) NK-8H5-E3 or (B,D,F) QQ-2A10-A5 in a checkerboard format. HCVcc infection was analyzed by luciferase reporter gene expression. Effects of antibody combinations on HCVcc infection were evaluated using the method of Prichard and Shipman.[32] Combination studies for each pair of compounds were performed in triplicate. The theoretical additive effect is calculated from the dose-response curves of individual compounds using the equation $z = x + y\ (1 - x)$, where x and y represent the inhibition produced by the individual compounds and z represents the effect produced by the combination of compounds. The theoretical additive surface is subtracted from the actual experimental surface, resulting in a horizontal surface that equals the zero plane when the combination is additive. A surface raising more than 20% above the zero plane indicates a synergistic effect of the combination, and a surface dropping lower than 20% below the zero plane indicates antagonism.

transfer of CE into the cell without internalization of HDL. At the same time, SR-BI also participates in HCV binding and entry into target cells. SR-BI is able to directly bind E2 and virus-associated lipoproteins but additional functions of SR-BI have been reported to be at play during HCV infection.[11,12,15,23] The results from this study highlight the importance of an SR-BI postbinding function for HCV entry and further extend the relevance of this function for HCV cell-to-cell transmission.

The molecular mechanisms underlying HCV cell-to-cell transmission are only partially understood. A recent study showed that SR-BI contributes to this process[37] and that E2–SR-BI interaction and/or SR-BI–mediated lipid transfer likely takes place during HCV dissemination, as antibodies and small molecule inhibitors targeting both SR-BI binding and lipid transfer reduce HCV cell-to-cell transmission.[9,17] However, which SR-BI functions are relevant for this process remain to be determined. Taking advantage of our novel mAbs uniquely inhibiting SR-BI postbinding activity required for HCV entry, we demonstrated that an E2 binding-independent postbinding function is involved in neutralizing antibody-resistant cell-to-cell transmission. E2-independent SR-BI function in HCV dissemination is in line with the observation that cell-to-cell transmission is largely insensitive to E2-specific antiviral mAbs.[37] Given that mSR-BI does not bind sE2 but mediates HCV entry and promotes cell-to-cell transmission, the postbinding function of SR-BI seems to be essential for HCV infection and dissemination, while the binding function may be dispensable. Furthermore, since HVR1-deleted HCV is less sensitive to inhibition by anti–SR-BI mAbs (Supporting Results and Supporting Fig. 4), HVR1–SR-BI interaction may play an important role during postbinding steps of HCV entry.

Previous studies using small molecule inhibitors indicated a role for SR-BI lipid transfer function in HCV infection and HDL-mediated entry enhancement.[12,13,23] Because inhibition of cell-free HCV entry and cell-to-cell transmission by our anti–SR-BI mAbs was associated with interference with lipid transfer, our data suggest that the SR-BI lipid transfer function may be relevant for both initiation of HCV infection and viral dissemination. Of note, our anti–SR-BI mAbs are the first anti–SR-BI mAbs that do not inhibit HDL binding to SR-BI. These data suggest that HCV entry and dissemination can be inhibited without blocking HDL–SR-BI binding. The further characterization of the SR-BI postbinding function will make it possible to determine whether the SR-BI–mediated postbinding steps of HCV entry and dissemination are directly linked to its lipid transfer function.

Using SR-BI chimeras, we demonstrate that the determinants relevant for HCV postbinding steps lie within the N-terminal half of the human SR-BI ectodomain (Supporting Results and Supporting Figs. 5 and 6). Amino acids 70-87 and residue E210 of SR-BI are required for E2 binding, while distinct protein regions are involved in HDL binding.[20,38] Although the SR-BI determinants involved in HDL binding and CE uptake have not yet been defined, a recent study reported that amino acid C323 is critical for these processes.[38] Given that our anti–SR-BI mAbs do not interfere with E2 and HDL binding, amino acids 70-87 and residues E210 and C323 are most likely not part of the targeted epitopes. Interestingly, the amino acid associated with cholesterol homeostasis[5] probably also lies outside these epitopes. The further characterization of these epitopes may make it possible to more thoroughly determine the regions of SR-BI relevant for its postbinding function during initiation of HCV infection and spread.

Finally, our data suggest that the SR-BI postbinding function is a highly relevant target for antivirals. Therapeutic options for a large proportion of HCV-infected patients are still limited by drug resistance and adverse effects.[1] Furthermore, a strategy for prevention of HCV liver graft infection is absent. Antivirals targeting essential host factors required for the HCV life cycle are attractive because they may increase the genetic barrier to antiviral resistance.[2,3] Indeed, our data demonstrate a marked synergy on the inhibition of HCV entry when combining antibodies directed against the viral envelope and SR-BI. These results suggest that combining molecules directed against viral and host entry factors is a promising strategy for prevention of HCV infection such as liver graft infection. The potent effect on cell-to-cell transmission and viral spread also opens a perspective of SR-BI–based entry inhibitors for treatment of chronic infection.

Small molecules and mAbs targeting SR-BI and interfering with HCV infection have been described.[12,17,26] A human anti–SR-BI mAb has been reported to inhibit HDL binding, to interfere with cholesterol efflux and to decrease HCVcc entry during attachment steps without having a relevant impact on SR-BI–mediated postbinding steps.[20,26] A codon-optimized version of this mAb has been demonstrated to prevent HCV spread *in vivo*,[9] underscoring the potential of SR-BI as an antiviral target. The mAbs generated in our study are highly novel in their function, as they do not interfere with sE2–SR-BI binding but inhibit HCV entry during postbinding steps of cell-free infection and cell-to-cell transmission. Furthermore, in

contrast to described anti–SR-BI mAbs,[26] these mAbs do not hinder HDL–SR-BI binding and only partially inhibit lipid transfer at concentrations significantly inhibiting HCV infection. Given their novel mechanism of action and their potential differential toxicity profile, QQ-4A3-A1, QQ-2A10-A5, QQ-4G9-A6, and NK-8H5-E3 define a novel class of anti–SR-BI mAbs for prevention and treatment of HCV infection.

Acknowledgment: We thank R. Bartenschlager (University of Heidelberg, Germany) for providing Luc-Jc1 expression vector; T. Wakita (National Institute of Infectious Diseases, Tokyo, Japan) for the JFH1 construct; S. K. H. Foung (Stanford University, Palo Alto, CA) for anti-E2 antibody CBH23; and C. M. Rice (The Rockefeller University, New York, NY) and F. V. Chisari (The Scripps Research Institute, La Jolla, CA) for Huh7.5 and Huh7.5.1 cells, respectively. We also thank A. H. Patel (MRC-University of Glasgow Centre for Virus Research, Glasgow, UK) for Huh7.5-GFP cells and AP33 antibody; J. Ball (University of Nottingham, Nottingham, UK) for providing plasmids for production of different HCVpp genotypes; and D. Trono (Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland) for pWPI plasmid. We also acknowledge E. Schnober (University of Freiburg, Freiburg, Germany) for contributing to sE2 binding assays and S. Durand (INSERM U748, Strasbourg, France), C. Bach (INSERM U748, Strasbourg, France), J. Barths (INSERM, University of Strasbourg, Strasbourg, France), C. Granier (INSERM U758, France), and S. Glauben (Aldevron Freiburg, Freiburg, Germany) for technical assistance.

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Table 1. Monoclonal antibodies directed against human SR-BI. Isotype, binding affinity to Huh7.5.1 cells (Kd_{app}) as well as inhibition of HCVcc infection (IC_{50}) and inhibition of lipid transfer of anti-SR-BI mAbs are shown. Huh7.5.1 cells were incubated with increasing concentrations of mAbs and Kd values were determined as half-saturating concentrations of the mAbs.IC_{50} was determined after incubation of Huh7.5.1 cells with serial dilutions of anti-SR-BI mAbs for 1h at room temperature before infection with HCVcc. The results represent means of three independent experiments performed in triplicate. Lipid uptake and efflux were assessed in Huh7 cells as described in Material and Methods in the presence of anti-SR-BI mAbs (20 µg/mL). The results are expressed as % inhibition of lipid transfer relative to cells incubated in the absence of antibody and represent means ± SD of three independent experiments. n. a. : not applicable

mAb	Isotype	Kd_{app} Huh7.5.1 (nM)	IC_{50} HCVcc (µg/mL)	Inhibition of HDL-CE uptake (mean % ± SD)	Inhibition of cholesterol efflux (mean % ± SD)
QQ-4A3-A1	rat IgG2b	1.0	0.7	44.18 ± 1.42	40.97 ± 0.92
QQ-2A10-A5	rat IgG2b	0.5	0.2	47.64 ± 1.2	40 ± 1.01
QQ-4G9-A6	rat IgG2b	0.5	1.0	44.64 ± 1.57	39.02 ± 1.14
PS-6A7-C4	rat IgG2b	n. a.	n. a.	10.24 ± 1.52	-2.52 ± 1.25
PS-7B11-E3	rat IgG2b	n. a.	n. a.	11.73 ± 2.1	5.04 ± 0.83
NK-8H5-E3	mouse IgG2b	7.4	8.0	56.28 ± 0.8	44.74 ± 0.55
NK-6B10-E6	mouse IgG1	n. a.	n. a.	1.28 ± 1.69	18.41 ± 0.81
NK-6G8-B5	mouse IgG1	n. a.	n. a.	5.64 ± 1.04	13.08 ± 0.77

Supplementary material

The post-binding activity of scavenger receptor BI mediates initiation of hepatitis C virus infection and viral dissemination

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Supplementary Material and Methods

Cell culture-derived HCV (HCVcc) and HCV pseudoparticles (HCVpp). Luciferase reporter chimeric HCVcc of genotypes 1-4 (H77/1a/R2a, Con1/1b/R2a, J8/2b/R2a, S52/3a/R2a and ED43/4a/R2a) and HCVpp of genotypes 1-6 (H77, HCV-J, JFH1, UKN3A1.28, UKN4.21.16, UKN5.14.4 and UKN6.5.340) have been described.[1-3] Patient-derived HCVpp (P02VJ) from a patient (P02) undergoing liver transplantation have been as described.[2,4] HCVpp bearing the envelope glycoproteins of strain H77, H77 deleted of hypervariable region 1 (HVR1) within E2 (ΔG384-N411) or H77 containing a point mutation within HVR1 (L399R) have been described.[5] Luc-Jc1 HCVcc lacking HVR1 (ΔHVR1) have been described.[6]

Investigation of the antiviral effects of antibodies and antibody combinations on HCV entry and infection. HCVcc and pseudoparticle infection and kinetic assays have been described.[2,7-10] Briefly, for infection experiments, Huh7.5.1 cells were pre-incubated in the presence or absence of antibodies for 1h at 37°C and infected at 37°C for 4h with HCVcc or pseudoparticles. 72h later infection was analyzed in cell lysates by quantification of luciferase activity using a Promega kit. For kinetic entry experiments, Huh7.5.1 were inoculated with HCVcc (10^4-10^5 $TCID_{50}$/mL) for 1h at 4°C in the presence or absence of compounds. Subsequently, cells were washed three times with ice cold PBS, supplied with fresh culture fluid pre-warmed to 37°C and supplemented with the respective compounds and shifted to 37°C. The compounds were removed after 4h and cells were supplied with fresh culture fluid without compounds and cultured an additional 48h at 37°C before quantification of luciferase activity in cell lysates. For combination experiments, each antibody was tested individually or in combination with the second antibody. Huh7.5.1 cells were pre-incubated with anti-SR-BI or control antibody for 1 h and then incubated for 4h at 37°C with HCVcc (Luc-Jc1) or HCVpp (P02VJ) (pre-incubated for 1h with or without anti-envelope antibodies).

Assessment of synergy. Synergy was assessed by two independent methods comprising the Combination Index and the method of Prichard and Shipman. The Combination Index (CI) was calculated as described.[11,12] A CI less than, equal to, and more than 1 indicates synergy, additivity, and antagonism, respectively[11]. The method of Prichard and Shipman was applied as described.[13] In brief, the theoretical additive effect is calculated from the dose-response curves of individual compounds by the equation $Z=X+Y(1-X)$ where X and Y represent the inhibition produced by the individual compounds and Z represents the effect produced by the combination of compounds. The theoretical additive surface is subtracted from the actual experimental surface, resulting in a horizontal surface that equals the zero plane when the combination is additive. A surface raising more than 20% above the zero plane indicates a synergistic effect of the combination and a surface dropping lower than 20% below the zero plane indicates antagonism. The antiviral assay was performed as described above except that the compound dilutions were added in a checkerboard format. Combination studies for each pair of compounds were performed in triplicate.

Chimeric human/mouse SR-BI and human SR-BI mutants. Retroviral vectors expressing human SR-BI (Z22555) or human SR-BI point mutants Q402R, E418R, Q402R-E418R and

G420H-G424H as well as mouse SR-BI (NM_016741) or human/mouse chimeric SR-BI were described previously.[14,15] Briefly, mouse SR-BI (NM_016741) or human/mouse chimeric SR-BI cDNAs were inserted in CNC MLV (murine leukemia virus) vector backbones (kind gift of M. Collins) harboring selectable marker genes for puromycine and G418, respectively. Using SR-BI sequence comparisons as well as structural features predictions, three regions in the SR-BI ectodomain were delineated between amino acid (aa) positions 38-215, 216-398 and 399-432. cDNAs encoding three human/mouse SR-BI chimeras were generated by PCR by swapping these three SR-BI regions. While the HHH and MMM SR-BI constructs refer to the wild-type human (H) and mouse (M) SR-BI molecules, respectively, the human/mouse SR-BI chimeras were denominated according to the origin of either SR-BI region, e.g., HMM bears region 1 from human SR-BI and regions 2 and 3 from murine SR-BI (Supplementary Figure 5).[15] All mutants were sequenced to ensure that the clones possessed only the expected mutation.[15] Retroviral vectors containing these cDNAs were produced from 293T cells as VSVG-pseudoparticles as described previously. Stable expression of either receptor in target cells was obtained by transduction with vector particle-containing supernatants of 293T producer cells, followed by antibiotic selection. CHO and BRL3A cells expressing human, mouse, human/mouse chimeric as well as mutant SR-BI were produced as described.[8,14,15]

Epitope mapping. BRL3A or CHO cells were transduced with retroviral vectors expressing human, mouse or human-mouse chimeric SR-BI or previously described human SR-BI mutants.[14,15]Transduced cells were selected using antibiotics and proper SR-BI expression was studied using flow cytometry and commercial anti-SR-BI antibodies. Anti-SR-BI mAb binding was assessed using flow cytometry.[14]

Immunoblotting. Huh7.5.1 cells were lysed with Glo lysis buffer (Promega) and 50 µg of protein of each sample were separated by 12% SDS-PAGE, transferred to HyBond-P nitrocellulose membranes (GE Healthcare) and then incubated with anti-SR-BI mAbs QQ-4A3-A1, QQ-2A10-A5, QQ-4G9-A6, PS-6A7-C4, NK-8H5-E3, NK-6B10-E6 and NK-6G8-B5 (5 µg/mL) or EP1556Y (Abcam, 1:100) and AP-labelled secondary antibodies.[8]

Supplementary Results

Anti-SR-BI antibodies do not interfere with sE2 binding to target cells. As HCV E2 directly binds hSR-BI, we assessed their ability to interfere with E2-SR-BI binding using recombinant soluble E2 (sE2) as a surrogate model for HCV and Huh7.5.1 cells as target cells. In contrast to a polyclonal anti-SR-BI rat serum[8] and an anti-CD81 mAb, none of the anti-SR-BI mAbs inhibited sE2-SR-BI binding (Supplementary Figure 1B-C, statistically not significant). Given that Huh7.5.1 cells express all known HCV receptors that may also contribute to sE2 binding, we also used rat BRL cells lacking SR-BI[14] to assess sE2 binding to exogenously expressed hSR-BI in the absence of other HCV receptors. Although one antibody (NK-8H5-E3) appeared to have a minor inhibitory effect in some experiments, inhibition of sE2 binding was not statistically significant and not robust compared to the polyclonal anti-SRBI rat serum (Supplementary Figure 1D). Surprisingly, rat anti-SR-BI mAbs increased sE2-SR-BI binding. It is conceivable that binding of the rat mAb to SR-BI results in a different interaction of sE2 with other HCV attachment factors on BRL cells such as heparan sulfate which subsequently enhances HCV attachment. Taken together, these data confirm the findings obtained for cellular attachment of HCVcc (Figure 2) and suggest that interference with E2 binding to target cells does not play a major role for the antiviral action of SR-BI-specific mAbs described in this study.

Functional impact of HCV HVR1 and SR-BI during post-binding steps of the viral entry process. The 27 amino acid long hypervariable region 1 (HVR1) at the N-terminus of HCV E2 has been shown to mediate E2 binding to SR-BI and also to contribute to HDL-mediated enhancement of HCV entry that is dependent on the lipid transfer function of SR-BI but independent of HDL binding.[14-16] Given this complex role of HVR1 in SR-BI-dependent HCV entry steps, we investigated the effect of anti-SR-BI mAbs inhibiting HCV post-binding steps on HCVcc and HCVpp deleted in HVR1 (ΔHVR1). Interestingly, HCVcc and HCVpp lacking

HVR1 were less sensitive to inhibition by anti-SR-BI mAbs (Supplementary Figure 4A-B, $p<0.01$) although requiring SR-BI for cell entry as cells lacking SR-BI are not permissive for ΔHVR1 HCV (data not shown). This was also confirmed using HCVpp L399R containing a point mutation within HVR1 (Supplementary Figure 4C, $p<0.01$). Taken together, these data suggest that HVR1 may play a role during SR-BI-mediated post-binding steps of the HCV entry process. Since anti-SR-BI antibodies did not interfere with cellular binding of sE2, it is conceivable that the functional role of HVR during SR-Bi mediated post-binding steps may be beyond direct E2-HVR1-SR-BI interactions.

Protein determinants relevant for HCV post-binding steps lie within the N-terminal half of the human SR-BI ectodomain. To map the protein determinants important for SR-BI post-binding function during HCV entry, we first performed cross-competition studies in order to determine whether these antibodies recognize overlapping or distinct epitopes. Labelled anti-SR-BI mAb NK-8H5-E3 was incubated with Huh7.5.1 cells in the presence of increasing concentrations of unlabelled anti-SR-BI mAbs. Cross-competition experiments with labelled versions of QQ-4A3-A1, QQ-2A10-A5 and QQ-4G9-A6 demonstrated that each of these mAbs reduced binding of unlabelled rat mAbs but not mouse mAb (Supplementary Figure 6A-C). Moreover, in contrast to unlabelled mouse NK-8H5-E3, none of the three unlabelled rat mAbs (QQ-4A3-A1, QQ-2A10-A5 and QQ-4G9-A6) reduced binding of NK-8H5-E3 to Huh7.5.1 cells, comparable to control isotype mAb (Supplementary Figure 6D). The mutual cross competition between the three rat mAbs suggests that they recognize overlapping or closely related epitopes on SR-BI while the mouse mAb recognizes a distinct epitope. To further define the epitopes targeted by these antibodies, we investigated their ability to bind to human-mouse SR-BI chimeras, where part of the mouse SR-BI ectodomain was replaced by the corresponding human sequence (Supplementary Figure 5A-C).[15] While the HHH and MMM SR-BI constructs refer to the wild-type human (H) and mouse (M) SR-BI molecules, respectively, the human/mouse SR-BI chimeras were denominated according to the origin of either SR-BI region, e.g., HMM bears region 1 from human SR-BI and regions 2 and 3 from murine SR-BI (Supplementary Figure 5B-C). The overall homology between human and mouse SR-BI is 80% (54 aa difference) (Supplementary Figure 5A). There are a total of 31, 14 and 9 different aa within the first, second and third region of the SR-BI human/mouse chimeras, respectively (Supplementary Figure 5A). The three rat anti-SR-BI mAbs QQ-4A3-A1, QQ-2A10-A5, QQ-4G9-A6 bind to HMM SR-BI, i. e. aa 38-215, with high affinity and also to MHM, i. e. 216-398, to a lesser extent while the mouse mAb NK-8H5-E3 only recognizes HMM SR-BI with high affinity (Supplementary Figure 5D). These data suggest that the epitope targeted by NK-8H5-E3 lies in the N-terminal half of the human SR-BI ectodomain, between aa 38 and aa 215, while the epitope(s) targeted by QQ-4A3-A1, QQ-2A10-A5, QQ-4G9-A6 probably lie more downstream within the SR-BI ectodomain. To further map residues within SR-BI contributing to antibody binding we used previously described SR-BI point mutants.[14] Interestingly, point mutation G420H and double mutations Q402R-E418R and G420H-G424H within human SR-BI markedly reduced binding of the four anti-SR-BI mAbs inhibiting HCV infection (Supplementary Figure 5E). These data suggest that aa 402, 418, 420 and 424 may be part of the epitopes of these antibodies or that these mutations may induce conformational changes within the epitopes. Finally, to further characterize the nature of the epitopes targeted by our panel of anti-SR-BI mAbs, we assessed the ability of the anti-SR-BI mAbs to bind to human SR-BI using SDS-PAGE and Western blot. Immunostaining of SR-BI by anti-SR-BI mAbs PS-6A7-C4, NK-6B10-E6 and NK-6G8-B5 suggest that the epitopes interacting with these antibodies, that do not inhibit HCV infection, probably include linear domains (data not shown). In contrast, none of the antibodies inhibiting HCV infection interacted with linear SR-BI in Western blot experiments suggesting that the antibodies inhibiting HCV infection likely recognize predominantly conformational epitopes (data not shown). Taken together, these data indicate that anti-SR-BI mAbs inhibiting HCVcc infection recognize conformational epitopes within the N-terminal half of the SR-BI ectodomain. Moreover, these data suggest that the N-terminal ectodomain of SR-BI contains protein determinants relevant for the SR-BI post-binding function in HCV entry.

Supplementary Figure legends

Supplementary Figure 1. Monoclonal antibodies specific for human SR-BI do not block HCV E2 binding. (A) BRL3A cells engineered to express mouse (m) or human (h) SR-BI were first incubated with monoclonal anti-SR-BI antibodies (20 µg/mL) for 1h at RT before bound antibodies were detected using PE-labelled secondary antibodies and flow cytometry. Results are expressed as net mean fluorescence intensity (ΔMFI). (B-C) Huh7.5.1 cells were pre-incubated with anti-CD81 (5 µg/mL), anti-SR-BI or control serum (1:100), anti-SR-BI (20 µg/mL) or control antibodies for 1h at room temperature (RT) before incubation with sE2 for 1h at RT. (B) sE2 binding was detected using mouse anti-His antibody followed by PE-labelled anti-mouse antibody and flow cytometry. (C) sE2 binding was detected using FITC-labelled mouse anti-His antibody and flow cytometry. Results are expressed as means ± SD % sE2 binding in the absence of antibody of three independent experiments performed in duplicate. (D) BRL cells engineered to express human SR-BI were pre-incubated with polyclonal anti-SR-BI or control (CTRL) serum (1:50), anti-SR-BI (20 µg/mL) or control (CTRL) antibodies for 1h at room temperature (RT) before incubation with sE2 for 1h at RT. sE2 binding was detected using FITC-labelled mouse anti-His antibody and flow cytometry. Results are expressed as means ± SD % sE2 binding in the absence of antibody of four independent experiments. * $P<0.01$

Supplementary Figure 2. Monoclonal anti-SR-BI antibodies block HCV cell-to-cell transmission and spread. (A-B) Quantification of HCV–infected target cells (Ti) after co–cultivation with HCV producer cells (Pi) during incubation with (A) control or anti-SR-BI mAb QQ-4G9-A6 (10 µg/mL) or (B) control or anti-SR-BI mAb QQ-2A10-A5 (10 µg/mL) in the presence of E2-neutralizing antibody AP33 (25 µg/mL) by flow cytometry. (C) Cell viability after long-term exposure to anti-SR-BI mAbs QQ-4G9-A6 and NK-8H5-E3. Cell viability was assessed using MTT assay after incubation of Huh7.5.1 cells for 14 days in the presence or absence of control or anti-SR-BI mAbs at 1, 10, or 100 µg/mL. Control medium and medium containing antibodies were replenished every 4 days. Data are expressed as % cell viability relative to cells incubated in the absence of mAb and represent means ± SD from one experiment.

Supplementary Figure 3. Genotype-independent inhibition of HCVpp infection by monoclonal anti-SR-BI antibodies. Inhibition of entry into Huh7.5.1 cells of HCVpp bearing envelope glycoproteins from genotypes 5 and 6. Huh7.5.1 cells were pre-incubated with control or anti-SR-BI mAbs (50 µg/mL) for 1h at 37°C before infection with HCVpp bearing envelope glycoproteins of strains UKN5.14.4 (5) or UKN6.5.340 (6) and VSV-Gpp. HCVpp entry was analyzed by luciferase reporter gene expression. Results are expressed as % HCVpp entry and represent means ± SD from 3 independent experiments performed in triplicate. * $P<0.01$

Supplementary Figure 4. Inhibition of HVR1-deleted HCVcc and HCVpp by monoclonal anti-SR-BI antibodies. Huh7.5.1 cells were pre-incubated with control or anti-SR-BI mAbs (20 µg/mL) for 1h at 37°C before infection with (A) Luc-Jc1 HCVcc deleted of HVR1 (ΔHVR1) or (B-C) HCVpp bearing the envelope glycoproteins of strain H77, (B) H77 deleted of HVR1 (ΔG384-N411) or (C) H77 containing a point mutation within HVR1 (L399R). HCVpp and HCVcc infection was analyzed by luciferase reporter gene expression. Results are expressed as % HCVpp entry or HCVcc infection and represent means ± SD from (A) one experiment performed in triplicate and (B-C) 3 independent experiments performed in triplicate. * $P<0.01$

Supplementary Figure 5. Binding of monoclonal anti-SR-BI antibodies to human, mouse or chimeric mouse and human SR-BI as well as human SR-BI lipid transfer mutants. (A) Alignment of amino acid sequences of mouse and human SR-BI. (B-C) Schematic representations of three human/murine SR-BI chimeras that were generated through PCR by swapping three SR-BI domains between amino-acid positions 38-215

(region 1), 216-398 (region 2) and 399-432 (region 3), respectively. While the HHH and MMM SR-BI constructs refer to the wild-type human (H) and murine (M) SR-BI molecules, respectively, the human/mouse SR-BI chimeras were denominated according to the origin of either SR-BI domain, e.g., HMM bears the region 1 from human SR-BI and the regions 2 and 3 from murine SR-BI.[15] (D) BRL3A cells engineered to express human (HHH), mouse (MMM) or chimeric mouse and human (HMM, MHM, MMH) SR-BI were first incubated with monoclonal anti-SR-BI antibodies (20 µg/mL) for 1h at room temperature before bound antibodies were detected using PE-labelled secondary antibodies. Results are expressed as means ± SD net mean fluorescence intensity (ΔMFI). (E) BRL3A cells engineered to express wild-type human SR-BI (SR-BI wt) or human SR-BI point mutants (G420H, Q402R, E418R, Q402R-E418R and G420H-G424H) were first incubated with monoclonal anti-SR-BI antibodies (20 µg/mL) for 1h at RT before bound antibodies were detected using PE-labelled secondary antibodies. Results are expressed as % binding of antibodies as compared to SR-BI wt and represent means ± SD from one out of 2 independent experiments.

Supplementary Figure 6. Competition of monoclonal anti-SR-BI antibodies for cellular binding. Huh7.5.1 cells were incubated with 0.1 µg/mL of biotinylated anti-SR-BI mAb (A) QQ-4A3-A1, (B) QQ-2A10-A5, (C) QQ-4G9-A6 or (D) NK-8H5-E3, together with increasing concentrations of unlabeled control or anti-SR-BI mAb (QQ-4A3-A1, QQ-2A10-A5, QQ-4G9-A6, NK-8H5-E3) as competitors. Following washing of cells with PBS, binding of labelled mAbs was determined by flow cytometry and is shown % binding relative to biotinylated mAb incubated in the absence of antibody.

Supplementary Figure 7. Combination of anti-SR-BI and neutralizing antibodies results in a synergistic activity in inhibiting HCV infection. Patient derived HCVpp P02VJ (A, C, E) or HCVcc (Luc-Jc1) (B, D, F) were pre-incubated with (A-B) anti-E1 (IGH526) or (C-D) anti-E2 (IGH461) mAbs or (E-F) purified heterologous anti-HCV IgG obtained from an unrelated chronically infected subject or isotype control IgG at the indicated concentrations for 1h at 37°C and added to Huh7.5.1 cells pre-incubated with increasing concentrations of control or anti-SR-BI mAbs (NK-8H5-E3). HCVpp and HCVcc infection was analyzed by luciferase reporter gene expression. Results are expressed as mean % HCVpp entry or HCVcc infection from a representative experiment. Synergy was assessed by the Combination Index calculated as described.[11, 12] Combination of anti-E1 or anti-E2 or patient-derived anti-HCV IgG with a sub-IC_{50} concentration of anti-SR-BI mAb - which exerts only minimal inhibitory effect on HCV infection - resulted in a synergistic activity in inhibition of HCVcc infection (CIs of 0.06 to 0.67). These combinations reduced the IC_{50} of anti-SR-BI mAb by up to 100-fold. (A, C, E) CI NK-8H5-E3 + anti-E1 (1 µg/mL): 0.30; CI NK-8H5-E3 + anti-E2 (1 µg/mL): 0.51; CI NK-8H5-E3 + anti-HCV IgG (1 µg/mL): 0.67 (B, D, F) CI NK-8H5-E3 + anti-E1 (0.01 µg/mL): 0.06; CI NK-8H5-E3 + anti-E2 (0.01 µg/mL): 0.25; CI NK-8H5-E3 + anti-HCV IgG (0.1 µg/mL): 0.14.

Supplementary References

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Figure S1

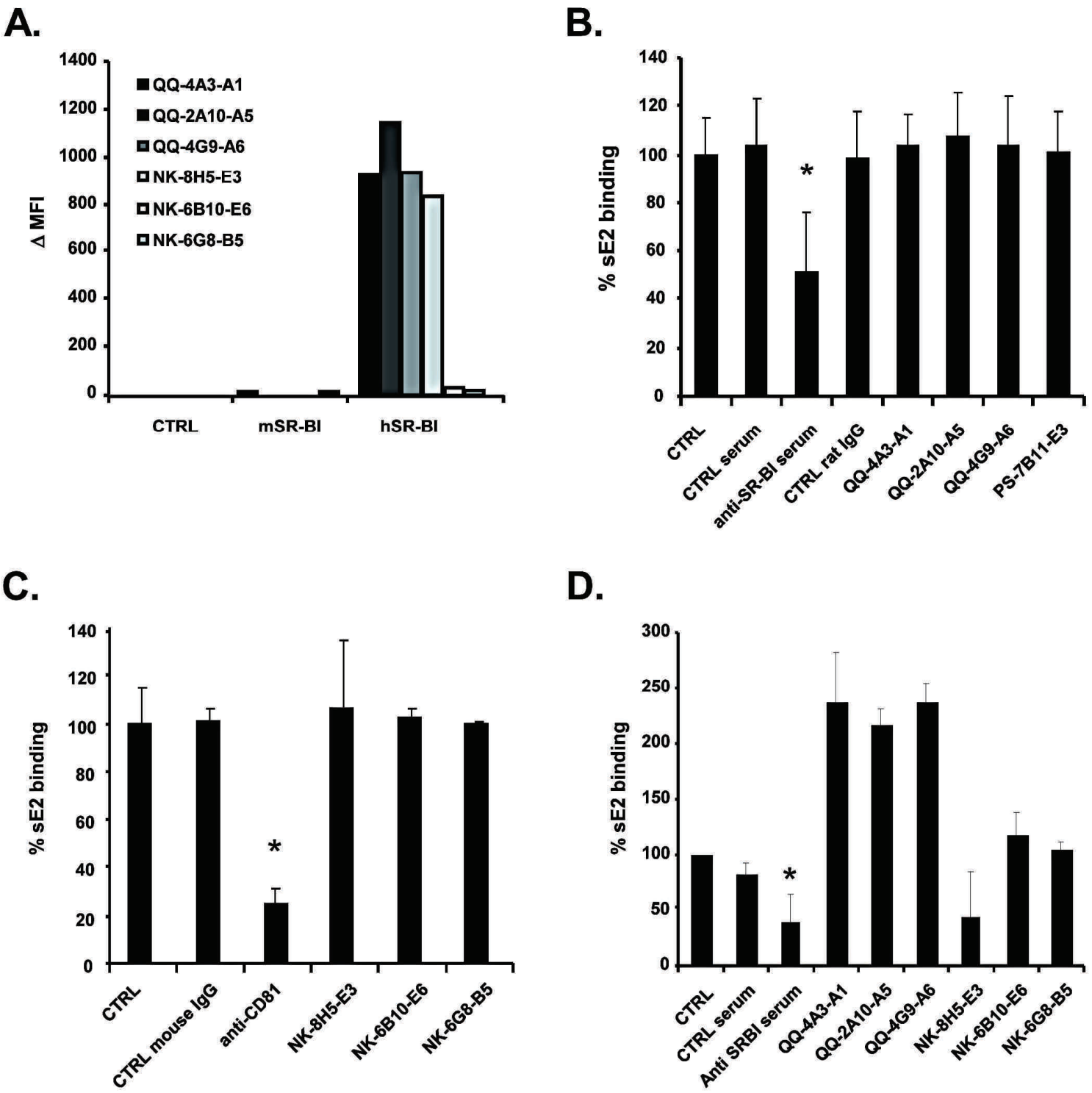
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Figure S2

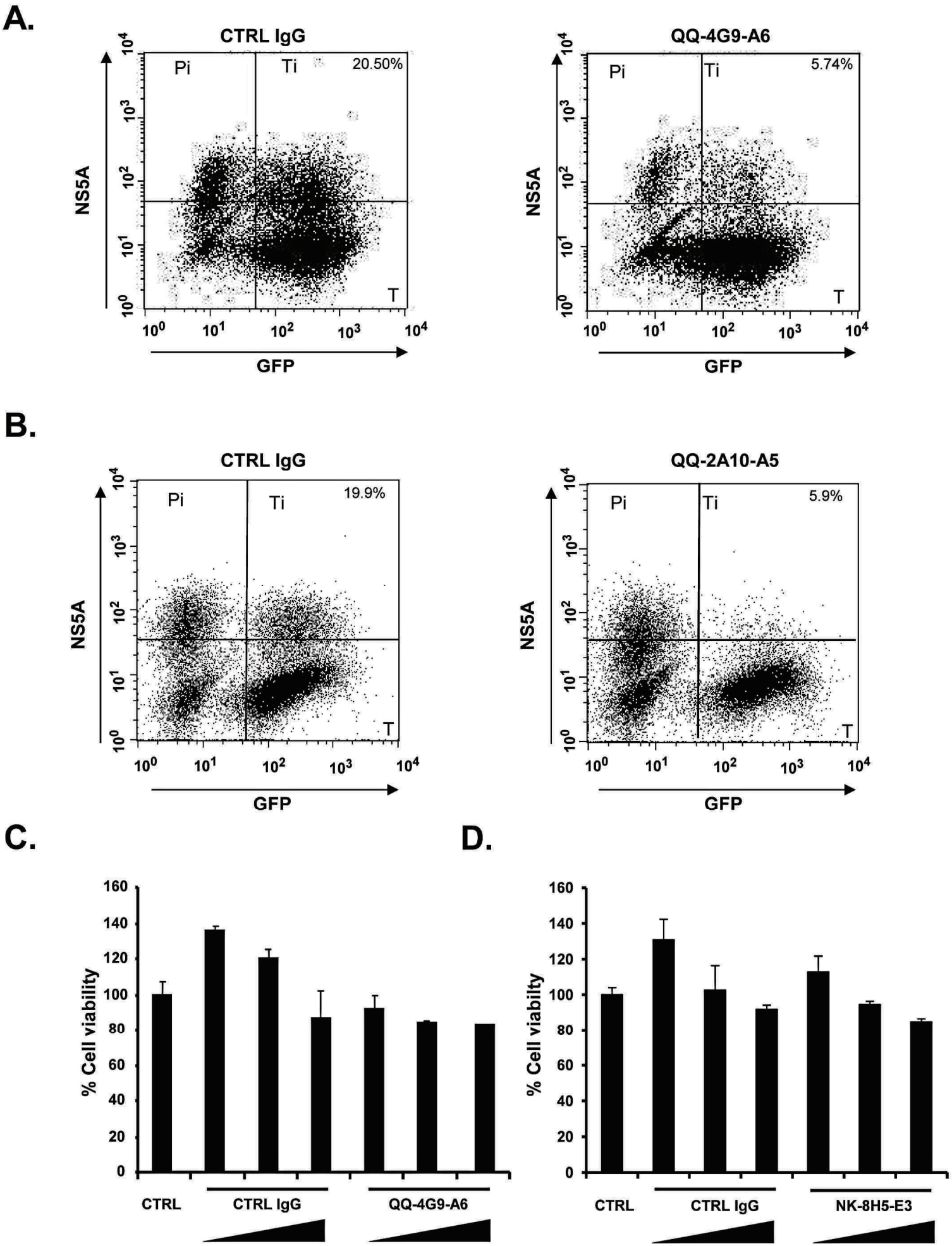
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Figure S3

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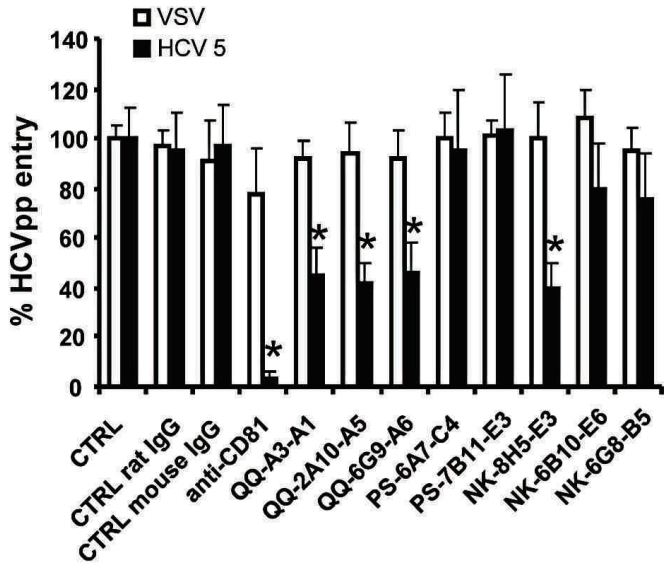
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B.

