Virology 429 (2012) 112–123

Contents lists available at SciVerse ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Significance of palmitoylation of CD81 on its association with tetraspanin-enriched microdomains and mediating hepatitis C virus cell entry

Yong-Zhe Zhu [1], Yuan Luo [1], Ming-Mei Cao [1], Yuan Liu, Xiao-Qing Liu, Wen Wang, Da-Ge Wu, Mo Guan, Qing-Qiang Xu, Hao Ren, Ping Zhao *, Zhong-Tian Qi *

Department of Microbiology, Shanghai Key Laboratory of Medical Biodefense, Second Military Medical University, Shanghai 200433, China

ARTICLE INFO

Article history:
Received 26 January 2012
Accepted 3 March 2012
Available online 4 May 2012

Keywords:
Hepatitis C virus
CD81
Palmitoylation
Tetraspanin-enriched microdomains
Cell entry

ABSTRACT

CD81, a co-receptor for hepatitis C virus (HCV), is a member of the tetraspanin superfamily and is heavily palmitoylated in the juxtamembrane cysteine residues. Palmitoylation plays an important role in protein–protein interactions and association with cholesterol-rich domains of membranes. In this study, Huh7 cells expressing wild-type or palmitoylation-defective CD81 were generated to analyze whether palmitoylation of CD81 is involved in HCV cell entry. Our data showed that de-palmitoylation of CD81 dramatically reduced its association with tetraspanin CD151, but did not influence CD81 partition in detergent-resistant membranes. Moreover, de-palmitoylated CD81 decreased the host cell susceptibility to HCV. Notably, CD151-specific antibodies and siRNA inhibited HCV cell entry, and detachment of CD81 with CD151 decreased the lateral movement of virus particle/CD81 complex to areas of cell–cell contact. These results suggest that palmitoylation of CD81 should facilitate HCV entry, at least in part, by regulating the association of CD81 with tetraspanin-enriched microdomains.

Introduction

Approximately 180 million people are infected worldwide by hepatitis C virus (HCV). A majority of those people are at risk for the development of chronic infection, which induces end-stage liver disease such as liver cirrhosis and hepatocellular carcinoma (Lauer and Walker, 2001). However, treatment options for chronic hepatitis C are limited and a vaccine is not available. Due to the serious consequences of its infection in humans, much effort has being made to understand the basic mechanisms of HCV cell entry and infectivity.

HCV cell entry is a multi-step process mediated by several receptors, involving scavenger receptor class B type I (SR-BI), tetraspanin CD81, and tight junction proteins claudin-1 (CLDN1) and occludin (OCLN) (Stamataki et al., 2008). CD81 is a member of the tetraspanin superfamily defined by four transmembrane domains, a conserved CCG motif, and four cysteine residues that form critical disulfide bonds in the large extracellular loop (LEL). CD81 interacts with HCV envelope (E2) glycoprotein via a series of discontinuous amino acid residues in LEL (Flint et al., 2006). Antibodies directed against CD81 as well as a soluble form of the CD81 LEL, are able to inhibit HCV entry into hepatocytes both *in vitro* (Bartosch et al., 2003; Molina et al., 2008) and *in vivo* (Meuleman et al., 2008). In conjunction with viral entry, a role for CD81 in post-binding steps of HCV infection has been demonstrated. Following virus binding, CD81-mediated signals allow the lateral movement of virus particle/CD81 complex and its delivery to areas of cell–cell contact (Brazzoli et al., 2008), which may initiate viral internalization process (Harris et al., 2010).

Tetraspanins associate with tetraspanin and non-tetraspanin proteins to many biological functions, including cell–cell adhesion, cell migration, signaling and proliferation (Hemler, 2008). These associations are thought to occur mainly in membrane compartments such as lipid rafts and constitute tetraspanin-enriched microdomains (TEMs). In most cell lines, CD81 is associated in a high stoichiometry with its partner proteins EWI-F or EWI-2 (Charrin et al., 2003). Both are members of the EWI family, a small Ig-domain family whose members have a single transmembrane domain and several extracellular Ig-domains, as well as a very short cytosolic tail (Stipp et al., 2001). Recently, it was shown that a truncated form of its partner EWI-2 (EWI-2wint) blocked HCV infection (Rocha-Perugini et al., 2008). This inhibitory effect depends on the interaction of EWI-2/EWI-2wint with CD81 (Montpellier et al., 2011). In addition to their direct and robust interaction with partner proteins, tetraspanins also associate with each other dynamically and less

* Corresponding authors. Fax: +86 21 81870988.
E-mail addresses: pnzhao@163.com (P. Zhao), qizt@smmu.edu.cn (Z.-T. Qi).
[1] These authors contributed equally to this work.

0042-6822/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.virol.2012.03.002

stoichiometrically. In human hepatocytes, CD81 is associated with CD151 and some other tetraspanins (Rocha-Perugini et al., 2009). However, it is not known whether these interactions play a role in HCV infection.

Palmitoylation occurs at the eight juxtamembrane cysteine residues of CD81 (Delandre et al., 2009). Covalent attachment of palmitate is a post-translational modification that influences protein–protein interactions and association with cholesterol-rich domains of membranes (Linder and Deschenes, 2003). The palmitoylation of the juxtamembrane cysteine residues is present in many tetraspanins, including CD9, CD63, CD81 and CD151 (Charrin et al., 2002). Mutagenesis of these cysteines, for example, abolished the palmitoylation of CD9 (Kovalenko et al., 2004) and of CD151 (Berditchevski et al., 2002), thus reducing their association with other tetraspanins.

Tetraspanins are mainly present in part in a detergent-resistant membrane (DRM) domain. These DRM are reported to be highly ordered cholesterol-enriched microdomains, also termed lipid rafts. These ordered membrane microdomains play an important role in the early steps of virus infection by providing a convenient platform for virus–receptor interactions (Lu et al., 2008; Carter et al., 2009; Medigeshi et al., 2008). Targeting of proteins such as src kinases (Dunphy and Linder, 1998), influenza hemagglutinin (Scheiffele et al., 1997) and caveolin (Uittenbogaard and Smart, 2000) to lipid rafts is dependent on palmitoylation. However, whether palmitoylation alters the association of CD81 with TEMs, and location of CD81 in lipid rafts, and whether these interactions play a role in CD81 mediating HCV cell entry remains to be addressed.

In this study, wild-type, partially or completely palmitoylation-defective CD81 were expressed in CD81-deficient Huh7 hepatoma cells. These cells permitted an examination of the effect of palmitoylation on the interaction of CD81 with TEMs and their role in mediating HCV entry. The data suggest that palmitoylation of CD81 strongly promotes HCV entry, at least in part by regulating the association of CD81 with tetraspanin-enriched microdomains.

Results

Association of CD81 with CD151 is dependent on palmitoylation

To examine the role of palmitoylation in the intermolecular association involving CD81, Huh7 cells were incubated with 2-bromopalmitate (2-BP), which is a potent inhibitor of palmitoylation. As shown in Fig. 1A, 2-BP treatment completely eliminated the incorporation of [^{3}H] palmitate into CD81. When total CD81 content was assayed, a faster migrating band was observed on polyacrylamide gels, which was consistent with the loss of palmitoyl moieties (Fig. 1A). Additional co-immunoprecipitations showed that the levels of CD81-associated CD151 and EWI-2 decreased by almost 95% and 10%, respectively, in 2-BP treated compared to untreated cells (Fig. 1A).

To further demonstrate the role of CD81 palmitoylation in protein–protein interactions, experiments were designed to examine the interactions of palmitoylation-deficient CD81 with CD151 and EWI-2. For transmembrane proteins, palmitoylation typically occurs on intracellular cysteine residues and proximal to transmembrane domains (Resh, 1999). CD81 contains eight juxtamembrane cysteines at position 6, 9, 80, 89, 97, 104, 227 and 228 (Fig. 1B). To evaluate the contribution of palmitoylation to HCV infectivity, CD81-deficient Huh7 cells were stably transfected with wild-type CD81 (wtCD81) or each of three cysteine mutants of CD81. Preliminary characterization of these proteins showed comparable levels of expression by FACS analysis (Fig. 1C). Importantly, CD81m5 incorporated only about 30% of [^{3}H] palmitate compared to wild type CD81, while CD81m8 incorporated only about 10% of [^{3}H] palmitate (Fig. 1C). These observations confirm that intracellular juxtamembrane cysteine residues, proximal to all four TM domains, are palmitoylated in CD81.

The effect of cysteine mutagenesis on associations of CD81 with other tetraspanin web members was analyzed by immunoprecipitation under experimental conditions that maintain tetraspanin–tetraspanin interactions (*e.g.* in 1% Brij97). As shown in Fig. 1D, the amount of CD81 co-immunoprecipitated with endogenous CD151 depended on the number of cysteines in the intracellular and transmembrane domains. Elimination of palmitoylation (CD81m8) almost completely abolished the interaction of CD81 with tetraspanin CD151 (Fig. 1D, upper panel), and slightly reduced the association with its partner, EWI-2 (Fig. 1D, lower panel). Hence, these data suggest that the mutation of juxtamembrane cysteine residues affects the association of CD81 with tetraspanin CD151.

Palmitoylation does not contribute to raft localization of CD81

Since palmitoylation promotes the association of most transmembrane proteins with lipid rafts (Dunphy and Linder, 1998; Scheiffele et al., 1997; Uittenbogaard and Smart, 2000), experiments were designed to investigate whether palmitoylation of CD81 played a role in its partition to DRM. Brij97 lysates of Huh7 cells expressing wild type or mutant CD81 were subjected to density equilibrium centrifugation in sucrose gradients, and the fractions then analyzed by western blotting. As shown in Fig. 2A, wtCD81 appeared in the low density fractions of a sucrose gradient. There was no difference in the distribution of wtCD81 and palmitoylation-deficient CD81, indicating that mutation of juxtamembrane cysteines did not change the flotation properties of CD81 (Fig. 2A). In control experiments, abundant endogenous caveolin-2 was in the low density fractions (Fig. 2A, Fractions 3–5) and CD71 marked the dense fractions (Fig. 2A, Fractions 9–12).

To further dissect the contribution of palmitoylation to CD81 raft localization, lysates from 2-BP-treated and untreated Huh7 cells were subjected to sucrose density gradient centrifugation. After pharmacological block of palmitoylation, wild-type CD81 exhibited similar distribution with sucrose gradients to their palmitoylation-deficient counterparts (Fig. 2B, upper panel). In contrast, 2-BP treatment shifted the distribution of caveolin-2 into the heavier fractions of the sucrose gradient (Fig. 2B, bottom panel).

To verify that the western blotting data reflected plasma membrane localization of CD81 mutants, confocal laser scanning microscopy was performed using antibodies against CD81 and caveolin-2. As shown in Fig. 2C, wtCD81 and CD81 mutants co-localized with caveolin-2. Taken together, these results show that blocking of palmitoylation does not have a significant influence on the raft localization of CD81.

Mutation of juxtamembrane cysteines in CD81 impairs HCV entry

To determine whether palmitoylation of CD81 alters its function as a HCV receptor, each CD81 mutant was analyzed for its capacity to mediate HCV-E2 binding, HCVpp entry and HCVcc infection. To test the capacity of these CD81 variants to interact with HCV glycoproteins, we used a soluble recombinant form of HCV E2 glycoprotein (sE2). As shown in Fig. 3A, sE2 bound to wtCD81 and to all CD81 mutants to similar extents, suggesting that removal of the palmitoylation does not affect binding of CD81 to the HCV envelope.

To examine the effect of CD81 palmitoylation on viral entry, Huh7 cells expressing wild type or mutant CD81 were infected with pseudotyped particles bearing HCV envelope proteins of different genotypes or VSV-G envelopes as control. Compared to