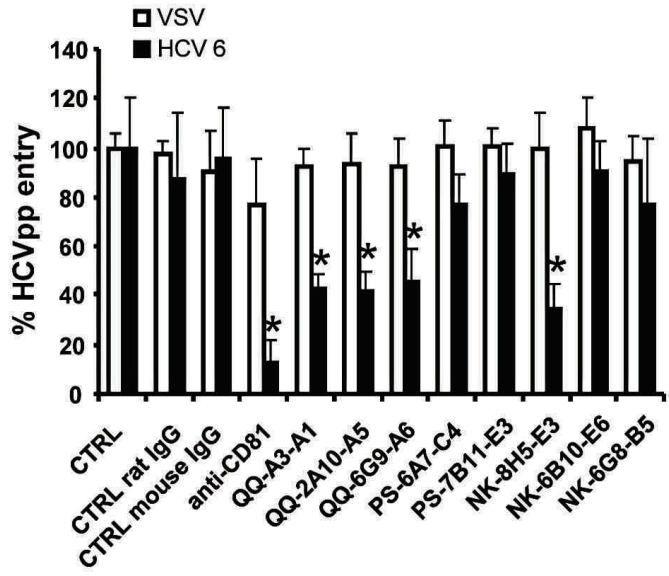
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Figure S4

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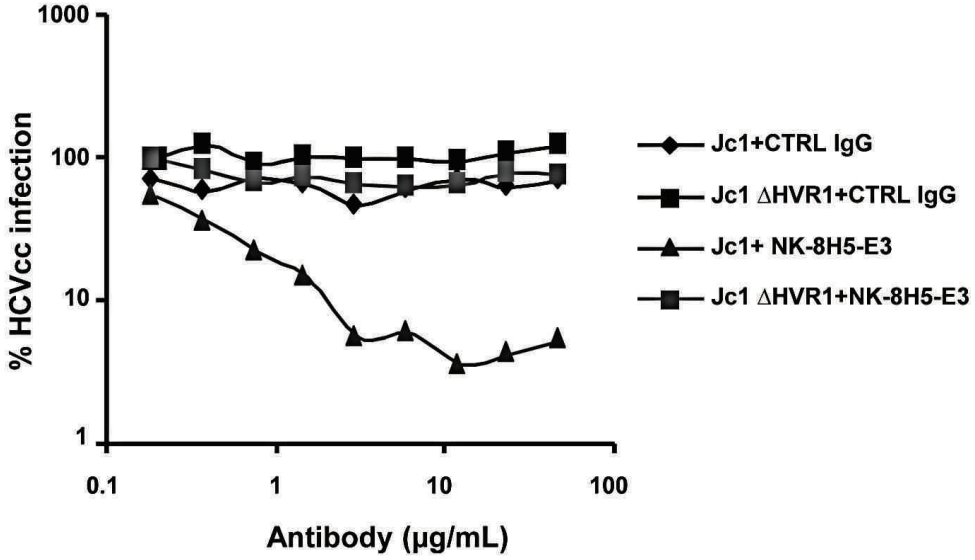

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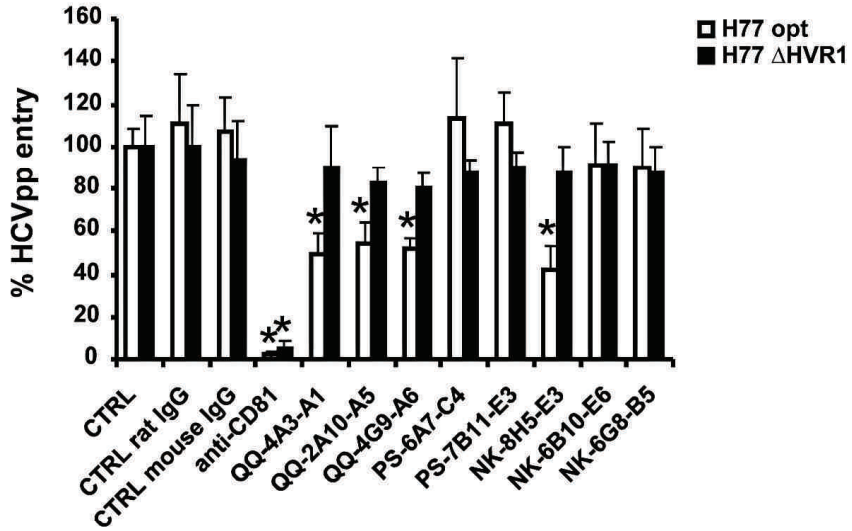

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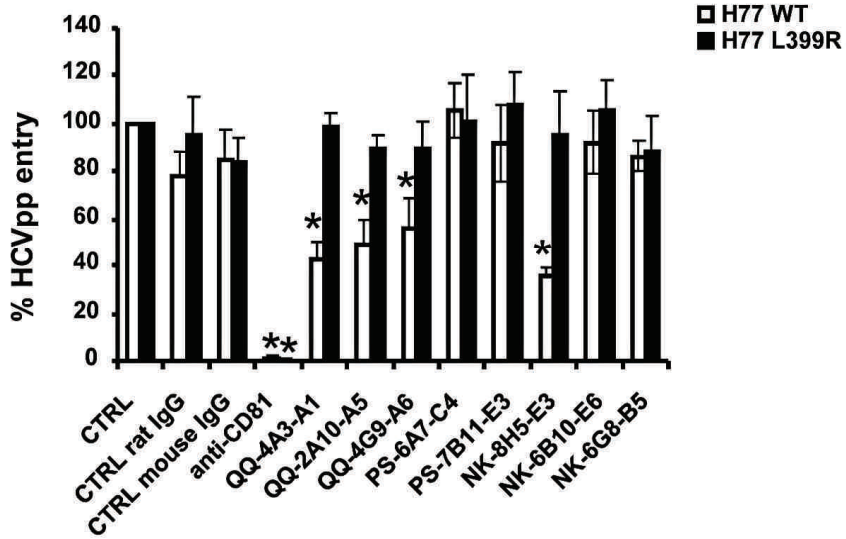
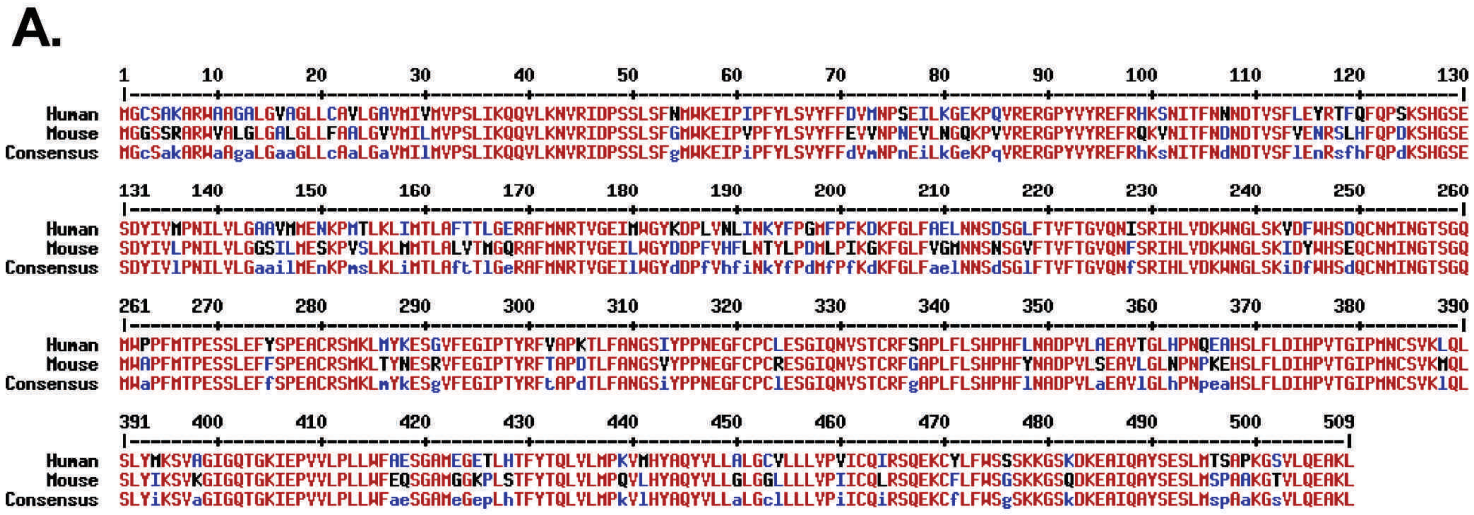


Figure S5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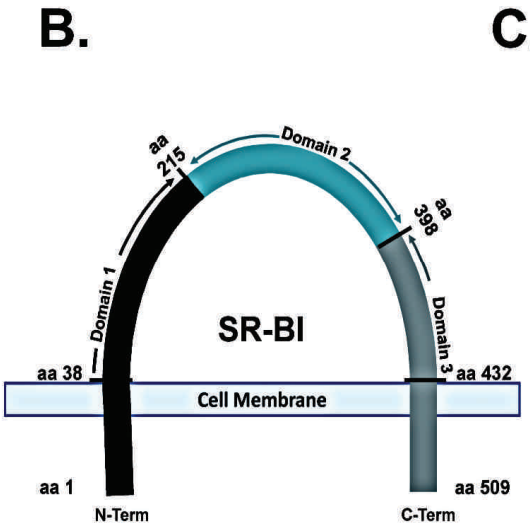
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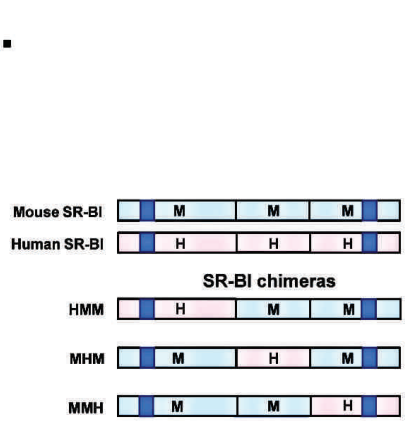
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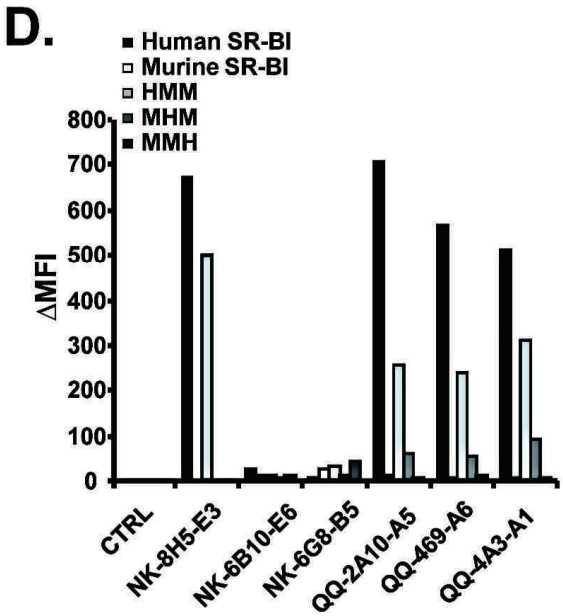
MHM
MMH


D.
Human SR-BI
Murine SR-BI
HMM
MHM
MMH
ΔMFI
CTRL
NK-8H5-E3
NK-6B10-E6
NK-6G8-B5
QQ-2A10-A5
QQ-469-A6
QQ-4A3-A1

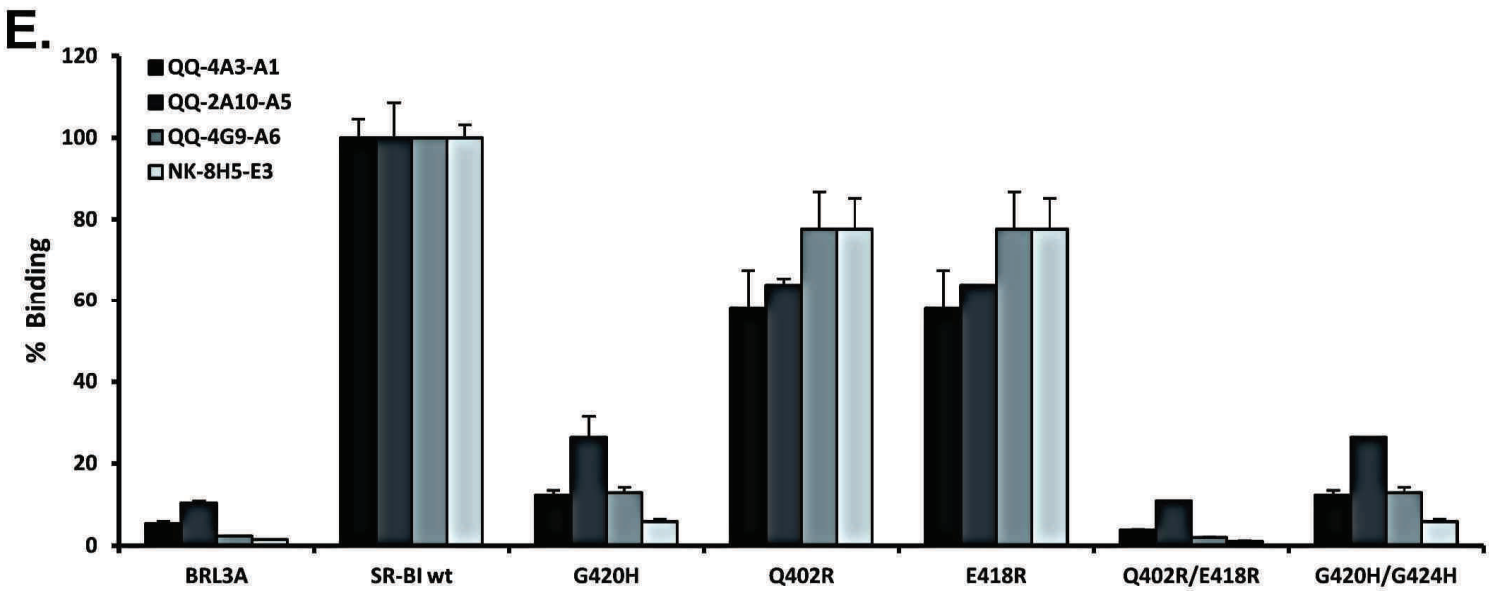
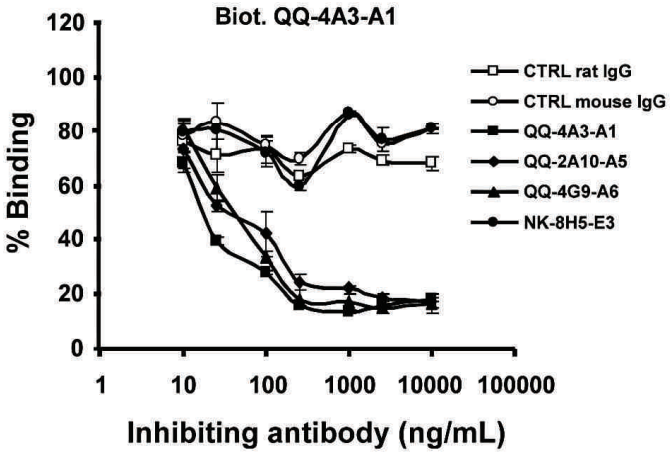
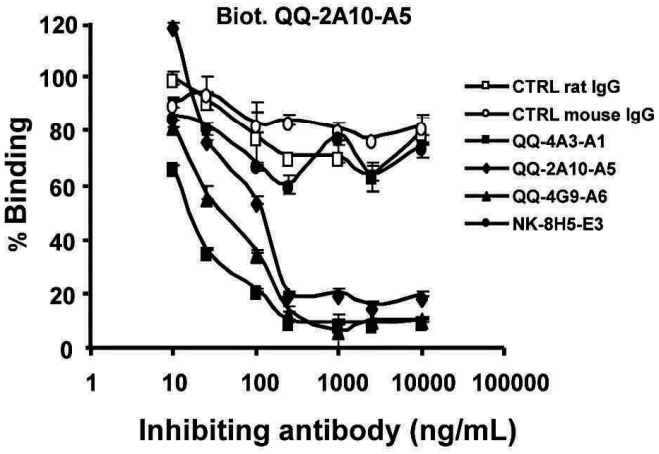

E.
QQ-4A3-A1
QQ-2A10-A5
QQ-4G9-A6
NK-8H5-E3
% Binding
BRL3A
SR-BI wt
G420H
Q402R
E418R
Q402R/E418R
G420H/G424H

Figure S6

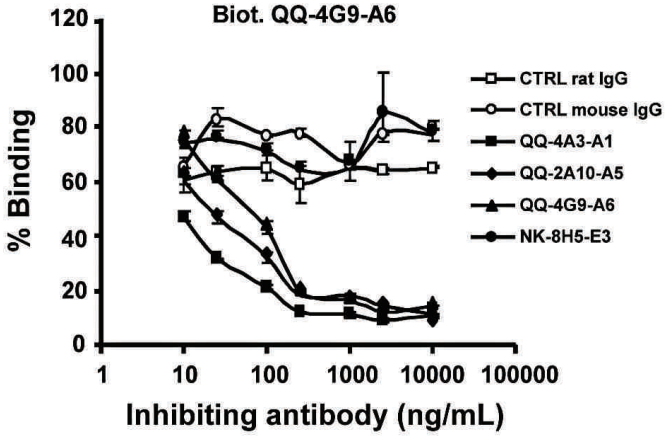
A.



B.



C.



D.

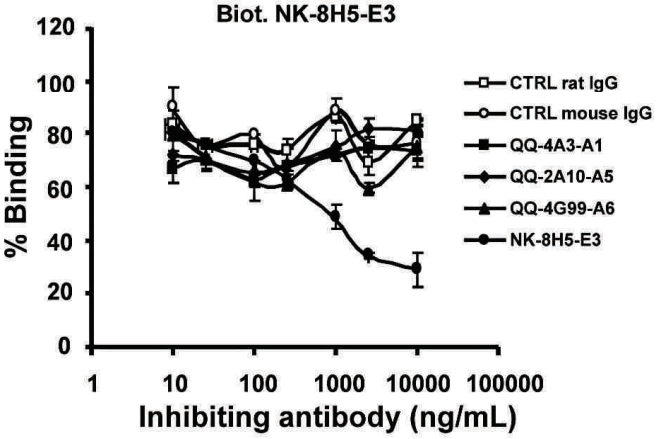
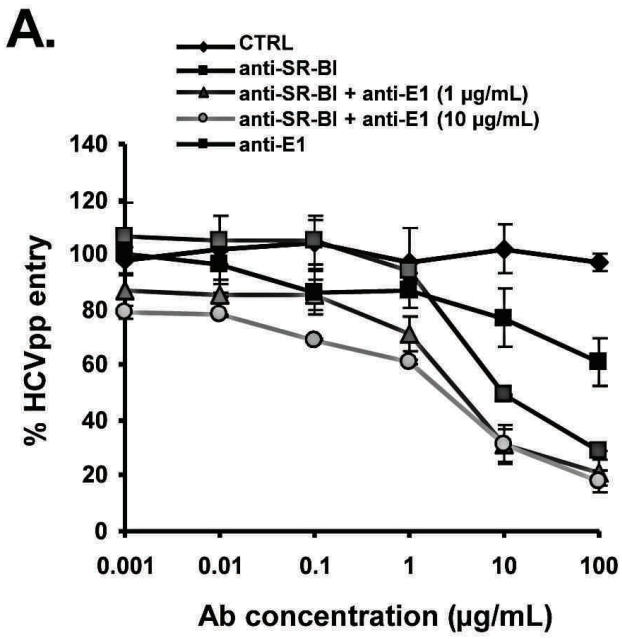
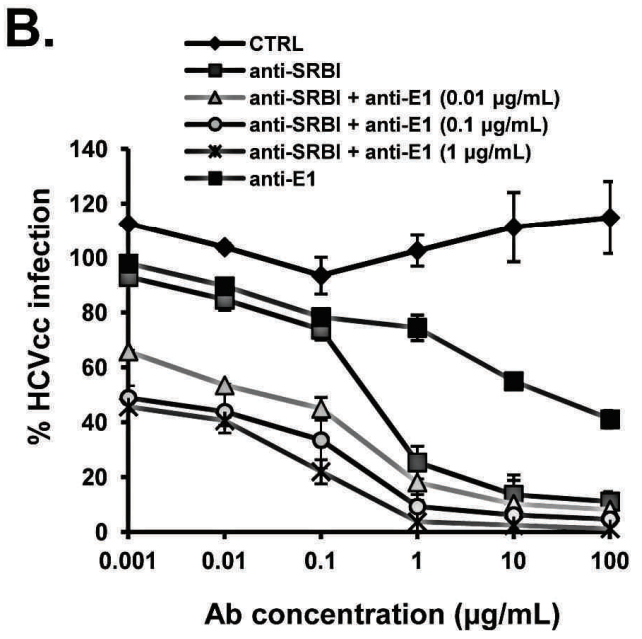
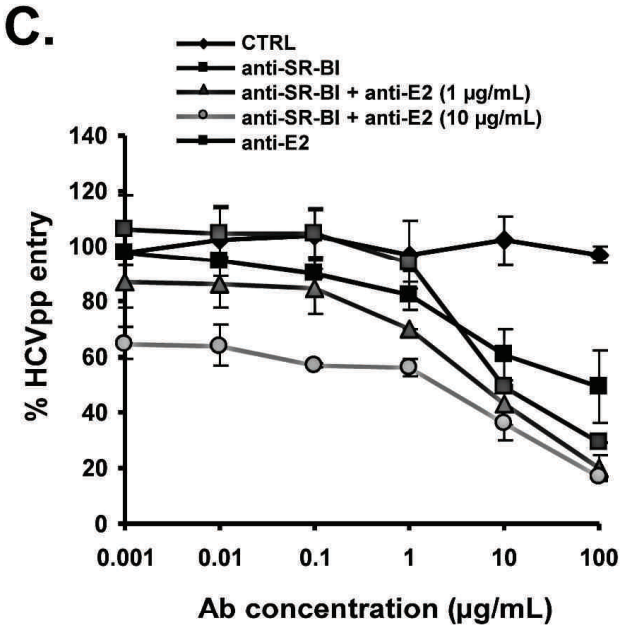
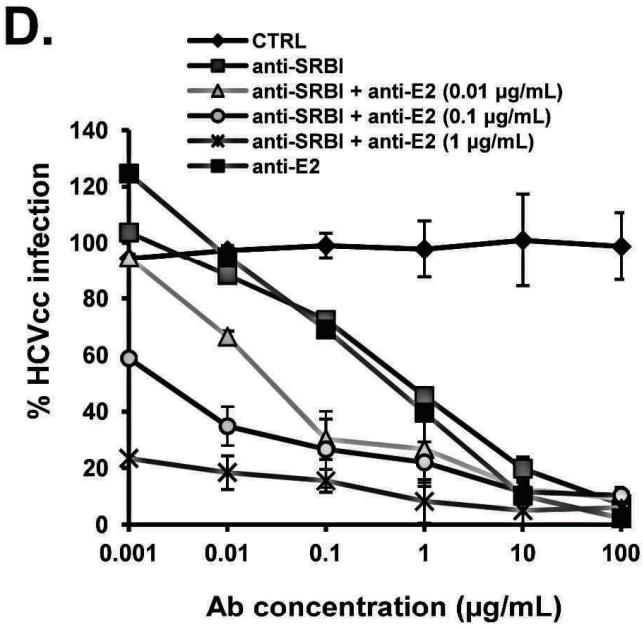


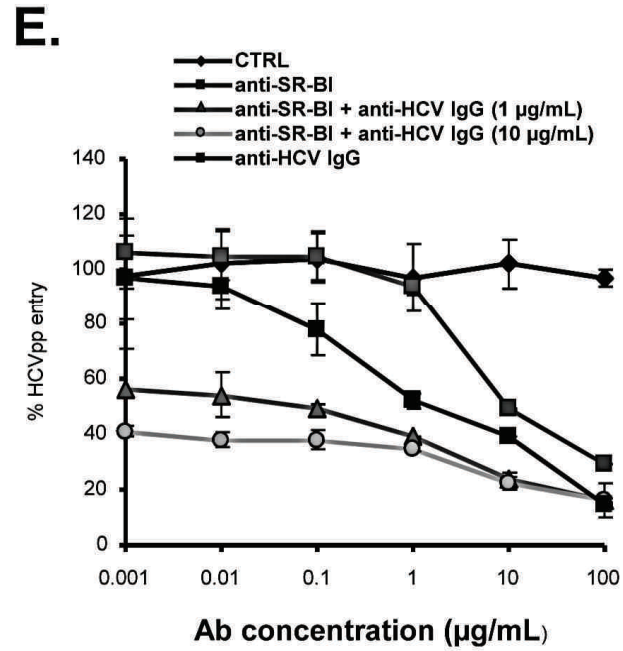
Figure S7

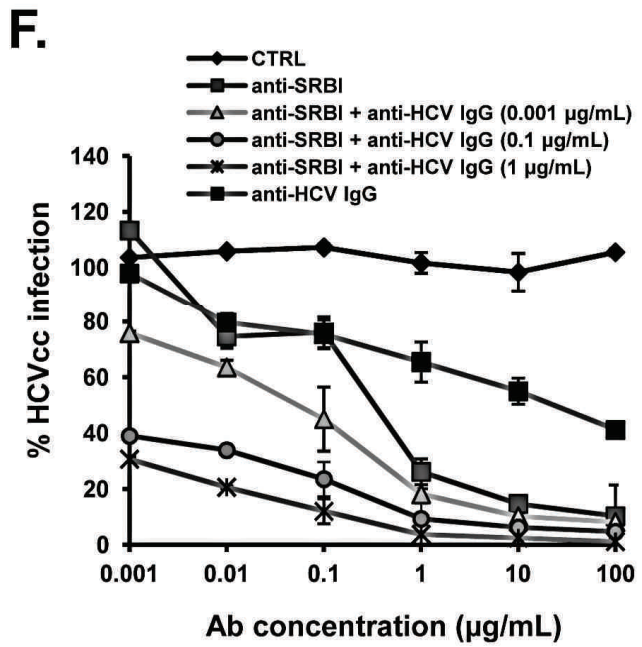

A.
CTRL
anti-SR-BI
anti-SR-BI + anti-E1 (1 µg/mL)
anti-SR-BI + anti-E1 (10 µg/mL)
anti-E1
% HCVpp entry
Ab concentration (µg/mL)


B.
CTRL
anti-SRBI
anti-SRBI + anti-E1 (0.01 µg/mL)
anti-SRBI + anti-E1 (0.1 µg/mL)
anti-SRBI + anti-E1 (1 µg/mL)
anti-E1
% HCVcc infection
Ab concentration (µg/mL)


C.
CTRL
anti-SR-BI
anti-SR-BI + anti-E2 (1 µg/mL)
anti-SR-BI + anti-E2 (10 µg/mL)
anti-E2
% HCVpp entry
Ab concentration (µg/mL)


D.
CTRL
anti-SRBI
anti-SRBI + anti-E2 (0.01 µg/mL)
anti-SRBI + anti-E2 (0.1 µg/mL)
anti-SRBI + anti-E2 (1 µg/mL)
anti-E2
% HCVcc infection
Ab concentration (µg/mL)


E.
CTRL
anti-SR-BI
anti-SR-BI + anti-HCV IgG (1 µg/mL)
anti-SR-BI + anti-HCV IgG (10 µg/mL)
anti-HCV IgG
% HCVpp entry
Ab concentration (µg/mL)


F.
CTRL
anti-SRBI
anti-SRBI + anti-HCV IgG (0.001 µg/mL)
anti-SRBI + anti-HCV IgG (0.1 µg/mL)
anti-SRBI + anti-HCV IgG (1 µg/mL)
anti-HCV IgG
% HCVcc infection
Ab concentration (µg/mL)

Publication n°2: A novel monoclonal anti-CD81 antibody produced by genetic immunization efficiently inhibits hepatitis C virus cell-cell transmission

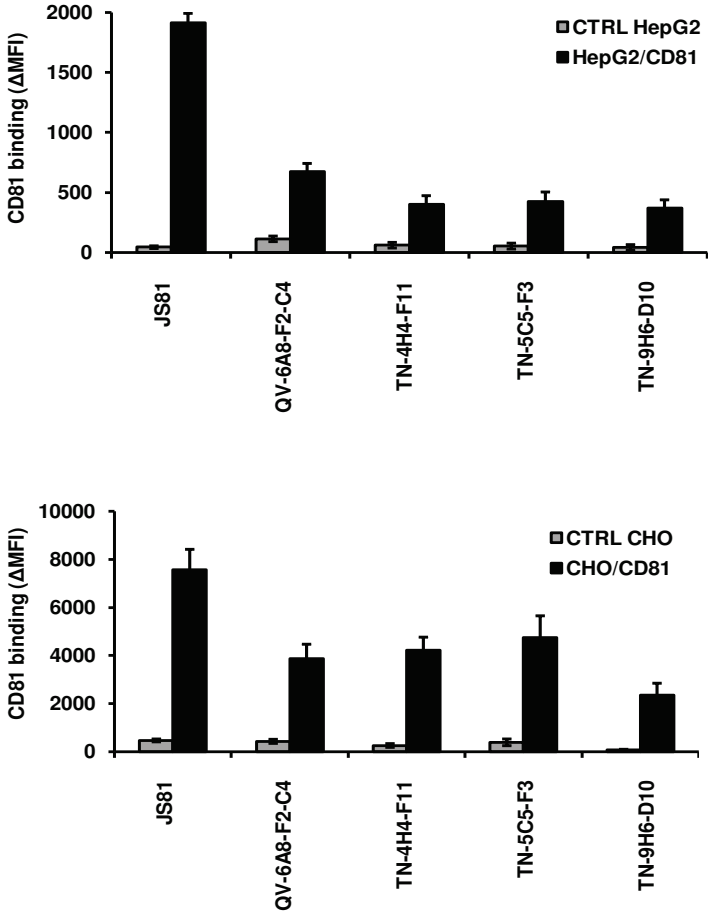
I. Fofana, F. Xiao, C. Thumann, M. Turek, L. Zona, F. Grunert, J. Thompson, M. B. Zeisel ,T. F. Baumert

PloS One 2013

CD81 a été le premier récepteur où l'on a pu démontrer une interaction directe avec sE2 (Pileri et al., 1998). L'interaction HCV/CD81 joue un rôle essentiel dans l'entrée virale et ce durant l'étape de post attachement (cf chapitre cycle viral) (Bertaux and Dragic, 2006; Koutsoudakis et al., 2006). En effet il a pu être démontré que CD81, via son association avec CLDN1, forme un complexe de corécepteur essentiel pour l'internalisation du HCV (Harris et al., 2008, 2010). Des voies indépendantes de CD81 ont été identifiées dans la transmission cellule à cellule (cf. chapitre précédent). Cependant, afin d'élucider plus en détail les mécanismes moléculaires qui sous tendent au rôle de CD81 dans l'infection et particulièrement sa propagation, le laboratoire a généré des anticorps monoclonaux anti-CD81 (QV-6A8-F2-C4, TN-9H6-D10, TN-5C5-F3, TN-4H4-F11) par immunisation génétique de rat.

Le but de cette étude a été de caractériser ces anticorps dans l'inhibition de l'entrée et la dissémination du HCV dans des cellules hépatocytaires humaines permissives.

Dans un premier temps, mon travail a consisté à caractériser la capacité de ces anticorps à reconnaître spécifiquement CD81 à la surface de cellules hépatocytaires humaines (HepG2) et non hépatocytaires ovariennes de hamster (CHO) exprimant ou non CD81. Les cellules HepG2 comme les cellules CHO n'expriment pas de manière naturelle le CD81 humain. J'ai pu démontrer que l'expression ectopique de CD81 dans ces cellules permettait la reconnaissance spécifique de CD81 par les anticorps anti-CD81 QV-6A8-F2-C4, TN-9H6-D10, TN-5C5-F3 et TN-4H4-F11 (Figure 13). L'anticorps anti-CD81 commercial murin JS-81 a été utilisé comme témoin positif de liaison.



<u>Figure 13</u> : Liaison d'anticorps monoclonaux anti-CD81 dirigés contre le CD81 à la surface des cellules Les cellules HepG2 et CHO, transfectées ou non avec un plasmide d'expression de CD81, ont été incubées avec différents anticorps anti-CD81 : CD81 QV-6A8-F2-C4, TN-9H6-D10, TN-5C5-F3 et TN-4H4-F11. La liaison de ces anticorps est mesurée par cytométrie en flux et les résultats sont exprimés en intensité de fluorescence moyenne soustraite de l'intensité de fluorescence moyenne des anticorps contrôles (ΔMFI).

Nous avons ensuite démontré que ces différents anticorps avaient la capacité d'inhiber l'infection par le HCV des génotypes 1 à 6 (publication en Annexe 1). Nous nous sommes ensuite concentrés à caractériser plus en détail l'anticorps QV-6A8-F2-C4, ayant le plus grand pouvoir d'inhibition et de liaison. J'ai pu ainsi démontrer que cet anticorps avait la capacité d'inhiber l'entrée du HCV dans les cellules Huh7.5.1 mais aussi dans les hépatocytes primaires humains (PHH). De manière intéressante, cet anticorps à le même pouvoir d'inhibition qu'un anticorps commercial bien caractérisé anti CD81 mais d'origine murine (JS81) (Figure 14).

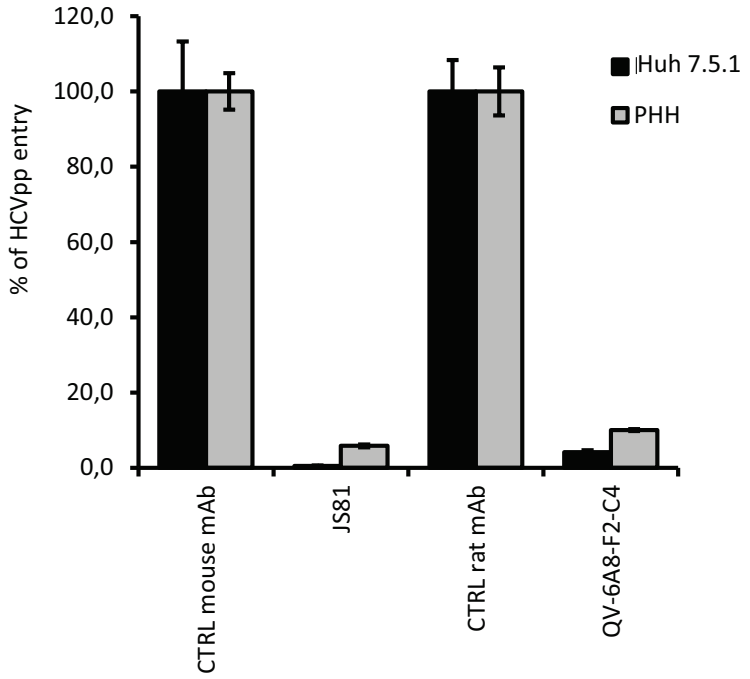


Figure 14 : Inhibition de l'entrée des HCVpp par les anticorps anti-CD81 QV-6A8-F2-C4 et JS81. Les cellules Huh7.5.1 ou les hépatocytes primaires humains (PHH) ont été préincubées avec les anticorps anti CD81 QV-6A8-F2-C4 ou JS81 ou des anticorps contrôles respectifs puis infectées avec des HCVpp de génotype 1b. L'infection des HCVpp est observée 72h post infection par détermination de l'activité luciférase intracellulaire. Les résultats sont exprimés en % d'entrée des HCVpp relativement à l'entrée des HCVpp en présence des anticorps contrôle.

Nous avons ensuite observé, grâce à des cinétiques d'infection, que le QV-6A8-F2-C4 comme le JS81 déjà décrit comme tel, ciblait l'étape d'entrée virale qui suivait la liaison du virus à la cellule hôte (publication en Annexe 1). De plus, des expériences de compétition croisée ont permis d'établir que ces deux anticorps reconnaissaient probablement des épitopes proches (publication en Annexe 1). L'étape d'entrée virale est une étape clé lors de la réinfection du foie après transplantation (Fafi-Kremer et al., 2010) et nous avions précédemment montré au laboratoire que l'interaction HCV/CD81 joue un rôle important dans ce processus (Fofana et al., 2012). Nous avons ainsi, par la suite, étudié l'effet de QV-6A8-F2-C4, en combinaison avec des anticorps anti-glycoprotéine d'enveloppe, sur l'infection d'un variant d'échappement isolé d'un patient atteint d'hépatite C chronique et ayant bénéficié d'une transplantation hépatique. Le laboratoire avait précédemment démontré que ce variant échappe à la réponse immunitaire autologue de l'hôte et ainsi, a la capacité de réinfecter le foie (Fafi-Kremer et al., 2010). L'utilisation conjointe de ces deux anticorps résulte en une diminution de l'IC_{50} de près de 100

fois avec un effet synergique sur l'inhibition démontrant l'intérêt potentiel de cibler à la fois CD81 et les protéines virales dans un cadre thérapeutique (publication en Annexe 1).

La transmission cellule à cellule est le mode de transmission viral essentiel pour la dissémination virale et le maintien de l'infection. Ce mode de transmission est résistant à la majorité des anticorps neutralisants (Brimacombe et al., 2011). Nous avons alors observé l'effet du QV-6A8-F2-C4 sur la transmission cellule à cellule en utilisant une expérience en cytométrie en flux (publication en Annexe 1) : QV-6A8-F2-C4 bloque efficacement la transmission du HCV cellule à cellule indiquant que cet anticorps a la capacité d'inhiber la dissémination virale. D'autre part, j'ai pu démontrer par des expériences d'immunofluorescence à plus long terme que cet anticorps avait la capacité de réduire fortement la dissémination virale (Figure 15). Il est important de noter que je n'ai pas observé de toxicité au cours de cette expérience sur 14 jours avec le QV-6A8-F2-C4 (Figure 15-16) contrairement au JS81, qui bien qu'ayant la capacité d'inhiber la dissémination virale, engendre une mortalité cellulaire à long terme de près de 50% (Figure 15).

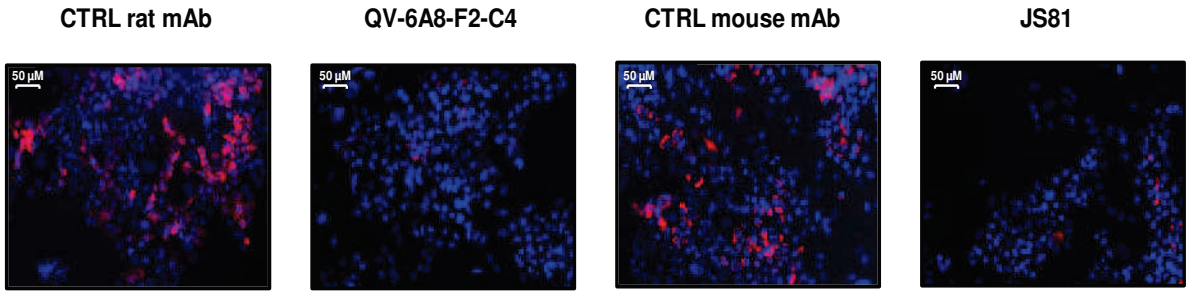


Figure 15 : Inhibition de la dissémination des HCVcc par les anticorps anti-CD81 QV-6A8-F2-C4 et JS81. Les cellules Huh7.5.1 ont été infectées avec des HCVcc Jc1. Après 2 jours d'infection, elles sont incubées en présence d'un anticorps contrôle ou d'un anticorps anti-CD81 (QV-6A8-F2-C4 ou JS81 (50µg/mL)). Puis elles sont cultivées durant 14 jours en présence des anticorps (50µg/mL). La dissémination virale est observée par immunofluorescence avec un marquage dirigé contre NS5A (QV-6A8-F2-C4) ou E2 (JS81).

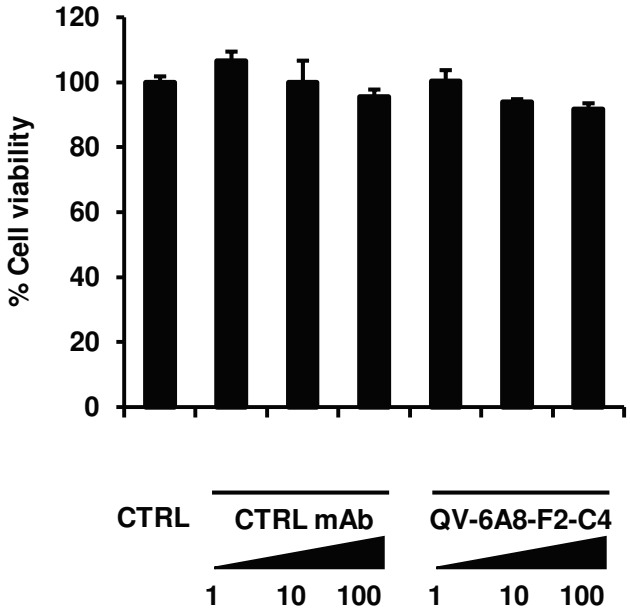


Figure 16 : Absence de toxicité de l'anticorps QV-6A8-F2-C4. Les cellules Huh7.5.1 ont été incubées en présence d'un anticorps contrôle ou de l'anticorps anti CD81 QV-6A8-F2-C4 à différentes concentrations (1 µg/mL, 10 µg/mL,100 µg/mL). Le milieu de culture contenant les anticorps a été remplacé tous les 4 jours. La viabilité cellulaire est déterminée après 14 jours par test au sel de tétrazolium MTT.

3. Discussion

L'infection et la dissémination virale sont des processus complexes. Le HCV va utiliser de nombreux facteurs de l'hôte afin de conduire son cycle viral et de faire persister son infection. L'étape d'entrée virale à été une des premières étapes du cycle à être étudiée et on a pu démontrer l'importance d'un certain nombre de facteurs de l'hôte dans cette étape comme entre autres les RTK EGFR et EphA2, SR-BI, CD-81, CLDN1 ou OCLN. La compréhension des mécanismes qui interviennent lors de l'interaction virale avec ces facteurs d'entrée est essentielle non seulement pour notre compréhension de l'infection par le HCV mais aussi d'un point de vue thérapeutique pour développer des traitements nouveaux.

Nous avons pu générer un panel d'anticorps monoclonaux reconnaissant spécifiquement CD81 ou SR-BI. Ces deux facteurs d'entrée ont largement été étudiés durant ces dernières années et leur importance dans l'infection virale par le HCV n'est plus à démontrer. En effet, l'inhibition de l'un ou l'autre de ces récepteurs résultant en une forte diminution de l'infection, faisant de

ces deux facteurs des cibles intéressantes pour le développent d'antiviraux. La confirmation dans nos études du potentiel d'inhibition de nos nouveaux anticorps par une diminution efficace de l'infection par le HCV en présence des anticorps que ce soit CD81 ou SR-BI en faisait alors des modèles d'études intéressants.

SR-BI est connu pour intervenir à plusieurs étapes du cycle viral. Différents résultats ont été observés avec différents modèles d'études. Si sE2 est un ligand pour SR-BI, il semble que la liaison de particules virales infectieuses à SR-BI ne soit pas nécessairement E2 dépendante (Maillard et al., 2006; Dao Thi et al., 2012a). En effet, il a pu être déterminé que SR-BI intervenait dans un premier temps lors de la liaison du virus à la surface de la cellule via l'interaction avec les lipoprotéines (Maillard et al., 2006; Dao Thi et al., 2012a). Dans un deuxième temps, il a été démontré que SR-BI intervenait dans l'étape de post attachement du virus et que ce mécanisme se faisait en potentielle coopération avec CD81 (Kapadia et al., 2007; Zeisel et al., 2007b). Plus récemment, une fonction supplémentaire de SR-BI à été identifiée, fonction qui interviendrait durant l'infection et augmenterait efficacité d'entrée via l'interaction entre HCV, SR-BI et les HDL (Dao Thi et al., 2012a), connus pour augmenter l'entrée et l'infection du HCV (Dreux et al., 2006; Voisset et al., 2006). Il était alors intéressant de déterminer quelle étape du cycle viral était ciblée par nos anticorps afin de déterminer leur mode d'action. Nous avons ainsi démontré, par des études d'attachement et des études des cinétiques d'infection permettant de suivre l'infection du HCV au cours du temps, que nos anticorps anti SR-BI n'intervenaient pas lors de la première étape de liaison du virus à la surface de la cellule hôte, mais au contraire intervenaient lors de l'étape suivant cette liaison appuyant l'importance de cette étape dans l'infection par le HCV. En utilisant ces mêmes techniques, nous avons pu démontrer que les anticorps anti-CD81 intervenaient durant la même période de post attachement confirmant la possibilité de la coopération de ces deux récepteurs lors de l'étape d'entrée virale.

La caractérisation des épitopes ciblés par les anticorps est souvent importante pour la détermination des fonctions liées aux différentes régions des facteurs d'entrée. Ainsi il a pu être démontré que l'acide aminé C323 possédait un rôle crucial dans la liaison de SR-BI aux HDL (Guo et al., 2011) De plus, la liaison sE2-SE-BI est dépendante d'une région particulière de SR-BI comprenant les acides aminés 70 à 87 et E210 (Catanese et al., 2010). Du fait que nos anticorps ne soient pas capables d'inhiber la liaison à sE2 et aux HDL, ces résultats suggèrent que l'épitope ciblé par nos anticorps ne comprend pas les acides aminés C323, E210 et 70 à 87. Sans caractériser exactement les épitopes reconnus par nos anticorps, nous avons cependant pu

déterminer que le site de liaison de nos anticorps était dans la partie N terminale de l'ectodomaine de SR-BI, région contenant sûrement les séquences responsables de l'homéostase du cholestérol (Vergeer et al., 2011). De plus, des études de compétition croisée nous ont permis de déterminer que les anticorps de rat anti-SR-BI (QQ-4A3-A1 ; QQ-2A10-A5 ; QQ-4G9-A6) reconnaissaient un épitope similaire ou proche alors que l'anticorps de souris anti-SR-BI ciblait un épitope distinct. De la même manière, nous avons aussi pu démontrer que l'anticorps de rat anti-CD81 QV-6A8-F2-C4 caractérisé dans notre étude reconnaissait le même épitope, ou un épitope très proche, que l'anticorps commercial JS81 au fort pouvoir inhibant. Ces résultats corroborent une partie de notre étude indiquant que ces deux anticorps anti-CD81 inhibent tous deux l'entrée du HCV à des étapes similaires. Il est intéressant de noter qu'un autre anticorps anti-CD81 (1D6) inhibe l'entrée du HCV à un temps différent (Bertaux and Dragic, 2006), suggérant que différents domaines de CD81 sont associés à différentes fonctions de CD81 durant l'infection par le HCV L'ensemble de ces données suggère que différentes régions d'un facteur d'entrée peuvent correspondre à une fonction propre.

L'initiation de l'infection virale se fait par la transmission libre du virus via le flux sanguin, l'attachement du virus à la surface de la cellule hôte puis son internalisation. Mais, pour le maintien de l'infection et pour la dissémination du virus, la voie principale de transmission semble être la transmission directe du virus de cellule à cellule. Cette voie de transmission n'est pas encore complètement caractérisée. Il était alors essentiel de déterminer l'action de nos anticorps sur cette voie de transmission. En effet, une étude d'un autre laboratoire avait démontré que SR-BI intervenait dans cette voie de transmission (Brimacombe et al., 2011) et que des anticorps ou des inhibiteurs, ciblant soit la liaison du HCV à SR-BI soit la fonction de transfert lipidique de SR-BI, engendraient une diminution de la transmission cellule à cellule (Meuleman et al., 2012; Syder et al., 2011). Nous avons pu déterminer dans un premier temps que nos anticorps anti-SR-BI avaient la capacité d'inhiber la transmission cellule à cellule du HCV. Et cela nous a permis de définir, grâce à nos anticorps ayant la particularité d'inhiber uniquement l'étape de post attachement du virus, que seule la fonction de post attachement E2 indépendante était indispensable à cette transmission cellule à cellule. Ceci est corroboré par le fait que le SR-BI murin, connu pour ne pas lier sE2, est capable de promouvoir la transmission cellule à cellule de la même manière que le SR-BI humain. Nous avons également pu démontrer que l'anticorps anti-CD81 que nous avions généré (QV-6A8-F2-C4) avait lui aussi la capacité d'inhiber la transmission cellule à cellule. Par conséquent il était logique d'examiner si ces anticorps anti-SR-BI anti-CD81 avaient une influence sur la dissémination virale, mécanisme

grandement dépendant de la transmission virale cellule à cellule. En effet, nous avons pu observer que la dissémination virale était fortement diminuée sur le long terme en cultivant les cellules infectées en présence de ces anticorps. Il est intéressant de noter que bien qu'une voie de transmission cellule à cellule indépendante de CD81 a été décrite (Witteveldt et al., 2009), il semble que CD81 reste un facteur important de cette transmission virale, suggérant que la dissémination virale se fait quand même principalement de manière CD81 dépendante.

En conclusion, nous avons pu identifier de nouveaux anticorps anti-CD81 et anti-SR-BI qui, après caractérisation fonctionnelle, diffèrent des anticorps préexistants autant dans leur mode d'action pour les anti-SR-BI que dans leur toxicité pour l'anti-CD81 QV-6A8-F2-C4. Ces anticorps ont la capacité à la fois d'inhiber l'infection, mais aussi de restreindre la dissémination virale du HCV. Ceci fait de ces anticorps des candidats intéressants pour le développement de nouvelles thérapies antivirales. De plus, la combinaison des anticorps anti-SR-BI ou anti-CD81 avec des anticorps anti glycoprotéines du HCV montre un effet synergique sur l'inhibition du HCV et une diminution de l'IC_{50} importante. Il serait alors intéressant de tester ces anticorps *in vivo* seuls ou dans le cadre d'une thérapie combinée.