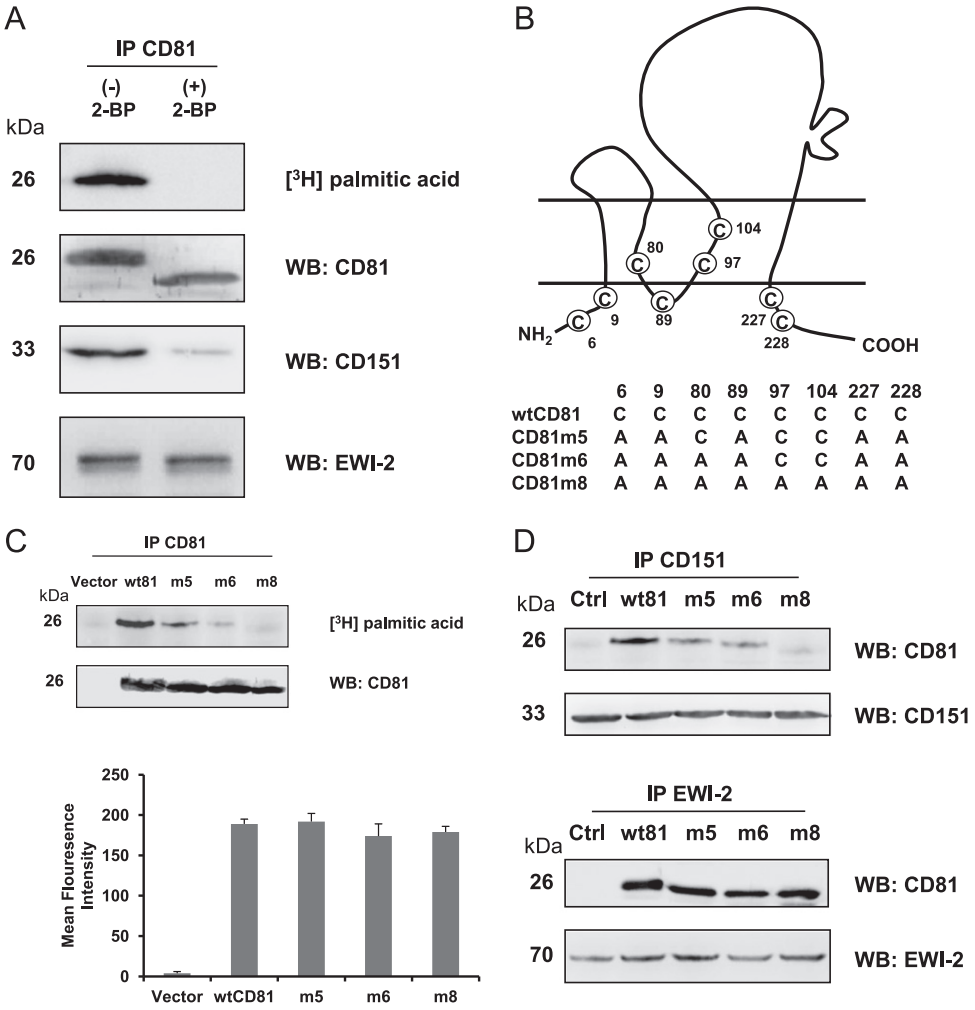

Fig. 1. Association of the palmitoylation-deficient CD81 with CD151 and EWI-2. (A) Huh7 cells were preincubated with 25 μM 2-BP or DMSO for 24 h and then labeled for 2 h with [^{3}H] palmitic acid in the continued presence of 2-BP or DMSO. Cells were lysed in a buffer containing 1% Brij97 and immunoprecipitated using anti-CD81 mAb. Following SDS-PAGE and transfer to PVDF membranes, the immunoprecipitated complexes were exposed to autoradiography (upper panel) and probed with mAb for detection of CD81, CD151 and EWI-2 (lower panels). (B) Schematic representation of CD81 with indication of the juxtamembrane cysteine residues, and their change in the mutant molecules. The numbers indicated correspond to the position of cysteines in the CD81 sequence. C and D, Huh7 cells expressing wtCD81 or CD81 mutants were labeled with [^{3}H] palmitic acid. Cells were lysed and immunoprecipitations were performed. (C) Cell lysates were immunoprecipitated with anti-CD81 mAb. The extent of [^{3}H] palmitate labeling was determined by autoradiography (upper panel). A fraction of the immunoprecipitates was analyzed by immunoblotting using anti-CD81 mAb (middle panel). Surface CD81 expression was analyzed by flow cytometry using 5A6 (lower panel). Results are reported as the mean ± SD of three independent experiments. (D) Cell lysates were immunoprecipitated with anti-CD151 or anti- EWI-2 mAb. The immunoprecipitates was analyzed by immunoblotting for the detection of CD81, CD151 and EWI-2. The results of a representative of three experiments are shown.

wtCD81, Huh7 cells expressing CD81m5 permitted HCVpp entry to about 75% that of cells expressing wtCD81, and for CD81m6 expressing cells, entry was reduced to 54% (Fig. 3B). When all eight cysteine residues in the juxtamembrane domain of CD81 were mutated to alanine, entry was reduced to 40%, but the infectivity of VSV pseudovirus was not altered significantly (Fig. 3B, VSV-G).

Additional work was conducted to evaluate the infectivity of HCVcc in Huh7 cells expressing wild type or individual mutant CD81 molecules. Again, Huh7 cells expressing CD81m8 reduced their infectivity to about 48% compared to Huh7 cells (Fig. 3C), which is similar to the assays above using HCVpp, suggesting that palmitoylation of CD81 contributes to viral entry during HCV infection.

siRNA and antibody specific for CD151 inhibit HCV entry

The above work supports the hypothesis that CD81 palmitoylation contributes importantly to mediating HCV entry in association with TEMs. In this context, the findings that CD81 and CD9 are closely related tetraspanins (Hemler, 2001), and that CD151 strongly binds to CD81 (Rocha-Perugini et al., 2009), imply that CD9 may also potentially play a role in HCV infection. When this was evaluated, the data showed low surface expression of CD9 in the presence of high expression levels of CD151 in CD81 negative Huh7 cells (data not shown), suggesting CD9 may not be operative.

To evaluate the role of CD151 upon infectivity, CD151 levels were depleted by siRNA treatment, and then the cells were challenged with HCV. Efficiency of siRNA-mediated knockdown of CD151 on the cell surface was analyzed by FACS assay. As shown in Fig. 4A, siRNA-1 reduced cell surface expression of CD151 to 24% in Huh7 cells (mean intensity), while a second CD151 specific siRNA (siRNA-2) showed similar results, reducing CD151 expression to about 29% of wild type levels. Importantly, infection of siCD151-treated cells by HCVpp and HCVcc was markedly reduced, as compared to control siRNA-treated cells (Fig. 4B and C). CD151-specific siRNAs reduced HCVpp entry of different genotypes to approximately 35% (siRNA-1) or 46% (siRNA-2) (Fig. 4B) and HCVcc infectivity to 41% (siRNA-1) or 49% (siRNA-2) in Huh7 cells (Fig. 4C). These results suggest that CD151

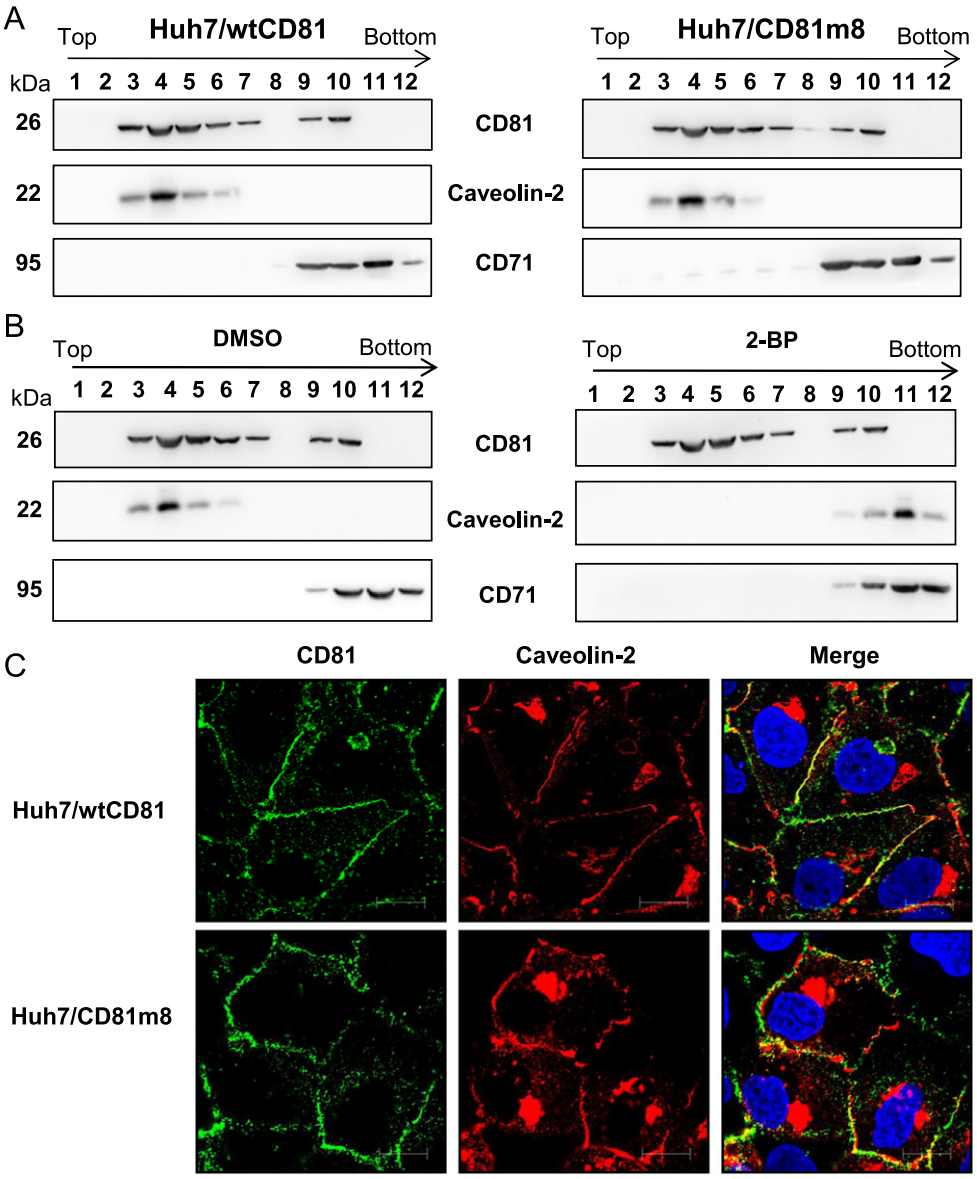

Fig. 2. Raft localization of the unpalmitoylated CD81 in Huh7 cells. (A) Huh7 cells expressing wtCD81 or CD81m8 were lysed with Brij97 and subjected to sucrose density grandient centrifugation. Gradient fractions were collected and analyzed by immunoblotting using the anti-CD81, caveolin-2 or CD71 antibody. The fractions are indicated at the top of each lane. (B) Huh7 cells preincubated with 25 μM 2-BP or DMSO for 24 h, and then subjected to sucrose gradient fractionation as described in the legend to A. The results of a representative of three experiments are shown. (C) Huh7 cells expressing wtCD81 or CD81m8 were cultured on the slides for 24 h. Cells were incubated with the mouse anti-CD81 antibody (5A6) and rabbit anti-caveolin-2 antibody for 1 h at 4 °C, followed by Alexa Flour 488-conjugated anti-mouse and Alexa Flour 555-conjugated anti-rabbit antibody for 1 h at 4 °C. Representative results are shown. The colocalization of red and green gives yellow staining. Bars, 10 μm.

should be an important mediator of HCV infection, independent of virus subtype.

Since siRNA-inhibition studies suggested that the tetraspanin CD151 is primarily involved in HCV infection, additional work was performed to determine whether pretreatment of Huh7 cells with CD151 specific antibody might inhibit infection. As shown in Fig. 4D, the anti-CD151antibody inhibited HCV entry into Huh7 in a dose-dependent manner. To further elucidate the entry steps targeted by the anti-CD151 antibody, the kinetics of entry were evaluated (Krieger et al., 2010) (Fig. 4E). Heparin, a well-characterized molecule shown to interfere with HCV infection predominantly during viral attachment (Barth et al., 2006; Zeisel et al., 2007), markedly blocked HCVcc infection when added prior to HCVcc binding, but inhibited HCVcc infection less efficiently when added post-binding (Fig. 4F). Similar to concanamycin A (an inhibitor of endosomal acidification), the anti-CD151 had an inhibitory effect when added at 60–90 min after infection. Taken together, these data suggest that CD151 is involved in HCV cell entry by modulating early and late steps of post-binding events.

Relocalization of HCV glycoproteins to tight junction protein, claudin-1, is affected by depalmitoylation of CD81 or by siCD151 treatment

Post-binding steps of HCV entry are mediated by the HCV entry factors SR-BI, CD81, CLDN1 and OCLN. To understand the mechanistic role of TEMs, experiments were designed to test whether disruption of the CD81–TEMs associations alter expression of the above factors. Treatment of parental Huh7 cells with siRNA targeting CD151 (Fig. 5A) or depalmitoylation of CD81 (data not shown) had no effect on HCV entry factor expression.

After the virus attachment, HCV bound to the surface of hepatocytes can be transported laterally to areas of cell–cell contact