PARTIE II : Etude des facteurs restreignant l'infection par le HCV aux hépatocytes humains

1. Introduction

Dans la deuxième partie de ma thèse, je me suis intéressée au déroulement du cycle viral dans sa globalité et aux facteurs pouvant être responsables de la spécificité tissulaire du HCV. L'objectif était d'établir une lignée cellulaire non hépatique pouvant supporter le cycle viral dans son intégralité. Les résultats sont présentés ci-dessous sous forme d'une publication originale (Da Costa, Turek, Felmlee et al., Journal of Virology 2012).

2. Résultats

Publication n°3: Reconstitution of the entire hepatitis C virus life cycle in nonhepatic cells

D. Da Costa *, M. Turek *, D. J. Felmlee *, E. Girardi, S. Pfeffer, G. Long, R. Bartenschlager, M. B. Zeisel$, T. F. Baumert$

* Ces auteurs ont contribué de manière équivalente
$ Ces auteurs ont contribué de manière équivalente

Journal of Virology 2012

La seconde partie de mon travail de thèse a visé à élucider les facteurs précis qui limitent l'infection du HCV aux hépatocytes humains. En effet, le tropisme du HCV est limité à l'homme et aux chimpanzés, et la présence d'une infection productive est spécifique des hépatocytes. Les lignées cellulaires hépatocytaires humaines Huh7 et ses dérivées sont les seules lignées cellulaires supportant le cycle viral du HCV *in vitro*. Si des progrès ont été accomplis au cours des dernières années afin de décrypter le cycle viral du HCV dans ces cellules, les facteurs limitant l'infection par le HCV aux hépatocytes humains n'ont pas encore été définis.

En utilisant les connaissances acquises ces dernières années sur les facteurs de l'hôte nécessaires pour l'interaction HCV/hépatocytes et des lignées cellulaires non hépatiques dérivées du rein humain qui sont non permissives au HCV (lignée cellulaire 293T), nous avons pu reconstituer entièrement le cycle viral du HCV dans les cellules 293T. En effet, l'expression des quatre principaux facteurs entrée du HCV, CD81, SR-BI, CLDN1 et OCLN, dans les cellules 293T rend ces cellules hautement permissives à l'entrée du HCV. De plus l'expression au sein des 293T de miR-122, un facteur clé de la réplication du HCV, m'a permis de démontrer que je pouvais obtenir une réplication robuste du HCV dans les cellules 293-4R/miR-122, qui n'est toutefois pas tout à fait équivalente à celle observée dans les Huh7.5.1 supportant la réplication de manière naturelle (Figure 2B et D-E de la publication n°3). En comparaison, j'ai pu démontrer qu'aucune réplication n'était observée dans les cellules n'exprimant pas de miR-122, démontrant l'importance de ce facteur dans la réplication (Figure 2 A-B, D-E). De manière intéressante, j'ai pu observer que le profil d'inhibition par différents anticorps ou inhibiteurs de l'infection par le HCV était le même dans les cellules 293T-4R/miR-122 que dans les cellules Huh7.5.1 (Figure 2F de la publication n°3). Enfin, j'ai pu observer que l'expression du facteur d'assemblage apoE ou de ses isoformes permettait l'achèvement du cycle viral démontré par la présence de l'assemblage et la libération de particules virales infectieuses à partir de ces cellules 293T-4R/miR-122/apoE (Figure 3B, D). J'ai pu alors montrer que la cinétique de production de ces particules virales dérivées des 293T-4R/miR-122/apoE était identique à celle des Huh7.5.1, bien que bien plus faible en termes de quantité (Figure 4A-B), et que les particules virales issues de ces cellules 293T-4R/miR-122/apoE étaient elles aussi caractérisées par le même schéma d'inhibition que les particules virales issues des cellules Huh7.5.1 (Figure 4C). Cette étude montre pour la première fois une véritable infection par le HCV de cellules non hépatiques et suggère que l'apport d'une série de facteurs définis confère aux cellules 293T la capacité de reconstituer entièrement le cycle viral du HCV.

Ces résultats nous ont permis de faire progresser nos connaissances sur les facteurs tissulaires spécifiques de l'infection par le HCV. Ils nous ont également apporté un outil précieux pour étudier l'assemblage et la libération du HCV dans un environnement non-hépatique.

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Reconstitution of the Entire Hepatitis C Virus Life Cycle in Nonhepatic Cells

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Hepatitis C virus (HCV) is a human hepatotropic virus, but the relevant host factors restricting HCV infection to hepatocytes are only partially understood. We demonstrate that exogenous expression of defined host factors reconstituted the entire HCV life cycle in human nonhepatic 293T cells. This study shows robust HCV entry, RNA replication, and production of infectious virus in human nonhepatic cells and highlights key host factors required for liver tropism of HCV.

Virus-host interactions that determine and restrict specific tissue and host tropisms have a complex evolutionary history and also have significant consequences for the pathogenesis of viral infection and human disease. Viral hepatitis is a major disease burden. Indeed, infection of hepatocytes by a variety of hepatotropic viruses from different orders and families can lead to tissue inflammation, fibrosis, and hepatocellular carcinoma. Hepatitis C virus (HCV), a member of the family *Flaviviridae*, is a prime example of a virus that causes chronic hepatitis worldwide. While HCV primarily infects hepatocytes of humans and chimpanzees, the virus has been shown to enter neuronal and endothelial cells of the blood-brain barrier. However, infection of these cells occurs at a low level, and production of infectious viruses is greatly diminished relative to that in liver-derived cells (9, 10). Unlike HCV, other members of the family *Flaviviridae* have a much broader tissue and species tropism. For example, dengue virus infects and replicates both in the midgut epithelia of *Aedes aegypti* mosquitoes and in human monocytes and hepatocytes (20, 25, 39). Moreover, a virus closely related to HCV was recently identified from respiratory samples from dogs (18). A large panel of host factors required for HCV has been identified so far (36). However, the key host factors mediating liver tropism of the virus and allowing reconstitution of the viral life cycle in human cells are still only partially understood.

Taking advantage of our current knowledge of host factors involved in HCV infection, we sought to engineer a human kidney cell line (293T) that would be capable of sustaining the entire HCV life cycle. The aim was to define host factors that are necessary and sufficient for the HCV life cycle, in order to understand the liver tissue specificity of HCV.

293T cells were obtained from ATCC and their identity was verified by genomic profile comparison to the LGC Standards database by short tandem repeat profiling as described previously (1) (Fig. 1A). In order to render them infectible by HCV, we used lentiviral vectors to express the four principal HCV host entry factors—claudin-1 (CLDN1), CD81, occludin (OCLN), and scavenger receptor class B type I (SR-BI) (2, 7, 34, 35)—by using previously described expression constructs and methods (3, 24). Four stable 293T cell lines were selected to express either CLDN1 alone, CD81/OCLN with or without CLDN1, or CLDN1/CD81/OCLN together with SR-BI (293T-4R). After verifying stable expression of these proteins using receptor-specific antibodies (Fig. 1B), we infected these cells with HCV pseudoparticles expressing the envelope glycoproteins of HCV genotype 1b (HCVpp; HCV-J strain described in reference 31). While CLDN1 expression alone conferred limited permissiveness for HCV infection, as previously described (7), expression of all four factors enhanced HCV entry to a level that was around 4-fold higher than that in Huh7.5.1 cells, which is the liver-derived model hepatoma cell line for studying HCV infection (Fig. 1C).

Genuine cell culture infection of HCV (HCVcc) was then investigated in 293T-4R cells using a chimeric virus composed of two genotype 2a isolates (designated Jc1 [19, 32]) and engineered for *Renilla luciferase* expression (JcR2a [38]). However, as shown in Fig. 2A, overcoming the HCV entry block was not sufficient for robust viral RNA replication in 293T cells.

Several studies have shown that micro-RNA 122 (miR122) is a liver-specific host factor critical for HCV replication (5, 16, 17, 28). Since Northern blot analyses demonstrated undetectable miR122 expression in 293T-4R cells (Fig. 2C), we investigated whether exogenous miR122 expression reconstituted viral RNA replication. Indeed, stable expression of this factor, by using miR122-encoding lentiviruses in the 293T-4R line, rendered the cells permissive for bona fide HCVcc infection, with replication to levels comparable to those seen with Huh7.5.1 cells, as assessed by luciferase reporter activity (Fig. 2B). Further confirmation of genuine infection was obtained by observing similar infectivity (determined as 50% tissue culture infective doses [$TCID_{50}$]) with HCVcc (Jc1) without a reporter gene, by detecting expression of viral protein NS5A (Fig. 2B). We verified expression of miR122 in transduced 293T-4R/miR122 cells, and the level was comparable

Received 30 April 2012 Accepted 3 August 2012
Published ahead of print 15 August 2012
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doi:10.1128/JVI.01066-12

A.

Loci Tested	AMELO	THO1	D5	D13	D7
ATCC Reference: CRL-1573 (HEK293)	*X, X*	*7, 9.3*	*8, 9*	*12, 14*	*11, 12*
293T cells	X, X	9.3, 9.3	8, 9	12, 13, 14	11, 12

Loci Tested	D16	CSF	VWA	TPOX
ATCC Reference: CRL-1573 (HEK293)	*9, 13*	*11, 12*	*16, 19*	*11, 11*
293T cells	9, 13	11, 12	16, 19	11, 11

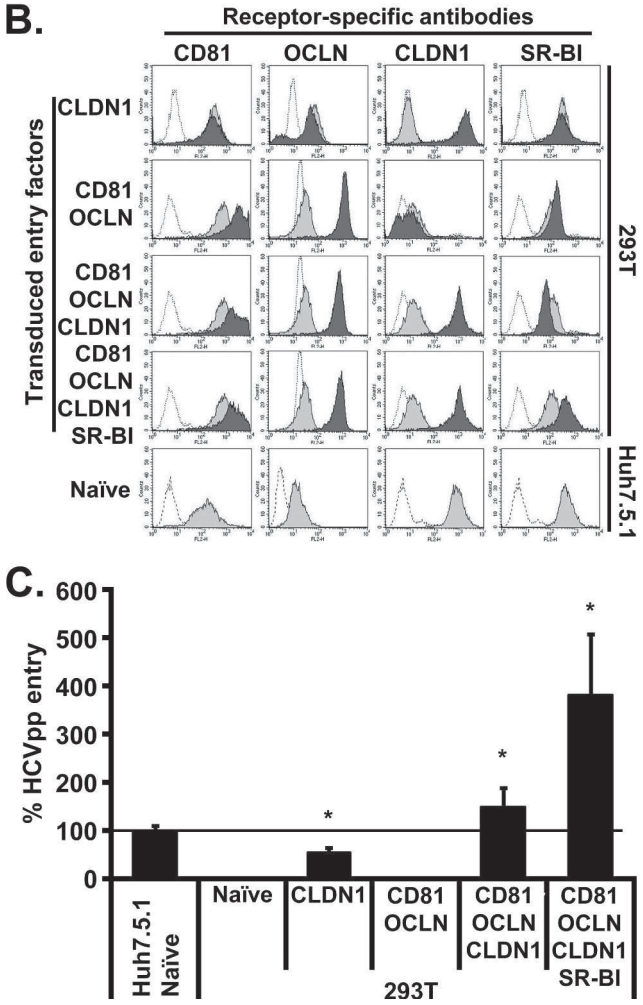

FIG 1 Expression of four HCV entry factors renders 293T cells highly permissive to HCVpp entry. (A) Short tandem repeat (STR) profile of the 293T cells used in this study (cell line authentication, LGC Standards) was performed as described previously (1). The names of tested loci are in bold, and peak positions from STR profile of 293T cells were compared to the LGC Standards database. (B) 293T cells (cultured in Dulbecco's modified Eagle medium with high glucose; Life Technologies) were transduced with lentiviruses (as described in reference 3) to express given HCV entry factors. After transduction, cells were selected with 12 μg/ml of blasticidin for 2 weeks. Blasticidin-resistant cells were assessed by flow cytometry using monoclonal antibodies (CLDN1 [11], OCLN [catalog no. 33–1500; Invitrogen], and SR-BI [Zahid et al., submitted for publication]) recognizing indicated entry factors. Entry factor-transduced cells (dark gray histograms) were compared to naïve 293T cells (light gray histograms) and isotype control antibody (catalog no. 10400C; Life Technologies) (white histograms with dashed lines). The *x* axis shows fluorescence intensity; the *y* axis shows the number of events. (C) Transduced 293T cells were assessed for HCVpp (genotype 1b; HCV-J strain; produced as described in reference 31) entry by determining luciferase activity 72 h postinfection as previously described (35). Results were first normalized to vesicular stomatitis virus pseudoparticle entry (VSV-Gpp; produced as described in reference 8) and then compared to those obtained with Huh7.5.1 cells (cultured as described in reference 41). Results are means and standard deviations (SD) from three independent experiments performed in triplicate. Entry is relative to entry into Huh7.5.1 cells, and 100% relative infectivity is represented by a solid line. Statistical analysis for entry factor expressing cells relative to naïve 293T cells was performed using the Student *t* test (*, $P < 0.05$).

to that in Huh7.5.1 cells, as assessed by Northern blotting (Fig. 2C), and the cell proliferation rates of the different cell lines were similar (data not shown). Kinetics of HCV replication in 293T-4R/miR122 cells matched those of Huh7.5.1 cells, suggesting that aside from miR122, cell factors present in human liver- and kidney-derived cells are equally efficient for replication, as assayed by luciferase reporter gene expression (Fig. 2D). Expression of viral proteins in infected cells was further confirmed using HCV core-specific immunofluorescence (Fig. 2E) and flow cytometry (data not shown).

To further confirm whether viral entry and replication in stably transduced 293T cells are mediated by the same host and virus factors as in human Huh7.5.1 hepatoma cells, we used well-characterized entry and replication inhibitors. Antibodies directed against the HCV entry factors CD81 (JS-81; BD Biosciences), CLDN1 (11), and SR-BI (M. N. Zahid, M. Turek, F. Xiao, V. L. D. Thi, M. Guérin, I. Fofana, P. Bachellier, J. Thompson, L. Delang, J. Neyts, D. Bankwitz, T. Pietschmann, M. Dreux, F.-L. Cosset, F. Grunert, T. F. Baumert, and M. B. Zeisel, submitted for publication) were effective in inhibiting infection (Fig. 2F). Moreover, both a polyclonal serum recognizing Apolipoprotein E (ApoE) (29) and a monoclonal antibody recognizing the low-density-lipoprotein (LDL) receptor binding domain of ApoE (37) effectively neutralized HCV infection of 293T-4R/miR122 cells (Fig. 2F). The same was true for the recently identified HCV entry inhibitor erlotinib, which targets the kinase activity of the host entry regulatory protein, epidermal growth factor receptor (EGFR) (Fig. 2F) (24). Likewise, the well-characterized inhibitors of HCV NS3 protease and polymerase telaprevir (VX950) and mericitabine (R7128) impaired HCV replication in 293T-4R/miR122 cells (Fig. 2F). These data demonstrate that HCVcc RNA replication in kidney-derived 293T-4R/miR122 cells is efficient and dependent on mechanisms similar to those in liver-derived Huh7.5.1 cells.

Despite efficient entry and RNA replication of 293T-4R/miR122 cells infected with recombinant HCVcc, these cells did not release infectious virions, suggesting that kidney-derived cells lack factors required for viral assembly and release. Therefore, we aimed to reconstitute virus production by expression of HCV assembly factors. HCV production shares factors involved in very-low-density lipoprotein (VLDL) assembly, a process that occurs exclusively in hepatocytes (13, 14, 27). While the necessity of apolipoprotein B (ApoB) in HCV production is controversial (15), ApoE is known to be critical and is incorporated into the virion (26). We therefore expressed the most common isoform of ApoE (ApoE3) in 293T-4R/miR122 cells by using a lentiviral vector encoding human ApoE3 as described previously (23) and confirmed its expression by flow cytometry using an ApoE-specific antibody (Fig. 3A). We then infected 293T-4R/miR122/ApoE cells. Subsequently, the production and release of viral particles was assessed by incubating naïve Huh7.5.1 cells with the supernatants from these cells. Indeed, 293T-4R/miR122/ApoE released infectious

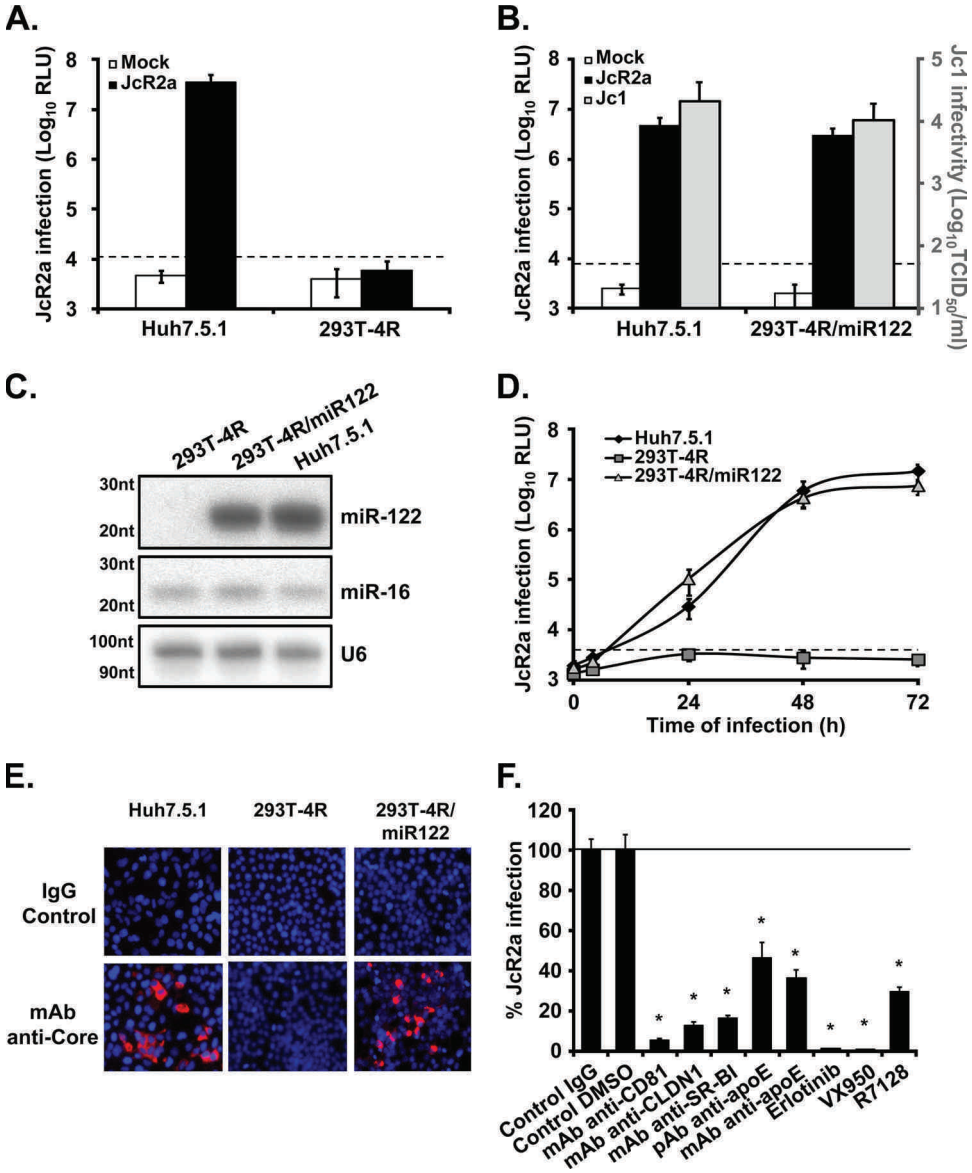

FIG 2 293T-4R cells support robust HCV infection upon miR122 expression. (A) Stable 293T-4R cells described in the legend to Fig. 1 were challenged with HCVcc (JcR2a; produced as described in reference 38) or were mock infected, and luciferase activity was assessed 72 h postinfection as described previously (38). Results are means and SD, in relative light units (RLU), from three independent experiments performed in triplicate. (B) 293T-4R cells were stably transduced using miR122-encoding lentiviruses (catalog no. mh15049; ABM) and 2.5 μg/ml of puromycin-resistant cells were selected over 2 weeks. 293T-4R/miR122 cells and Huh7.5.1 cells were then infected with HCVcc or mock infected for 6 h. Infection was assayed by monitoring luciferase activity 72 h postinfection. Results are means and SD from three independent experiments performed in triplicate. Jc1, an HCVcc without a luciferase reporter (32), was likewise used to infect Huh7.5.1 and 293T-4R/miR122 cells, and its infectivity was assessed by limiting-dilution assay ($TCID_{50}$) by detecting viral protein NS5A using immunohistochemistry, represented as gray bars (22). Results are expressed as means and SD from three independent experiments. (C) Northern blots of miR122 and miR-16, and U6 RNA as a loading control, extracted from 293T-4R cells, 293T-4R cells stably expressing miR122, and Huh7.5.1 cells as positive control. Northern blotting using a miR122-specific probe were performed as described previously (30). Oligonucleotide lengths (in nucleotides [nt]) are indicated on the left. (D) 293T-4R, 293T-4R/miR122, and Huh7.5.1 cells were incubated side by side with HCVcc (JcR2a), and luciferase activity was monitored every 24 h over a 72-h period. Results are means and SD from three independent experiments performed in triplicate. (E) Huh7.5.1, 293T-4R, and 293T-4R/miR122 cells were infected for 72 h, and HCV core protein (core antibody C7-50; Thermo Scientific), or nonspecific IgG as a control (catalog no. 10400C; Life Technologies), was observed by immunofluorescence; nuclei were stained using DAPI (4′,6′-diamidino-2-phenylindole). (F) 293T-4R/miR122 cells were preincubated for 1 h at 37°C with the indicated entry inhibitors, antivirals, or controls (20 μg/ml of monoclonal antibodies [MAb], anti-CD81 [JS81; BD Biosciences], anti-CLDN1 [11], and anti-SR-BI [Zahid et al., submitted]; 1:200 dilution of polyclonal antibody [PAb] anti-ApoE [catalog no. 178479; Calbiochem]; 20 μg/ml anti-ApoE MAb [37]; 10 μM erlotinib [catalog no. E-4997; LC Laboratories]; 1 μM protease inhibitor telaprevir VX950; 1 μM polymerase inhibitor mericitabine R7128 [both synthesized by Acme Bioscience Inc.]; and 0.7% dimethyl sulfoxide [DMSO]) and then infected with HCVcc (JcR2a) in the presence of the given entry inhibitors or antivirals. Cell lysates were assessed for luciferase activity 72 h postinfection. Results are means and standard errors of the means from three independent experiments performed in triplicate. Values are relative to controls, and 100% relative infectivity is represented by a solid line. In panels A, B, and D, detection limits are represented by dashed lines. Statistical analysis relative to control was performed using the Student *t* test (*, $P < 0.05$).

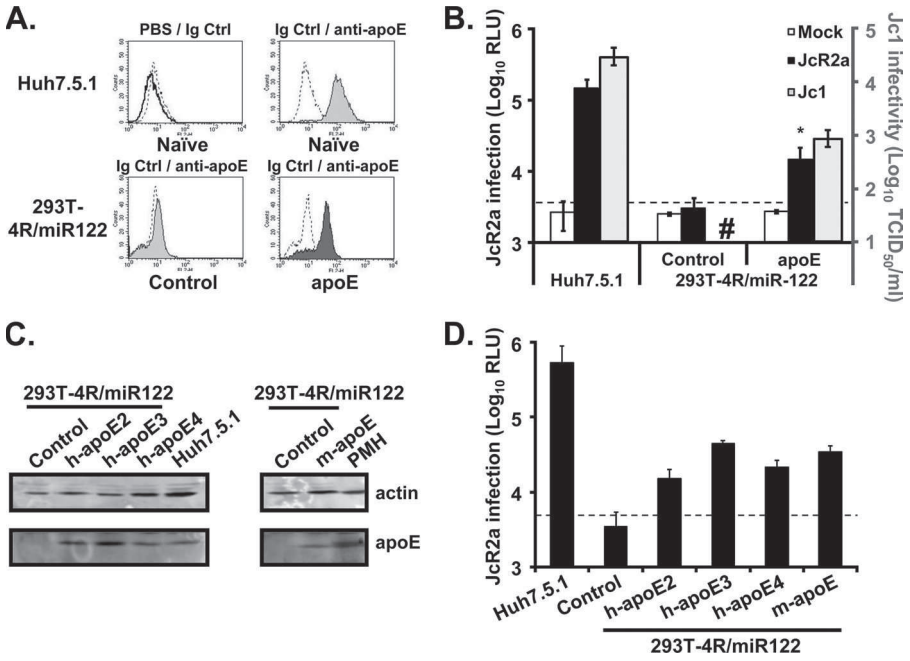

FIG 3 Infectious HCV particles are released from 293T-4R/miR122 cells upon ApoE expression. (A) 293T-4R/miR122 cells were transduced with an ApoE3-encoding lentiviral vector described in reference 23. At 72 h posttransduction, both transduced and untransduced cells were stained for flow cytometry analysis. ApoE expression was analyzed using a specific ApoE antibody (clone D6E10, catalog no. ab1906; Abcam) (untransduced cells are represented as light gray histograms, and transduced cells are shown by dark gray histograms), and an isotype antibody (catalog no. 10400C; Life Technologies) was used as a control (white histograms with dashed lines). Huh7.5.1 cells were used for control of ApoE expression, and PBS is the control for the isotype antibody (histogram with the thick black outline). (B) Transduced 293T-4R/miR122/ApoE cells were infected with HCVcc (JcR2a or Jc1). At 6 h postinfection, cells were washed three times with PBS, and fresh culture medium was added. At 72 h postinfection, medium from infected cells was passaged onto naïve Huh7.5.1 cells. Lysates of JcR2a-infected cells were assessed for luciferase activity 72 h postinfection. Results are means and SD from three independent experiments performed in triplicate. The detection limit is represented by a dashed line. The infectivities of Jc1 derived from infected Huh7.5.1 or 293T-4R/miR122/ApoE cells were assessed by limiting-dilution assay ($TCID_{50}$) by detecting NS5A by immunohistochemistry, represented as gray bars. Results are means and SD from three independent experiments. #, levels were below the limit of detection. Statistical analysis relative to the control was performed using the Student *t* test (*, $P < 0.05$). (C) 293T-4R/miR122 cells were transduced with the indicated ApoE isoform-encoding lentiviral vectors (24) or mock transduced (control). At 72 h posttransduction, cells were either lysed or seeded for HCVcc infection. Cell lysates were assessed for ApoE expression by Western blotting either by using ApoE antibody (clone D6E10, catalog no. ab1906; Abcam) for human ApoE (h-ApoE) expression or by using a mouse ApoE-specific antibody for mouse ApoE (m-ApoE) expression (catalog no. ab20874; Abcam). Huh7.5.1 and primary mouse hepatocytes (PMH) were used as controls for human and mouse ApoE expression, respectively. (D) The different ApoE isoform-expressing 293T-derived cells were assessed for their capacity to produce infectious virus by infecting them with HCVcc (JcR2a), and 72 h postinfection, supernatants of infected 293T-derived cells were passaged onto naïve Huh7.5.1 cells. At 72 h after infection was initiated, Huh7.5.1 cells were lysed and luciferase activity assessed. Results are means and SD from a representative experiment performed in triplicate. The dashed line represents the detection limit.

HCV particles as shown by a marked and highly significant increase in infectivity (as assessed by luciferase activity of JcR2a virus and $TCID_{50}$ of Jc1 virus without a reporter gene) of the supernatant compared to the supernatant of 293T-4R/miR122 cells without ApoE expression (Fig. 3B). Although the production of infectious particles was lower than in Huh7.5.1 cells studied in side-by-side experiments, these data indicate that ApoE is a key factor for virus production in reconstituting the viral life cycle in nonhepatic cells. This diminished HCV production was not due to diminished replication levels, as ApoE-transduced cells had HCV replication levels similar to those of 293T-4R/miR122 cells prior to ApoE expression (data not shown). To test if HCV produced by these cells is reliant only on the human ApoE3 isoform or could use other forms of ApoE, we similarly transduced human ApoE2 and ApoE4 isoforms, as well as murine ApoE (Fig. 3C). Viruses produced from 293T cells expressing these ApoE isoforms and the mouse ortholog had similar infectivity compared to human ApoE3 isoform (Fig. 3D).

Focusing on the most common ApoE isoform (ApoE3), we further characterized the kinetics and attributes of these viruses. First, we confirmed that HCV particles from engineered 293T cells could establish infection by monitoring the increase in HCV genomes over time in Huh7.5.1 target cells after exposure to the supernatant of HCVcc-infected 293T-4R/miR122/ApoE cells (Fig. 4A). Next, we characterized the kinetics of HCV RNA production from infected 293T-4R/miR122/ApoE cells by measuring HCV RNA in the media at serial time points following infection (Fig. 4B). Interestingly, the levels of HCV RNA released into the culture media of 293T-4R/miR122/ApoE cells was similar to levels of HCV RNA in the media of Huh7.5.1 cells after 72 h, whereas cells that were not transduced with ApoE released minimal amounts of HCV RNA, likely due to previously reported nonspecific release of HCV RNA during replication (Fig. 4B) (33). These data suggest that the specific infectivity differs between virus produced from Huh7.5.1 cells and 293T cells engineered to express essential host factors. An estimation of the specific infectivity of the released viruses ($TCID_{50}$/HCV RNA genomes) revealed approximately a 30-fold difference between the differently derived

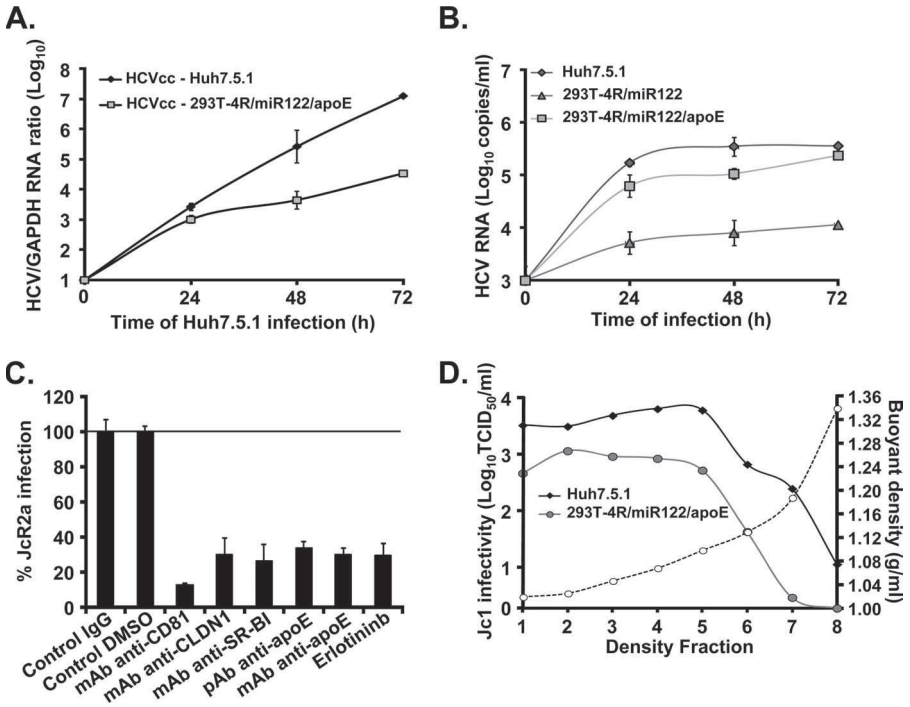

FIG 4 Characterization of HCVcc derived from 293T-4R/miR122/ApoE cells. (A) Culture media from Jc1-infected 293T-4R/miR122, 293T-4R/miR122/ApoE, and Huh7.5.1 cells were passaged onto naïve Huh7.5.1 target cells. Total RNA from these Huh7.5.1 target cells was extracted at the indicated time points, and HCV RNA was quantitated by reverse transcription-quantitative PCR (RT-qPCR) as described previously (11). Values were normalized to the value for the internal control gene (GAPDH gene). Results are means and SD from an experiment performed in quadruplicate. (B) HCV RNA production was measured by infecting 293T-4R/miR122, 293T-4R/miR122/ApoE, and Huh7.5.1 cells side by side with HCVcc (Jc1). RNA from supernatants of infected cells was extracted at the indicated time points, and HCV RNA was quantitated by RT-qPCR. Results are means and SD from an experiment performed in triplicate. (C) Culture media of infected 293T-4R/miR122/ApoE cells were harvested 72 h postinfection and passaged onto naïve Huh7.5.1 cells that had been preincubated with either control IgG, DMSO, or the indicated entry inhibitors. Results are mean percentages of HCV infection (as assessed by luciferase activity) relative to the control and SD from a representative of two independent experiments performed in triplicate, and 100% relative infectivity is represented by a solid line. The virus used was JcR2a with a $TCID_{50}$ of 10^5 to 10^6/ml. (D) Density distributions of infectious 293T-4R/miR122/ApoE- and Huh7.5.1-derived HCVcc (Jc1) were determined by overlaying 0.5 ml of culture medium on a 5-ml, 4-to-40% iodixanol step gradient and ultracentrifuging samples for 16 h at 40,000 rpm on an SW-55 rotor (Beckman Coulter). Fractions were carefully harvested from the top of each tube, and density was determined by weighing 0.5 ml of each fraction. Each fraction was assayed for infectivity by $TCID_{50}$ by detecting NS5A as described previously (22).

viruses (1/900 for Huh7.5.1-derived virus and 1/26,000 for 293T-4R/miR122/ApoE-derived virus). It should be noted that HCV particles produced from 293T-4R/miR122/ApoE cells proved to have a route of infection similar to that of liver-derived HCVcc, in that entry into Huh7.5.1 cells was neutralized by well-characterized HCV entry inhibitors, including CD81-, SR-BI-, CLDN1-, and ApoE-specific antibodies and erlotinib (Fig. 4C). Fractionating the virus by iodixanol density gradients revealed that the infectious virions produced from 293T-4R/miR122/ApoE cells have a buoyant density similar to those from Huh7.5.1 cells (Fig. 4D).

The data presented here demonstrate that *trans*-expression of OCLN, CD81, CLDN1, SR-BI, miR122, and ApoE endows 293T human kidney-derived cells with the capacity to support the complete HCV life cycle. Expression of four principal entry factors and miR122 generated cells with higher entry levels than and similar replication kinetics to those of the extensively optimized Huh7.5.1 cells (4, 41). It should be noted in this context that the recently identified entry factor EGFR is also expressed in 293T cells (data not shown) (24, 40). We confirmed that expression of CLDN1 alone appears to be sufficient for infection of 293T cells (7) and expand these findings by showing that high-level expression of the four canonical HCV entry factors makes previously impenetrable cells fourfold more permissive than Huh7.5.1 cells. These observations were confirmed by HCVcc infection of 293T cells engineered to express miR122 in addition to variable sets of entry factors (data not shown). While the present study focused on engineering a human cell line for infection, it has been demonstrated that concomitant high-level expression of the four human entry factors is required for robust HCV entry into mouse hepatocytes *in vivo* (6). Since none of the identified entry factors are exclusively expressed in the liver, it is likely that the combined expression of these host factors at substantial levels allows the virus to productively infect the human liver, rather than a single liver-specific entry factor restricting HCV infection.

Investigators have shown that miR122 expression increases HCV replication in mouse embryonic fibroblasts and other hepatoma cell lines such as HepG2 cells (17, 21, 28). Furthermore, HEK-293 cells modified to express miR122 are capable of sustaining selectable HCV subgenomic replicons, although expression of mutated miR122, at sites required for HCV RNA binding, can also sustain these replicons (5). We demonstrate here *de novo* replication following an infection event of a nonhepatic cell line engineered to express HCV host factors. Our data also demonstrate that there is no restrictive factor of HCV entry and viral RNA replication that is present in 293T cells. HCV entry and replication in human blood brain barrier endothelial and neuronal cells have

been described (9, 10). In contrast to the kidney-derived cells described here, HCV replication in blood-brain barrier endothelial cells occurred via a miR122-independent mechanism but at a diminished level (9). Thus, the cell lines developed in this study may be useful as a tool to further understand the molecular mechanisms of extrahepatic infection.

The production of HCV in 293T-4R/miR122/ApoE cells was diminished relative to that in Huh7.5.1 cells but markedly and significantly higher than that in cells without ApoE expression. This demonstrates that apart from ApoE, all the other factors necessary for the production of infectious particles are present in 293T cells, yet additional host factors may increase efficient production levels. The cell line generated in this study is likely to allow further discovery of the minimal set of host factors required for robust viral production. Additional relevant factors enhancing viral production may be ApoB (27), DGAT1 (13), or microsomal triglyceride transfer protein (MTP) (12, 14). Notably, ApoE has recently been demonstrated to be essential for virus production; ApoE-deficient mouse hepatocytes with *trans* expression of HCV RNA and proteins along with ApoE are able to produce high levels of infectious virions (23).

In summary, this study demonstrates that a small set of defined host factors is sufficient to reconstitute the complete viral life cycle in nonhepatic cells. These results advance our knowledge of tissue-specific factors for HCV infection and provide novel tools to elucidate host and restriction factors for the HCV life cycle.

ACKNOWLEDGMENTS

This work was supported by the European Union (ERC-2008-AdG-233130-HEPCENT, INTERREG-IV-Rhin Supérieur-FEDER-Hepato-Regio-Net 2009), an EASL fellowship to D.J.F., ANRS (2011/132), the Laboratoire d'Excellence HEPSYS (Investissement d'Avenir; ANR-10-LAB-28), an ANRS fellowship to E.G., INSERM, CNRS, and the Université de Strasbourg.

We thank T. Pietschmann (Division of Experimental Virology, TWINCORE, Hannover, Germany) for providing the lentiviral vectors encoding HCV entry factors, F.-L. Cosset for providing plasmids for the production of HCVpp, D. Trono (Ecole Polytechnique Fédérale de Lausanne, Switzerland) for pWPI plasmid, R. Milne for monoclonal ApoE antibody, M. Harris for HCV NS5A antibody used for immunohistochemistry, and F. Chisari for Huh7.5.1 cells. We acknowledge Sarah Durand (INSERM U748, Strasbourg) and Charlotte Bach (INSERM U748, Strasbourg) for excellent technical work. We are thankful to Heidi Barth (INSERM U748, Strasbourg) and Catherine Schuster (INSERM U748, Strasbourg) for helpful discussions.

M.B.Z. and T.F.B. designed and supervised the research. D.D.C., M.T., D.J.F., E.G., S.P., G.L., R.B., M.B.Z., and T.F.B. performed research. D.D.C., M.T., E.G., S.P., M.B.Z., and T.F.B. analyzed data. R.B. provided important ideas for the initiation and execution of this study and provided reagents. D.D.C., M.T., M.B.Z., D.J.F., and T.F.B. wrote the manuscript.

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3. Discussion

Le HCV a un tropisme cellulaire qui restreint son infection aux hépatocytes. Durant ces dernières années, nos connaissances sur les étapes limitantes du cycle infectieux se sont approfondies et ont permis d'établir un consensus scientifique quant aux étapes importantes pour le déroulement de l'infection : l'entrée, la réplication ou l'assemblage de la particule virale. Il était alors intéressant de déterminer s'il est possible, en réunissant les conditions optimums d'entrée, de réplication et d'assemblage, de conduire le cycle viral dans son intégralité dans une lignée cellulaire non hépatique.

Nous nous sommes alors intéressés à l'établissement d'un modèle de cellules non hépatiques, les cellules 293T (cellules humaines embryonnaires dérivées du rein), capables d'effectuer le cycle viral du HCV dans son intégralité. Deux études concomitantes à la nôtre ont montré que l'expression du facteur de réplication miR-122 dans les cellules hépatocytaires Hep3B ou HepG2-CD81, normalement réfractaires à la réplication et à la production virale, rendait ces cellules compétentes pour la production virale (Kambara et al., 2012; Narbus et al., 2011). Cependant, contrairement à notre étude, ces études se sont focalisées sur des cellules hépatocytaires humaines non permissives au HCV alors que notre but était d'obtenir une infection productive dans des cellules humaines non hépatocytaires.

Il a été démontré que l'entrée virale dépendait d'un certain nombre de facteurs, dont entre autre CD81, SR-BI, CLDN1 et OCLN. Les cellules 293T expriment de manière naturelle CD81, SR-BI et OCLN pais pas CLDN1 et sont ainsi résistantes à l'entrée du HCV (Evans et al., 2007). En accord avec une étude précédente (Evans et al., 2007), nous avons pu observer que l'expression de CLDN1 dans les cellules 293T conférait une permissivité aux HCVpp qui correspondait à environ 50% de celle observée dans les cellules Huh7.5.1. Ces résultats démontrent que CLDN1 est un facteur important de l'entrée mais que son expression seule dans les cellules 293T ne permet pas d'obtenir une permissivité identique à celle observée dans les cellules Huh7.5.1. Ces résultats suggèrent que d'autres facteurs d'entrant sont absents ou exprimés de manière sous optimale dans ces cellules. Afin de tester cette hypothèse, nous avons ensuite surexprimé d'autres facteurs d'entrée dans les cellules 293T-CLDN1. La surexpression de CD81 et OCLN dans ces cellules 293T exprimant CLDN1 permet d'établir une permissivité aux HCVpp égale à celle des Huh7.5.1. Ceci peut suggérer que lorsque OCLN et CD81 sont surexprimés, l'équilibre stœchiométrique de distribution des récepteurs est rétabli à l'identique de celui observé dans les Huh7.5.1. La surexpression supplémentaire de SR-BI permet même

d'augmenter l'entrée des HCVpp, phénomène aussi retrouvé dans les cellules Huh7.5.1 lorsqu'on surexprime SR-BI (Fofana et al., 2012; Grove et al., 2007; Schwarz et al., 2009). Ceci nous a permis d'obtenir des cellules 293T exprimant ou surexprimant les quatre récepteurs principaux de l'entrée CD81, SR-BI, CLDN1 et OCLN (293T-4R) hautement permissives à l'entrée du HCV. Il serait bien sûr aussi intéressant de déterminer si l'ajout des nouveaux facteurs ou cofacteurs d'entrée récemment identifiés, comme NPC1L1 ou EGFR, peuvent moduler cette permissivité.

Cependant bien que ces cellules soient permissives à l'entrée des HCVpp, elles ne permettent pas d'observer de la réplication virale avec les HCVcc. Le microARN spécifique au foie, miR-122, est un facteur essentiel de la réplication (Jopling et al., 2005). Il est intéressant de noter que miR122 a un rôle anti-tumoral (Hsu et al., 2012; Tsai et al., 2012), c'est pourquoi de nombreuses cellules hépatocytaires n'expriment pas miR-122, les cellules hépatocytaires Huh7.5.1 constituant une exception. Il a pu être démontré dans le cas des cellules hépatocytaires Hep3B (Kambara et al., 2012) et HepG2 CD81 (Narbus et al., 2011) que l'expression de miR-122 permet la restauration de la réplication virale. Nous avons également observé que l'expression de miR-122 dans les cellules 293T-4R permet d'obtenir une cinétique de réplication similaire à celle des Huh7.5.1. Cependant, l'efficacité de réplication est environ réduite de moitié par rapport à celle observée dans les cellules Huh7.5.1. L'ensemble de ces résultats confirme le rôle essentiel de miR-122 dans la réplication du HCV. Mais le fait que la restauration de la réplication ne soit pas équivalente à celle des Huh7.5.1 laisse suggérer que d'autres facteurs doivent agir en coopération pour avoir une réplication optimale. Il serait ainsi intéressant d'étudier l'expression d'autres facteurs intervenant dans la réplication virale dans les cellules 293T-4R/miR122, comme par exemple les cyclophillines ou la PI4K et d'évaluer la réplication virale suite à une éventuelle (sur)expression de ces protéines.