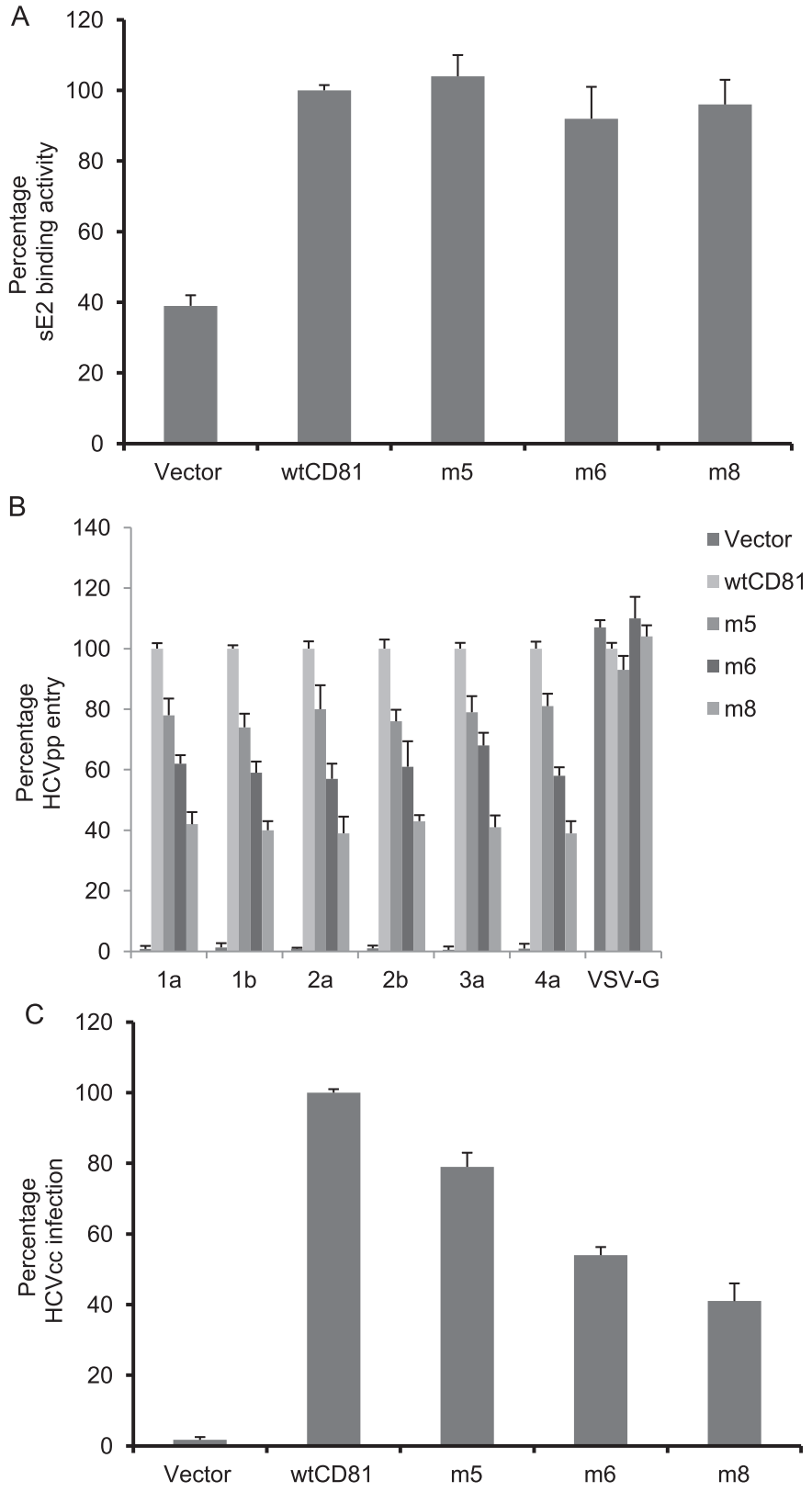

Fig. 3. Role of CD81 palmitoylation in HCV entry. (A) E2 binding activity of CD81 mutants was determined using soluble E2 protein (sE2). The results of sE2 binding were expressed as the mean percentages of mean fluorescence intensity detected in wtCD81 or CD81 mutant-expressing Huh7 cells which were incubated with sE2. The value of wtCD81 was set as 100%. Results are reported as the mean ± SD of three independent experiments. (B and C) Effect of CD81 mutations on HCVpp entry and HCVcc infectivity. Huh7 cells expressing wtCD81 and CD81 mutants were infected with HCVpp of different genotypes or HCVcc of JFH-1 strain. (B) The results were expressed as percentages of infectivity relative to infectivity of HCVpp on cells expressing wtCD81. Results are reported as the mean ± SD of three independent experiments. (C) Efficiency of HCVcc infection was determined as the percentage of HCV NS5A-positive cells relative to infectivity of HCVcc on cells expressing wtCD81. Results are reported as the mean ± SD of three independent experiments.

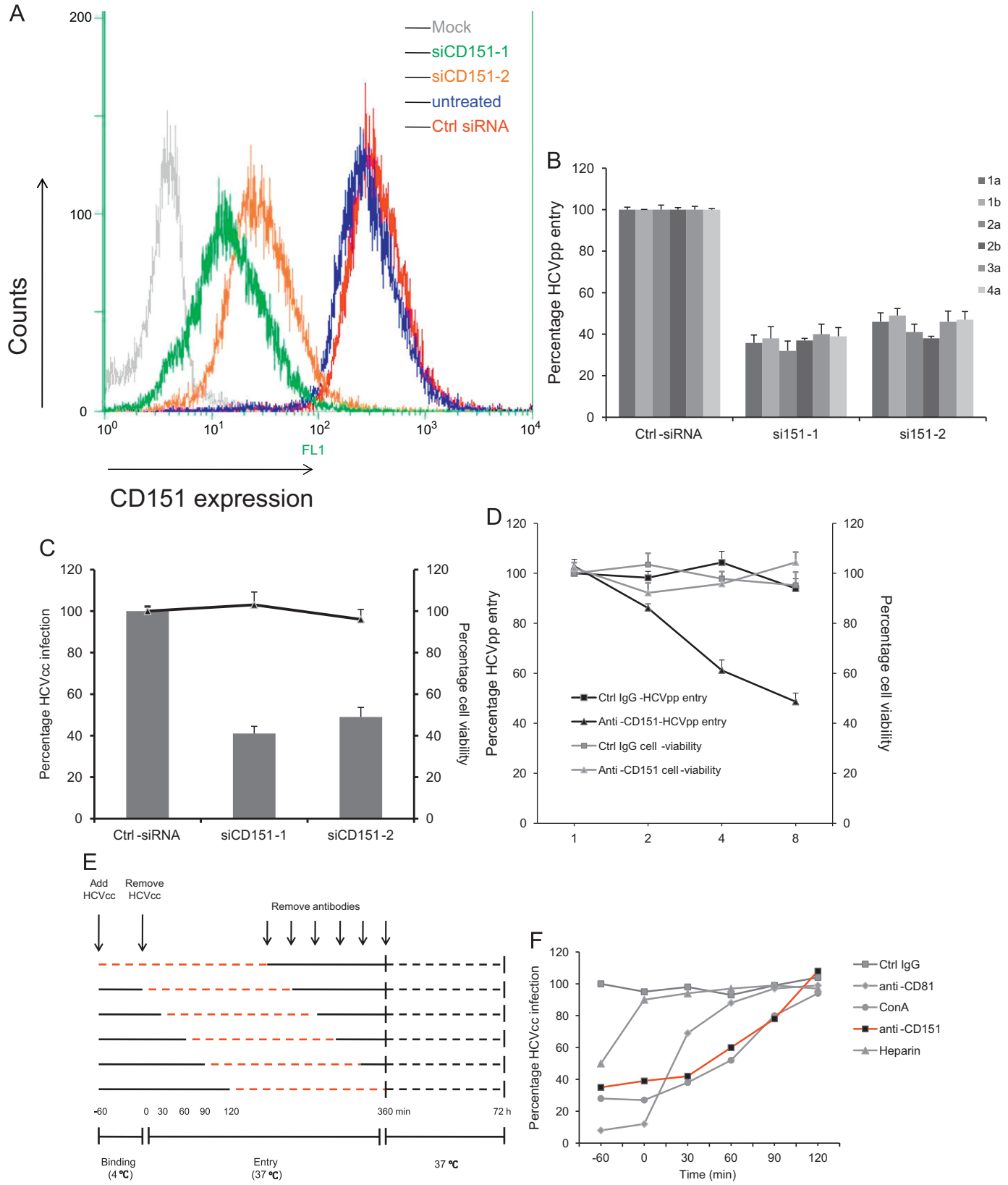

Fig. 4. Role of tetraspanin CD151 in HCV entry. (A) Flow cytometry analysis of the siRNA mediated knockdown of cell surface exposed tetraspanin CD151 in Huh7 cells. (B and C) Huh7 cells were transfected with siRNA as indicated for 48 h and then HCVpp (B) and HCVcc (C) infection assay was performed; infection rate and cell viability of the control siRNA was set to 100%. Results are reported as the mean ± SD of three independent experiments. (D) Percentage of HCVpp entry into Huh7 cell preincubated with anti-CD151 or mouse IgG control is shown (means ± SD from three independent experiments in triplicate). (E) Schematic drawing of kinetics of anti-CD151 mAb-mediated inhibition of HCVcc. (F) Time course of HCVcc infection of Huh7 cells after incubation with anti-CD151 mAb. Results are reported as the mean ± SD of three independent experiments.

mediated solely by CD81, where it comes into contact with the co-receptor claudin-1 (Brazzoli et al., 2008). We therefore used recombinant HCV E2 to examine the lateral migration of CD81-associated tetraspanin. For this purpose, Huh7 cells were cultured on coverslips overnight to achieve a confluent layer and then stimulated with soluble HCV E2 at 37 °C. At indicated time after the addition of sE2, cells were fixed and stained with specific antibodies. Indeed, while CD81 and cell-bound HCV E2 were detected in a diffuse pattern along the whole cellular surface at 10 min, after 60 min of engagement at 37 °C, they concentrated at areas of cell–cell contact (Fig. 5B). A similar relocalization of CD151 was observed (Fig. 5B), indicating the involvement of CD151 in the lateral movement of HCV E2.

Next, similar experiments were designed to test whether the association of CD81 with TEMs interfered with the relocalization of virus to CLDN1 using confocal microscopy. In Huh7-wtCD81 cells, cell-bound E2 accumulated at areas of cell–cell contact after 60 min of engagement at 37 °C, co-localizing with claudin-1 (Fig. 5C). However, on the membrane surface of cells expressing CD81m8, relocation of E2 to CLDN1 was markedly decreased (Fig. 5C). HCV E2 was detected in a diffuse pattern along the cell surface (Fig. 5C). There was also reduced level of E2 accumulation at the boundaries in siCD151-treated Huh7 cells, while the majority of cell-bound E2 was delivered to CLDN1 in mock-treated cells (Fig. 5D). These data suggest that the association of CD81 with TEMs plays a role in mediating the relocalization of virus to tight junction protein claudin-1.

Discussion

HCV cell entry involves a set of receptors. Among them, CD81 directly interacts with HCV envelope glycoprotein and also