Par ailleurs, nous avons observé qu'en incubant les cellules 293T-4R/miR-122 avec des inhibiteurs ou des anticorps dirigés contre les facteurs d'entrée du HCV lors de l'infection par les HCVcc, le profil d'inhibition obtenu était identique à celui observé dans les cellules Huh7.5.1, démontrant que la voie d'infection suivie par les particules virales dans les cellules 293T-4R/miR-122 est identique à celle observée dans les cellules Huh7.5.1

Les cellules 293T-4R/miR-122, bien que susceptibles à l'entrée et la réplication du HCV, ne permettent pas la production de particules virales. Il manque donc très probablement un facteur essentiel à la cellule non hépatocytaire pour permettre l'assemblage de nouvelles particules

virales. De nombreuses études ont démontré qu'apoE jouait un rôle clé lors de cette étape d'assemblage (Benga et al., 2010; Jiang and Luo, 2009). En effet, l'expression d'apoE dans nos cellules 293T-4R/miR-122 nous a permis d'observer une légère mais significative production de particules virales. Ceci démontre qu'apoE possède bien un rôle dans l'étape d'assemblage de la particule virale mais aussi que d'autres facteurs importants sont nécessaires à la production optimale des virions ou qu'à l'opposé certains facteurs restrictifs sont peut-être présents. Il est intéressant de noter que le titre d'ARN viral dans le surnageant des cellules 293T-4R/miR-122/apoE est pratiquement identique à celui du surnageant des cellules Huh75.1. Cependant, si l'on utilise ce même surnageant pour réinfecter des cellules Huh7.5.1, on observe que le surnageant des 293T-4R/miR-122/apoE est moins infectieux: en effet, l'infection avec ces surnageants est presque de trois log inférieure à celle observée avec les surnageants des cellules Huh7.5.1. On peut expliquer cela par le fait que l'ARN viral peut être excrété sans pour autant être encapsidé dans une particule virale infectieuse.

De manière intéressante, bien que le rôle et l'action des différentes isoformes d'apoE dans l'infection par le HCV soit sujet à controverse (Hishiki et al., 2010; Long et al., 2011), nous n'avons pas observé de différence quant à la production de particules virales résultant de l'expression des différentes isoformes d'apoE ou même de son orthologue murin dans les cellules 293T-4R/miR-122.

Nous avons ensuite pu démontrer, en utilisant des anticorps ou des inhibiteurs des facteurs d'entrée, que l'infection avec ces particules virales dérivées des 293T pouvait être inhibée de la même manière que celles dérivées des cellules Huh7.5.1. Un point important est que leur profil sur un gradient de densité est similaire à celui observé pour les particules dérivées des Huh7.5.1.

En conclusion, nous avons pu démontrer que la reconstitution du cycle viral dans son intégralité dans des cellules non-hépatiques est possible si l'on réunit les conditions essentielles d'entrée, de réplication et d'assemblage. Il est à noter que pour le moment nous n'avons pas pu rétablir une vraie conduction du cycle viral identique à celle observée dans les cellules hépatiques, l'assemblage viral étant sous optimal dans nos cellules 293T-4R/miR-122/apoE. L'étape d'assemblage est une des étapes du cycle viral la moins bien caractérisée à ce jour. La mise au point du modèle cellulaire 293T-4R/miR-122/apoE possédant ce que nous appellerons les facteurs minimums du cycle infectieux du HCV pourrait justement permettre d'identifier et d'étudier les facteurs nécessaires à un assemblage d'une efficacité équivalente à celui observé dans les cellules Huh7.5.1. Il serait ainsi intéressant d'étudier dans ces cellules l'expression de

facteurs de l'hôte ayant été décrits comme jouant un rôle dans l'assemblage, comme par exemple apoB ou DGAT1, et d'évaluer ensuite la production de particules virales suite à une éventuelle (sur)expression de ces protéines.

IV. CONCLUSIONS ET PERSPECTIVES

Au cours de ma thèse, nous avons pu dans un premier temps, démontrer l'importance des facteurs d'entrée de l'hôte SR-BI et CD81 non seulement dans l'initiation, mais aussi dans la dissémination virale de l'infection par le HCV. Nous avons pu démontrer que des anticorps spécifiques dirigés contre ces deux facteurs d'entrée avaient la capacité d'inhiber la transmission virale libre importante lors de l'initiation de l'infection, mais aussi d'inhiber la transmission virale cellule à cellule, responsable en grande partie de la dissémination virale et du maintien de l'infection par le HCV. Ces résultats nous ont permis de mieux caractériser les mécanismes de fonctionnement des facteurs d'entrée CD81 et SR-BI au cours de l'infection virale par le HCV. Par ailleurs, nous avons montré que ces deux facteurs d'entrée représentent de bonnes cibles thérapeutiques potentielles. Il serait alors intéressant, dans l'avenir, de tester l'efficacité de nos nouveaux anticorps anti-SR-BI et anti-CD81 sur l'infection par le HCV *in vivo,* seuls ou en combinaison, dans des modèles murins.

Dans un second temps, nous nous sommes intéressés à caractériser et à mieux comprendre la spécificité cellulaire du HCV. Nous avons ainsi pu, en nous basant sur les connaissances préexistantes des facteurs cellulaires principaux nécessaires au bon déroulement du cycle viral dans les hépatocytes, dépasser les différentes étapes limitantes d'entrée, de réplication et d'assemblage du virus restreignant la conduction du cycle infectieux du HCV dans les cellules non hépatocytaires. Nous avons ainsi obtenu un modèle cellulaire non hépatocytaires (293T-4R/miR122/apoE) permettant de reproduire l'entrée du HCV, la réplication et aussi l'assemblage. Cependant nous avons démontré que certaine étapes du cycle viral comme la réplication ou l'assemblage, restaient non optimales dans ce modèle, suggérant que l'expression de facteurs additionnels sera nécessaire afin de permettre le bon déroulement de ces étapes. Il serait alors intéressant dans le futur d'utiliser ce modèle cellulaire comme un modèle minimum permettant, par criblage par exemple, d'identifier de nouveaux facteurs essentiels à ces étapes de réplication et d'assemblage du HCV.

En conclusion, l'ensemble de nos travaux a permis de mieux caractériser les mécanismes ou les facteurs qui sous-tendent l'infection par le HCV. Une meilleure caractérisation de ces interactions virus-hôte permettra à terme de développer de nouveaux modèles d'études du HCV et de mettre au point de nouvelles stratégies antivirales.

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VI. ANNEXES

Annexe 1:

Novel monoclonal anti-CD81 antibodies produced by genetic immunization efficiently inhibit hepatitis C virus cell-cell transmission

I. Fofana, F. Xiao, C. Thumann, M. Turek, L. Zona, F. Grunert, J. Thompson, M. B. Zeisel and T. F. Baumert

PLoS One 2013

Les résultats de cette publication sont discutés dans la partie 1 des résultats de cette thèse.

Par ailleurs, durant ma thèse, il m'a aussi était possible de travailler sur deux autres projets participant à notre meilleur compréhension des facteurs d'entrée et des mécanismes d'infection se déroulant lors de l'infection par le HCV. Les résultats de ces projets ont été publiés dans deux publications présentées en annexe 2 et 3.

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A Novel Monoclonal Anti-CD81 Antibody Produced by Genetic Immunization Efficiently Inhibits Hepatitis C Virus Cell-Cell Transmission

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Abstract

Background and Aims: Hepatitis C virus (HCV) infection is a challenge to prevent and treat because of the rapid development of drug resistance and escape. Viral entry is required for initiation, spread, and maintenance of infection, making it an attractive target for antiviral strategies.

Methods: Using genetic immunization, we produced four monoclonal antibodies (mAbs) against the HCV host entry factor CD81. The effects of antibodies on inhibition of HCV infection and dissemination were analyzed in HCV permissive human liver cell lines.

Results: The anti-CD81 mAbs efficiently inhibited infection by HCV of different genotypes as well as a HCV escape variant selected during liver transplantation and re-infecting the liver graft. Kinetic studies indicated that anti-CD81 mAbs target a post-binding step during HCV entry. In addition to inhibiting cell-free HCV infection, one antibody was also able to block neutralizing antibody-resistant HCV cell-cell transmission and viral dissemination without displaying any detectable toxicity.

Conclusion: A novel anti-CD81 mAb generated by genetic immunization efficiently blocks HCV spread and dissemination. This antibody will be useful to further unravel the role of virus-host interactions during HCV entry and cell-cell transmission. Furthermore, this antibody may be of interest for the development of antivirals for prevention and treatment of HCV infection.

Citation: Fofana I, Xiao F, Thumann C, Turek M, Zona L, et al. (2013) A Novel Monoclonal Anti-CD81 Antibody Produced by Genetic Immunization Efficiently Inhibits Hepatitis C Virus Cell-Cell Transmission. PLoS ONE 8(5): e64221. doi:10.1371/journal.pone.0064221

Editor: Stephen J. Polyak, Inserm, France

Received January 29, 2013; **Accepted** April 12, 2013; **Published** May 21, 2013

Funding: This work was supported by Inserm, University of Strasbourg, the European Union (ERC-2008-AdG-233130-HEPCENT, INTERREG-IV-Rhin Supérieur-FEDER-Hepato-Regio-Net 2009 and 2012), the chair of excellence program of the Agence Nationale de la Recherche France (ANR-05-CEXC-008), ANRS (2009/183, 2011/132, 2012/239), Laboratoire d'excellence LabEx HEPSYS (Investissement d'Avenir; ANR-10-LAB-28), the Direction Générale de l'Offre de Soins (A12027MS) and Aldevron (Pro Inno II (KA0690901UL8)). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: FG and JT are employees of Aldevron GmbH, a company who participated in the development of the anti-CD81 antibodies within a collaborative research agreement with the University of Strasbourg and Inserm. FG and JT have been notified regarding PLOS ONE policies and stated that their participation does not alter their adherence to all the PLOS ONE policies on sharing data and materials.

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Introduction

Hepatitis C virus (HCV) is a major cause of chronic hepatitis worldwide. The current therapy against HCV infection based on pegylated interferon-alfa (PEG-IFN-α) and ribavirin does not allow to cure all patients. Although the addition of a direct-acting antiviral (DAA) targeting HCV protein processing - telaprevir or boceprevir- to the standard of care improves sustained virological response in genotype 1 infected patients, toxicity of the individual compounds and development of viral resistance remain major challenges [1]. To date, a vaccine is not available and the absence of preventive strategies is a major limitation for patients undergoing liver transplantation (LT) for HCV-related end-stage liver disease. Re-infection of the graft is universal and characterized by accelerated progression of liver disease [2]. Efficacy and tolerability of IFN-based therapies are limited in LT recipients [3,4] and potentially life-threatening drug-drug interactions limit the use of DAAs in these patients if combined with immunosuppressive agents [5]. Thus, there is an urgent need for novel antiviral preventive and therapeutic strategies.

HCV entry is a multifactorial process involving several host cell factors, including the four main entry factors CD81, scavenger receptor class B type I (SR-BI), claudin-1 (CLDN1) and occludin (OCLN), as well as co-entry factors such as epidermal growth factor receptor (EGFR), ephrin receptor A2 (EphA2), and the Niemann-Pick C1-Like 1 (NPC1L1) cholesterol absorption receptor [6,7]. This process thus provides numerous targets for antivirals. Targeting viral entry offers the advantage to combat viral infection at the very first steps of virus infection and before the virus starts to produce genomic material that will persist in infected cells. Proof-of-concept studies showed that entry inhibitors

efficiently prevent or delay HCV infection *in vitro* and *in vivo* [6]. Viral entry inhibitors are thus unique and feasible antiviral candidates to prevent HCV infection in transplant recipients where entry has been shown to be a key determinant for infection of the liver graft [8,9]. Furthermore, since entry is also required for dissemination and maintenance of infection [10], this approach may allow treating persistent infection as well.

CD81 is a member of the tetraspanin family of proteins, containing a small extracellular and a large extracellular loop (LEL). CD81 was the first HCV host factor to be identified by its ability to interact with a soluble form of HCV E2 (sE2) [11]. The HCV-CD81 interaction and its role in HCV infection have then been extensively studied using various model systems. The CD81 LEL plays an important role in this process [12,13]. CD81 is an essential HCV host factor as silencing of CD81 expression in hepatoma cells inhibits HCV entry while CD81 expression in HCV-resistant hepatoma cell lines confers susceptibility to HCV entry [14,15,16,17]. Although CD81 binds sE2 *in vitro*, it has a central role in HCV entry of viral particles during post-binding steps [18,19,20]. Indeed, CD81 associates with CLDN1 to form co-receptor complexes that are crucial for HCV internalization [20,21,22] and disruption of these complexes prevents HCV infection [23,24,25]. CD81 contributes to the species specificity of HCV infection as mouse cell lines and mouse hepatocytes become permissive to HCV entry upon expression of human CD81 and OCLN *in vitro* and *in vivo* [26,27]. Furthermore, HCV mutants able to use mouse CD81 for cell entry have also been described [28]. Noteworthy, studies demonstrating that anti-CD81 antibodies can prevent HCV infection using uPA-SCID mice underscore the relevance of targeting CD81 for prevention of HCV infection [29].

In addition to cell-free virus entry, where CD81 has been described as an essential factor [12,14,15,16,17,30,31], HCV uses direct cell-cell transfer to infect neighbouring cells and persist in the presence of virus-neutralizing antibodies [10]. This process also seems to require several HCV host factors including CD81, SR-BI, CLDN1, OCLN, EGFR, EphA2 and NPC1L1 [10,25,32,33] but has been less extensively characterized than cell-free entry. Although a CD81-independent route of HCV spread has been described [10,34,35], the exact role of CD81 in viral cell-cell transmission remains unknown.

In this study, we produced and functionally characterized a novel panel of monoclonal antibodies (mAbs) directed against CD81 generated by genetic immunization which specifically and dose-dependently inhibit HCV infection at post-binding steps of the viral entry process. In addition to inhibiting cell-free HCV infection, one antibody was also able to completely block neutralizing antibody-resistant HCV cell-cell transmission and viral dissemination.

Materials and Methods

Cell lines

Culture of Huh7.5.1 [31], Huh7.5-GFP+ [34], HEK 293T [36], Chinese hamster ovary (CHO) [24] and HepG2 [37] cells has been described.

Antibodies

Anti-CD81 mAbs were raised by genetic immunization of Wistar rats using an eukaryotic expression vector encoding the full-length human CD81 cDNA as previously described [9], according to proprietary Aldevron technology (Aldevron, Freiburg, Germany). Animal maintenance and immunization of rats to generate mAbs against CD81 were carried out by a certified animal facility in Germany (MfD Diagnostics GmbH) according to DIN EN ISOO 9000:2000 standards, the regulations of the German Animal Act of 18 May 2006 (BGBI. I S. 1206) and the regulations of European Union guidelines 86/609/EWG of 24 November 2006 and according to the European Agreement of 18 March 1986 for protection of animal trials and other for scientific purposes used vertebrates (Act of 11 December 1990 (BGBI. II S. 1486). The protocol was reviewed by the MfD Diagnostics GmbH animal care committee. For immunization, the animals were anaesthetized using isofluorane. This standard technology does not create animal discomfort. The animals were sacrificed by trained personnel by CO_2 gas and their draining lymph nodes removed as sources for the antibody-producing B-lymphocytes. Immediately following animal death, final bleeds were carried out by cardiopuncture. Antibodies were selected by flow cytometry for their ability to bind to human CD81 expressed on the cell surface of CHO cells transfected with pcDNA3.1-hCD81. Mouse anti-CD81 JS81 antibody was obtained from BD Biosciences. Rat anti-SR-BI (QQ-6G9-A6) and control rat mAbs have been reported [38]. Anti-E2 (IGH461, Innogenetics; AP33, Genentech; CBH23, a kind gift from S.K.H. Foung) mAbs and human anti-HCV IgG have been described [36,39]. NS5A-specific antibody (Virostat), anti-rat IgG alkaline phosphatase (AP) antibody, phycoerythrin (PE)-anti-rat antibody have been described [25,34].

Binding to cell surface CD81

CHO cells were transduced with lentiviruses to express hCD81 and selected with 250 μg/ml of G418 [40]. HepG2 cells were transfected with a plasmid to express hCD81 and selected with 80 μg/ml of hygromycin [24]. Cells were then analyzed by flow cytometry for CD81 expression. Briefly, 2×10^5 cells were stained with mAbs specific for hCD81 (monoclonal rat TN-4H4-F11, TN-9H6-D10, TN-5C5-F3, QV-6A8-F2-C4, 20 μg/ml) or with control mAb in PBS for 1 h at room temperature. Primary bound antibodies were detected with secondary polyclonal antibodies coupled to phycoerythrin (Beckman coulter, 1/100) for 45 minutes at 4°C in PBS. After washing, the cells were fixed with 2% PFA and analyzed by flow cytometry (BD LSR II Flow Cytometer) [38]. Results are expressed as net mean fluorescence intensities (ΔMFI).

HCVcc and HCVpp production and infection

HCVcc (Luc-Jc1 and Jc1) [18] and HCVpp (H77, HCV-J, JFH1, UKN3A1.28, UKN4.21.16, UKN5.14.4, UKN6.5.340, P02VJ) [8,41] were produced as described [8,25]. Patient-derived HCVpp were produced from serum of a patient undergoing LT using full-length E1E2 expression constructs generated from circulating HCV as described [8,36]. The study was approved by the Strasbourg University Hospital Institutional Review Board and written informed consent was obtained from all patients (ClinicalTrial.gov Identifier: NCT00213707). Huh7.5.1 cells were pre-incubated with antibodies for 1 h and then incubated with HCVpp or HCVcc for 4 h at 37°C. Analysis of viral infection was performed by detection of luciferase activity as described [18,24,25]. For combination experiments, anti-CD81 (QV-6A8-F2-C4) mAb was tested individually or in combination with the second antibody. Huh7.5.1 cells were pre-incubated with anti-CD81 or control mabs for 1 h and then incubated for 4 h at 37°C with HCVcc or HCVpp (pre-incubated for 1 h with or without anti-envelope antibodies) as described [38]. Synergy was assessed using the combination index (CI) [38,42,43]. A CI less than 0.9, between 0.9 and 1.1, and more than 1.1 indicates synergy, additivity, and antagonism, respectively [38,42,43]. Cell viability was assessed using a MTT test [9,25].

Kinetic assays

HCVcc kinetic entry assays were performed in Huh7.5.1 cells using anti-CD81 QV-6A8-F2-C4, anti-CD81 JS81, anti-SRBI QQ-4G9-A6 or control mAbs added at different time-points during or after viral binding as described [24,38,39].

Cross-competition

Competition between anti-CD81 mAbs JS81 and QV-6A8-F2-C4 for cellular binding was measured by a cell-based ELISA and labeled antibodies: Huh7.5.1 or CHO-CD81 cells were incubated for 60 min with 0.1 μg/ml biotinylated JS81 (Sulfo-NHS-LC-Biotin; Thermo Scientific) together with increasing concentrations of unlabeled QV-6A8-F2-C4 as competitor. Following washing with PBS, binding of biotinylated antibody was detected by incubation with streptavidin labeled with horseradish peroxidase. Curves determined by measurement of binding in the presence of an isotype-matched control were compared to those determined in the presence of the competing antibody.

Cell-cell transmission of HCV

Cell-cell transmission of HCV was assessed as described [25,34]. Briefly, producer Huh7.5.1 cells were electroporated with HCV Jc1 RNA (Pi) and cultured with naive target Huh7.5-GFP+ cells (ratio of 1:2) in the presence or absence of anti-CD81 or control mAbs (10 μg/ml). An HCV E2–neutralizing mAb (AP33, 25 μg/ml) was added to block cell-free transmission [34]. After 24 h of co-culture, cells were fixed with paraformaldehyde, stained with an NS5A-specific mAb (0.1 μg/ml) and analyzed by flow cytometry [25,34]. Total and cell-cell transmission was defined as percentage HCV infection of Huh7.5-GFP+ target cells (Ti) in the absence (total transmission) or presence (cell-cell transmission) of an HCV E2-specific mAb [25].

Immunofluorescence of viral dissemination

Virus spread was assessed by visualizing Jc1-infected Huh7.5.1 cells by immunofluorescence using anti-NS5A (Virostat) or anti-E2 (CBH23) mAbs as described [25]. In these long-term experiments, cells are plated and infected at low density and cell growth between control- and anti-CD81 antibody-treated cells was ascertained by enumeration of cells (by cell counting and by immunofluorescence staining of cell nuclei using DAPI) as described [38].

Results

Production of anti-CD81 monoclonal antibodies directed against cell surface CD81

To further explore CD81 as a target for antiviral strategies, we generated anti-CD81 mAbs by genetic immunization using a full-length human CD81 cDNA expression vector. Four mAbs (QV-6A8-F2-C4, TN-9H6-D10, TN-5C5-F3, TN-4H4-F11) were selected that reacted with native human CD81 expressed on HepG2 and CHO cells (Fig. 1A–B). To characterize the nature of the epitopes recognized by the four different anti-CD81 mAbs, we performed immunoblot analyses using Huh7.5.1 cells which express high levels of endogenous CD81 on their cell surface. Immunoblot analyses under reducing conditions demonstrated no staining of CD81 by anti-CD81 mAbs suggesting that the anti-CD81 mAbs most likely recognize predominantly conformational epitopes or their affinity to linear epitopes is low (data not shown).

Inhibition of HCV infection by anti-CD81 monoclonal antibodies

To investigate whether these antibodies inhibit HCV infection, Huh7.5.1 cells were pre-incubated with anti-CD81 mAbs and infected with chimeric luciferase reporter virus Luc-Jc1 (genotype 2a). As shown in Fig. 2A, anti-CD81 mAbs inhibit Luc-Jc1 infection of Huh7.5.1 cells in a dose-dependent manner (IC_{50} of 0.7–8 μg/ml). In contrast, an isotype control mAb had no effect. Among these antibodies, anti-CD81 mAb QV-6A8-F2-C4 most efficiently inhibited HCVcc infection with an IC_{50} of 0.7 μg/ml. To investigate whether anti-CD81 mAbs were effective against other HCV genotypes, we analyzed their inhibition of HCVpp bearing envelope glycoproteins from HCV genotype 1b. All four anti-CD81 mAbs inhibited HCVpp genotype 1b entry in a dose-dependent manner (Fig. 2B). Anti-CD81 mAb QV-6A8-F2-C4, displaying the lowest IC_{50} against HCVcc from genotype 2, was also characterized by the lowest IC_{50} against HCVpp from genotype 1b (IC_{50} of 4 μg/ml). Inhibition of HCVcc infection and HCVpp entry by QV-6A8-F2-C4 was in a similar range as inhibition of infection by the commercially available anti-CD81 mAb JS81 (IC_{50}s of 0.5 and 2 μg/ml, respectively). Interestingly, the IC_{50} of all these anti-CD81 mAbs were higher on inhibition of HCVpp entry than HCVcc infection, suggesting that these mAbs may act on another step of the viral life cycle in addition to cell-free entry. Noteworthy, these antibodies also blocked the infectivity of HCVpp bearing the envelope glycoproteins from HCV genotypes 2–6 (Fig. 2C). Taken together, these data indicate that anti-CD81 mAbs efficiently block HCV infection in a pan-genotypic manner.

Anti-CD81 monoclonal antibody QV-6A8-F2-C4 inhibiting HCV infection targets post-binding steps of viral entry

CD81 has been demonstrated to participate in post-binding steps of the viral entry process [18,19,44]. To investigate the HCV entry steps targeted by our anti-CD81 mAbs, we investigated the inhibitory capacity of anti-CD81 mAbs in kinetic entry studies [24,39,45]. To allow virus binding, Luc-Jc1 HCVcc were first incubated with Huh7.5.1 cells for 1 h at 4°C in the presence or absence of antibodies. Then the temperature was shifted to 37°C to allow continuation of the viral entry process. Antibodies were added at different time-points after the temperature shift to assess their ability to inhibit the course of HCV entry. Anti-CD81 mAb JS81 and anti-SR-BI mAb QQ-4G9-A6, two antibodies that have been previously reported to inhibit HCV post-binding steps [18,38], were used side-by-side. As shown in Fig. 3A, anti-CD81 mAb QV-6A8-F2-C4 inhibited HCVcc infection at post-binding steps similarly to results obtained with anti-CD81 mAb JS81 and anti-SR-BI mAb QQ-4G9-A6, while a control mAb had no effect. Noteworthy, cross-competition experiments on Huh7.5.1 and CHO-CD81 cells demonstrated that QV-6A8-F2-C4 and JS81 recognize similar epitopes on CD81 (Fig. 3B–C). Furthermore, anti-CD81 mAbs TN-9H6-D10, TN-5C5-F3 and TN-4H4-F11 also inhibited HCV entry at post-binding steps, albeit at lower levels (Fig. 3A). Taken together, these data indicate that anti-CD81 mAb QV-6A8-F2-C4 blocks HCV entry during post-binding steps.

Synergy between anti-CD81 and anti-HCV envelope antibodies on inhibiting HCV escape variant infection

We have previously demonstrated that viral entry is a key determinant for HCV re-infection during LT and that HCV-CD81 interactions play an important role in this process [8,46]. Moreover, we have demonstrated that receptor-specific antibodies

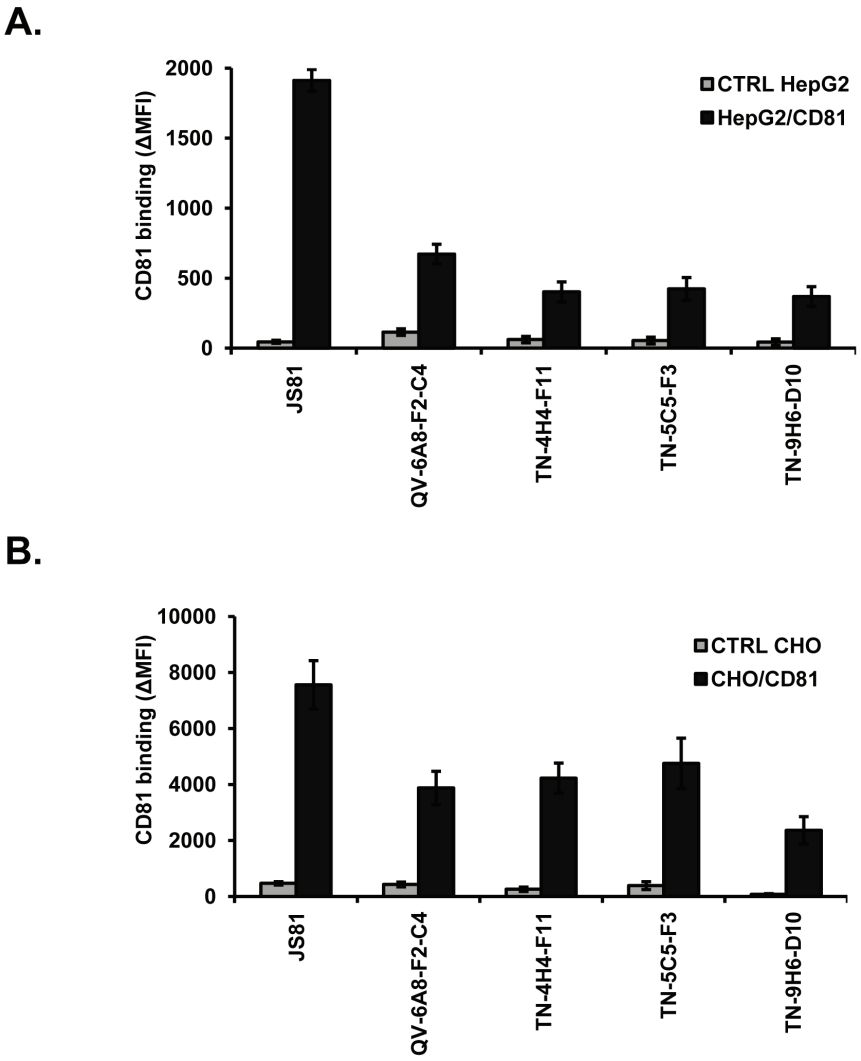

Figure 1. Production of CD81-specific mAbs directed against cell surface CD81. (A) HepG2 and HepG2 cells expressing human CD81 as well as (B) CHO and CHO cells expressing human CD81 were incubated with indicated anti-CD81 mAbs (20 μg/ml) and antibody binding was assessed using flow cytometry. Results are expressed as mean fluorescence intensity (ΔMFI) ± SEM of a pool of three independent experiments performed in duplicate.
doi:10.1371/journal.pone.0064221.g001

or kinase inhibitors specifically inhibit entry of highly infectious HCV escape variants that are resistant to autologous host responses and re-infect the liver graft [8,9,25,38]. To assess the clinical relevance of anti-CD81 mAbs to inhibit HCV escape variants, we determined the effect of anti-CD81 mAb QV-6A8-F2-C4 on entry into Huh7.5.1 cells of HCVpp bearing the envelope glycoproteins of a highly infectious HCV strain selected during LT (P02VJ) [8]. As shown in Fig. 4A, entry of patient-derived HCVpp P02VJ into Huh7.5.1 cells was efficiently inhibited by anti-CD81 mAb QV-6A8-F2-C4 in a dose-dependent manner. Since we have previously demonstrated that combining anti-receptor mAbs, such as anti-CLDN1 or anti-SR-BI mAbs, with anti-E2 mAb or purified heterologous anti-HCV IgG resulted in a marked synergistic effect [9,38], we next investigated whether the combination of envelope-specific antibodies and anti-CD81 mAb QV-6A8-F2-C4 also results in an additive or synergistic effect on the inhibition of HCV infection. Thereto, we pre-incubated patient-derived HCVpp with anti-E2 mAb IGH461 or purified heterologous anti-HCV IgG (1 or 10 μg/ml) and studied their ability to inhibit HCVpp entry in cells pre-incubated with increasing concentrations of anti-CD81 mAb QV-6A8-F2-C4. Each antibody was tested alone and in combination to determine the combination index (CI) [38,43] allowing to conclude about additivity or synergy. As shown in Fig. 4 and Table 1, combination of anti-CD81 and anti-HCV envelope antibodies resulted in a synergistic effect on inhibition of HCVpp P02VJ entry as well as of HCVcc infection decreasing the IC_{50} of anti-CD81 mAb by up to 100-fold. Taken together, these data indicate that targeting CD81 may hold promise for design of novel antiviral strategies targeting both viral and host entry factors.

Anti-CD81 monoclonal antibody QV-6A8-F2-C4 inhibits neutralizing antibody-resistant HCV cell-cell transmission and viral dissemination

While cell-free HCV infection is crucial for initiation of infection, direct cell-cell transmission, that is largely resistant to the majority of described neutralizing antibodies, is believed to be most relevant for viral spread and maintenance of infection

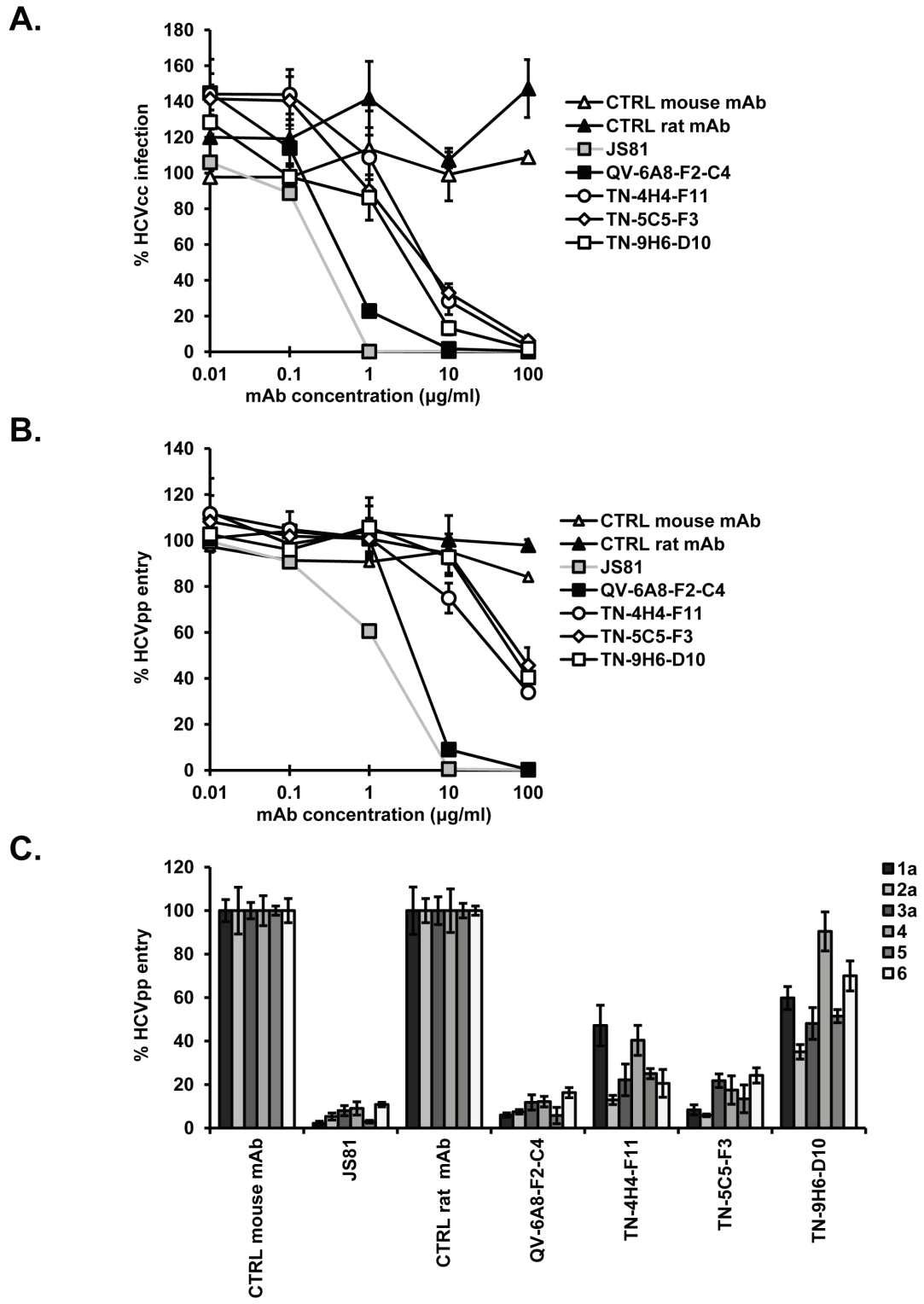

Figure 2. Anti-CD81 mAbs dose-dependently inhibit HCV infection. (A–B) Dose-dependent inhibition of HCV infection by anti-CD81 mAbs. Huh7.5.1 cells were pre-incubated with increasing concentrations of anti-CD81 or isotype control (CTRL IgG) mAbs for 1 h at 37°C before infection with (A) HCVcc (Luc-Jc1 (2a)) or (B) HCVpp (HCV-J (1b)). Three days later, viral infection was quantitated by assessing the expression of luciferase reporter gene. Results are expressed as % HCVcc infection or % HCVpp entry and represent means ± SD of one representative experiment performed in triplicate. (C) Inhibition of infection of HCVpp bearing envelope glycoproteins from genotypes 1–6. Huh7.5.1 cells were pre-incubated with a fixed concentration (100 µg/ml) of antibodies before infection with HCVpp (strains H77 (1a), JFH1 (2a), UKN3A1.28 (3a), UKN4.21.16 (4), UKN5.14.4 (5), UKN6.5.340 (6)). Means ± SD from a representative experiment performed in triplicate are shown.
doi:10.1371/journal.pone.0064221.g002

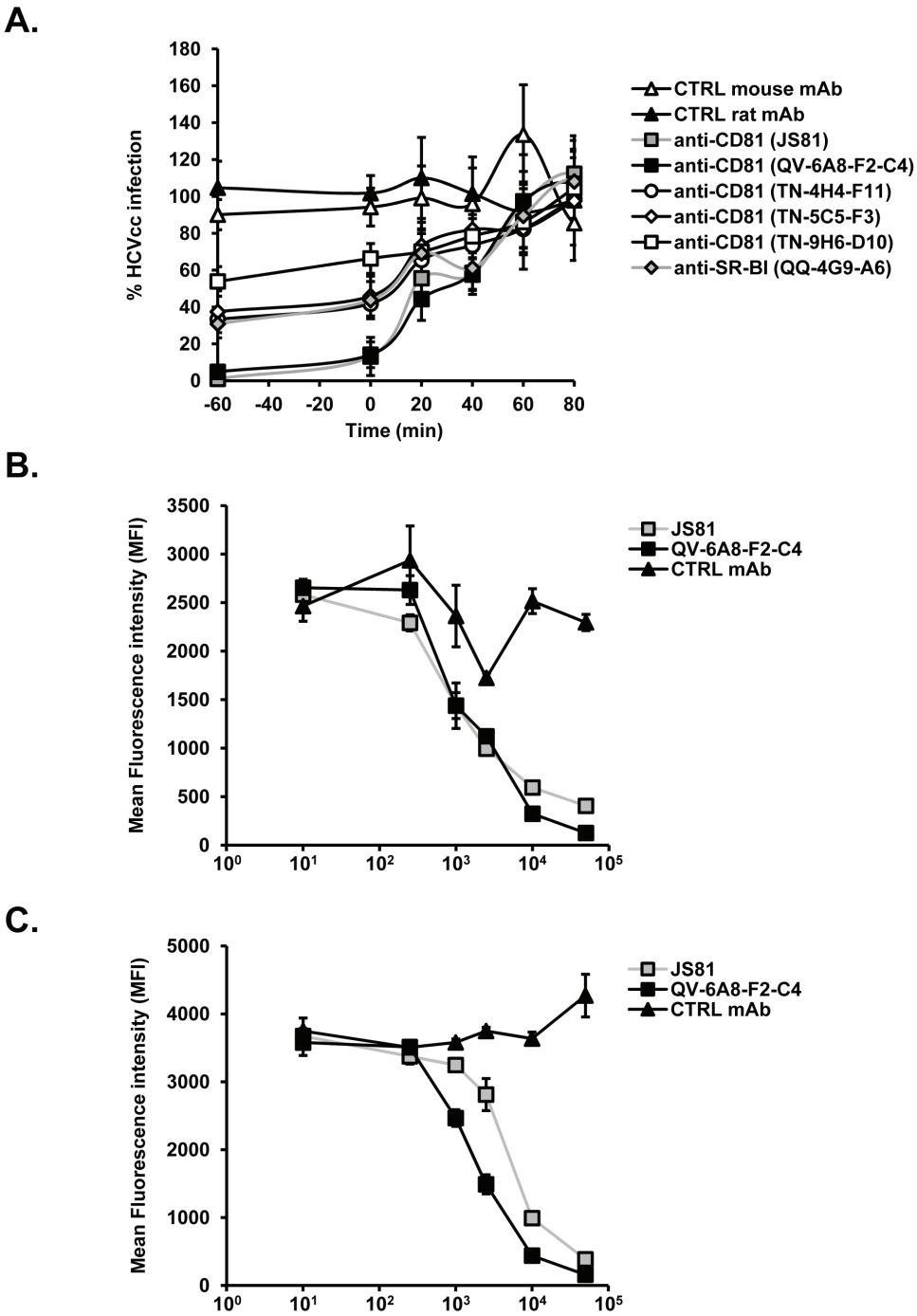

Figure 3. Anti-CD81 mAbs inhibit HCV infection at post-binding steps of viral entry. (A) Kinetics of HCVcc entry into human hepatoma cells. To discriminate between virus binding and post-binding events, Luc-Jc1 HCVcc binding to Huh7.5.1 cells was performed in the presence or absence of anti-CD81 mAbs QV-6A8-F2-C4, TN-9H6-D10, TN-5C5-F3 and TN-4H4-F11 (20 μg/ml), anti-CD81 mAb JS81 (5 μg/ml), anti-SR-BI mAb QQ-4G9-A6 (20 μg/ml) or respective control mAbs (20 μg/ml) for 1 h at 4°C, before cells were washed and incubated for 4 h at 37°C with mAbs added at different time-points during infection as described [24,39]. Compounds were then removed and cells were cultured for an additional 48 h in the absence of mAbs before measuring HCV infection by luciferase assay. Results are expressed as % HCVcc infectivity relative to cells incubated in the absence of mAb and represent means ± SD from two independent experiments performed in triplicate. (B–C) Competition of anti-CD81 mAbs JS81 and QV-6A8-F2-C4 for cellular binding. (B) Huh7.5.1 or (C) CHO-CD81 cells were incubated with 0.1 μg/ml biotinylated anti-CD81 mAb JS81 together with increasing concentrations of unlabeled anti-CD81 mAb QV-6A8-F2-C4 as competitor. Following washing of cells in PBS, binding of labeled antibody was detected as described in Methods and is shown as mean fluorescence intensity (MFI).
doi:10.1371/journal.pone.0064221.g003

[10,32]. To investigate the ability of anti-CD81 mAb QV-6A8-F2-C4 to interfere with neutralizing antibody-resistant viral spread, we used a well-described assay, where cell-free HCV entry is efficiently reduced by more than 90% using a neutralizing anti-E2 mAb, to assess HCV cell-cell transmission [25,34]. Anti-CD81 mAb QV-6A8-F2-C4 efficiently blocked HCV cell-cell transmis-

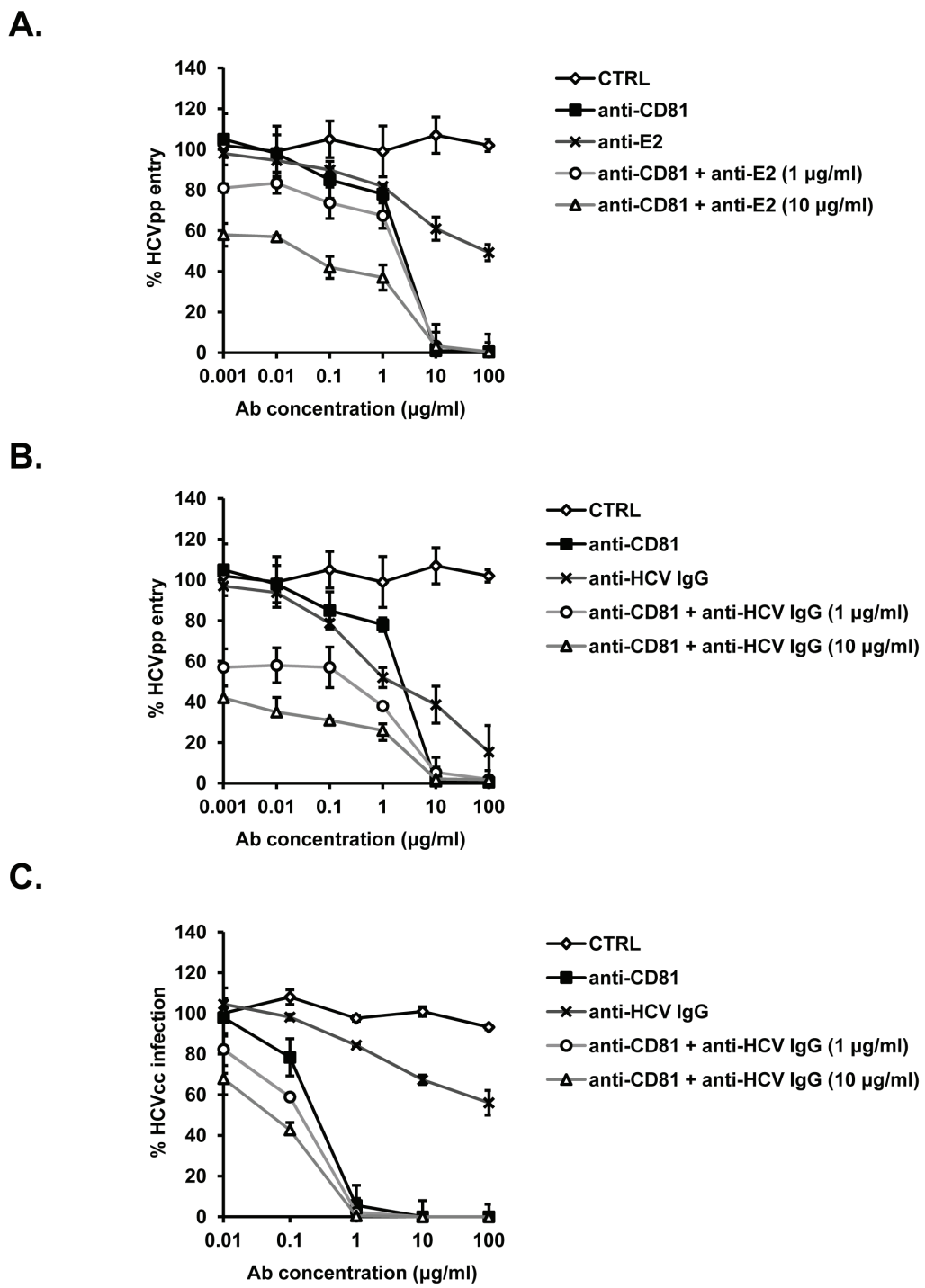

Figure 4. Synergy between anti-CD81 and anti-envelope antibodies in inhibiting HCV infection. (A–B) Patient derived HCVpp P02VJ or (C) HCVcc-Luc Jc1 were pre-incubated with (A) anti-E2 mAb (IGH461) or (B–C) purified heterologous anti-HCV IgG (1 or 10 µg/ml) obtained from an unrelated chronically infected subject or isotype control IgG for 1 h at 37°C and added to Huh7.5.1 cells that had been pre-incubated with increasing concentrations of control or anti-CD81 mAb QV-6A8-F2-C4. In anti-envelope antibody monotherapy setting, HCVpp or HCVcc were in parallel pre-incubated with increasing concentrations of anti-E2 mAb or anti-HCV IgG. HCVpp entry and HCVcc infection were analyzed by luciferase assay. Results are expressed as % HCVpp entry or HCVcc infection and represent means ± SD from a representative experiment performed in triplicate.
doi:10.1371/journal.pone.0064221.g004

sion (Fig. 5A–B) indicating that this antibody may prevent viral dissemination *in vitro*. Furthermore, we next assessed whether this anti-CD81 mAb can prevent viral spread when added post-infection. Thereto, cell cultures were first infected with HCV and antibodies were subsequently added to the cells 48 h after infection. Medium or medium supplemented with control mAb or anti-CD81 mAb QV-6A8-F2-C4 was replenished every 4 days until the end of the experiment and HCVcc infection was monitored over 14 days. The anti-CD81 mAb efficiently inhibited HCV spread over 2 weeks in a dose-dependent manner (Fig. 5C)

Table 1. Synergistic effect of anti-envelope and anti-CD81 antibodies on inhibition of HCV infection.

Virus	Compound 1	IC_{50} (μg/ml)	Compound 2	IC_{50} for combination (μg/ml)	CI
HCVpp	anti-CD81	2.5±0.3	1 μg/ml anti-E2	1.5±0.06	0.61±0.04
		2.5±0.3	10 μg/ml anti-E2	0.03±0.01	0.16±0.008
		2.5±0.3	1 μg/ml anti-HCV IgG	0.2±0.06	0.75±0.04
		5±0.6*	10 μg/ml anti-HCV IgG	1±0.06*	0.45±0.02
HCVcc	anti-CD81	0.25±0.03	1 μg/ml anti-HCV IgG	0.15±0.03	0.61±0.2
		0.25±0.03	10 μg/ml anti-HCV IgG	0.05±0.006	0.29±0.04

HCVpp of strains P02VJ or HCVcc-Luc Jc1 were pre-incubated with anti-E2 mAb IGH461 or purified heterologous anti-HCV IgG (1 or 10 μg/ml) obtained from an unrelated chronically infected subject or isotype control IgG for 1 hour at 37°C and added to Huh7.5.1 cells pre-incubated with serial dilutions of anti-CD81 QV-6A8-F2-C4 or rat isotype control mAbs. HCVpp entry and HCVcc infection were analyzed by luciferase assay. The Combination Index (CI) was calculated as described [42,43]. A CI less than 0.9, between 0.9 and 1.1, and more than 1.1 indicates synergy, additivity, and antagonism, respectively. CI for anti-CD81 mAb in combination with 10 μg/ml anti-HCV IgG in HCVpp entry inhibition was calculated for an IC_{75} as the combination resulted in an inhibition below the IC_{50} and is indicated by a star (*). IC_{50} of anti-envelope antibodies: anti-E2, 70±5 μg/ml (for HCVpp); anti-HCV IgG, 40±3 μg/ml (for HCVpp), 120±6 μg/ml (for HCVcc).

doi:10.1371/journal.pone.0064221.t001

without affecting cell viability as assessed using a MTT test (Fig. 5D). We also assessed Jc1 spread in Huh7.5.1 cells via immunostaining of infected cells after several days of incubation in the presence of anti-CD81 mAbs QV-6A8-F2-C4 and JS81. While 67.6±11.8% of cells incubated with control rat mAb stained positive for NS5A, incubation with QV-6A8-F2-C4 markedly reduced the number of NS5A-positive (7.63±4.8%) cells without displaying any significant cell mortality (Fig. 5E). Incubation of cells with JS81 reduced the number of E2-positive cells (2.3±2.5%) compared with cells incubated with control mouse mAb (60.4±12.5%) (Fig. 5F). However, the number of total cells was significantly and reproducibly reduced in JS81-treated cells which could be due to a cytotoxic or to an anti-proliferative effect. Taken together, these data indicate that anti-CD81 mAb QV-6A8-F2-C4 blocks viral spread by interfering with HCV cell-cell transmission and dissemination without any detectable toxic effect in cell culture models for HCV infection.

Discussion

In this study we report the successful production of anti-CD81 mAbs using DNA immunization which potently inhibit cell-free HCV infection from different genotypes in a dose-dependent manner and block cell-cell transmission and dissemination. Production of mAbs using DNA immunization has been reported to induce higher avidity antibodies than protein immunization [47], which may be advantageous for the development of antibodies efficiently inhibiting HCV infection. Indeed, among the four anti-CD81 mAbs generated in this study, anti-CD81 mAb QV-6A8-F2-C4 showed very effective inhibition of HCVcc infection and HCVpp entry during HCV-CD81 post-binding interaction(s).

The CD81 LEL has been shown to play an important role in the entry process as soluble recombinant forms of CD81 LEL are able to inhibit HCV infection [12,13,15]. The amino acid residues within the CD81 LEL and HCV E2 involved in E2-CD81 binding have been extensively characterized [11,44,48,49,50]. Interestingly, our studies identify anti-CD81 mAbs with different inhibition profiles on HCV infection. Anti-CD81 mAb QV-6A8-F2-C4 which most efficiently inhibited HCV infection was characterized by binding to cell surface-expressed human CD81 and mutual cross-competition between QV-6A8-F2-C4 and the well-characterized commercially available anti-CD81 antibody JS81 suggests that they recognize similar epitopes on CD81.

These novel anti-CD81 antibodies may be very useful for investigators studying the HCV entry process. Indeed, a panel of antibodies inhibiting HCV entry with different efficacy and recognizing different epitopes is of interest as it may be used to (i) further decipher structural and functional domains in CD81 which are crucial for inhibition and (ii) to more deeply dissect its mechanistic role in the entry process. This will allow a better understanding of CD81 regions binding to envelope glycoprotein E2 or domains involved in the formation of the CD81-CLDN1 complex [22]. Furthermore, the antibodies are useful to study CD81 expression by flow cytometry.

The identification of novel anti-CD81 antibodies may also be relevant for the development of novel antiviral antibodies for prevention and treatment of HCV infection. CD81 may be an attractive therapeutic target for the development of HCV entry inhibitors as it is a key player in the HCV entry process. Small molecules and mAbs targeting CD81 and interfering with HCV infection have previously been described [6]. So far, the effect of the majority of these compounds has been solely assessed on cell-free HCV entry [6]. While cell-free viral entry is undoubtedly essential for initiation of infection, direct cell-cell transmission probably constitutes the dominant mechanism of viral spread and thus persistence of infection [10,32]. Direct cell-cell transfer has an important impact for the development of antivirals as this process allows viral spreading by escaping extracellular neutralizing antibodies as well as defined antibodies interfering with host cell entry factors [10,32]. Most of the known HCV entry factors are involved in this process [10,51]. In addition to CD81-dependent HCV cell-cell transmission, a fraction of viral spread appears to be independent of CD81 [10,34,35]. Noteworthy, the anti-CD81 mAb QV-6A8-F2-C4 described in our study not only inhibited cell-free HCV entry but also efficiently and dose-dependently blocked cell-cell transmission and viral spread, providing novel options for the development of efficient anti-HCV therapeutics interfering with this process.

Entry inhibitors, such as anti-CD81 mAbs, are ideal to be applied for the prevention of HCV re-infection in the transplantation setting where currently no clinical option exists to protect HCV-negative transplanted livers from re-infection [3,4]. An anti-CD81 antibody inhibiting HCV infection *in vitro* has already been demonstrated to prevent HCV infection in the human liver-chimeric Alb-uPA/SCID mouse model [29]. This suggests that targeting CD81 may be an efficient strategy to prevent HCV infection e. g. in transplant recipients where entry has been shown

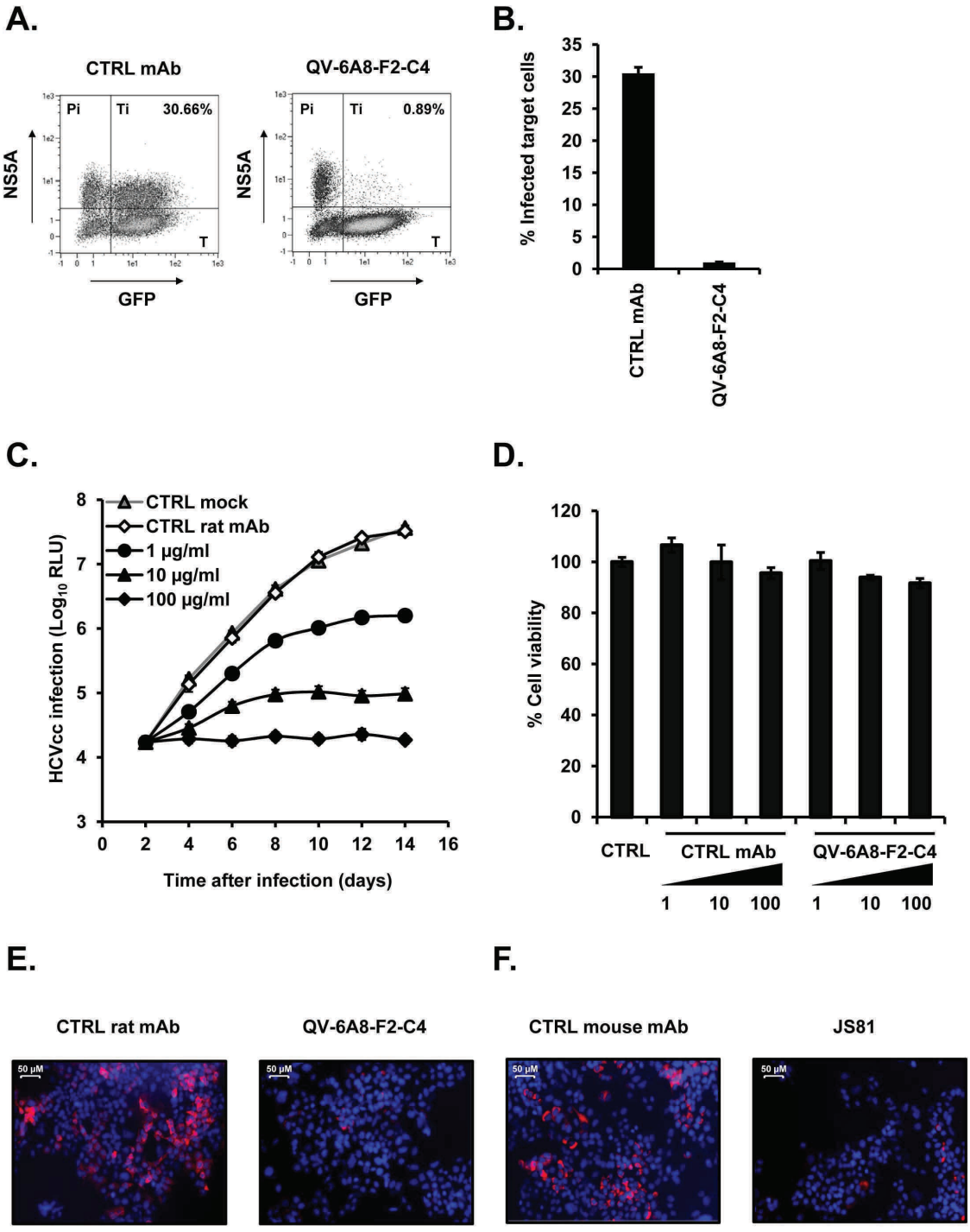

Figure 5. Anti-CD81 mAb inhibits HCV cell-to-cell transmission and viral spread. (A) Quantification of HCV-infected target cells (Ti) after co–cultivation with HCV producer cells (Pi) during incubation with control or anti-CD81 QV-6A8-F2-C4 mAbs (10 µg/ml) in the presence of neutralizing anti-HCV E2 mAb (AP33, 25 µg/ml) by flow cytometry. (B) Percentage of infected target cells is shown as histograms and is represented as means ± SD from three experiments. (C) Long-term analysis of HCVcc infection in the presence or absence of control or anti-CD81 QV-6A8-F2-C4 mAbs at the indicated concentrations. Antibodies were added 48 h after HCVcc infection and control medium or medium containing mAbs were replenished every 4 days. Luciferase activity was determined in cell lysates every 2 days. Data are expressed as Log_{10} RLU and represent means ± SD of three experiments performed in duplicate. (D) Cell viability after long-term exposure to anti-CD81 mAb QV-6A8-F2-C4. Cell viability was assessed using MTT assay after incubation of Huh7.5.1 cells for 14 days in the presence or absence of control or anti-CD81 mAbs at 1, 10, or 100 µg/ml. Data are expressed as % cell viability relative to cells incubated in the absence of mAb and represent means ± SD from one experiment performed in triplicate. (E–F) Virus spread in the presence or absence of anti-CD81 mAbs QV-6A8-F2-C4 (E) and JS81 (F). Antibodies (50 µg/ml) were added 48 h after HCVcc (Jc1) infection and control medium or medium containing antibodies were replenished every 4 days. HCV-infected cells were visualized 9 days post-infection via immunofluorescence using anti-NS5A (E) or anti-E2 (CBH23) (F) antibodies. The percentage of infected cells was calculated as the number of infected cells relative to the total number of cells as assessed by 4′,6-diamidino-2-phenylindole (DAPI) staining of the nuclei.
doi:10.1371/journal.pone.0064221.g005

to be a key determinant for infection of the liver graft [6,8,46]. In this study, we demonstrate that anti-CD81 mAbs efficiently inhibited the entry of highly infectious HCV escape variants that are resistant to autologous host responses and re-infect the liver graft. Interestingly, combination of HCV envelope-specific antibodies with a CD81-specific mAb resulted in a synergistic activity on the inhibition of HCVcc infection and HCVpp escape variant entry. The combination decreased the concentration needed to achieve a 50% antiviral activity of the individual compounds up to 100-fold. The ability of anti-CD81 mAbs to block entry of HCV escape variants and the marked synergy with anti-envelope antibodies on inhibiting HCV entry indicate that the novel CD81-specific mAbs are prime candidates for prevention of liver graft infection. Furthermore, entry inhibitors may also be efficient antivirals for treatment of HCV infection [52,53]. Indeed, the ability of anti-CD81 mAb QV-6A8-F2-C4 to block cell-cell transmission and dissemination post-infection without any detectable toxicity suggests that targeting CD81 may also hold promise for the treatment of chronic infection in combination with other antivirals. A potential challenge for the clinical development of anti-CD81 antibodies could be adverse effects. Indeed, CD81 is ubiquitously expressed on the surface of various cell types. Antibodies binding to CD81 may alter the function, expression or signaling of the receptor resulting in side effects. Interestingly, using anti-CD81 mAb QV-6A8-F2-C4, no toxic effects were detected in MTT-based cellular assays (Fig. 5D). However, further *in vivo* studies are needed to address toxicity in hepatic and extrahepatic tissues.

In conclusion, we identified and functionally characterized a novel panel of anti-CD81 mAbs generated by DNA immunization which efficiently inhibit HCV infection and dissemination. These antibodies will be useful for the molecular investigations of virus-host interactions during the HCV entry process and the characterization of CD81 expression in cell lines, primary cells and tissues. Furthermore, one antibody is an interesting and relevant candidate for the development of novel preventive and improved therapeutic antiviral strategies against HCV infection.

Acknowledgments

We thank R. Bartenschlager (University of Heidelberg, Germany), F.-L. Cosset (Inserm U758, ENS Lyon, France) and J. Ball (University of Nottingham, UK) for providing plasmids for production of HCVcc and HCVpp, C. M. Rice (The Rockefeller University) and F. V. Chisari (The Scripps Research Institute, La Jolla, CA) for Huh7.5 and Huh7.5.1 cells, respectively, A. Patel (University of Glasgow, UK) for Huh7.5-GFP+ cells, S. K. H. Foung (Stanford University,Palo Alto, CA) for anti-E2 mAb CBH23, D. Trono (Ecole Polytechnique Fédérale de Lausanne, Switzerland) and T. Pietschmann (TWINCORE, Centre for Experimental and Clinical Infection Research, Germany) for lentiviral expression constructs. We acknowledge excellent technical assistance of L. Heydman (Inserm U1110, France) and S. Glauben (Aldevron GmbH, Germany).

Author Contributions

Conceived and designed the experiments: TFB. Performed the experiments: IF FX CT MT LZ RGT FG JT MBZ TFB. Analyzed the data: IF FX CT MT LZ RGT FG JT MBZ TFB. Wrote the paper: IF MBZ TFB.

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Annexe 2

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

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Nature Medicine 2011

Dans le but de déterminer des protéines kinases importantes dans l'entrée du HCV, notre laboratoire a conduit des criblages à ARN interférant qui ont permis l'identification de deux récepteurs à tyrosine kinase (RTK), EGFR et EphA2, comme nouveau facteurs d'entrée du HCV**.** Dans ce projet, j'ai déterminé si l'expression des différents facteurs d'entrée du HCV (CD81, SR-BI, CLDN1, OCLN, EGFR, EphA2) était modulée après silencing des RTK ou après inhibition de leur activité kinase dans des cellules Huh7.5.1 (Figure 4b). Mes résultats montrent que l'expression d'aucun autre facteur d'entrée n'est affectée par la réduction de l'expression ou l'inhibition de l'activité kinase d'EGFR ou d'EphA2. Ceci indique donc que l'inhibition de l'entrée du HCV observée suite au silencing ou à l'inhibition de ces RTK est bien due à la diminution de l'expression ou de l'activité des RTK et pas à une diminution de l'expression d'un autre facteur essentiel à l'entrée du HCV. J'ai également confirmé la spécificité de ces résultats de silencing par une autre méthode en mettant au point la technique de « silencing-rescue » (Figure 1 e,f , figure S4 e, f): en co-électroporant des siRNA dirigés contre EGFR ou EphA2 et des plasmides d'expression codant EGFR ou EphA2 qui sont résistant à l'action des siRNA, j'ai montré que l'effet inhibiteur des siEGFR et siEphA2 sur l'entrée du HCV peut être rétablie par l'expression exogène de la protéine correspondante. Finalement, j'ai également pu observer l'effet du silencing de CDC2 par western blot (figure S3 b) et observer son effet sur l'infection par le HCV (Figure S3 c, d) et j'ai pu observer que l'inhibition de l'infection due au silencing de CDC2 n'était pas génotype dépendante (Figure s3 d). J'ai enfin vérifié si la spécificité des résultats de silencing par « silencing rescue » (Figure s3 e). De manière intéressante j'ai pu aussi démontrer que l'expression d'EGFR humain dans des cellules murines permissives à l'entrée du HCV (car exprimant les 4 facteurs d'entrée OCLN, CLDN1, CD81 et SR-BI) permettait d'accroitre fortement la permissivité de ces cellules aux HCVpp. (Figure S5 a, b, c)

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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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Received 7 December 2010; accepted 3 March 2011; published online 24 April 2011; doi:10.1038/nm.2341

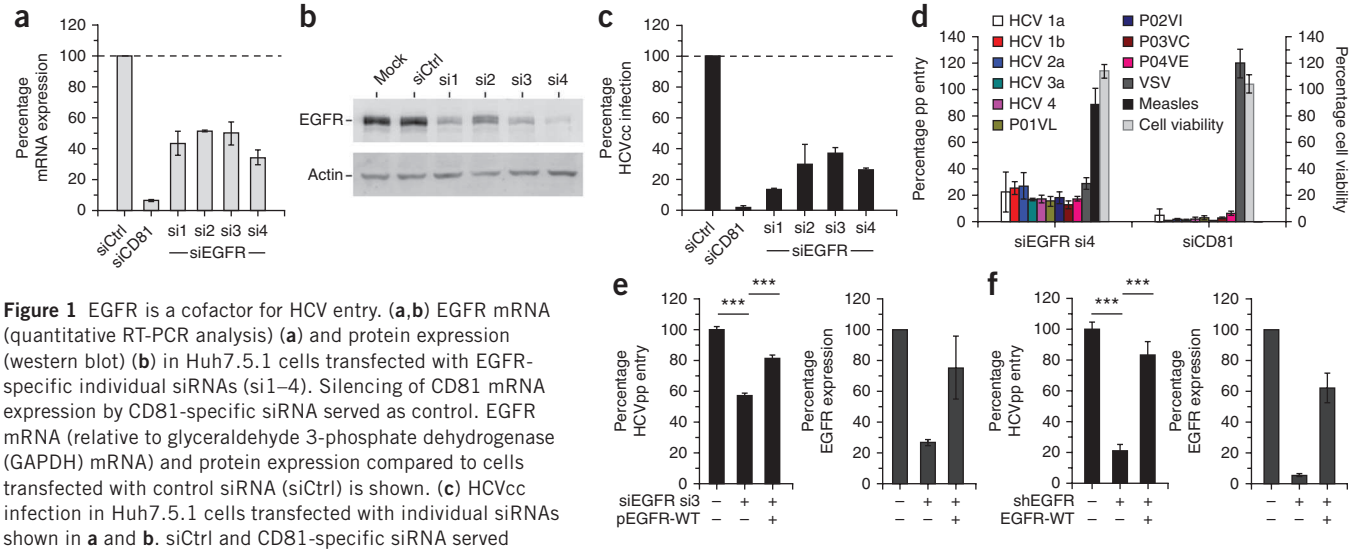

Figure 1 EGFR is a cofactor for HCV entry. (**a**,**b**) EGFR mRNA (quantitative RT-PCR analysis) (**a**) and protein expression (western blot) (**b**) in Huh7.5.1 cells transfected with EGFR-specific individual siRNAs (si1–4). Silencing of CD81 mRNA expression by CD81-specific siRNA served as control. 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

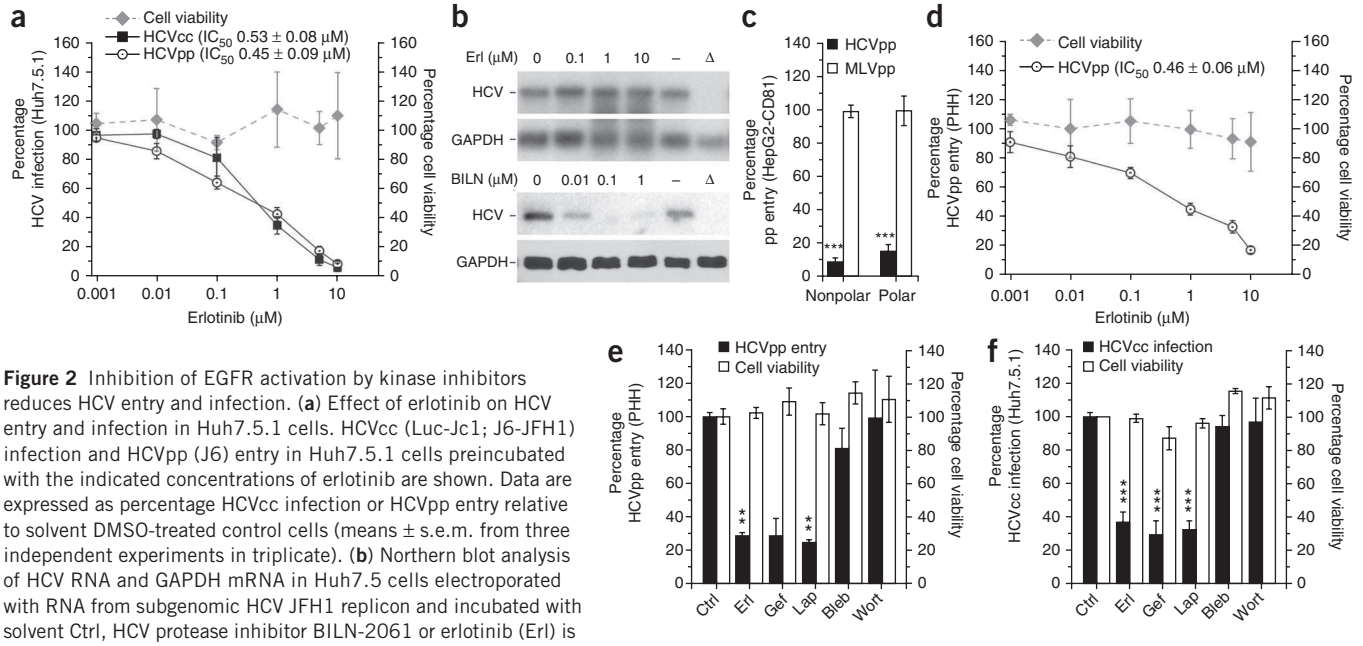

Figure 2 Inhibition of EGFR activation by kinase inhibitors reduces HCV entry and infection. (**a**) Effect of erlotinib on HCV entry and infection in Huh7.5.1 cells. HCVcc (Luc-Jc1; J6-JFH1) infection and HCVpp (J6) entry in Huh7.5.1 cells preincubated with the indicated concentrations of erlotinib 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

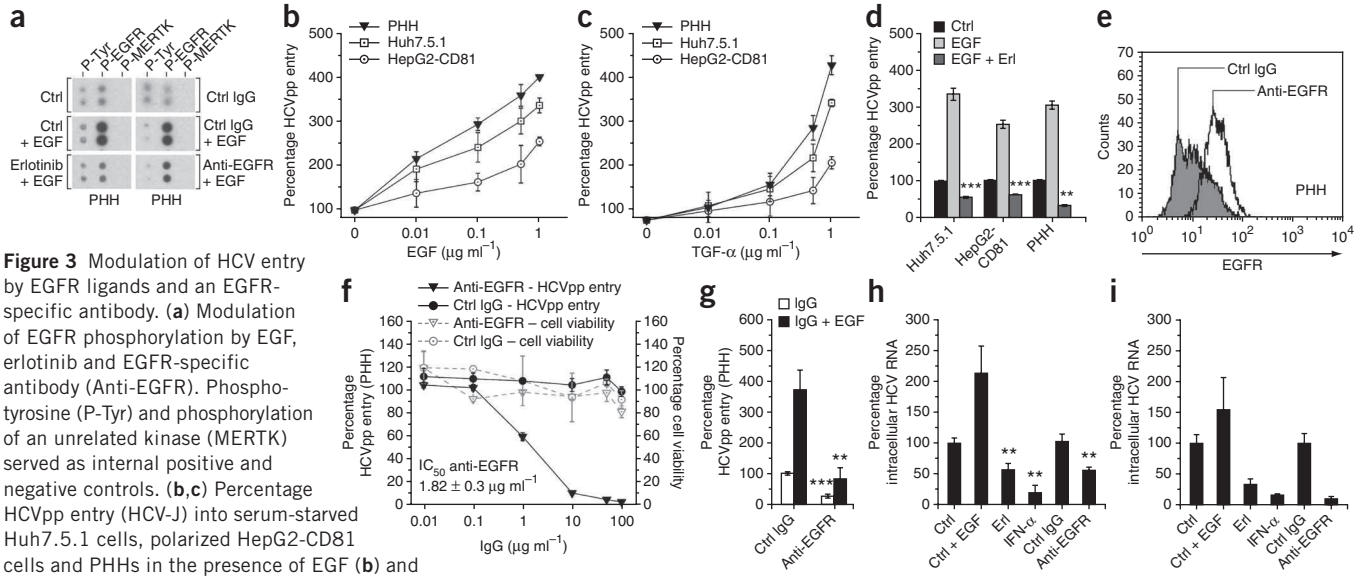

Figure 3 Modulation of HCV entry by EGFR ligands and an EGFR-specific antibody. (**a**) Modulation of EGFR phosphorylation by EGF, erlotinib and EGFR-specific antibody (Anti-EGFR). Phosphotyrosine (P-Tyr) and phosphorylation of an unrelated kinase (MERTK) served as internal positive and negative controls. (**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.

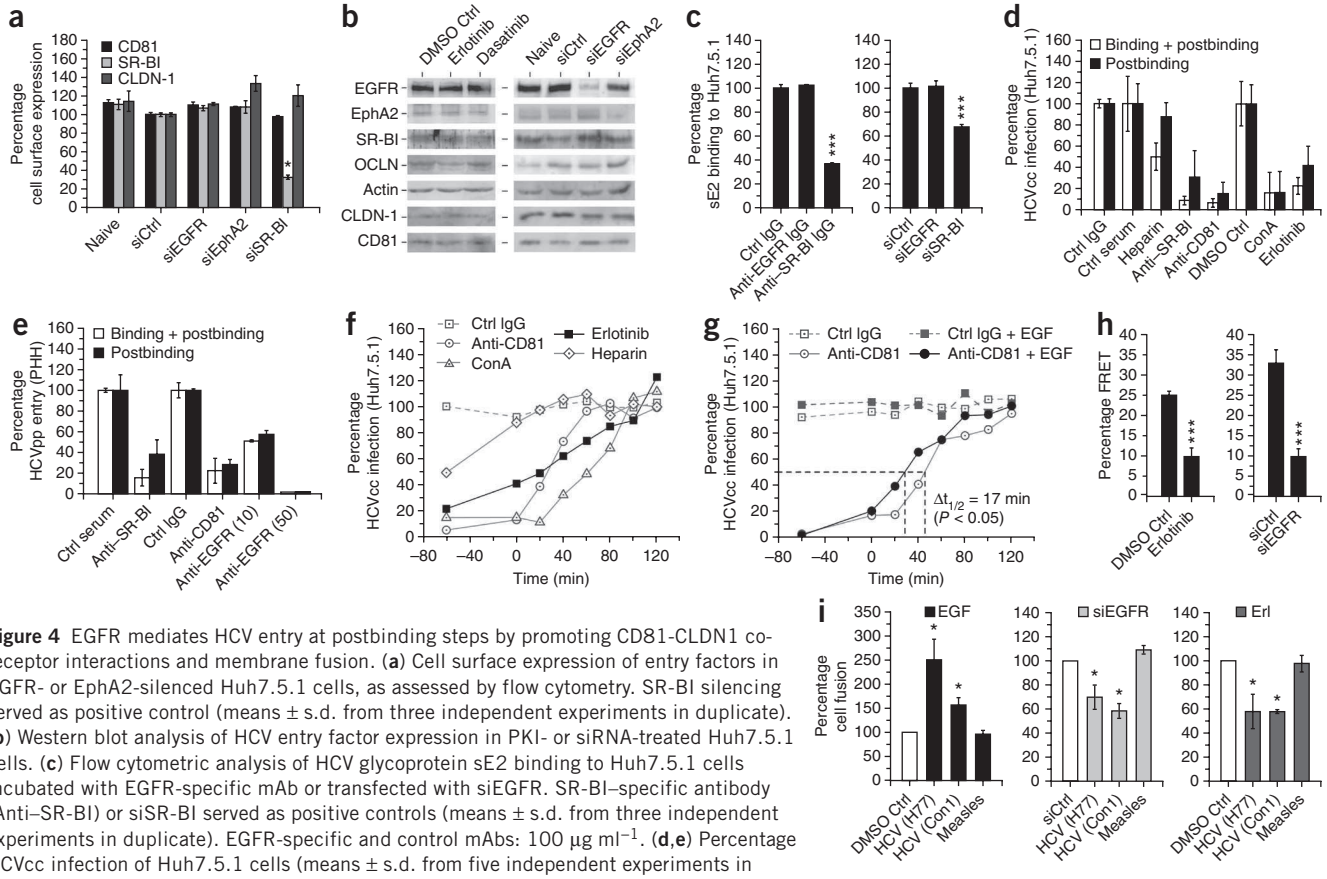

Figure 4 EGFR mediates HCV entry at postbinding steps by promoting CD81-CLDN1 co-receptor interactions and membrane fusion. (**a**) Cell surface expression of entry factors in EGFR- or EphA2-silenced Huh7.5.1 cells, as assessed by flow cytometry. SR-BI silencing served as positive control (means ± s.d. 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

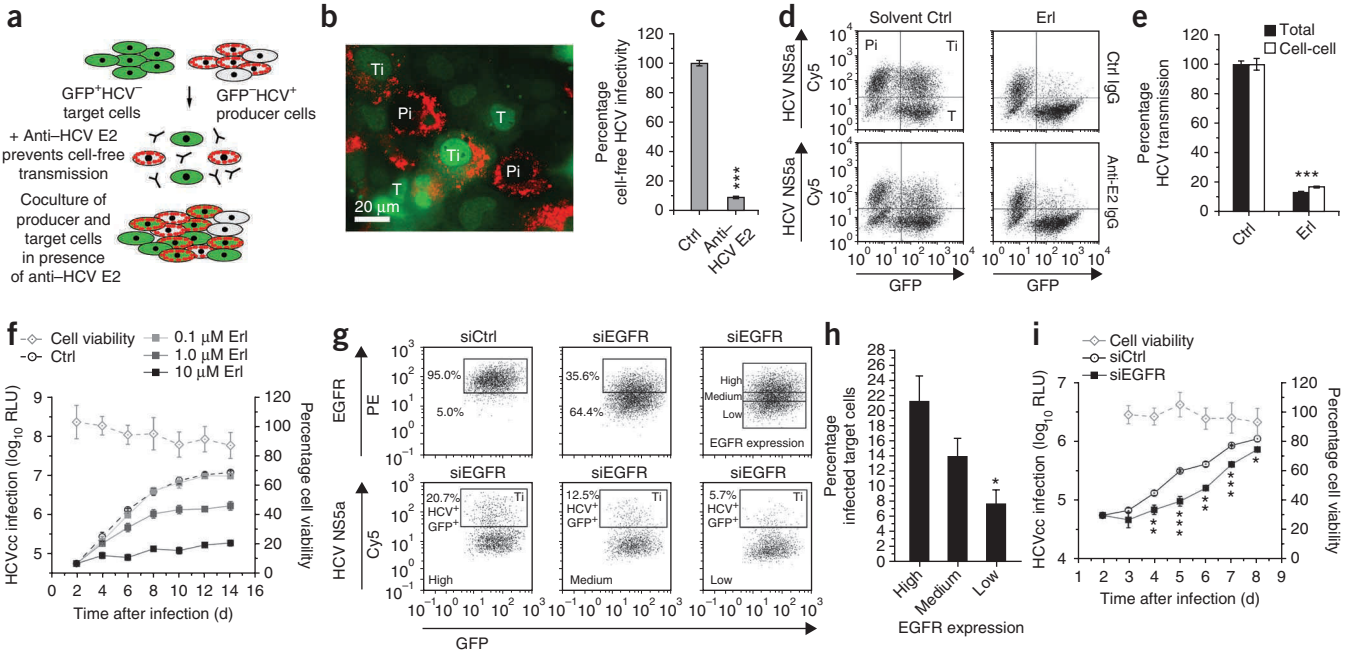

Figure 5 Functional role of EGFR in viral cell-to-cell transmission and spread. (**a**) Experimental setup. HCV producer cells cultured with uninfected target cells[26] were incubated with siEGFR or PKIs. Cell-free HCV transmission was blocked by an E2-neutralizing antibody (Anti–HCV E2, 25 µg ml^{-1})[26]. 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

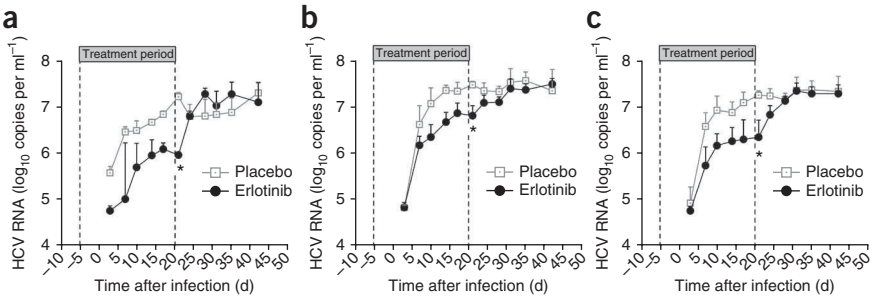

Figure 6 Erlotinib modulates HCV kinetics and inhibits infection *in vivo*. Chimeric uPA-SCID mice repopulated with PHHs[27,28] were treated with erlotinib or placebo during infection with human-derived HCV as indicated by the bar and dashed lines. Serum HCV load was analyzed at the timepoints indicated. 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.

ACKNOWLEDGMENTS

This work was supported by the European Research Council (ERC-2008-AdG-233130-HEPCENT), INTERREG-IV-Rhin Supérieur-FEDER-Hepato-Regio-Net 2009, Agence Nationale de la Recherche (ANR-05-CEXC-008), Agence Nationale de Recherche sur le Sida 2008/354, Région Alsace, Institut National du Cancer, the Institut de Génétique et de Biologie Moléculaire et Cellulaire, Institut National de la Santé et de la Recherche Médicale, Centre National de la Recherche Scientifique, Université de Strasbourg, the US National Insititutes of Health (1K08DE020139-01A1), the UK Medical Research Council and the Wellcome Trust. We acknowledge A.-L. Morand, L. Froidevaux, A. Weiss, L. Poidevin, S. Durand and E. Soulier for excellent technical work. We thank R. Bartenschlager (University of Heidelberg) for providing Jc1 and Luc-Jc1 expression vectors, J. Ball (University of Nottingham) for UKN2A.2.4, UKN3A1.28 and UKN4.21.16 expression vectors, T. Wakita (National Institute of Infectious Diseases, Japan) for JFH1 constructs, C.M. Rice (The Rockefeller University) for Huh7.5 cells, F.V. Chisari (The Scripps Research Institute) for Huh7.5.1 cells, E. Harlow (Harvard University) for CDC2 expression plasmids and M. Tanaka (Hamamatsu University) for EphA2 expression plasmids.

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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doi:10.1038/nm.2341

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

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SUPPLEMENTARY RESULTS

RNAi screen identifies kinases with functional impact on HCV entry. To identify host cell kinases involved in HCV entry, we performed a small interfering RNA (siRNA)–based screen silencing 691 human kinases and associated proteins in Huh7 hepatoma cells to comprehensively identify cellular kinases regulating HCV entry. 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) (**Supplementary Fig. 1**). False positive results due to toxicity were excluded using MTT–based cell viability test (**Supplementary Table 2**). To exclude non–specific off–target effects by siRNA pools, hits were validated using four individual siRNAs in a third screen (**Supplementary Fig. 1**). 106 kinases were identified by the primary HCVpp screen (**Supplementary Table 1**) and, of these, 95 were confirmed by infection with HCVcc. 58 of the 95 kinases passing the secondary screen were validated in the third screen (**Supplementary Table 2**).

The genome–wide RNAi kinase screen identified 58 kinases with impact on HCV entry and initiation of HCV infection (**Supplementary Table 2**). These kinases constituted 11.2% of the human kinome[1]. This percentage is in a similar range as previously published RNAi screens targeting human immunodeficiency virus (HIV) (4.3% of human kinome)[2] and West Nile Virus (6.4% of human kinome)[3]. Using a genome–wide RNAi screen, Li and co–workers identified 407 host genes required for HCVcc (JFH1) infection[4]. Among these hits were 14 kinases (2.7% of the human kinome); four of these kinases (29%) were confirmed in our screen. These kinases were CHKA, RYK, PTK2B and PI4KA. Thus, our screen identified 54 novel cellular kinases as host factors for HCV entry, including EGFR, EphA2, and CDC2, which had not been identified previously[4]. The relatively small overlap of hits in the two HCV RNAi screens is not unusual and has been observed for other RNAi screens identifying host factors for HIV infection[5]. Reasons include the use of different siRNA libraries (Qiagen vs. Dharmacon) and screening formats

Nature Medicine doi:10.1038/nm.2341

(96 wells vs. 384 wells). Our screen was performed in a 96–well screening format with a particular focus on avoiding microplate edge effects, which increased the robustness and reproducibility (Z–value). This allowed us to use a more advanced statistical approach for threshold considerations with respect to significance analysis and false discovery rate considerations of each observed hit (see **Supplementary Methods**). In contrast to the genome–wide screen which was limited to a detailed bioinformatic analysis of the identified hits[4], our study for the first time provides a comprehensive functional analysis of kinases required predominantly for HCV entry by assessing the effect of gene silencing on HCVpp and VSVpp entry and HCVcc infection. Most importantly, we identified EGFR and EphA2 as novel co–factors for HCV entry, elucidated their functional relevance within the HCV entry process and identified them as targets for antiviral therapy.

Bioinformatic analyses identify kinase networks involved in HCV entry. For a classification of the known biological functions of the identified 58 kinases, we performed a bioinformatic analysis using the Ingenuity Pathways database[3]. This analysis revealed a high representation of genes involved in cell death (58.6%), amino acid metabolism, post–translational modification and small molecule biochemistry (51.7%), cancer (50.0%), cellular growth and proliferation (44.8%), and cell cycle as listed by percent frequency (**Supplementary Fig. 2a**). When classifying kinases with an impact on HCV but not VSV entry (**Supplementary Table 2**), amino acid metabolism, post–translational modification and small molecule biochemistry (19%), cell death (19%), cell morphology and development (15.5%), cellular function and maintenance (15.5%), cellular function and organization (13.8%), and cell signaling (12.1%), emerged among the top six categories as listed by percent frequency (**Supplementary Fig. 2b**).

Next, the identified hits were analyzed for known and predicted protein interactions using the STRING database[6]. STRING represents a meta–database mapping of all known protein–protein interactions onto a common set of genomes and proteins[6]. Analysis of the 58 kinases identified in the RNAi screen 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 (**Supplementary Fig. 2c**). Key interactions were confirmed using the IntAct database[7]. However, STRING analysis resulted in the most detailed network, which is due to the fact that STRING contains the largest collection of data sets for protein–protein interactions. Furthermore, STRING provides detailed and integral quality–scores which are of great importance in keeping the rate of false positives as low as possible[6]. To further confirm the validity of the identified network, we randomly selected 1000 groups of 58 kinases (from a collective of 691 kinases and associated molecules) and compared the connectivity of these random collections to our identified network presented in **Supplementary Fig. 2**. The subsequent comparison revealed that none of the randomly chosen sets of 58 kinases shows a single instance (P<0.001) of our identified network or anything similar (matching at least 20% of the network in **Supplementary Fig. 2c**). Taken together, these results demonstrate that the network depicted in

Supplementary Fig. 2c is the result of extensive and reproducible bioinformatic analyses and highly distinct form a random product.

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*) (**Supplementary Fig. 2c**). Silencing by kinase–specific siRNAs reduced HCVpp entry by 4.39 fold for EGFR, 3.05 fold for EphA2, 14.96 fold for CDC2, 9.76 fold for CDK4 and 6.36 fold for CDK8 (**Supplementary Table 2**).