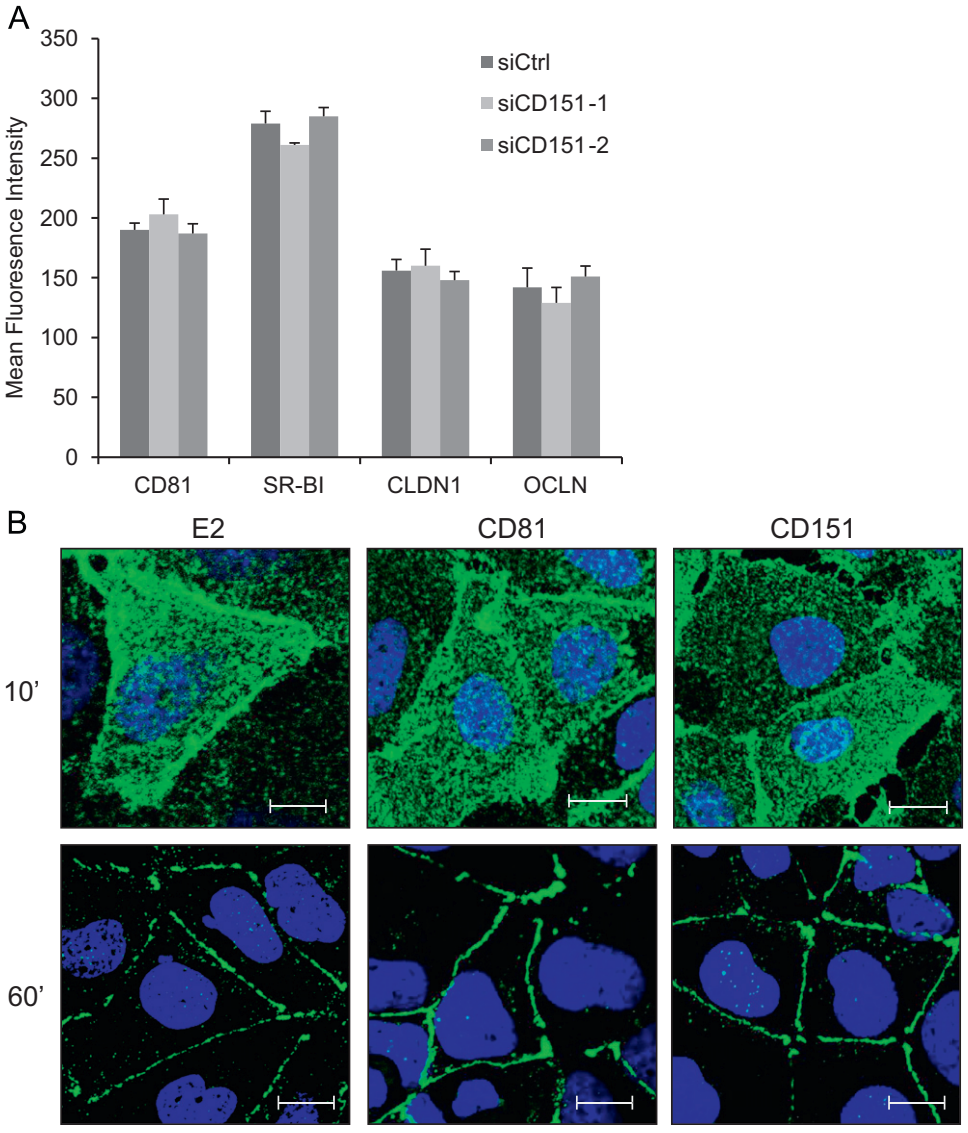

Fig. 5. Effect of depalmitoylation of CD81 or siCD151 treatment on the lateral movement of HCV E2. (A) Cell surface expression of entry factors in siCD151-treated Huh7 cells, as assessed by flow cytometry. Results are reported as the mean ± SD of three independent experiments. (B) Huh7 monolayers grown on slides were incubated with soluble HCV E2 glycoprotein for indicated minutes at 37 °C. Cells were then fixed and stained with antibodies against HCV E2, CD81 and CD151, followed by an anti-mouse-Alexa Fluor 488 secondary antibody (green). Nuclei were stained with DAPI (blue). Bars, 10 μm. (C) Huh7 cells expressing wtCD81 or palmitoylation-deficient CD81 (CD81m8) were cultured on slides overnight to achieve a confluent layer and then stimulated with soluble HCV E2 at 37 °C for 60 min. Cells were then fixed and stained with antibodies against HCV E2 and claudin-1, followed by an anti-rabbit-Alexa Fluor 555 secondary antibody (red) and an anti-mouse-Alexa Fluor 488 secondary antibody (green), as indicated. Areas of colocalization appear yellow. Bars, 10 μm. (D) Soluble HCV E2 was allowed to bind to Huh-7 cells at 37 °C for 60 min. Cells were then fixed and stained with antibodies against HCV E2 and claudin-1 as described in legend to C. Bars, 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

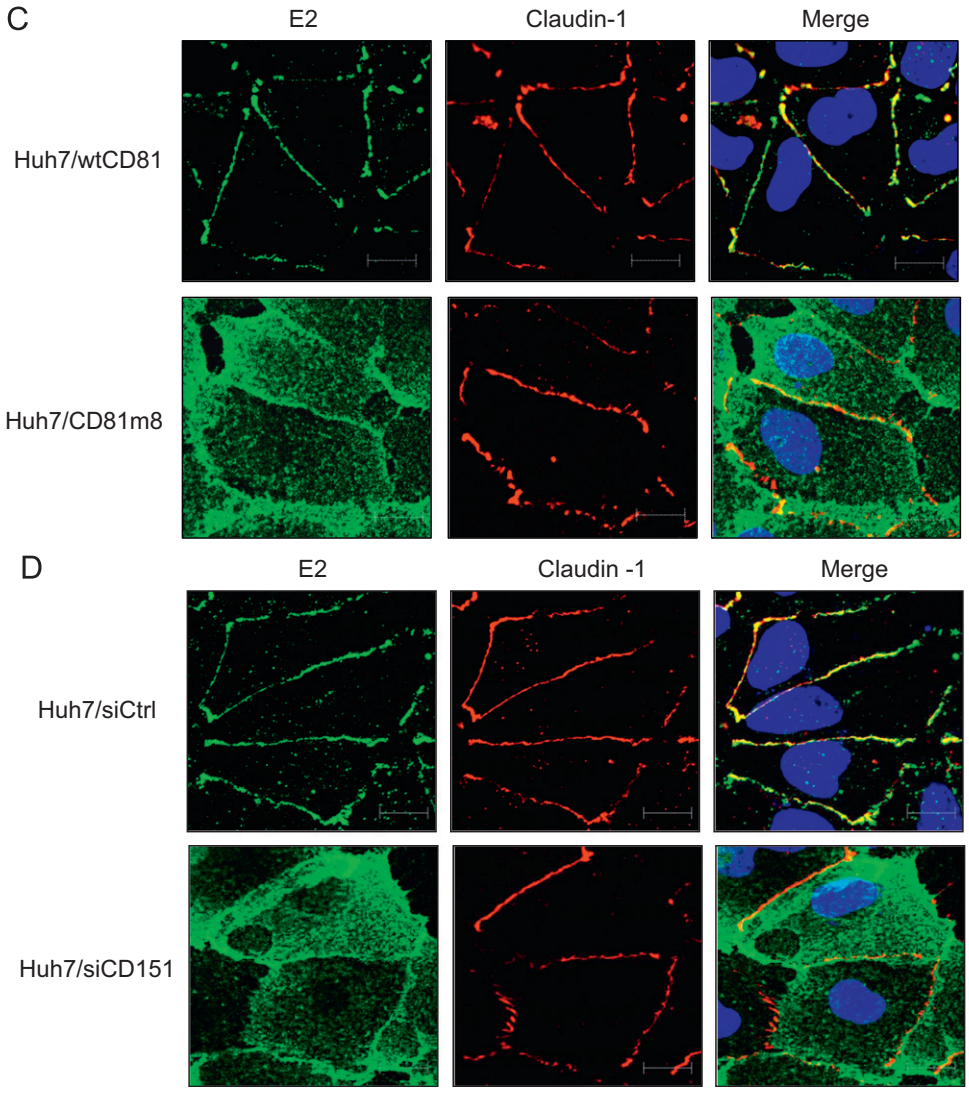

Fig. 5. (*continued*)

mediates the lateral movement of virus particle to areas of cell–cell contact. CD81 belongs to the tetraspanin superfamily, members of which interact with each other to participate in various biological roles. The work herein set out to characterize the essential requirements of CD81 palmitoylation for its association with TEMs and HCV entry.

To generate palmitoylation-deficient CD81 proteins, Bertaux and his colleagues mutated five cysteines located in the intracellular domains of CD81 (Bertaux and Dragic, 2006). However, the predicted three-dimensional conformation of CD81 showed another three cysteines at the transmembrane domains where it would be available for palmitoylation (Seigneuret, 2006). In this study, we constructed the mutants for all of the related eight cysteines to alanines to eliminate palmitoylation. Palmitoylation of tetraspanins affects protein–protein interactions, suggesting a key role in the assembly of the tetraspanin web. Co-immunoprecipitations of CD81 mutants showed that the association of CD81 with endogenous CD151 correlated with the level of palmitoylation. The finding that 2-BP prevented CD81–tetraspanin interactions further supported the important role of palmitoylation for the association of CD81 with TEMs. TEMs protein associations can be divided into three categories. In the first category (Level 1), each tetraspanin forms primary complexes with a limited number of molecular partners, which are stable in Triton X-100, and are likely to be direct, such as with CD81/EWI-2 (Charrin et al., 2001) and CD151/$\alpha 3\beta 1$ complex (Yauch et al., 2000). Most tetraspanin–tetraspanin associations (such as CD9, CD63, CD81, CD151), formed between members of the family, are Level 2 interactions (Lozahic et al., 2000). These secondary associations can be disrupted by Triton X-100 but are retained in Brij97 or other less hydrophobic detergents (Boucheix and Rubinstein, 2001). Tetraspanins associate indirectly with additional proteins, which have been referred to Level 3 interactions. These complexes are not disrupted in milder detergents, such as 1% CHAPS. These include tetraspanin associations with various signaling molecules and cytoskeletal structures (Shi et al., 2000; Levy and Shoham, 2005). Our results showed that EWI-2 interaction with unpalmitoylated CD81 was only slightly decreased. One possibility is that other domains of CD81, such as LEL (Shoham et al., 2006), could also be involved in its association with partner proteins. Importantly, the interaction between CD81 and EWI-2 has been proposed to be independent of CD81 palmitoylation (Montpellier et al., 2011). However, in the latter study, only six of eight potential cysteines were mutated. Together these results suggest that the maintenance of tetraspanin–tetraspanin interactions is generally regulated by palmitoylation.