Cell cycle control and HCV entry. STRING analysis pointed to a network that included 12 kinases involved in cell cycle regulation (**Supplementary Fig. 2c** and **Supplementary Table 2**), including CDC2, CDK4, CDK8, cholin kinase alpha (CHKA), cholin kinase beta (CHKB), cyclin–dependent kinase inhibitor 1B (CDKN1B), CDC28 protein kinase regulatory subunit 1B (CKS1B), ataxia telangiectasia mutated protein (ATM), polo–like kinase 1 (PLK1), polo–like kinase 3 (PLK3), aurora kinase B (AURKB), inhibitor of kappa light polypeptide gene enhancer kinase B in B–cells (IKBKB). Although we cannot exclude the possibility that these molecules were identified because of intrinsic properties of the cell division–dependent hepatoma model system, several observations support a specific role of cyclin–dependent kinases (CDKs) for HCV entry: first, silencing of kinases potently inhibited HCVpp entry and HCV infection (**Supplementary Table 2** and **Supplementary Fig. 3a–d**). Second, after gene silencing (apart from experiments with CHKA and IKBKB), no cytotoxicity was observed as measured by the cellular metabolite MTT (**Supplementary Table 2**). This suggests that the silencing of kinases was not due to non–specific toxic effects. Third, silencing/rescue experiments further confirmed a functional role for CDC2 in HCV entry (**Supplementary Fig. 3e**). Fourth, *Flavopiridol* — a well–characterized inhibitor of CDKs– markedly inhibited HCVpp entry in the absence of any detectable cytotoxic effects in PHH (**Supplementary Fig. 3f**). These data suggest that the effect of CDKs is not related to either the model target cell line or the pseudoparticle entry assay, and is relevant to HCV entry. It is well known that CDKs play an important role in the life cycle of HIV and herpes viruses. These include regulation of HIV transcription by CDK9[8] and the activation by Kaposi sarcoma–associated herpes virus of CDK4 and CDK6 that regulate microfilament organization and cell morphology[9]. Thus, it is conceivable that similar mechanisms apply for HCV entry.

Kinases involved in integrin signaling and HCV entry. Furthermore, the screen identified kinases involved in cell adhesion and integrin signaling: focal adhesion kinase (PTK2), focal adhesion kinase 2 (PTK2B), and integrin–linked kinase (ILK), all of these kinases regulate cell adhesion and cell–matrix interaction[10,11] (**Supplementary Table 2**). It has been shown that CD81 — a key HCV entry factor — and other tetraspanins are associated with adhesion receptors of the integrin family and regulate integrin–dependent cell migration[12]. It is thus conceivable that

functional integrin signaling might be a prerequisite for HCV entry factor trafficking and localization on the cell surface — and therefore for HCV entry. In this context a number of tetraspanins, including CD81, are associated with type II phosphatidylinositol 4–kinase and it is suggested that this may facilitate the assembly of signaling complexes by tethering these enzymes to integrin heterodimers[12]. It is of interest to note that silencing of phosphatidylinositol 4–kinase type 2 alpha (PI4KII) impaired HCV entry and infection (**Supplementary Table 1**). Moreover, it is known that integrin signaling plays a pivotal role in the entry of other viruses such as adenovirus, hantavirus and herpesviruses (for review see[13]): HCV may therefore have another integrin–dependent entry mechanism.

EGFR and EphA2 do not mediate HCV entry by modulation of cell polarity. EphA2 and EGFR are involved in regulation of cell polarity[14-17] and polarization has been shown to restrict HCV entry[18,19]. Since blocking kinase function inhibited HCV entry, we investigated whether PKI treatment modulated HepG2 polarization. *Dasatinib* reduced HepG2 polarization and *Erlotinib* had no effect (**Supplementary Fig. 12a**). TJ integrity was not affected (**Supplementary Fig. 12b**). These data indicate that the marked inhibition of HCV entry cannot be explained by a PKI–induced decrease in polarization.

Modulation of HCV entry by EphA2–specific ligands and antibodies. Since EphA2 is constitutively active and its degradation is modulated by membrane–bound ligands during cell–cell contact[20-22], the investigation of ligand–induced activation of EphA2 in cell culture models is technically more complex. To address this question, we used soluble model ligands ephrin–A1 and –A3 which have been reported to mimic some but not all mechanisms of cell–cell contact[21]. Ephrin–A1 and –A3 have been shown to induce the degradation of cell surface EphA2[20,21]. The addition of ephrin–A1 or –A3 but not of control ligand resulted in a small but highly reproducible and significant (P<0.0005) decrease of HCV entry that was dependent on target cell density (**Supplementary Fig. 9a**). To further address the role of EphA2 ligand binding domain for HCV entry, we produced polyclonal antibodies to the EphA2 extracellular loop by genetic immunization. The polyclonal antibodies specifically bound to the native EphA2 extracellular domain as demonstrated by the specific binding of the antibodies to non–permeabilized BOSC and CHO cells transfected to express human EphA2 (**Supplementary Fig. 9b** and data not shown) and the HCV permissive hepatoma cell line Huh7.5.1 (**Supplementary Fig. 9c**). Pre–incubation of PHH with antibodies to EphA2 significantly (P<0.005) inhibited HCV entry, suggesting that engagement of the EphA2 extracellular domain contributes to its effect on HCV entry (**Supplementary Fig. 9d**).

Clinical approved PKIs inhibit entry of highly diverse HCV escape variants. *Erlotinib* and *Dasatinib* inhibited HCV infection at dose ranges (IC_{50} 0.45–0.53 μM) similar to mean plasma concentrations of patients during cancer treatment (*Dasatinib* ~0.2 μM; *Erlotinib* ~4 μM)[23-25]. Furthermore, *Flavopiridol* inhibited HCV entry (IC_{50} 0.005 μM) in concentrations well below

clinical use in cancer treatment (~2 μM[26]) (**Supplementary Fig. 3f**). Therefore, we further assessed the potential of PKIs as antivirals by investigating their impact on infection of 14 HCV strains isolated from 6 patients undergoing liver transplantation[27]. These variants re–infecting the liver graft ("escape variants") were characterized by high infectivity and marked resistance to autologous host neutralizing responses[27]. Pre–incubation of cells with approved kinase inhibitors markedly and significantly (P<0.0005) inhibited entry of HCV escape variants in PHH and Huh7.5.1 cells without decreasing cell viability (**Supplementary Fig. 6**, data not shown). In contrast, pre–incubation of cells with *Blebbistatin*, an unrelated small molecule inhibitor did not decrease entry of HCV isolates (**Supplementary Fig. 6**). These data demonstrate that *Erlotinib*, *Dasatinib* and *Flavopiridol* inhibit entry of highly infectious HCV escape variants, which are resistant to autologous neutralizing antibodies.

Clinical implications of identified RTKs for HCV pathogenesis and treatment. *In vivo*, expression of EphA2 and EGFR has been shown to be elevated in HCV–induced hepatocellular carcinoma (HCC)[28,29]. However, a detailed analysis of EGFR and EphA2 expression in the hepatocytes of HCV–infected patients *in vivo* is not yet available. TGF–α expression is elevated in the liver of HCC or chronically HCV infected patients[30]. In *in vitro* model systems, EGFR expression is increased in HCV infected cells[31]. Furthermore, HCV non–structural protein NS5A has been reported to alter EGFR trafficking[32] and HCV NS3/4A protease activates EGFR–induced signal transduction[33]. HCV core protein has been reported to enhance expression of TGF–α – an EGFR ligand[34]. Finally, HCV NS4B has been shown to enhance EphA2 expression[35]. Taken together, these findings suggest that HCV may not only use RTKs as co–factors for HCV entry but at the same time modulates their expression and function. Further studies are underway to investigate the relevance of virus–induced regulation of EGFR expression and signaling for HCV entry and pathogenesis of HCV infection (e.g. the presence of virus–induced positive feedback loops).

Furthermore, our results have important clinical implications for the prevention and treatment of HCV infection. PKIs and antibodies to RTKs are well established and approved drugs for cancer treatment and have a well characterized and manageable safety profile in humans[36,37]. Similar to standard of care or direct acting antivirals (DAAs) in development, the clinical use of these inhibitors is limited by adverse effects. Next generation EGFR kinase inhibitors with improved safety profiles[38] may address this limitation and thus are interesting antiviral candidates in the future. Although the antiviral potency of PKIs appears to be lower than DAAs targeting viral protein processing and replication, PKIs and monoclonal antibodies to RTKs are very attractive and clinically relevant antiviral compounds since they target complementary host factors required for viral infection. Indeed, there is increasing evidence that targeting essential host factors will increase the genetic barrier for viral resistance[39-41] and that ultimately a combination of complementary antivirals will be required to prevent antiviral resistance[41]. This concept is further supported by our results demonstrating that PKIs efficiently inhibit entry of escape viruses that are resistant to patients' immune responses (**Supplementary**

Fig. 6). Thus, the development of PKIs specifically targeting the proviral function of RTKs and the combination of PKIs with DAAs or standard of care may further increase their antiviral activity in eradicating HCV. Finally, a case report describing HCV clearance during *Erlotinib* treatment of a patient with metastatic hepatocellular carcinoma and chronic HCV infection provides further clinical evidence for an antiviral effect of PKIs *in vivo*[42].

SUPPLEMENTARY METHODS

Genome–wide RNAi kinase HCV entry screen. Screening was performed at the High Throughput Screening platform of the Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC) in Illkirch, France. The library used for this screen was the Human Kinase siRNA Set Version 2.0 (pool of four siRNAs) and individual siRNAs were obtained from Qiagen. A functional HCV entry siRNA screen targeting 691 cellular kinases and associated proteins was established as outlined (**Supplementary Fig. 1**). For each target 3.5 pmol siRNA was reverse transfected in 5,000 Huh7 cells 0.3 cm^{-2} using INTERFERin reagent (Polyplus). The effect of gene silencing on viral entry was investigated three days after siRNA transfection using HCVpp (H77; genotype 1a)[27,43] harboring a luciferase reporter gene. Impact on VSVpp entry was analyzed side–by–side. Virus entry was assessed two days after infection by measuring reporter gene luciferase activity in cell lysates using the Bright Glo Luciferase assay system (Promega) and a Mithras LB 940 luminometer (Berthold Technologies). Hits were validated independently using four different single siRNAs silencing the same target mRNA. Validation using HCVcc strain Luc–Jc1[44] ($TCID_{50}$ 10^3 mL^{-1}–10^4 mL^{-1}) was performed in Huh7.5.1 cells using the same protocol as described above. All siRNA screens were performed in 96–well cell culture microplates. Luciferase results were normalized by protein content of the lysates using DC Protein Assay (Bio–Rad). To minimize non–specific effects due to evaporation, outside wells were not used for the screens but were filled with phosphate buffered saline (PBS). Non–specific effects of gene silencing due to changes in cell proliferation were normalized by measuring the protein content of the individual well. The quality of the established high–throughput screens, the individual plate designs as well as the amount of replicates were assessed in pilot experiments by calculating the Z–factor[45]. The HCVpp screens (Z=0.37) were performed in duplicates with 60 of 96 central plate positions used for the screen. The HCVcc validation screens (Z=0.47) were performed in triplicates with 32 of 96 central plate positions used for the screen. As an internal quality control of gene silencing and HCVpp and HCVcc infection, positive and negative control siRNAs (targeting CD81 and GFP, respectively) were transfected side–by–side on each plate. Cytotoxic effects on cells were assessed in triplicates by analyzing the ability to metabolize 3–(4,5–dimethylthiazol–2–yl)–2,5–diphenyltetrazolium bromide (MTT) as described (**Methods**).

Statistical analysis: hit selection. The impact of gene silencing was defined by an increase or decrease of HCVpp entry expressed as the ratio of entry compared to the experimental mean value of entry into control transfected cells (siRNA targeting GFP). The local false discovery rates (fdr) for all comparisons for each gene were determined using the library "fdrtool"[46]. Resulting fdr–value were examined for their distribution in order to define meaningful cut–offs (data not shown). Finally, for HCVpp a threshold of fdr<0.001 (corresponding to a maximum p–value of 1.04×10^{-4}), for HCVcc a threshold of fdr<0.05 (corresponding to a maximum p–value of 2.05×10^{-2}) and for VSVpp a threshold of fdr<0.08 (corresponding to a maximum p–value of 8.03×10^{-2}) were chosen as stringent parameters based on the underlying frequency distributions

(data not shown). All candidates chosen for further validation are highlighted in **Supplementary Table 1**. To address potential off–target effects by pooled siRNAs, candidate genes were validated if HCV entry was reduced ≥50% compared to control transfected cells by at least two individual siRNAs (**Supplementary Table 2**).

Gene ontology and gene annotation. Gene ontology terms and gene associations were obtained from Human Kinase siRNA Set Version 2.0 validated by Ingenuity Pathways database (Mountainview, CA, USA). Biological function analysis of the identified kinases was performed using Ingenuity Pathways database[3]. Biological function terms were accepted if they were significantly enriched with a p–value $<10^{-5}$ as calculated by Ingenuity Pathways database. Additionally, the identified hits were analyzed for known and predicted protein interactions using STRING meta–database that maps all interaction evidence onto a common set of genomes and proteins[6]. The interactions addressed include direct (physical) and indirect (functional) associations derived from numerous sources, including experimental repositories, computational prediction methods and public text collections[6].

HCV strains for production of HCVpp and HCVcc. HCVpp from strains H77, HCV–J, JFH1, J6, UKN2A.2.4, UKN3A1.28, UKN4.21.16, P01VL, P02VH, P02VI, P02VJ, P03VC, P04VC, P04VD, P04VE, P05VD, P05VE, P05VF, P06VG, P06VH, P06VI[27,43,47] and HCVcc[44,48] (strains JFH1, Jc1, Luc–Jc1) were produced as described.

siRNAs and expression plasmids used for rescue experiments and functional studies. siEGFR si3 (Hs–EGFR_6, 5'–CAUCCAAUUUAUCAAGGAATT–3') and si4 (Hs–EGFR_12, 5'–GGAACUGGAUAUUCUGAAATT–3'), siEphA2 si4 (Hs–EPHA2_8, 5'–GGACAGACAU AUAGGAUAUTT–3'), siCDC2 (Hs–CDC2_14, 5'–GGUUAUAUCUCAUCUUUGATT–3') were obtained from Qiagen. siCTRL, siCD81, siSR–BI have been described[48]. Lentiviral expression plasmids pLKO–shEGFR[49], pWPI–EGFRWT[49], pEGFR–L858R[50], and expression plasmid pEphA2–WT[51] and pCDC2–WT (Addgene plasmid 1886)[52] have been described.

Protein kinase inhibitors, ligands and antibodies. *Erlotinib*, *Gefitinib*, *Lapatinib* and *Dasatinib* were obtained from IC Laboratories, *Flavopiridol* and Concanamycin A from Sigma, BILN–2061 from Boehringer Ingelheim and IFN–α–2a from Roche. All other small molecules and DMSO (used at a final concentration 0.7% for incubation of PKIs and control experiments) were obtained from Merck. Recombinant EGF and TGF–α were obtained from Sigma and soluble tagged ephrin ligands and tag controls from R&D. Production and purification of soluble His–tagged HCV E2 glycoprotein has been described[53]. Antibodies to EGFR (528), CD81 (5A6), EphA2 (C–20), and occludin (H–279) were obtained from Santa Cruz; antibody to E2 (AP33) from Genentech, antibody to EGFR (LA–1) from Millipore; antibody to NS5A from Virostat; antibody to CD81 (JS81) from BD; antibody to CLDN1 (1C5–D9) from Abnova; antibody to occludin (OC–3F10) from Zymed; antibody to actin (EP1123Y) from Abcam; antibody to CDC2 from Cell Signaling; antibody to His_5 from Qiagen, PE–conjugated antibody to mouse and Cy3–

conjugated antibody to mouse from Jackson ImmunoResearch, alkaline–phosphatase (AP)–labeled secondary antibodies from GE Healthcare. Polyclonal antibody to human EphA2 was raised by genetic immunization of Wistar rats by an expression vector containing the full–length human EphA2 cDNA as described previously for CLDN1-specific antibodies[54]. Polyclonal SR–BI–specific antibodies used for E2binding and postbinding experiments has been described[48,55].

Cell lines and primary hepatocytes. The sources and culture conditions for BOSC, CHO, 293T, Huh7, Huh7.5, Huh7.5.1 and HepG2–CD81 cells have been described[19,43,56,57]. Primary human hepatocytes (PHH) were isolated and cultured as described[54]. The mouse hepatoma cell line AML12 (#CRL–2254) was obtained from ATCC. The AML12 4R cell line was created by lentiviral gene transfer using lentiviruses transducing individual human CD81, SR–BI, CLDN1 and OCLN genes and subsequent selection of transduced cells with blasticidin and G418 as described previously[58]. The AML12 4R–hEGFR+ cell line was created by lentiviral gene transfer using vector pEGFR–L858R transducing active human EGFR[50].

HCV infection of primary hepatocytes. One day following PHH isolation and plating, PHH were washed with PBS and pre–incubated in the presence or absence of EGF, TGF–α, PKIs, EGFR–specific antibody or EphA2–specific serum for 1 h at 37 °C in William's E medium. Then, HIV–based HCVpp (J6)[54], HCVcc (J6–JFH1; Jc1, genotype 2a/2a, $TCID_{50}$: 10^5 mL^{-1}–10^6 mL^{-1})[59] or serum–derived HCV (HCV–positive infectious serum, genotype 1b described in ref.[40]) were added for 4 h at 37 °C. Following infection, fresh medium was added. HCVpp entry was assessed by measurement of luciferase activity 72 h postinfection as described[27,54]. HCVcc and infection with serum–derived HCV were assessed by HCV–specific qRT–PCR of purified intracellular HCV RNA as described[40].

Analyses of mRNA and protein expression. Cellular mRNA was extracted using RNeasy extraction kit (Qiagen) and quantified by qRT–PCR using Fastlane Cell Sybr Green kit (Qiagen)[48]. Western blots of cell lysates using protein–specific antibodies was performed following GE Healthcare protocols using Hybond–P membranes and visualized using ECF substrate and Typhoon Trio high performance fluorescence scanner (GE Healthcare). Immunostaining of HCV–infected cells was performed as described[60]. Kinase expression in liver was further confirmed by GeneAtlas database (BioGPS, Novartis Research Foundation, http://biogps.gnf.org) and is indicated (**Supplementary Table 2**).

Analysis of IC_{50} for PKIs and EGFR–specific antibody. IC_{50} was derived by logistic regression[61] using OriginPro (OriginLabs). IC_{50} values are expressed as median of three independent experiments ± standard error of the median.

Analysis of HCV postbinding steps and entry kinetics. HCV postbinding steps and entry kinetics were investigated as described[48,54]. Briefly, HCVcc (Luc–Jc1; genotype 2a/2a) binding to

Huh7.5.1 cells or HCVpp (P01VL, genotype 1b) binding to PHH was performed for 1 h at 4 °C in the presence or absence of heparin (250 μg mL^{-1}), control or anti–CD81 (5 and 10 μg mL^{-1}) and anti–EGFR IgG (10 and 50 μg mL^{-1}), anti–SR–BI or control serum (1:50), DMSO (0.7%) or PKIs (10 μM), Concanamycin A (ConA, 25 nM), before cells were washed and incubated with the indicated compounds for 4 h at 37 °C. HCVcc infection and HCVpp entry were assessed by luciferase reporter gene assay and expressed relative to control infections without addition of inhibitors as described[48,54,62,63]. For the study of HCV entry kinetics, compounds were added every 20 min for up to 120 min after viral binding. To assess the effect of EGF on HCVcc entry kinetics, serum–starved Huh7.5.1 cells were pre–incubated in serum–free medium in the presence or absence of EGF (1 μg mL^{-1}) prior to HCVcc binding and entry in the presence or absence of EGF (1 μg mL^{-1}). Complete medium supplemented with EGF (1 μg mL^{-1}) was added after the 4 h incubation period at 37 °C for 48 h.

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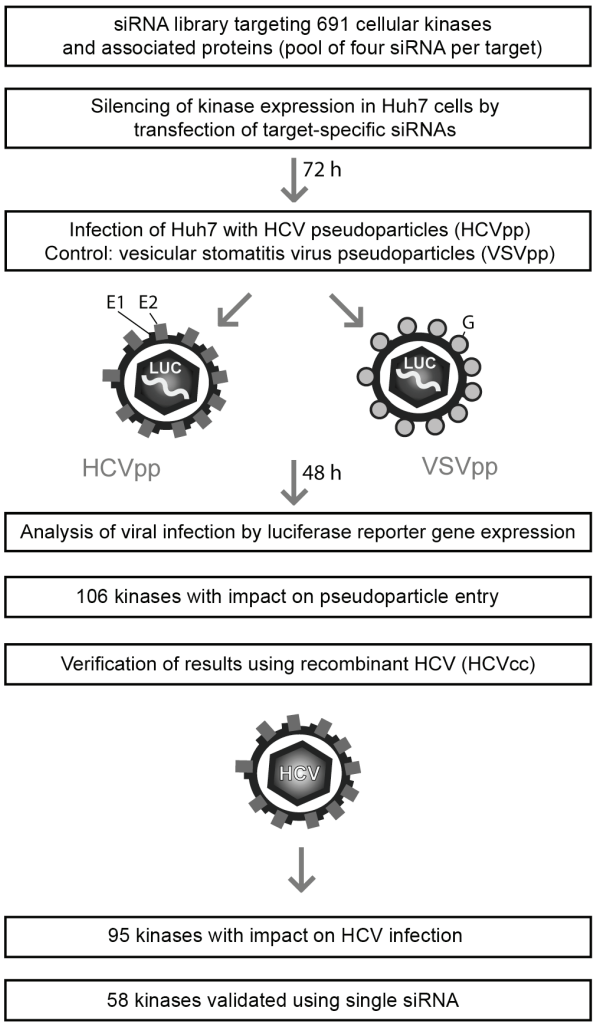
SUPPLEMENTARY TABLE LEGENDS

The contents of **Supplementary Tables 1** and **2** are provided as datasets in the online supplementary information.

Supplementary Table 1. Effect of silencing of 691 cellular kinases and associated proteins on HCVpp and VSVpp entry (primary screen). Using the Human Kinase RNAi Set Version 2.0 (pool of four siRNAs, Qiagen) and the Huh7–HCVpp system as a high–throughput model system for HCV entry we determined the impact of kinase gene silencing on entry of HCVpp (H77; genotype 1a) and VSVpp. Results are expressed as fold change of particle entry caused by gene silencing compared to particle entry into control siRNA–transfected cells (fold change = –1/fold infection, if the entry was reduced after kinase silencing; e.g. 50% decreased particle entry equals a fold infection of 0.5 and fold change of –2; e.g. 50% increased particle entry equals a fold infection of 1.5 and fold change of 1.5). Local false discovery rates (fdr) for each gene were determined using fdr analysis. Fdr threshold for hit selection (HCVpp entry) was <0.001, fdr threshold for HCV specificity (VSVpp entry) was <0.08. We identified a panel of 106 cellular kinases chosen for further validation (highlighted in yellow). Silencing of 42 kinases only decreased HCVpp infection (column J "HCV") but did not inhibit entry of VSVpp. Silencing of 64 kinases inhibited both HCVpp and VSVpp entry (column J "HCV+VSV"). For verification, we re–analyzed the screening results using ≥2 s.d. from the plate mean as measure of threshold for hit selection (average measure) (the analysis strategy used by Brass et al.[2]). Using this method at least the same kinases would have been identified for subsequent validation screening (column K).

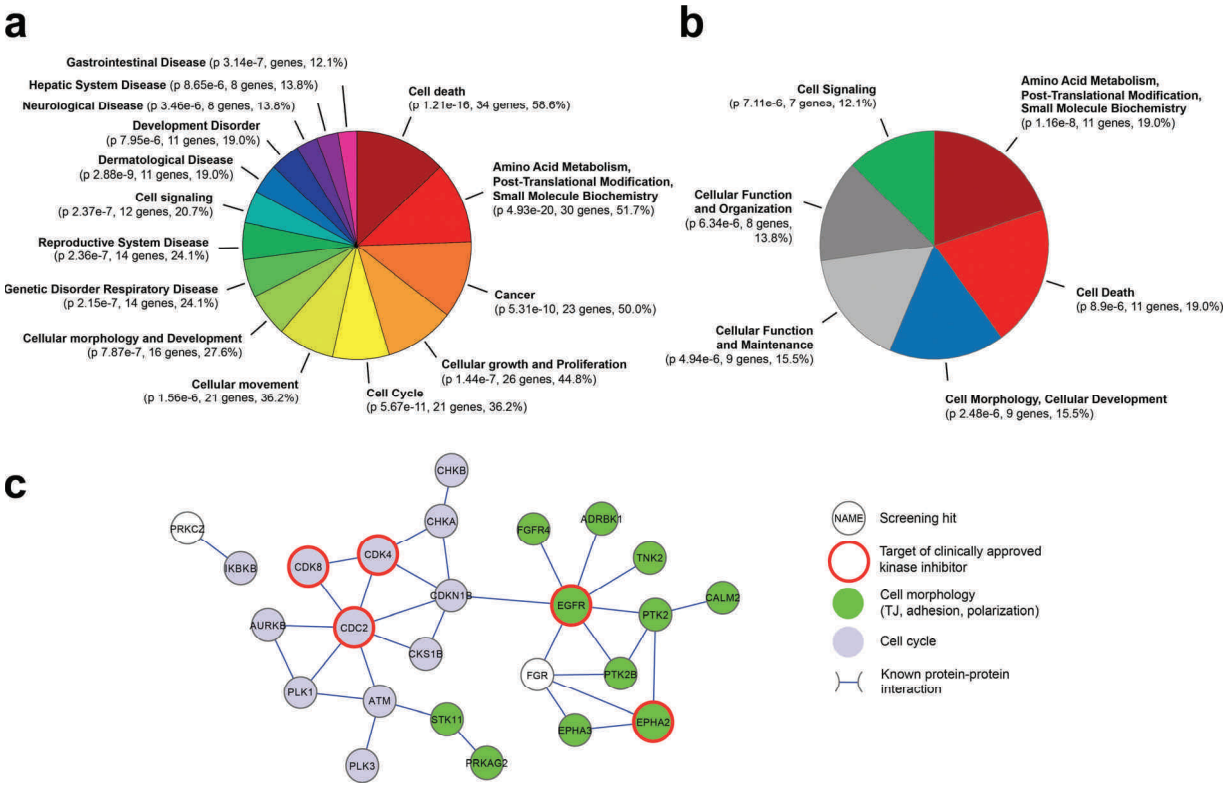
Supplementary Table 2. Cellular kinases modulating HCV entry identified by the RNAi kinase screen. Using the Human Kinase RNAi Set Version 2.0 (Qiagen), four individual siRNAs per target, we determined the impact of gene silencing on HCVpp, VSVpp and HCVcc infection and on cell viability (MTT). Results are expressed as fold change of pseudoparticle entry in cells with silenced kinase expression compared to control siRNA–transfected cells (fold change = –1/fold infection, if the entry was reduced after kinase silencing; e. g. 50% decreased particle entry equals a fold infection of 0.5 and fold change of –2; e. g. 50% increased particle entry equals a fold infection of 1.5 and fold change of 1.5). Local false discovery rates (fdr) and p–values for each gene were determined using fdr analysis. Following statistical analysis of the results from the primary and secondary screens and validation with individual siRNAs, we identified a panel of 58 cellular kinases exhibiting a significant (**Supplementary Methods**) impact on HCV entry and HCVcc infection that were validated by at least 2 of four individual siRNA. 18 kinases had an impact on HCV entry but not on entry of VSV (highlighted in blue). Kinase expression in liver was confirmed by GeneAtlas database (BioGPS, Novartis Research Foundation, http://biogps.gnf.org) and indicated in column N: relative gene expression is + = lower, ++ = higher, +++ = 3 fold higher, ++++ = 10 fold higher than the median expression in all investigated tissues (>60).

SUPPLEMENTARY FIGURES

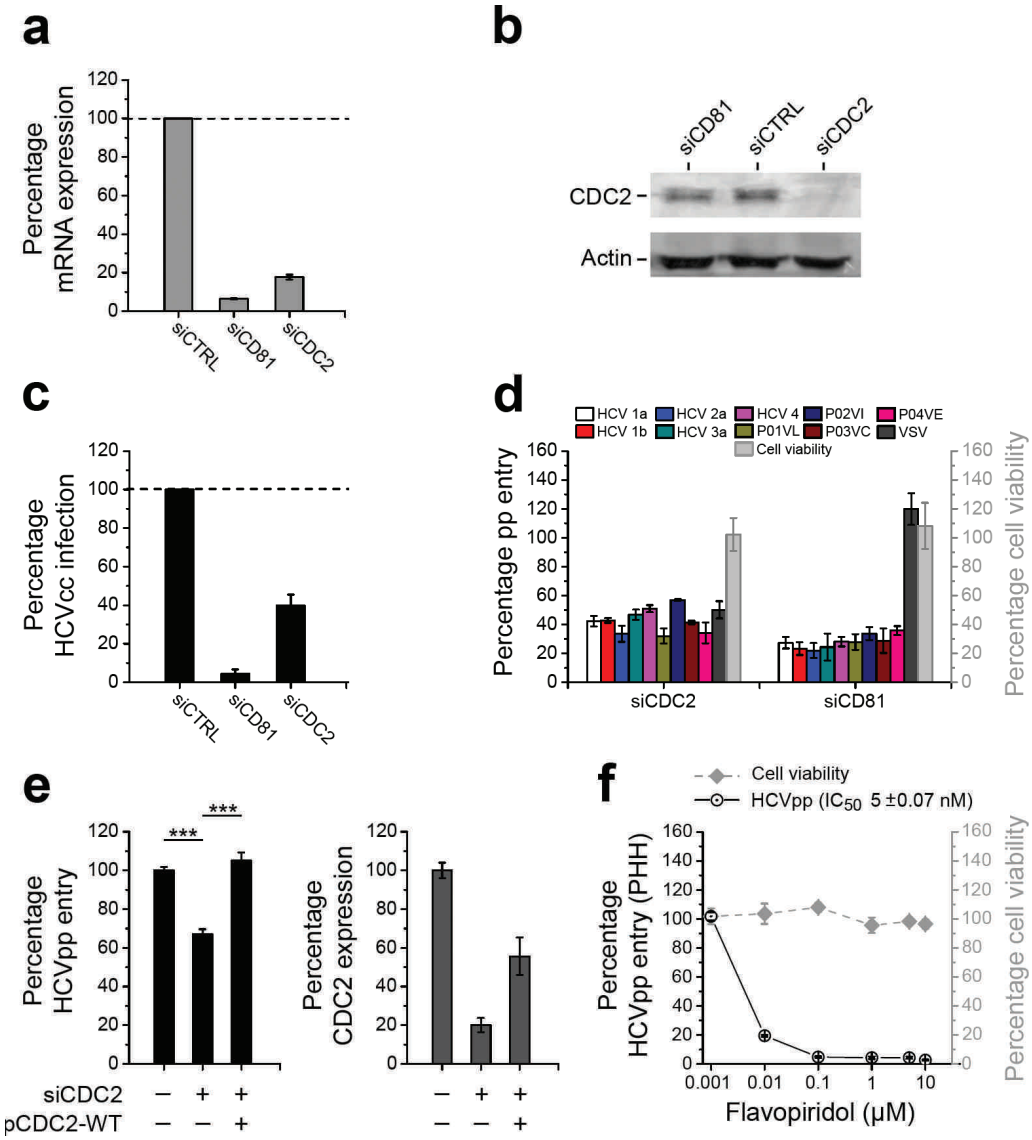


Supplementary Fig. 1. Schematic outline of the functional RNAi HCVpp entry screen used to identify HCV entry factors. Protein kinase expression was silenced in Huh7 hepatoma cells by target–specific siRNAs. Retroviral HCV pseudotyped particles (HCVpp) bearing HCV envelope glycoproteins (E1, E2) on their viral surface and harboring a luciferase reporter gene were used to analyze the impact of gene silencing on HCV entry into Huh7 cells. A decrease or increase in luciferase expression compared to the control siRNA–transfected cells indicated modulation of HCVpp entry by the corresponding target gene or genes. The effects of gene silencing on the infection of vesicular stomatitis virus derived pseudoparticles (VSVpp) were studied in side–by–side experiments. A RNAi library consisting of 691 siRNA pools was used to screen for cellular kinases and associated proteins with impact on entry of HCVpp. 106 candidates were identified in the primary RNAi screen. Pertinence to the infectious viral life cycle was verified for 95 candidate genes using recombinant HCVcc. 58 cellular kinases were validated by at least 2 individual siRNAs, thereby minimizing false positive hit selection due to off–target effects.

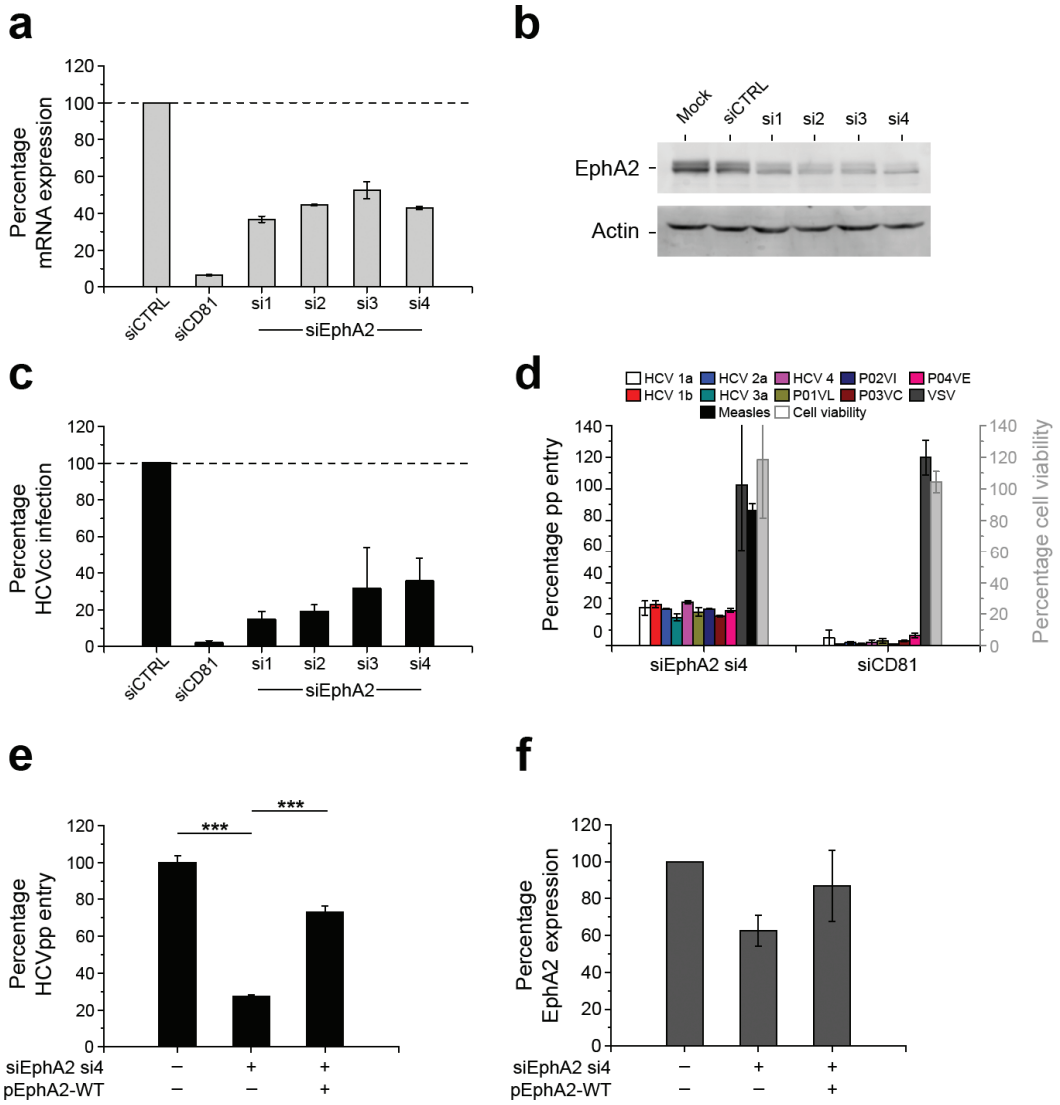
Nature Medicine doi:10.1038/nm.2341



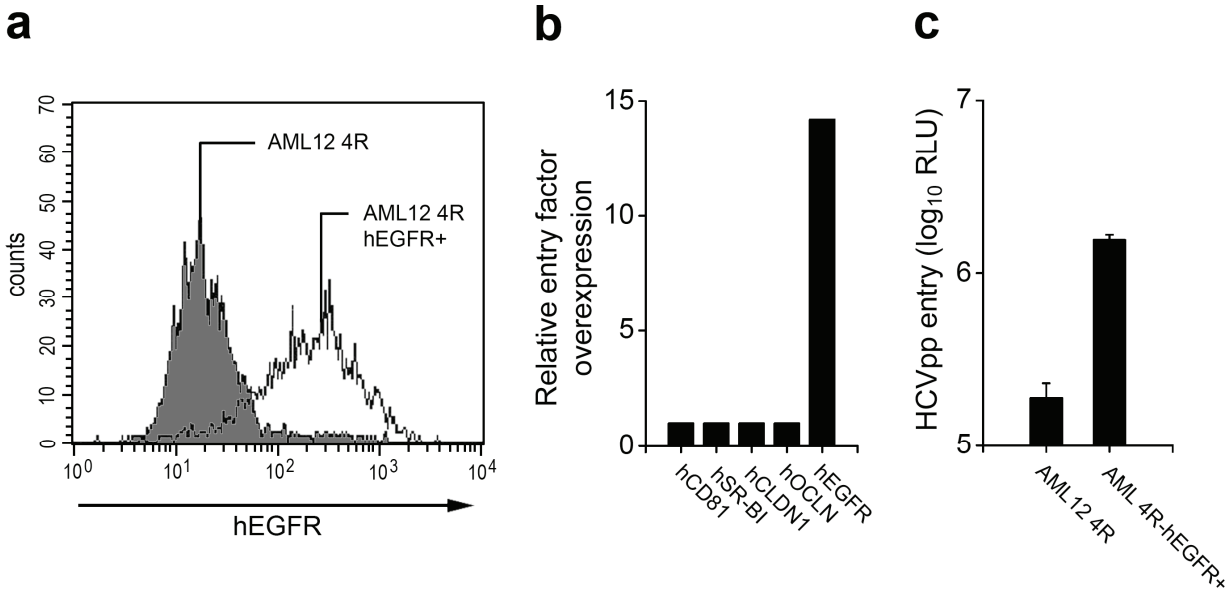
Supplementary Fig. 2. Biological process and protein association network analyses of the identified cellular kinases with marked impact on HCV entry. (**a**) The 58 identified cellular kinases involved in HCV entry and (**b**) the 18 identified kinases with impact on HCV but not on VSV entry were analyzed using the Ingenuity Pathways database. This analysis identified terms with the most prevalent biological processes associated with the identified candidate kinases within an organism (threshold $P<10^5$). The most significant terms of biological function were listed in the order of percentage frequency. (**c**) Protein association network of the 58 kinases involved in HCV entry identified by STRING analysis. Lines connecting kinases show direct (physical) and indirect (functional) associations derived from numerous sources, including experimental repositories, computational prediction methods and public text collections[6]. Kinases targeted by clinical licensed PKIs (red circles), kinases involved in the regulation of cell morphology including tight junctions, adhesion, cell polarity (green), cell cycle progression (blue) are highlighted.



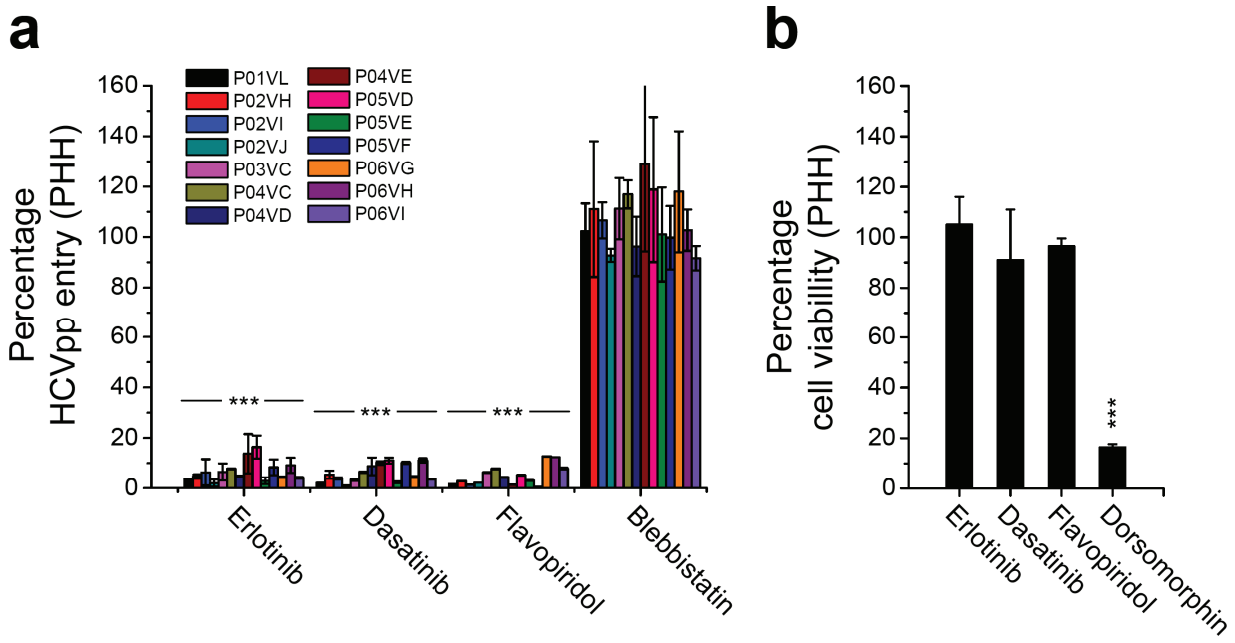
Supplementary Fig. 3. Cell division cycle 2 kinase (CDC2) is a co–factor for HCV entry. (**a**) Silencing of CDC2 expression in HCV permissive cells. **(a)** CDC2 mRNA (quantification by qRT–PCR relative to GAPDH mRNA) and (**b**) protein expression (Western blot) in Huh7.5.1 cells transfected with an individual CDC2–specific siRNA compared to control siRNA (siCTRL) is shown. Silencing of CD81 expression by CD81–specific siRNA served as control. (**c**) HCVcc infection in Huh7.5.1 cells with silenced CDC2 expression (as shown in panels **a,b**). (**d**) Entry of HCVpp containing envelope glycoproteins of various isolates[27,64] in Huh7.5.1 cells with silenced CDC2 expression (as shown in panels **a,b**). Data are expressed as percent pp entry relative to siCTRL–transfected cells. Cell viability in siCDC2–treated cells assessed using MTT test is shown as mean ± SEM. (**e**) Rescue of HCV entry in cells with silenced CDC2 expression by exogenous CDC2. HCVpp entry and CDC2 protein expression in Huh7.5.1 cells co–transfected with CDC2–specific siRNA and cDNA encoding for RNAi–resistant CDC2 is shown. Different transfection protocols (poly–cationic transfection (**a–d)** versus electroporation (**e**) are responsible for the apparent differences in Hs–CDC2_14–siRNA efficacy on reducing HCVpp entry. Protein expression was quantified using Image Quant analysis of Western blots. Data are expressed as percentage HCVpp entry relative to CTRL cells or as percentage CDC2 expression normalized for ß–actin expression (means ± SEM). *** P<0.0005. (**f**) Effect of *Flavopiriol*, a CDC2–inhibitor, on HCVpp entry into PHH. Cell viability was assessed using MTT assay.



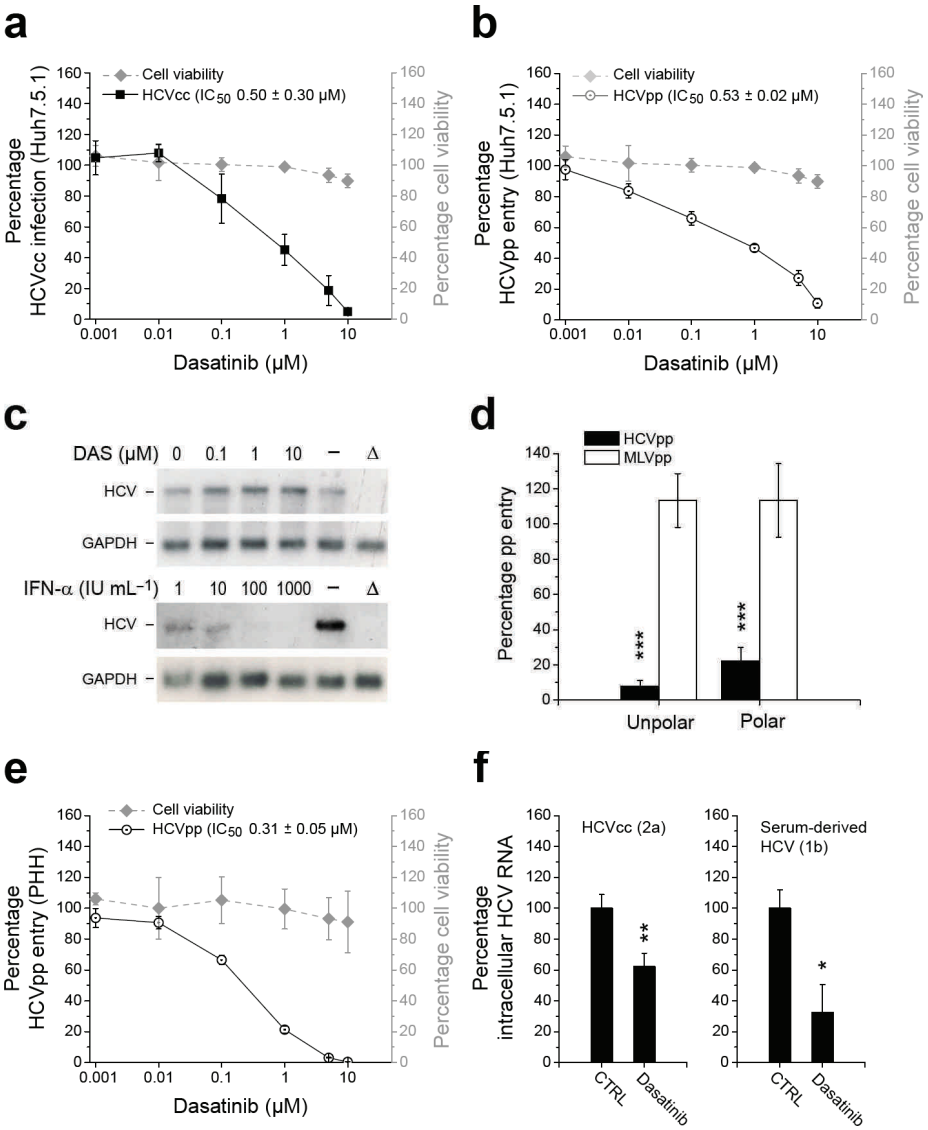
Supplementary Fig. 4. Ephrin receptor A2 (EphA2) is a co–factor for HCV entry. (a,b) Silencing of EphA2 expression in HCV permissive cells. **(a)** EphA2 mRNA (qRT–PCR analysis) and **(b)** protein expression (Western blot) in Huh7.5.1 cells transfected with EphA2–specific individual siRNAs (si1–si4). Silencing of CD81 mRNA expression by CD81–specific siRNA served as control. EphA2 mRNA (relative to GAPDH mRNA) and protein expression compared to cells transfected with control siRNA (siCTRL) is shown. **(c,d)** Inhibition of HCV infection and entry in cells with silenced EphA2 expression. **(c)** HCVcc infection in Huh7.5.1 cells transfected with individual siRNAs shown in panels **a,b**. siCTRL and CD81–specific siRNA served as internal controls. Data are expressed as percent HCVcc infection relative to siCTRL–transfected cells. **(d)** Entry of HCVpp containing envelope glycoproteins of various isolates in Huh7.5.1 cells transfected with siRNA si4. Analysis of VSV and measles virus pp entry or cells transfected with siCD81 served as controls. Data are expressed as percentage pp entry relative to siCTRL–transfected cells. **(d,e)** Rescue of HCV entry in cells with silenced EphA2 expression by exogenous EphA2. HCVpp entry **(e)** and EphA2 protein expression **(f)** in Huh7.5.1 cells co–transfected with EphA2–specific individual siRNA si4 and a cDNA encoding for siRNA si4–resistant EphA2 (pEphA2–WT)[51]. Protein expression was quantified using Image Quant analysis of Western blots. Data are expressed as percentage HCVpp entry relative to CTRL cells or as percentage EphA2 expression normalized for ß–actin expression. *** P<0.0005.



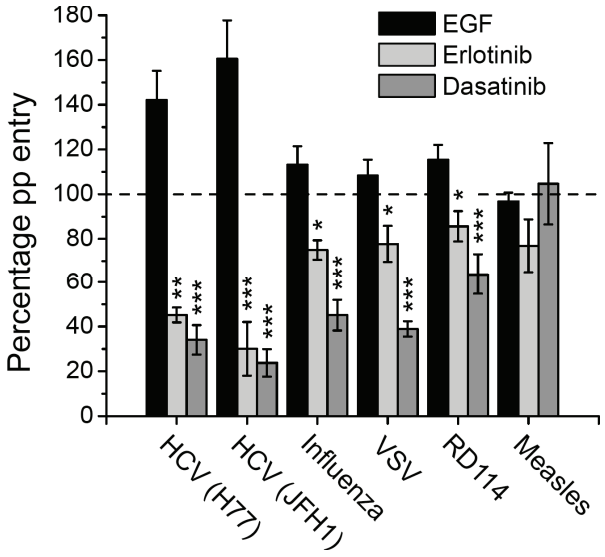
Supplementary Fig. 5. Expression of human EGFR enhances HCV entry into HCVpp permissive mouse hepatoma cells expressing human entry factors. Mouse hepatoma AML12 4R cells stably expressing human entry factors CD81, SR–BI, CLDN1, and OCLN were transduced with lentiviruses expressing human EGFR–L858R (hEGFR)[50]. Cell surface hEGFR expression (**a**) assessed by flow cytometry and (**b**) human CD81, SR–BI, CLDN1, OCLN and EGFR expression shown as fold expression relative to mock transduced AML12 4R cells. (**c**) HCVpp entry into mouse hepatoma cells engineered to express human EGFR. HCVpp entry was quantified in AML12 4R and AML12 4R–hEGFR+ cells in side–by–side experiments. Results are expressed as relative light units (RLU).



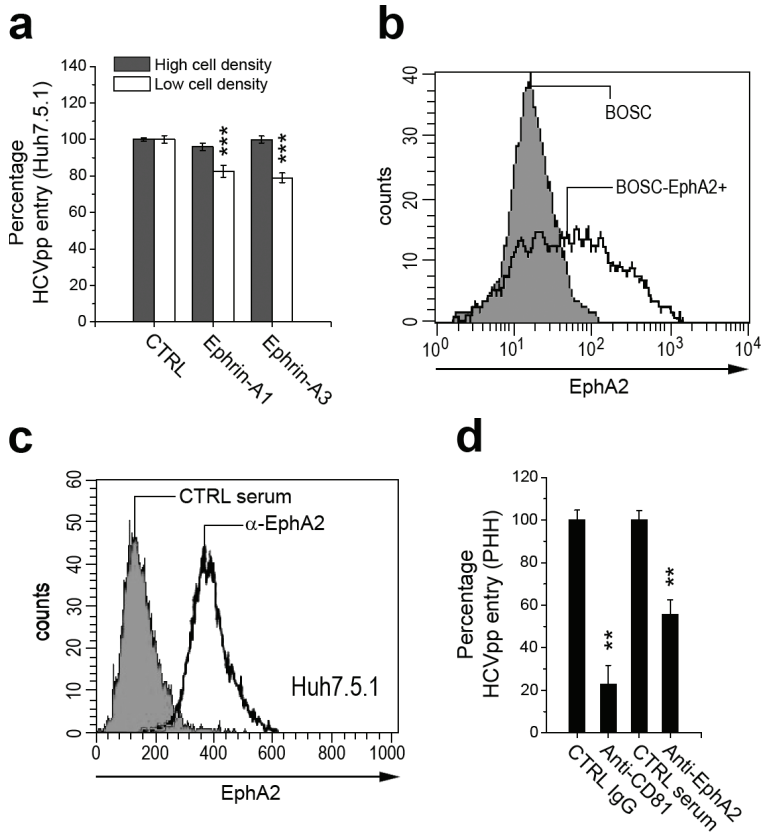
Supplementary Fig. 6. *Erlotinib*, *Dasatinib,* and *Flavopiridol* inhibit entry of HCV escape variants which are resistant to autologous host immune responses. HIV–based HCVpp bearing envelope glycoproteins from HCV escape variants P01VL, P02VH, P02VI, P02VJ, P03VC, P04VC, P04VD, P04VE, P05VD, P05VE, P05VF, P06VG, P06VH, P06VI isolated during liver transplantation from six different HCV–infected patients[27] were produced as described (**Supplementary Methods**)[27]. (**a**) Effect of *Dasatinib*, *Erlotinib,* or *Flavopiridol* on entry of HCV escape variants. HCVpp entry into PHH pre-incubated with PKIs (10 μM) is shown. Incubation of cells with *Blebbistatin* (10 μM) served as negative control. (**b**) Analysis of cell viability in PKI–treated cells. Cell viability was assessed by MTT assay. Incubation with *Dorsomorphin* (10 μM), an unrelated PKI, served as positive control for cell viability. ***, $P<0.0005$.



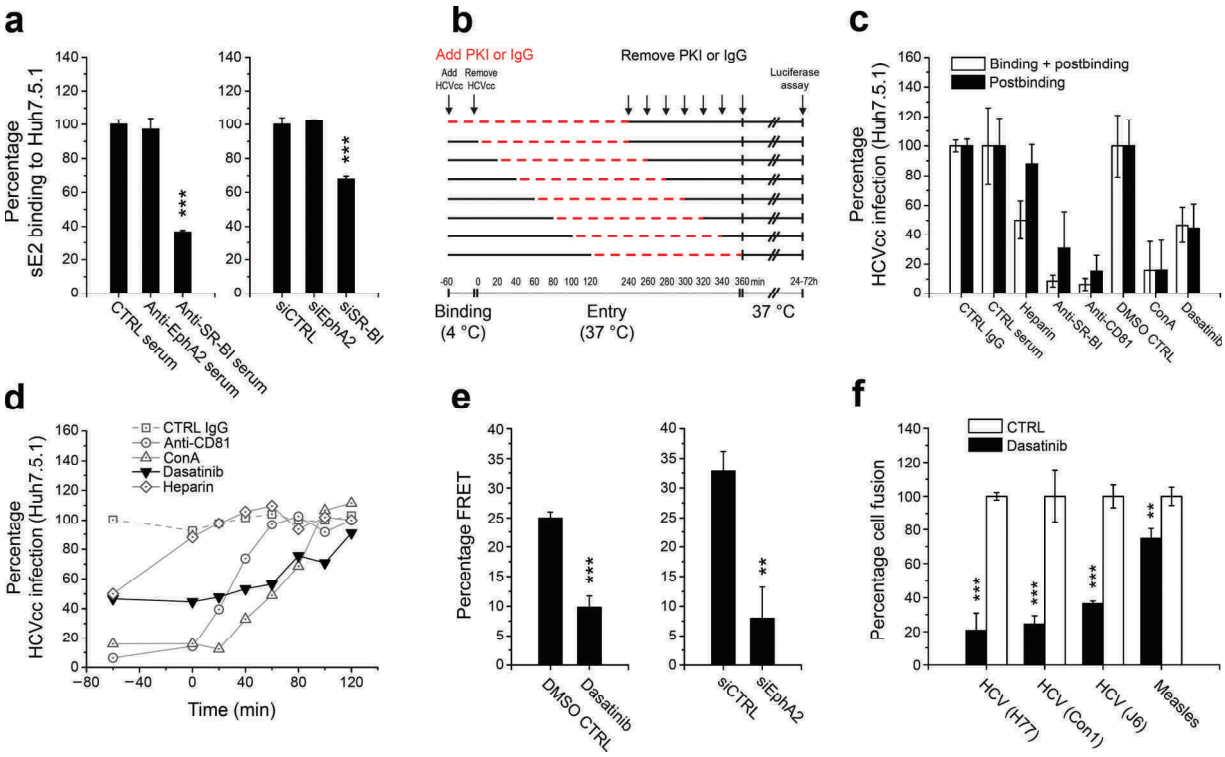
Supplementary Fig. 7. Dose–dependent inhibition of HCV entry and infection by *Dasatinib* – a clinically approved inhibitor of EphA2 kinase. (**a,b**) Effect of *Dasatinib* on HCVpp entry and HCVcc infection. (**a**) HCVcc infection and (**b**) HCVpp entry in *Dasatinib*– or CTRL–treated Huh7.5.1 cells was assessed as described[27,40]. Cell viability was assessed using MTT assay. IC_{50} values are expressed as median of three independent experiments ± standard error of the median. (**c**) Effect of *Dasatinib* on HCV replication. Following electroporation with HCV RNA from the subgenomic HCV JFH1 replicon or replication incompetent HCV RNA (GND, Δ) Huh7.5 cells were incubated with solvent CTRL, *Dasatinib,* or interferon–α (IFN–α–2a) at the indicated concentrations. HCV RNA and GAPDH mRNA were analyzed by Northern blot. (**d**) Effect of *Dasatinib* on HCVpp entry into polarized HepG2–CD81 cells. HCVpp and MLVpp entry was analyzed in non–polarized and polarized HepG2–CD81 cells pre–incubated with *Dasatinib* (means ± SEM). (**e**) Effect of *Dasatinib* on HCVpp entry into PHH. HCVpp entry was assessed in PHH pre–treated with *Dasatinib*. Viability of treated cells was assessed using MTT assay. IC_{50} values are expressed as median of three independent experiments ± standard error of the median. (**f**) Effect of *Dasatinib* on HCV infection of PHH. Intracellular HCV RNA in PHH infected with HCVcc[59] or serum–derived HCV[40] in *Dasatinib* or solvent CTRL–treated PHH was analyzed by qRT–PCR[40]. Cell viability was assessed using MTT assay. *, $P<0.05$, **, $P<0.005$; ***, $P<0.0005$. Unless otherwise indicated: *Dasatinib* 10 μM.



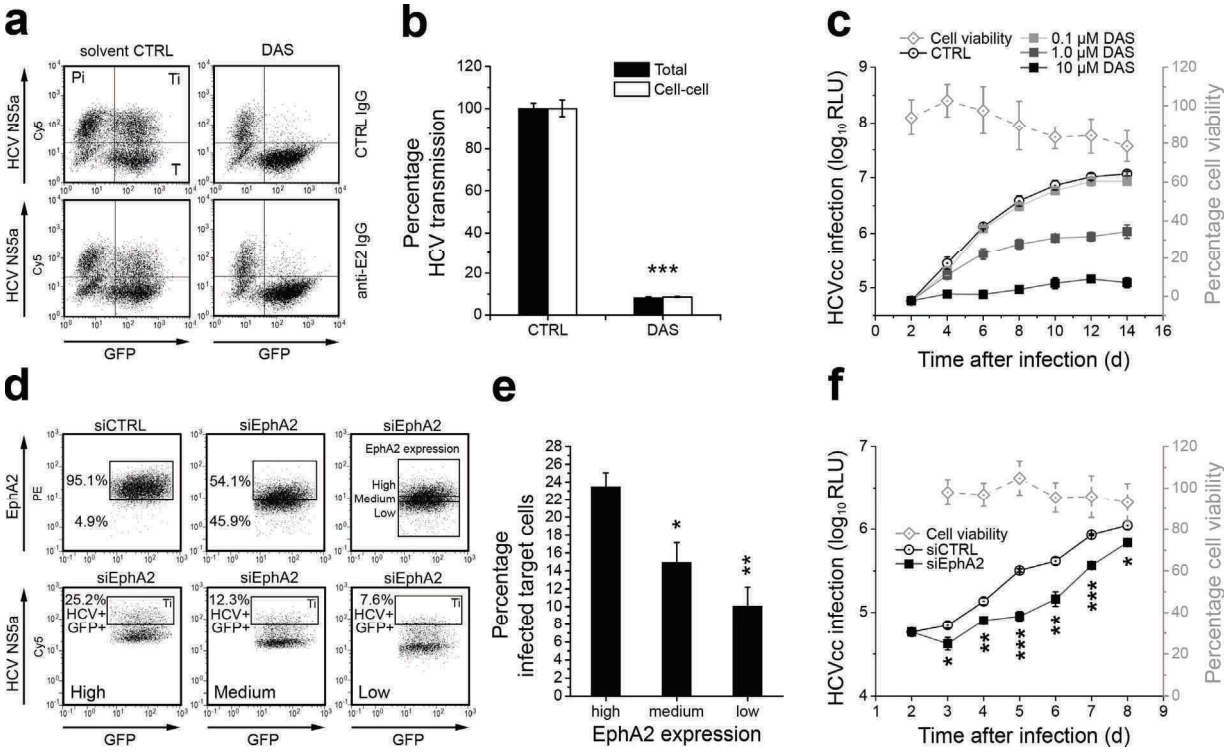
Supplementary Fig. 8. Effect of EGF and PKIs on entry of pseudoviruses expressing envelope proteins from HCV, VSV, influenza, measles and feline leukemia virus. Infection of serum–starved Huh7.5 cells incubated with EGF (1 μg mL^{-1}) or PKI (10 μM) with pseudotyped particles expressing envelope glycoproteins from HCV strains H77 and JFH1, influenza, vesicular stomatitis virus (VSV), endogenous feline leukemia virus (RD114) and measles virus. *, $P<0.05$; **, $P<0.005$; ***, $P<0.0005$.



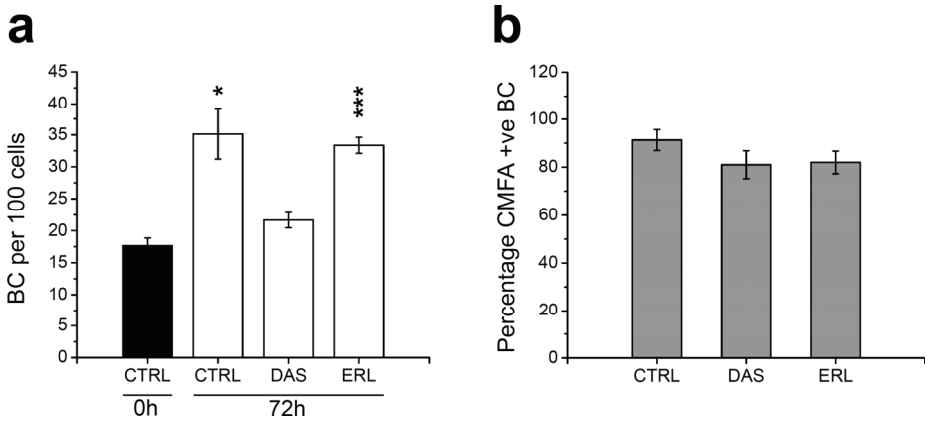
Supplementary Fig. 9. EphA2–specific ligands and antibodies modulate HCV entry. (**a**) Effect of EphA2–specific ligands ephrin–A1 and –A3 and cell–density on HCVpp entry. HCVpp entry into cells seeded at high or low density treated with 1 μg mL^{-1} soluble Fc–tagged ephrin–A1 and –A3 or Fc–tag (CTRL)[65] is shown. (**b**) Specific binding of rat serum to human EphA2 (dilution 1:5,000) to cell surface human EphA2 expressed on BOSC cells. Flow cytometric analysis of BOSC cells transfected with a human EphA2 expression construct (non shaded histograms; "BOSC–EphA2+") or a an empty control vector (pcDNA3; "BOSC", grey histograms)[54]. (**c**) Specific binding of EphA2–specific serum (dilution 1:100) to native EphA2 expressed on the surface of Huh7.5.1 cells. Flow cytometric analysis of non–permeabilized Huh7.5.1 cells incubated with EphA2–specific (non shaded histograms) or control serum (grey histograms) is shown. (**d**) Effect of EphA2–specific serum on HCVpp entry into PHH. HCVpp entry into PHH in the presence or absence of antibody to CD81 (10 μg mL^{-1}) or serum to EphA2 (dilution 1:50) is shown. **, $P<0.005$; ***, $P<0.0005$.



Supplementary Fig. 10. EphA2 mediates entry at a postbinding step by promoting the formation of CD81–CLDN1 co–receptor association(s). (**a**) EphA2 and binding of soluble HCV glycoprotein sE2 to HCV permissive cells. sE2 binding to Huh7.5.1 cells incubated with EphA2–specific serum or siEphA2 were analyzed by flow cytometry. SR–BI–specific antibodies and SR–BI silencing served as positive controls (all antibodies diluted at 1:100). (**b,c**) Effect of *Dasatinib* on HCV binding and postbinding steps. (**b**) Experimental setup. After HCVcc binding to Huh7.5.1 cells for 1 h at 4 °C in the presence or absence of antibodies, where HCVcc bind to the cells but do not efficiently enter, the inoculum is removed and the cells are shifted to 37 °C to allow synchronous viral entry[48,54,62,63]. Dashed lines indicate the presence of compounds. Data are expressed relative to HCVcc infection without compound. (**c**) To discriminate between virus binding and postbinding events, HCVcc binding to Huh7.5.1 cells was performed in the presence or absence of indicated compounds at 4 °C, before cells were washed and incubated with compounds at 37 °C. (**d**) HCV entry kinetics. Time–course of HCVcc infection of Huh7.5.1 cells following addition of the indicated compounds at different time–points during infection (**Supplementary Methods**). (**e**) Effect of *Dasatinib* and EphA2 silencing on CD81–CLDN1 association(s). FRET of CD81–CLDN1 co–receptor associations in HepG2–CD81 cells incubated with *Dasatinib* or EphA2–specific siRNA are shown (means ± SEM). (**f**) Effect of *Dasatinib* on membrane fusion. Viral glycoprotein–dependent fusion of 293T cells with Huh7 cells pre–incubated with *Dasatinib* or control is shown. **, $P<0.005$; ***, $P<0.0005$. *Dasatinib:* 10 µM.



Supplementary Fig. 11. EphA2 plays a functional role in HCV cell–cell transmission and spread. The experimental set–up of the HCV cell–cell transmission assay is described in **Fig. 5**. (**a**) Relative quantification of HCV–infected target cells (Ti) after co–cultivation with HCV producer cells (Pi) during *Dasatinib* treatment in the absence (cell–free and cell–cell transmission) and presence (cell–cell transmission) of HCV E2–specific antibody. (**b**) Total and cell–cell transmission were defined as HCV infection of Huh7.5–GFP+ target cells (Ti) in the absence (total transmission, black bars) or presence (cell–cell transmission, white bars) of HCV E2–specific antibody. (**c**) Effect of *Dasatinib* on viral spread. Long–term HCVcc infection of Huh7.5.1 cells incubated with *Dasatinib* 48 h post–infection at the indicated concentrations. Medium with solvent (CTRL) or PKI was replenished every 2nd day. Cell viability was assessed using MTT test. (**d**) EphA2 expression in target cells with silenced EphA2 expression. Cell surface EphA2 expression was analyzed by flow cytometry and target cells were divided in three groups displaying high, medium and low EphA2 expression. (**e**) Cell–cell transmission in HCV–infected GFP+ target cells with high, medium and low EphA2 expression was quantified by flow cytometry as described in **Fig. 5**. (**f**) Effect of EphA2 silencing on viral spread. Long–term analysis of HCVcc infection in Huh7.5.1 transfected with EphA2–specific or control siRNA 24 h post–infection. Cell viability was assessed using MTT test. DAS= *Dasatinib* 10 µM (unless otherwise stated). *, $P<0.05$; **, $P<0.005$; ***, $P<0.0005$.



Supplementary Fig. 12. Effect of PKIs on polarization and tight junction integrity of HCV permissive HepG2–CD81 cells. (**a**) Polarity in HepG2–CD81 cells incubated with PKIs. HepG2–CD81 cells were incubated in the presence of *Erlotinib* or *Dasatinib* for 72 h, fixed in 3% paraformaldehyde and stained for bile canaliculi (BC) that expressed marker MRP2. The polarity index (BC per 100 cells) was assessed by quantifying the number of MRP2 positive BC per 100 cell nuclei for five fields of view on 3 replicate cover slips. (**b**) Tight junction (TJ) integrity in HepG2–CD81 cells incubated with PKIs. TJ integrity was assessed after 72 h PKI treatment by determining the frequency of BC retaining marker dye CMFDA as described previously[18]. Means ± SEM are shown. *, $P<0.05$; ***, $P<0.0005$. PKI: 10 μM, DAS= *Dasatinib*, ERL= *Erlotinib*.

Annexe 3

HRas signal transduction mediates hepatitis C virus cell entry by triggering the assembly of the host tetraspanin receptor complex

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Cell Host&Microbe. 2013

Notre laboratoire a ensuite conduit des criblages additionnels afin de définir les voies de signalisation intracellulaires activées par l'EGFR importantes dans l'entrée du HCV. Nous avons ainsi identifié Hras comme facteur clé pour la constitution du complexe de récepteur CD81/CLDN1 dépendante d'EGFR**.** Il a pu ainsi être démontré qu'EGFR activait principalement la voie des MAP kinase dont les partenaires de signalisation sont Hras, b/c Raf, MEK puis ERK. Durant cette étude de nombreux inhibiteurs ont été utilisés et leur activité biologique à du être confirmée. J'ai ainsi pu vérifier l'activité biologique de l'inhibiteur de la kinase ERK (Fr180204) (Figure S2b) ceci en mesurant l'expression de PCNA qui est connue pour être régulé par la voie de la MAP kinase**.** Au sein de ce projet j'ai pu évaluer la spécificité du silencing des différentes formes de ras (Hras Kras Nras) en cellules hépatocytaires (Figure 4A) et en PHH (figure S3 a). Et il a pu être démontré que, bien que toutes les formes de ras soient silencer, seul le silencing de Hras avant un effet sur l'infection par le HCV. J'ai alors analysé l'éventuelle modulation de leur expression sous l'effet du silencing de l'une ou l'autre des formes de ras. J'ai ainsi démontré que le silencing de Hras n'affectait pas l'expression des autres formes de ras, Kras et Nras (Figure S3 b). Des expériences de protéomiques ont révélé que l'intégrine beta 1 (ITGB1) et Rap2b faisait partie du complexe de protéines CD81-CLDN1. Il était alors intéressant de voir si ITGB1 ou Rap2b pouvaient avoir un rôle sur l'infection par le HCV : En utilisant des anticorps bloquant ITGB1, j'ai démontré l'importance de l'ITGB1 au cours de l'infection par les HCVpp et HCVcc dans les cellules Huh7.5.1 (Figure 5 f). Il en est de même pour Rap2b. (Ces résultats ont aussi été observés en silencing pour les HCVpp). J'ai ainsi vérifié la spécificité de ces silencing en quantifiant l'expression de l'ITGB1 ou Rap2b après silencing. (Figure 5 d, e)

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

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http://dx.doi.org/10.1016/j.chom.2013.02.006

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).

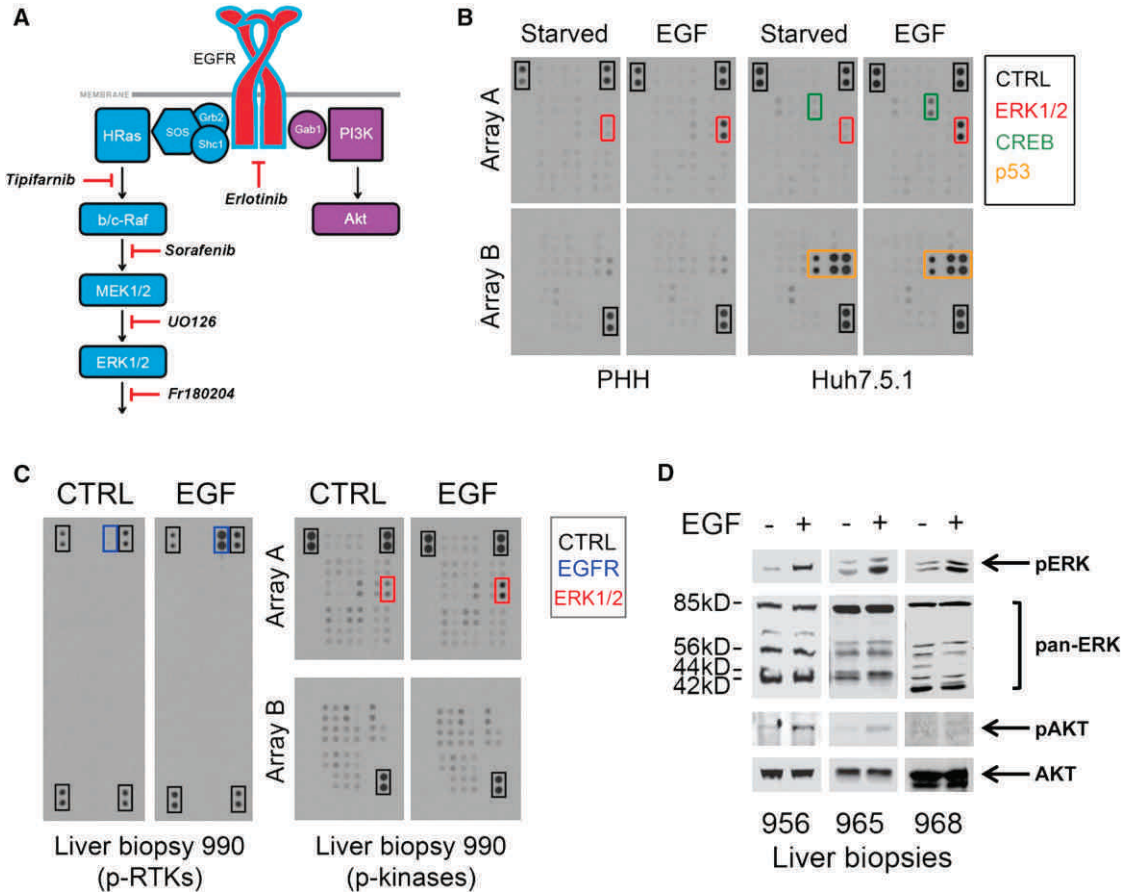

Figure 1. EGFR Signaling Pathways in Human HCV-Permissive Liver Cells, Hepatocytes, and Patient-Derived Liver Tissue

(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

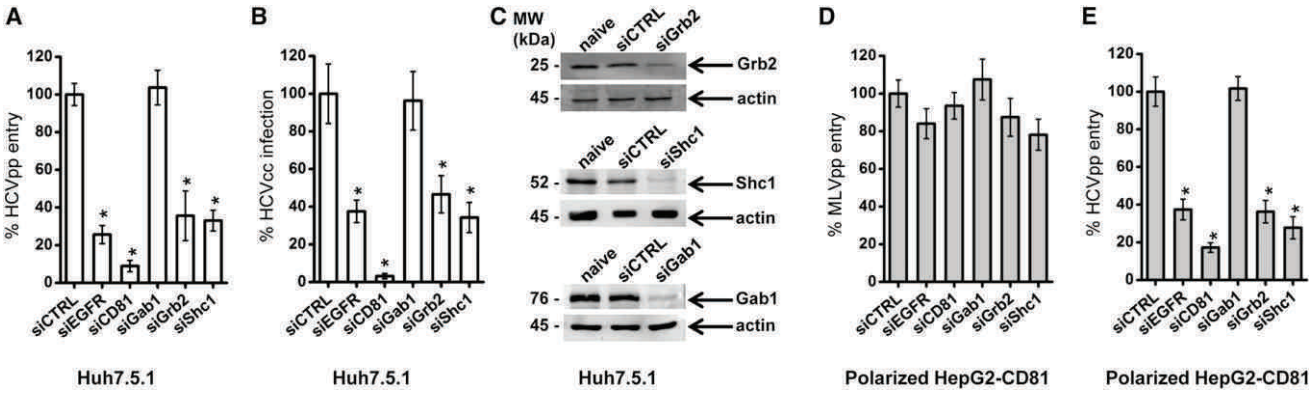

Figure 2. EGFR Adaptors Grb2 and Shc1 Are Relevant for HCV Entry

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

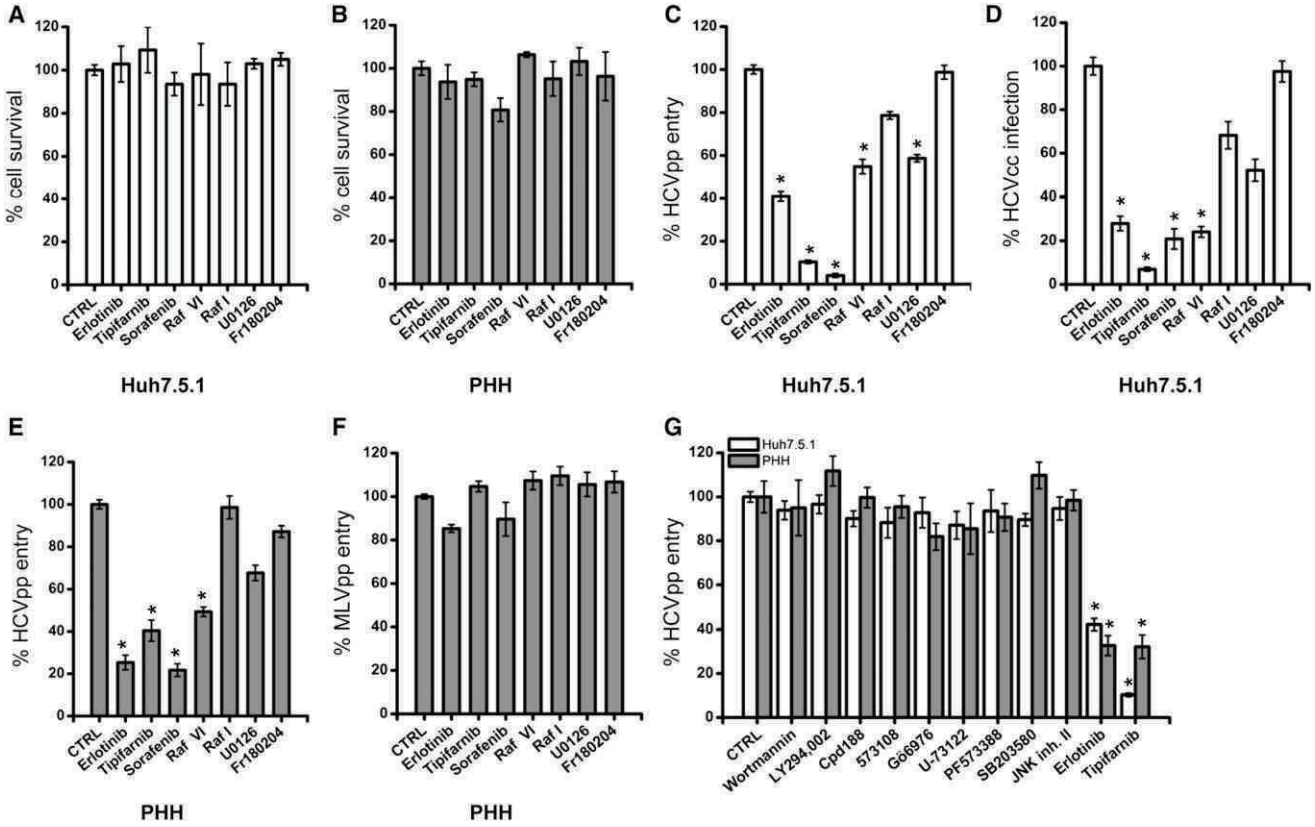

Figure 3. Upstream MAPK Are Relevant for HCV Entry in Hepatoma Cells and Human Hepatocytes

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

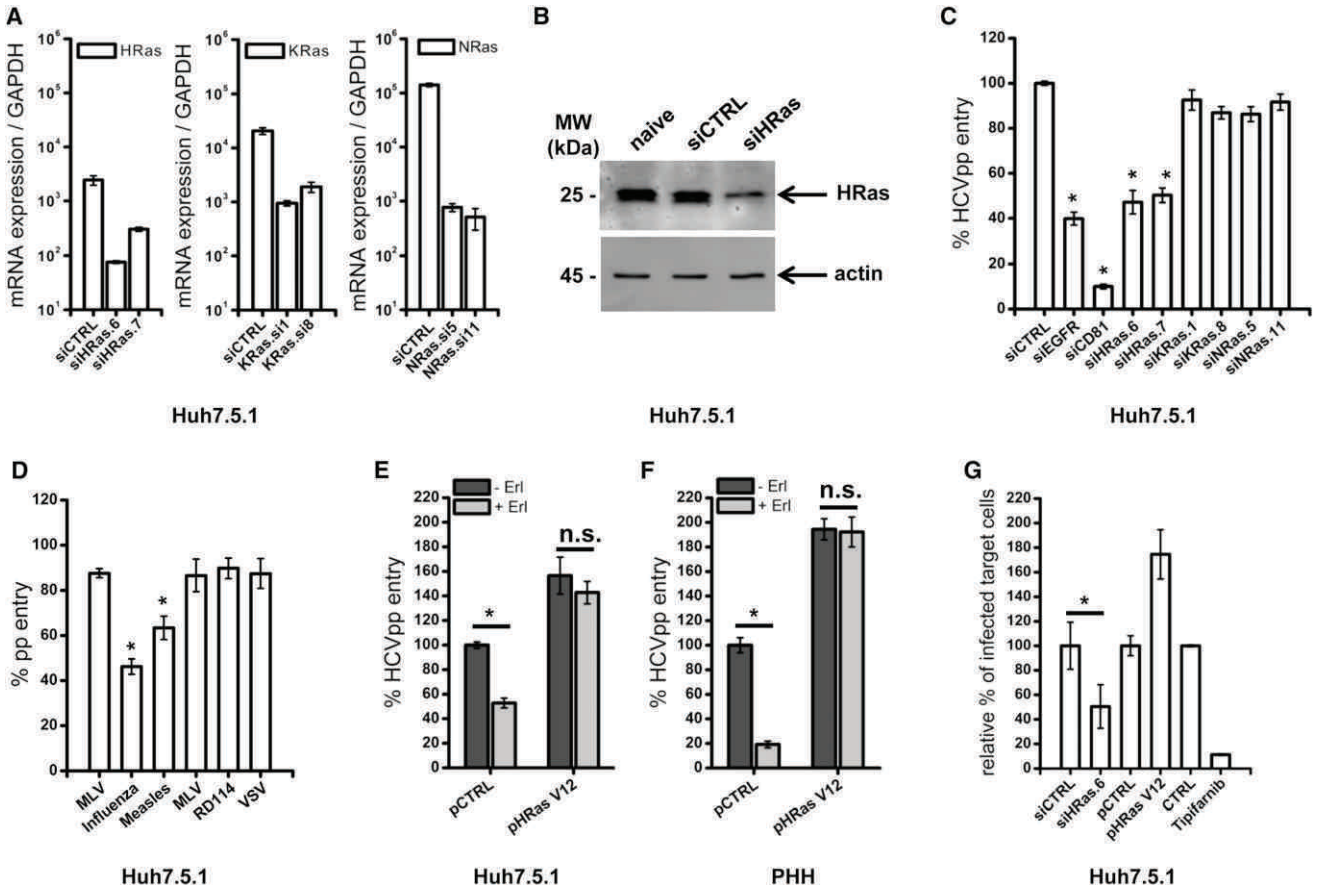

Figure 4. HRas Is a Host Cell Factor for HCV Entry

(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).

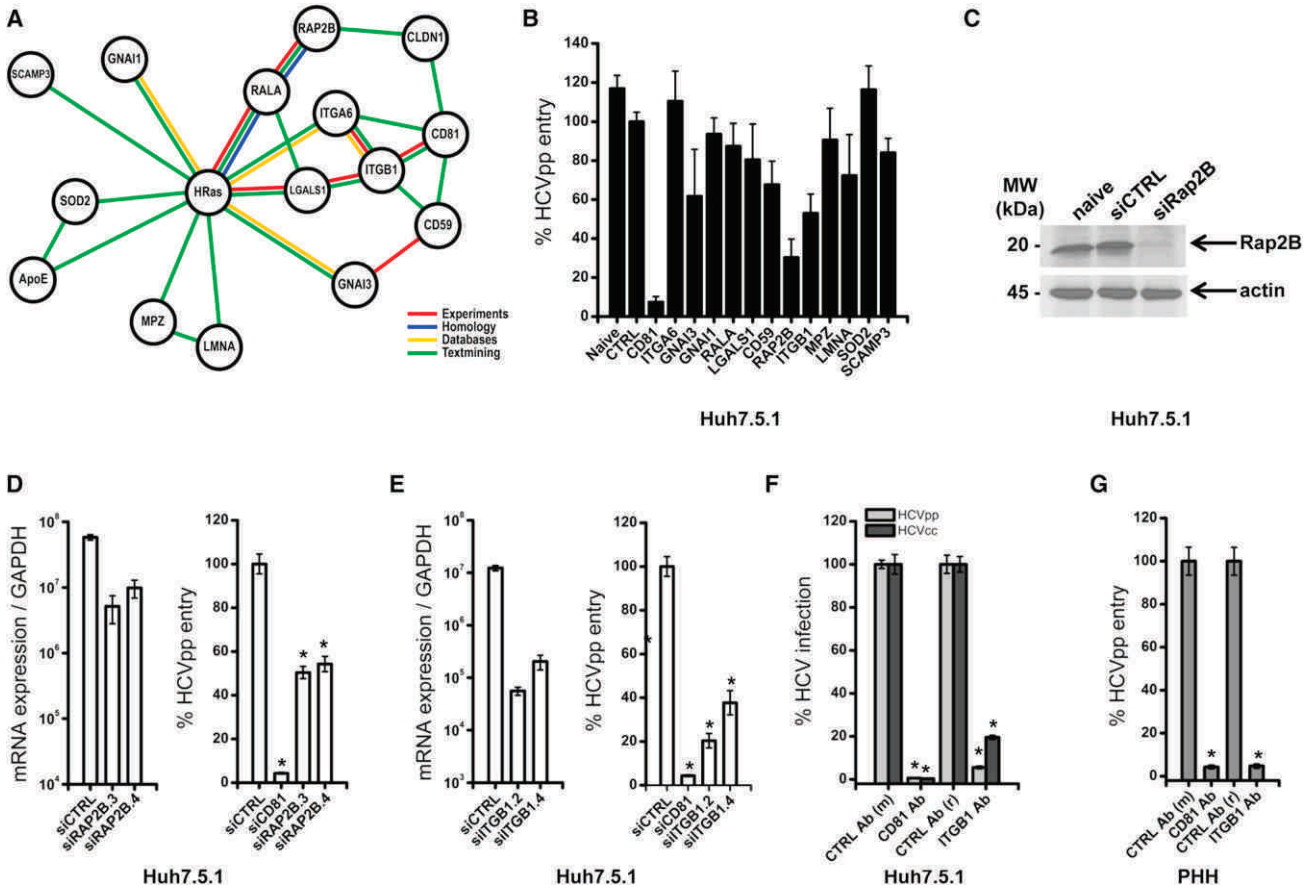

Figure 5. Functional Analysis of HRas-CD81-Associated Proteins Identifies Integrin Beta1 and Rap2B as Previously Undiscovered HCV Entry Factors

(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

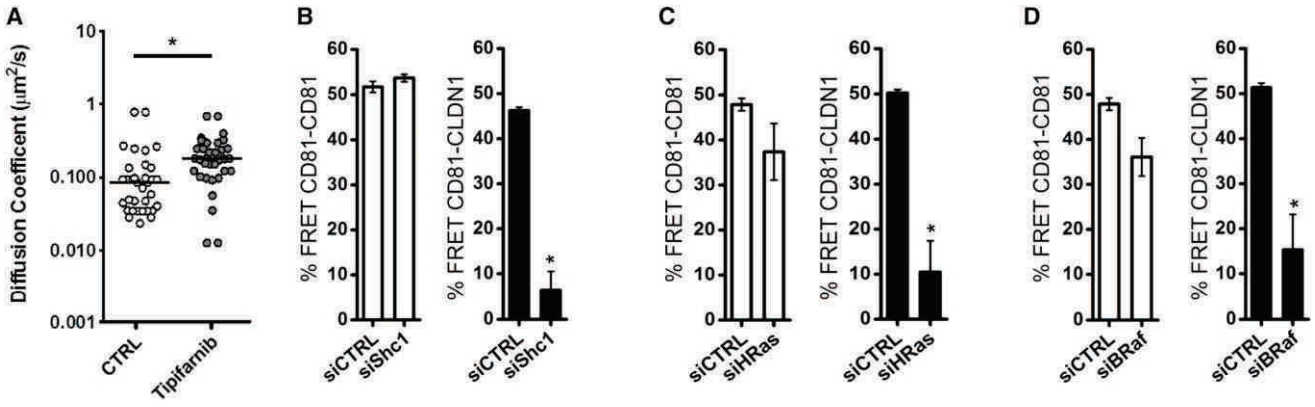

Figure 6. HRas Is Required for Lateral Diffusion of CD81 Promoting CD81-CLDN1 Associations

(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.

ACKNOWLEDGMENTS

This work was supported by the European Union (ERC-2008-AdG-233130-HEPCENT and INTERREG-IV-Rhin Supérieur-FEDER A9 and A30), Inserm, Laboratoire d'Excellence HEPSYS (ANR-10-LAB-28), ANRS (2010/304, 2011/132, 2012/239), and DGOS; J.A.M. acknowledges funding from the Wellcome Trust. The authors thank P. Pessaux and P. Bachellier (Strasbourg University Hospitals) for liver resections; R. Bartenschlager (University of Heidelberg, Heidelberg, Germany) for HCV strain Luc-Jc1; F.V. Chisari (The Scripps Research Institute, La Jolla, CA), C.M. Rice (Rockefeller University, New York, NY), and A. Patel (University of Glasgow, Glasgow, UK) for Huh7.5.1 and Huh7.5-GFP cells; E. Campeau and C. Beausejour for HRas V12 plasmid; D. Trono for pWPI plasmid; and C. Schuster (Inserm U1110, Strasbourg, France) for HepG2-CD81 cells. We thank A. Weiss, C. Bach, and M. Parnot for excellent technical work and Y. Couté for his helpful contributions to the SILAC analysis. L.Z. and J.L. as well as N.S.-A. and C.T. contributed equally. L.Z., J.L., F.L.N., D.S., I.H., H.J.H., A.B., M.B.Z., J.A.M., L.B., F.H.T.D., M.H.H., and T.F.B. designed experiments. L.Z., J.L., C.T., H.J.H., A.B., N.S.-A., R.G.T., F.X., M.T., S.C.D., J.F., and F.H.T.D. performed experiments. F.L.C. provided essential reagents. L.Z., J.L., F.L.N., N.S.-A., D.S., I.H., H.J.H., J.A.M., L.B., F.H.T.D., M.H.H., M.B.Z., and T.F.B. analyzed data. L.Z., J.L., F.L.N., M.B.Z., J.A.M., and T.F.B. wrote the manuscript.

Received: August 2, 2012
Revised: January 3, 2013
Accepted: February 11, 2013
Published: March 13, 2013

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Cell Host & Microbe, Volume *13*

Supplemental Information

HRas Signal Transduction Promotes

Hepatitis C Virus Cell Entry by Triggering

Assembly of the Host Tetraspanin Receptor Complex

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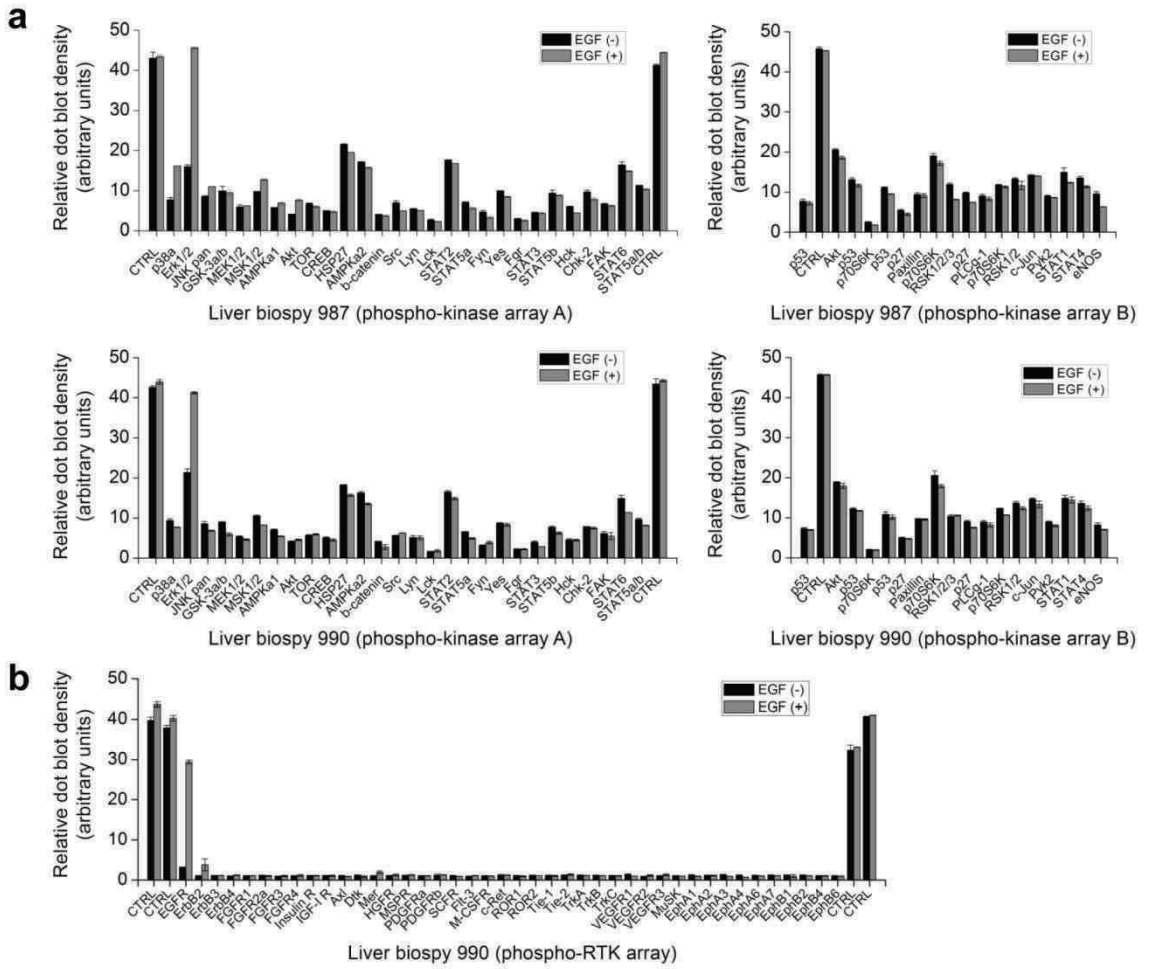
a
Relative dot blot density (arbitrary units)
EGF (-)
EGF (+)
Liver biospy 987 (phospho-kinase array A)
Liver biospy 987 (phospho-kinase array B)
Liver biospy 990 (phospho-kinase array A)
Liver biospy 990 (phospho-kinase array B)
b
Liver biospy 990 (phospho-RTK array)
c
% phosphorylation of ERK
15 min.
30 min.
60 min.
CTRL
Starved
ERL
EGF
ERL+EGF
d
% phosphorylation of AKT
e
% phosphorylation
p-AKT
p-ERK
f
counts
p-ERK
p-Akt
naive
starved
ERL
EGF
ERL+EGF
g
EGF (µg/mL)
0
0.005
0.01
0.1
0.5
1
p-ERK
ERK
p-AKT
AKT
Actin
Huh7.5.1
PHH

Figure S1. EGFR predominantly induces MAPK signaling in Huh7.5.1 cells, primary human hepatocytes and patient-derived liver tissue, Related to Figure 1
(a,b) Freshly isolated liver tissue of liver biopsies (987, 990 – shown in Fig. 1c) was incubated with EGF (1 µg/mL; 15 min) or mock control *ex vivo*. Following cell lysis, liver cell kinase phosphorylation was analyzed using a phospho-kinase array **(a)** and a phospho-RTK array **(b)** comprising the kinases and signal transducers indicated on the x-axis. Blots were exposed to hyperfilms and films were digitalized using a flatbed scanner and analyzed using Image J software (NIH). The relative dot-blot density of the phosphorylated proteins in samples incubated with EGF compared to mock-treated liver tissue was quantified by elliptical selection of individual dots and measuring standard deviation and integrated density. Means ± s.d. from two dots spotted with the same capture antibody are shown. **(c-f)** Phosphoflow analysis of the phosphorylated forms of ERK1/2 **(c,e,f)** and AKT **(d,e,f)** using phospho-specific antibodies in Huh7.5.1 cells **(c,d,f)** or PHHs incubated with EGF **(e)**. Cells were serum-starved for 12 h prior incubation with pervanadate (CTRL), that prevents dephosphorylation of kinases by phosphatases and results in the accumulation of the phosphorylated forms of ERK1/2 and AKT, in combination with the EGFR-inhibitor erlotinib (10 µM), the EGFR-ligand EGF (1 µg/mL), or both compounds for 15, 30 and 60 min for Huh7.5.1 cells or for 15 min for PHHs. Cells were then fixed, permeabilized, and incubated successively with antibodies directed against pAKT or pERK1/2, biotinylated secondary antibodies and a streptavidin-phycoerythrin solution. ERK1/2 and AKT phosphorylation was then assessed using flow cytometry. Data are expressed as percentage phosphorylation of ERK1/2 or AKT relative to untreated starved cells (means ± s.d. from two experiments in triplicate for Huh7.5.1, n=6 or means ± s.d. from three experiments for PHHs, n=3). **(f)** FACS histograms displaying the results shown in **(c)** and **(d)** after 15 min of incubation with EGF. **(g)** Immunoblot analysis of ERK1/2 and AKT phosphorylation in serum-starved Huh7.5.1 cells (left panel) and PHHs (right panel) incubated with EGF. Cells were incubated for 15 min with EGF at various concentrations prior to cell lysis. ERK and AKT phosphorylation as well as total ERK, AKT and actin expression were analyzed by immunoblot with specific antibodies.

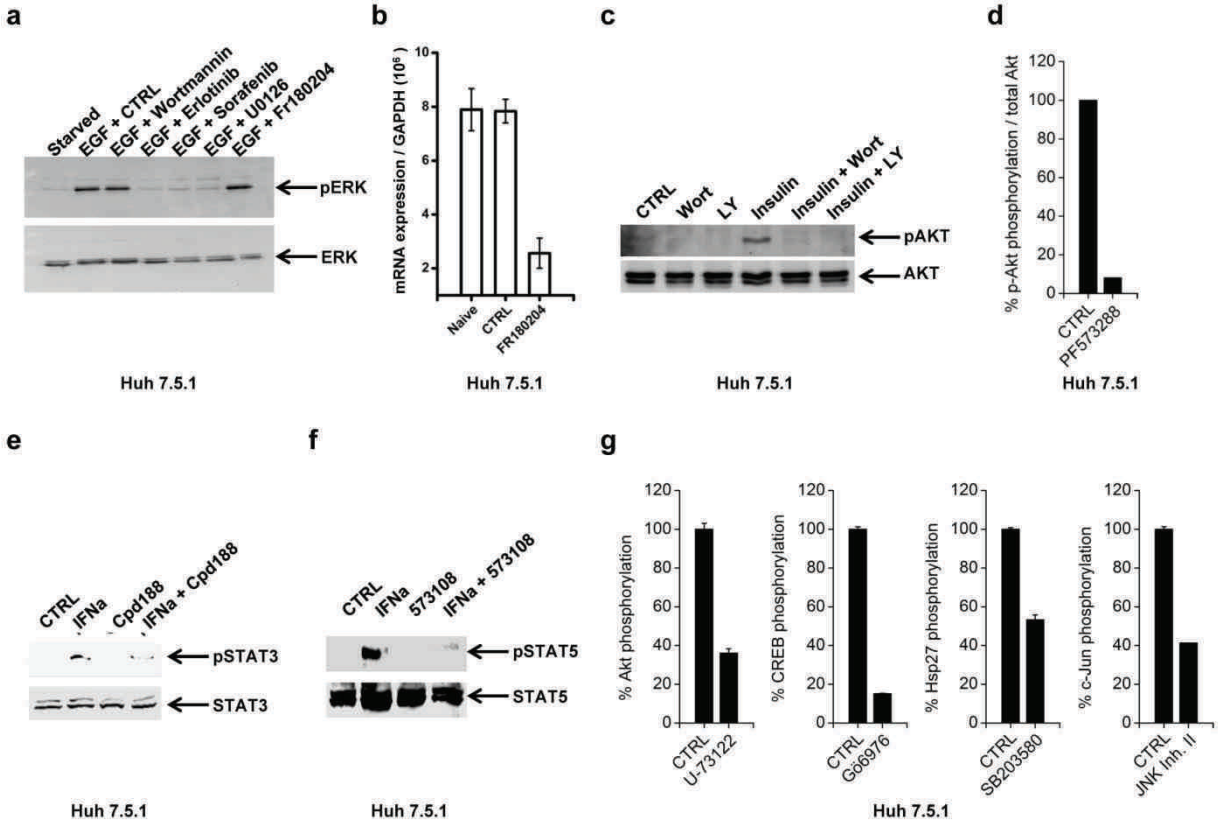

Figure S2. Functional confirmation of the biological activity of small molecule inhibitors targeting signal transduction pathways, Related to Figure 3

(a) The biological activity of MAPK inhibitors erlotinib (Lupberger et al., 2011; Shepherd et al., 2005), sorafenib (Bruix et al., 2012), U0126 (Duncia et al., 1998), Fr180204 (Ohori et al., 2005) and PI3K inhibitor wortmannin (Nakae et al., 1999) were assessed in starved Huh7.5.1 cells treated with EGF (1 µg/mL) and DMSO control or the indicated inhibitors (10 µM) by detection of ERK1/2 phosphorylation. ERK1/2 expression and phosphorylation were measured by specific antibodies targeting pERK1/2 or ERK1/2 by immunoblotting. **(b)** The biological activity of Fr180204 inhibiting ERK1/2 kinase activity (Ohori et al., 2005) was assessed measuring the expression of proliferating cell nuclear antigen (PCNA) which is regulated by MAPK signaling (Lupberger et al., 2006). mRNA expression after 24 h in starved Huh7.5.1 cells treated with Fr180204 inhibitor (10 µM) compared to control treated cells. mRNA expression of PCNA is shown as relative to GAPDH expression. **(c)** The biological activity of PI3K inhibitors wortmannin (Wort) (Nakae et al., 1999) and LY294,002 (LY) (Casagrande et al., 1998) (10 µM) was assessed by inhibition of AKT phosphorylation induced by insulin (100 nM, 1 h) as described (Nakae et al., 1999). Detection of pAKT compared to total AKT in Huh7.5.1 cells was performed by immunoblot. **(d)** The biological activity of FAK inhibitor PF573288 (Slack-Davis et al., 2007) (10 µM) was confirmed by inhibition of basal pAKT phosphorylation (Huang et al., 2002). Detection of pAKT compared to total AKT in Huh7.5.1 cells was performed by immunoblot and quantified using Image Quant. **(e,f)**

The biological activity of STAT3 inhibitor Cpd188 (Xu et al., 2009) **(e)** or STAT5 inhibitor 573108 (Muller et al., 2008) **(f)** (10 μM each) was assessed by their effect on IFNa-induced (1000 U/mL, 15 min) STAT3 or STAT5 phosphorylation, respectively. **(g)** The biological activity of PLC inhibitor U-73122 (Bleasdale et al., 1990) (inhibiting pAKT), PKC inhibitor Gö6976 (Martiny-Baron et al., 1993) (inhibiting pCREB), p38 inhibitor SB203580 (Davies et al., 2000) (inhibiting pHsp27) or JNK inhibitor II (Han et al., 2001) (inhibiting pc-Jun) (10 μM for each inhibitor) was confirmed by inhibition of PMA-induced (0.5 μg/mL, 1 h) phosphorylation of the respective target substrate protein in Huh7.5.1 cells. Substrate phosphorylation was measured using phospho-kinase array and relative dot-blot density of this array was quantified using ImageJ software (NIH) (means ± s.d. from two dots spotted with the same capture antibody). All experiments were performed in Huh7.5.1 cells using the inhibitor concentration indicated. Inhibitors were added for 1 h to the cells and phosphorylation was assessed using the human phospho-kinase array in cell lysates as described in Experimental Procedures. Data are expressed as percentage of substrate protein phosphorylation in the presence of PMA and inhibitor compared to PMA-treated cells alone. Relative dot blot densities are normalized by the positive controls spotted on each array.

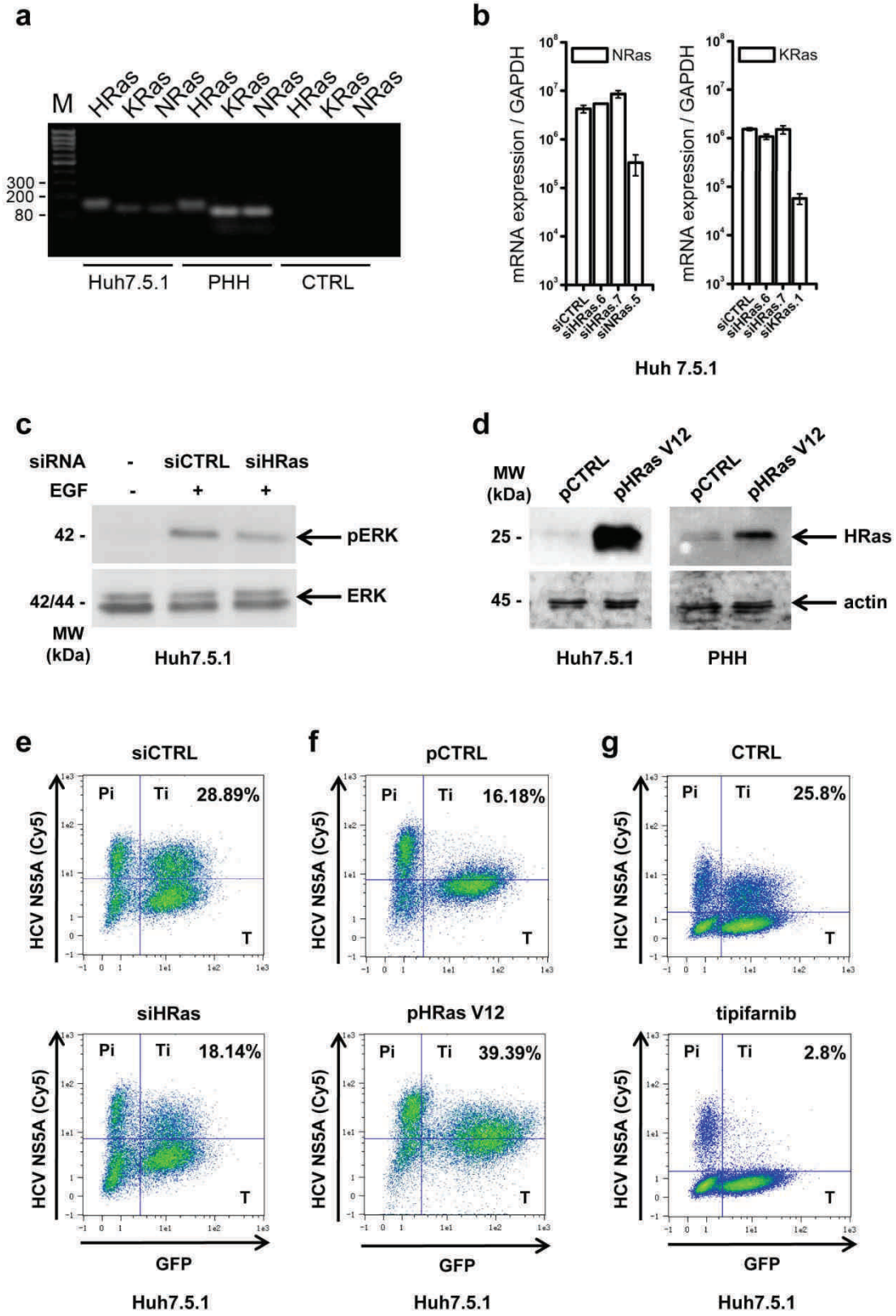
a
M
HRas
KRas
NRas
HRas
KRas
NRas
HRas
KRas
NRas
300
200
80
Huh7.5.1
PHH
CTRL
b
mRNA expression / GAPDH
NRas
KRas
siCTRL
siHRas.6
siHRas.7
siNRas.5
siKRas.1
Huh 7.5.1
c
siRNA - siCTRL siHRas
EGF - + +
42 -
pERK
42/44 -
ERK
MW (kDa)
Huh7.5.1
d
MW (kDa)
pCTRL
pHRas V12
pCTRL
pHRas V12
25 -
HRas
45 -
actin
Huh7.5.1
PHH
e
siCTRL
Pi
Ti
28.89%
T
HCV NS5A (Cy5)
siHRas
18.14%
GFP
Huh7.5.1
f
pCTRL
16.18%
pHRas V12
39.39%
Huh7.5.1
g
CTRL
25.8%
tipifarnib
2.8%
Huh7.5.1

Figure S3. Cellular HRas expression and its effects on HCV cell-cell transmission, Related to Figure 4

(a) Expression of HRas, KRas and NRas in Huh7.5.1 cells and PHHs. HRas, KRas and NRas mRNA expression in Huh7.5.1 cells and in PHHs was analyzed by RT-PCR using isoform-specific primers. The RT-PCR products were subjected to gel electrophoresis using 2 % agarose gel. The respective RT-PCR products are indicated. **(b)** HRas silencing is isoform-specific and does not impair the expression of KRas and NRas. Huh7.5.1 cells were transfected with siHRas.6 or siHRas.7 or siKRas.1 or siNRas.5 for 72 h prior to detection of mRNA expression of KRas and NRas by RT-PCR. mRNA expression of KRas and NRAs compared to GAPDH after silencing. (means ± s.d. from one representative experiment in duplicate, n=2). **(c)** HRas silencing reduces ERK phosphorylation after EGF stimulation. Huh7.5.1 cells were transduced with siHRas.6 for 72 h. The cells were serum starved for 6 h and then incubated with EGF (1 µg/mL) for 10 min prior to cell lysis. ERK phosphorylation and total ERK expression were analyzed by immnunoblot with specific antibodies. **(d)** Enhancement of HRas expression in Huh7.5.1 cells and PHHs 72 h after transduction with control lentiviruses (pCTRL) or lentiviruses expressing a trans-dominant active HRas mutant (pHRas V12). HRas expression was assessed using an HRas-specific antibody in cell lysates. **(e-g)** Modulation of HRas expression and function alters HCV cell-cell transmission in Huh7.5.1 cells. HCV producer cells (Lupberger et al., 2011) were cultured with **(e)** HRas-silenced GFP-positive target cells (siHRas.6) or with GFP-positive target cells transfected with control siRNA (siCTRL) or with **(f)** HRas lentivirus-transduced target cells (pHRas V12) or with **(g)** target cells transduced with control expression plasmid (pWPI) or with uninfected GFP-positive target cells incubated with tipifarnib (10 µM) or mock control. Cell-free HCV transmission was blocked by incubation of cells with patient-derived neutralizing anti-HCV IgG (Fofana et al., 2012) as described (Lupberger et al., 2011; Witteveldt et al., 2009). HCV-infected GFP-positive target cells were detected by staining using an HCV non structural protein 5A (NS5A)-specific antibody and quantified by flow cytometry as described in Experimental Procedures. The percentage of HCV infected GFP-positive target cells is indicated. Total cells = 100 %, Pi = HCV RNA-electroporated Huh7.5.1 producer cells, T = GFP-expressing Huh7.5 target cells with absent infection and Ti = GFP+HCV NS5A+ HCV-infected target cells.

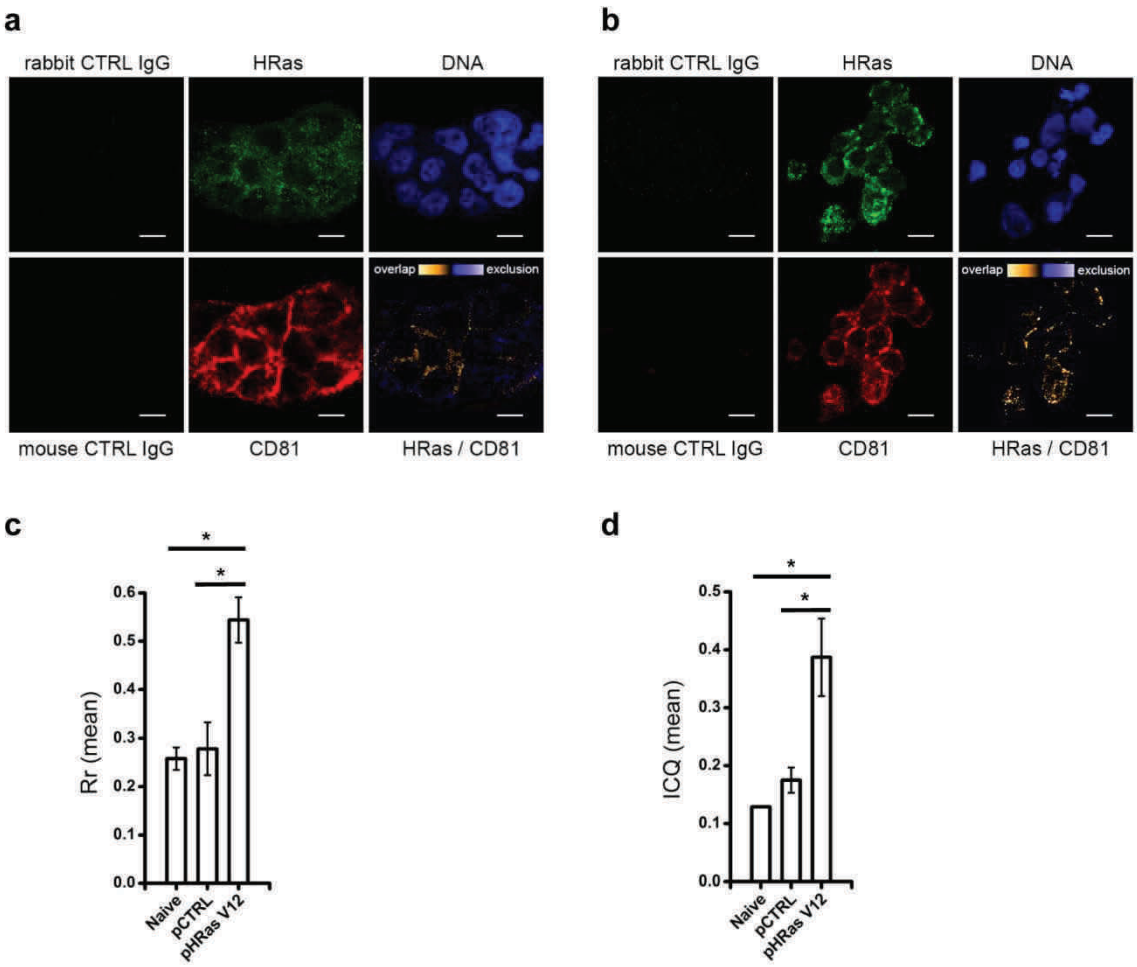

Figure S4. Co-localization of HRas with CD81 in Huh7 cells, Related to Table 1
(a-d) Confocal laser scan microscopy analysis of HRas (green) and CD81 (red) co-immunostaining in Huh7 cells. DAPI staining of DNA (blue) served as nuclear marker for cells. Cells were fixed with 4 % para-formaldehyde, permeabilized, and immunolabeled with rabbit polyclonal anti-HRas and mouse monoclonal anti-CD81 antibodies. Following washing bound antibodies were visualized using Alexa Fluor 488 goat anti-rabbit IgG or Alexa Fluor 594 goat anti-mouse IgG and confocal laser scan microscopy. Scale bar represents 10 µm. Co-localization was measured by Image J software (NIH) and calculated according to Pearson coefficient (Rr) and Intensity Correlation Quotient (ICQ) (Bolte and Cordelieres, 2006; Brown et al., 2010; French et al., 2008; Zinchuk et al., 2007). **(a)** Co-localization of CD81 and endogenous HRas in naïve Huh7 cells. **(b)** Overexpression of transdominant active HRas V12 results in an increase of HRas co-localization with endogenous CD81. Huh7 cells were transduced for 48 h with control (pCTRL) or HRas V12 lentiviruses. **(c,d)** Co-localization of endogenous CD81 with active HRas is significantly and reproducibly increased as calculated by analysis of variance using F-test from Pearson's correlation coefficients (Rr) **(c)** or Intensity Correlation Quotients (ICQ) **(d)**, respectively. (means ± s.d. from three independent experiments, n=3). *p<0.005.

Table S1. Functional siRNA HCVpp entry screen of EGFR binding partners in Huh7.5.1 cells, Related to Figure 2

Target name	Mean % HCVpp entry	SD
Naive	116.29	10.16
CTRL	100.00	10.48
CD81	6.62	2.53
GRB2	43.57	13.31
GAB1	70.14	2.25
SHC1	45.28	14.36
PTPN11	70.64	10.87
PLCG1	64.03	13.83
RASA1	79.12	18.43
CSK	58.28	5.97
SH3BP1	52.30	2.37
STAT3	59.34	10.37
STAT5A	66.91	3.69
STAT5B	44.64	1.06
VAV2	92.68	10.74
CBL	136.12	18.44
EPS15	124.09	13.14

Expression of EGFR binding partners were silenced in Huh7.5.1 cells using target-specific siRNAs (onTarget plus pool of four siRNA). 72 h later cells were infected with HCVpp (genotype 1b) and HCVpp infection was analyzed as described in Supplementary Experimental Procedures. Data are expressed as percentage HCVpp entry relative to siCTRL-transfected cells (mean ± s.d.). A threshold of five times the median of standard deviations on a plate (equals 47.96 residual infectivity) was chosen as a stringent parameter defining positive screening hits (Brass et al., 2008).

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Protein kinase inhibitors, ligands and antibodies. Erlotinib was obtained from LC Laboratories. Sorafenib, Fr180204, Raf-1 Kinase Inhibitor I, Raf Kinase Inhibitor VI, U-73122, JNK inhibitor II, SB203580, Gö6976, STAT5 inhibitor (573108) and DMSO (used at a final concentration 0.7 % for incubation of protein kinase inhibitors and control experiments) were obtained from Merck. Tipifarnib and wortmannin were obtained from Selleckchem. U0126 and Cpd188 were obtained from Calbiochem. PF573288 was obtained from Pfizer. Recombinant EGF, LY294,002 and the β-actin-specific antibody were obtained from Sigma; antibody to EGFR (clone 528) and to Rap2B (sc-81915) from Santa Cruz; antibody to actin (EP1123Y) and HRas (Y132) from Abcam. Antibodies specific for CD81 (JS81), HRas (clone 18), Shc1, Grb2 (clone 81), and integrin beta1 (CD29, clone 9E67) were obtained from BD Transduction Laboratories. Gab1- and ERK1/2-specific antibodies as well as phospho-specific antibodies directed against pERK1/2 (Thr202, Tyr204) (E10) were obtained from Cell Signaling. Anti-HCV IgG has been described (Fofana et al., 2012). Antibodies p-ERK1/2 (Thr202, Tyr204) (E10) labeled with Alexa Fluor 488 and p-AKT (Ser473) (D9E) labeled with Alexa Fluor 647 used for dynamic phosphoflow analyses were obtained from Beckman Coulter. All other antibodies labeled with the fluorophores Alexa Fluor 488 and Alexa Fluor 594 were obtained from Invitrogen. Antibody specific for CD81 (TS81) for immunoprecipitation and immunofluorescence was obtained from Diaclone. Antibody specific for claudin-1 (1C5–D9) was obtained from Abnova. Alkaline–phosphatase (AP)–labeled secondary antibodies were obtained from GE Healthcare. Secondary antibodies IRDye IgG (800CW and 680RD) were obtained from LI-COR Biosciences.

Infection of primary human hepatocytes. One day following PHH isolation and plating (Krieger et al., 2010), PHHs were washed with PBS and pre–incubated in the presence or absence of ligand or inhibitors for 1 h at 37 °C in William's E medium. Then, lentiviral-based HCVpp or VSVpp were added for 4 h at 37 °C and the plates were centrifuged the first hour at 37 °C at 400 x g. Following infection, fresh medium was added.

Analyses of protein expression. Immunoblots of cell lysates using protein–specific antibodies were performed following GE Healthcare protocols using Hybond–P membranes and visualized using ECF substrate and Typhoon Trio high performance fluorescence scanner (GE Healthcare). Immunoblots of biopsies were analyzed using Odyssey Infrared Imaging System (LI-COR). Phospho-array analysis was performed using Proteome Profiler Human Phospho-kinase Array and Human Phospho-RTK 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).

Functional RNAi HCV entry screens. Custom Plate (pool of four siRNA) libraries were obtained from Dharmacon for EGFR binding partner and STRING network siRNA screens. For each target 5.25 pmol siRNA was reverse transfected in 5,000 Huh7.5.1 cells per well of 96–well microplate using INTERFERin reagent (Polyplus). The effect of gene silencing on viral entry was investigated 72 h after siRNA transfection using

HCVpp (genotype 1b) harboring a luciferase reporter gene. To minimize non–specific effects due to evaporation, outside wells were not used for the screens but were filled with phosphate buffered saline (PBS). As an internal quality control of gene silencing and HCVpp entry, positive control siRNA (siCD81) was transfected side–by–side on each plate. Hit selection after siRNA screening: the impact of gene silencing was defined by an increase or decrease of HCVpp entry expressed as the ratio of entry compared to the experimental average value of entry into control-transfected cells (irrelevant siRNA). For HCVpp entry a threshold of five times the median of standard deviations inhibition on a plate was chosen as a stringent parameter defining screening hits similar as described (Brass et al., 2008).

MTT assay. Cytotoxic effects on cells were assessed by analyzing the ability to metabolize 3–(4,5–dimethylthiazol–2–yl)–2,5–diphenyltetrazolium bromide (MTT) as described elsewhere (Lupberger et al., 2011).

siRNAs and expression plasmids used for rescue experiments and functional studies. Custom onTARGET plus (pool of four individual siRNA) siRNA libraries comprising EGFR binding partners and CD81 associated proteins were obtained from Dharmacon. Individual onTarget plus siRNAs siITGB1.2 (J-004506-06, 5'-GGU AGA AAG UCG GGA CAA A-3'), siITGB1.4 (J-004506-08, 5'-GGG CAA ACG UGU GAG AUG U-3'), RAP2B.3 (J-009052-07, 5'-CAA UAG GGC CUG UUG UUU A-3'), RAP2B.4 (J-009052-08, 5'-GAA AUU AGG AAC ACU GCU A-3') were obtained from Dharmacon. siGab1 (Hs–Gab1_6, 5'–TAG ATG CTG GAT TGA CAT TTA–3'), siGrb2 (Hs–Grb2_1, 5'–CTG GTA TTC TCT CTA TGC AAA–3'), siShc1 (Hs–Shc1_9, 5'–AAG AGC CAC CTG ACC ATC AGT–3'), siHRas.6 (Hs–HRas_6, 5'–CCG GAA GCA GGT GGT CAT TGA–3'), siHRas.7 (Hs–HRas_7, 5'–CAC AGA TGG GAT CAC AGT AAA–3'), siKRas.1 (Hs–KRas_1, 5'–GAC GAT ACA GCT AAT TCA GAA–3'), siKRas.8 (Hs–KRas2_8, 5'–AAG GAG AAT TTA ATA AAG ATA–3'), siNRas.5 (Hs–NRas_5, 5'–AAC CTG TTT GTT GGA CAT ACT–3'), siNRas.11 (Hs–NRas_11, 5'–AGG GAG CAG ATT AAG CGA GTA–3') and siBRaf (Hs-BRAF_4, 5'-TAG AGT CTT CCT GCC CAA CAA-3') were obtained from Qiagen. siCTRL, siCD81 and siEGFR (Hs-siEGFR_6) have been described (Lupberger et al., 2011; Zeisel et al., 2007). Lentiviral expression plasmids pWPI, a lentiviral cDNA expression vector containing a co-cistronic GFP reporter (plasmid 12254) and pLenti CMV/TO RasV12 Puro (HRas V12, plasmid 22262) were obtained from Addgene.

Silencing, inhibition, and ligand experiments. Silencing was performed using INTERFERin (Polyplus) and DharmaFECT4 (Dharmacon) as described (Lupberger et al., 2011). Screening was performed at the High Throughput Screening (HTS) platform of the Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC) in Illkirch, France. Inhibitors, antibodies or ligand were added 1 h prior to HCVpp entry or HCVcc infection and during entry/infection unless otherwise stated. Experiments with EGFR ligand were conducted on serum-starved cells.

Cell-cell transmission of HCV. Cell-cell transmission of HCV was assessed as previously described (Lupberger et al., 2011; Witteveldt et al., 2009). Briefly, Huh7.5.1

producer cells were electroporated with HCV Jc1 RNA and cultured with naïve Huh7.5-GFP target cells, or target cells with silenced HRas expression, or overexpressed transdominant active HRas V12, or in the presence or absence of tipifarnib (10 µM). Cell-free transmission was blocked by patient-derived anti-HCV IgG (Fofana et al., 2012) as described (Lupberger et al., 2011).

Analyses of mRNA expression. Total RNA was extracted from Huh7.5.1 cells and PHHs using RNeasy Mini Kit (Qiagen). Gene expression in the total RNA extracts was assessed using two step RTqPCR. The reverse transcription on total RNA extract was made using MAXIMA reverse transcriptase (Thermo Scientific). RTqPCR for expression of HRas, KRas, NRas, PCNA and GAPDH was made using SOLARIS qPCR Master mix in combination of qPCR Gene Expression Assay (Thermo Scientific) and RT-PCR was performed using Corbett Rotor Gene 6000 (Qiagen). All RTqPCR steps were performed following protocols provided with the different kits. Serial dilutions of a standard were included for each gene to generate a standard curve to allow calculation of the input amount of cDNA for each gene. All values were normalized by GAPDH expression.

Analyses of 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. All the patients that donated liver tissue were male, between 39 and 57 years old and diagnosed with non-alcoholic steatohepatitis (liver biopsies 956, 968), primary sclerosing cholangitis (liver biopsy 965), hepatitis B (liver biopsy 987), or toxic hepatitis (liver biopsy 990). Biopsy liver tissues were analyzed as described (Dill et al., 2012). 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 mechanic homogenization followed by 20 min incubation on ice. Lysates were cleared by centrifugation at 4 °C and 21,000 x g for 5 min prior to immunoblot analysis of 30 µg protein per sample. For *ex vivo* stimulation, liver tissues were incubated for 15 min with 1 µg/mL EGF. Liver tissue was grinded in liquid nitrogen and lysed using lysis buffer 6 (R&D Systems) supplemented with protease and phosphatase inhibitors. Lysates were cleared after 20 min incubation on ice by centrifugation and analyzed by phospho-array.

Proteomic analyses of tetraspanin complexes and microdomains. Stable isotope labeling with amino acids in cell culture (SILAC) was performed as described (Ong et al., 2002). In brief, HepG2-CD81 cells and control HepG2 were incubated with either light isotope-labeled $^{12}C_6$-lysine/$^{12}C_6$-arginine or heavy isotope-labeled amino-acids $^{13}C_6$-lysine/$^{13}C_6$-arginine (ThermoScientific). It was confirmed that during 6-8 rounds of mitosis at least 98 % of proteins incorporated labeled amino-acids and that during the experiment labeled arginine was not transformed into proline. Then, 1.2×10^8 cells HepG2 and HepG2-CD81 were lysed using brij97 (10 mM Tris pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 % brij97) in the presence of protease inhibitors. The two

lysates were pooled and CD81-associated proteins were co-immunoprecipitated as described (Andre et al., 2006). Isolation of CD81-containing TEMs was performed using the CD81-specific antibody TS81 coupled covalently to sepharose 4B beads (Amersham Bioscience, Saclay, France). The beads were washed five times with lysis buffer and proteins were eluted using 1 % Triton X-100. The proteins were separated by 12 % SDS-PAGE under non-reducing conditions and directly trypsin-digested in gel pieces as described (Andre et al., 2006). Resulting peptides were analyzed by liquid chromatography mass spectrometry using LTQ-Orbitrap Velos spectrometer (Thermoscientific) coupled with nano-HPLC liquid chromatography (Ultimate 3000, Dionex) and identified using the software MaxQuant (http://maxquant.org) by comparing the results with a human protein database and a control database containing scrambled sequences. For each protein, the identification was considered as valid when at least 2 different peptides were sequenced by mass spectrometry. Proteins specifically associated with CD81 were designated by an exclusive ^{12}C or ^{13}C labeling, whereas contaminating proteins were labeled similarly with both light and heavy isotopes ^{12}C and ^{13}C, which appeared as doublets in the mass spectra. A peak volume ratio $^{13}C/^{12}C$ (heavy/light) >2 was defined as threshold for potential CD81-associated proteins.

Fluorescence recovery after photobleaching (FRAP). Cells transduced to express AcGFP-CD81 were plated onto Mattek glass bottomed dishes at a density of 1.5 x 10^4 cells/cm^2 and imaged using a 100x Plan Apochromat 1.4NA oil immersion objective on a Zeiss LSM 780 confocal microscope with a GaAsP spectral detector. 16 bit images were attained with optimal pixel resolution. Tagged proteins were excited with the argon 488 laser and initial images acquired with low laser power (0.1-1 % transmission), photobleaching was performed with full laser power for 20 interations on selected circular regions of interest (ROI) identified in the planar membrane. Subsequent recovery images were collected using low laser power at 0.18 second intervals for approximately 2 min, until ROI recovery had reached a plateau. Only a single Z-section was bleached and imaged, typically the basal side of the cell contacting the cover slip. The mean fluorescent intensity over time was obtained for the photo-bleached ROI, a background ROI (containing no cells) and an unbleached ROI in the cell of interest. ROIs were selected using the Zeiss Zen analysis software. Changes in fluorescence following photobleaching were normalized for fluctuations in image capture, laser power and overall fluorescence loss in the cells by subtracting the background and unbleached ROI. The values obtained for the photo-bleached ROI were converted to relative fractional recovery, where the pre-bleach fluorescence intensity values equal to 100 %. Data were imported into GraphPad Prism and fitted using an exponential decay algorithm, Y = Span (1-exp (-K*X)) + plateau. The span and plateau were used to calculate mobile fraction. The diffusion coefficient (D) was calculated using a simple two-dimensional diffusion model for a circular bleach ROI: D = 0.224 × (radius2/t1/2) (Axelrod et al., 1976). Bootstrap Monte Carlo sampling demonstrated that a minimum of 10 cells and 100 ROIs were required to represent the population.

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Marine TUREK

Etude des facteurs cellulaires responsables de l'initiation et de la dissémination du virus de l'hépatite C

Résumé

Le VHC est une cause majeure de cancer du foie. Le traitement actuel est caractérisé par à un cout élevé, la présence de toxicité et l'émergence de résistance virale. Dans la 1ère partie de ma thèse, je me suis intéressé à l'entrée virale. L'entrée est nécessaire pour l'initiation ; la dissémination et le maintien de l'infection et représente ainsi une cible intéressante dans le développement de thérapies antivirales : CD81 et SRBI sont les 1ers facteurs décrits comme importants pour l'entrée : Nous avons confirmé leur rôle clé dans l'entrée et les étapes suivant l'entrée. De plus ; nous avons montré leur rôle crucial dans la transmission cellule/cellule. Le VHC infecte principalement les hépatocytes, nous avons étudié en seconde partie de ma thèse le tropisme restreint du VHC aux hépatocytes. En définissant les facteurs essentiels à l'infection de cellules non hépatiques et en développant un modèle cellulaire afin d'identifier de nouveaux facteurs d'assemblage et de réplication du VHC.

Resume

HCV infection is the leading cause of chronic liver disease. The current SOC is still limited by high costs, toxicity and emergence of viral resistance. In the first part of my thesis we focused our work on viral entry. Viral entry is required for initiation, spread, and maintenance of infection, and thus is a promising target for the development of new antiviral therapies. CD81 and SR-BI are the first entry factors identified as important for HCV entry. In our work we confirmed their crucial role in entry, especially at the post-binding step. In addition we proved their key role in viral dissemination through the cell-cell transmission. As HCV mainly infects hepatocytes, we studied in the second part of my thesis, the restricted cellular tropism of HCV to hepatocytes and we defined the minimal host factors rendering non hepatic cell lines susceptible to HCV infection by the establishment of a powerful tool to identify new assembly and replication factors.