Since palmitoylation is important for many transmembrane proteins partitioning into lipid rafts, we detected the requirement of palmitoylation for the raft localization of CD81. The results

showed that elimination of the acylation acceptor cysteines does not significantly affect the location of CD81 in DRM. In support of this, treatment of 2-BP yields in a shift of caveolin-2 to non-raft domains but does not shift the raft localization of CD81. Consistent with the results herein, depalmitoylation of multiple members of the tetraspanin family including CD9 (Charrin et al., 2002), CD151 (Berditchevski et al., 2002), and CD63 (Israels and McMillan-Ward, 2010) is insufficient to promote them partitioning to non-raft regions. These data indicate that the role of tetraspanin palmitoylation in maintenance of TEMs is distinct from that of raft localization.

Compared with wtCD81, palmitoylation-deficient CD81 mutants exhibited similar sE2 binding, but markedly reduced the susceptibility of Huh7 cells to HCV. This inhibition probably occurs at cell entry steps, since CD81 mutants impair the infection of single-cycle HCV pseudoparticles of diverse genotypes. CD81 partitioning in lipid raft is important for HCV entry (Kapadia et al., 2007). As mutation of palmitoylation sites has no significant impact on the raft localization of CD81, it is hypothesized that the unpalmitoylated CD81 mediated reduced HCV entry by affecting its association with TEMs. Besides the tetraspanin–tetraspanin interaction, the direct interaction between tetraspanins and their partner proteins also results in the modulation of their functions. CD81 functions in infectious diseases can therefore be affected by the association with the proteins EWI-F, EWI-2 or EWI-2wint. EWI-F acts as a negative regulator of Plasmodium yoelii infection by interacting with CD81 and regulating its function (Charrin et al., 2009). EWI-2wint, a cleavage product of EWI-2, needs to interact with CD81 to exert its inhibitory effect on HCV infection (Rocha-Perugini et al., 2008). However, about 60% reduction of HCV entry seems not to be fully explained by only 10% detachment of EWI-2 with depalmitoylated CD81. Therefore, it is hypothesized that the interaction of CD81 with other tetraspanins could benefit HCV infection. As expected, siRNA-mediated knockdown or specific antibody targeting CD151 exert a strong inhibitory effect on HCV entry. Furthermore, treatment of the cells with anti-CD151 antibody inhibited HCV infection at late steps of HCV entry. Applying confocal microscopy, it was shown that CD151 played a functional role in the relocalization of HCV E2/CD81 complex to areas of cell–cell contact.

After virus binding, CD81 triggered Rho-GTPase-dependent actin rearrangement that allows the delivery of virus–receptor complex to areas of cell–cell contact (Brazzoli et al., 2008). In this context, the study herein provides preliminary data showing that depalmitoylation of CD81 or treatment of the cells with CD151 specific siRNA reduced the relocalization of HCV E2/CD81 complex to tight junction protein CLDN1. Data from the present work extend recent observations that TEMs involved in the infection process of various viruses, such as HIV (Gordon-Alonso et al., 2006), HTLV-1 (Pique et al., 2000), HPV (Spoden et al., 2008), although tetraspanins are not receptors for these viruses. The association of CD81 with TEMs may facilitate the signaling pathways that permit the uptake of the active entry complex efficiently, which is consistent the role of tetraspanins in providing specific functional links to cytoskeletal or signaling proteins (Stipp et al., 2001; Yunta and Lazo, 2003; Levy and Shoham, 2005; Sala-Valdes et al., 2006). A recent study has showed that in chronic hepatitis C (CHC)-infected liver tissues, CD151 was found in the E2-immunoprecipitated complexes (Sanz-Cameno et al., 2008), suggesting that virions may preferentially interact with CD81-associated TEMs. The finding that anti-CD151 antibody mainly recognizes the LEL, implies that the extracellular domains of both tetraspanins are also involved in their association. CD151 antibody could impair the interaction between the extracellular domains of both tetraspanins by steric hindrance, resulting in decrease the signaling pathways and redistribution of HCV/CD81 complexes.

Recently, Vera et al. took advantage of a monoclonal antibody that specifically recognizes a subset of mouse CD81 molecules associated with TEMs (MT81w) to investigate the role of TEM-associated CD81 in HCV infection. In contrast to the present study, these investigators found that TEMs-associated CD81 is not essential for viral entry. The observed discrepancy may be due to the species-specific CD81 used. A number of experiments have demonstrated the failure of mouse CD81 to mediate HCV entry (Flint et al., 2006; Ploss et al., 2009). Among the HCV receptors, CD81 and OCLN represent the minimal human-specific entry factor (Ploss et al., 2009). The inhibitory effect of the antibody targeting TEMs-associated CD81 on HCV infectivity may yield a negative result by using the mouse CD81 model. Besides the different species of CD81, It is also possible that the lower affinity of MT81w may lead to an underestimate of the ratio of CD81 engaged in TEMs (Silvie et al., 2006).

In conclusion, this study demonstrates the important role of CD81 palmitoylation in association with TEMs and mediating HCV cell entry. These findings provide novel insights into understanding of the very early steps of HCV infection and the development of novel antiviral strategies targeting viral entry.

Materials and methods

Cells and antibodies

CD81-deficient Huh7 hepatoma cells (Huh7 CD81$^-$) were provided by Jin Zhong (Institut Pasteur of Shanghai, Chinese Academy of Sciences, Shanghai, China) (Zhong et al., 2006). Huh7 and HEK 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen). CD81 (5A6), anti-CD71 (3B8 2A1) and CD151 (11G5a) monoclonal antibodies were purchased from Santa Cruz Biotechnology. Caveolin-2 antibody was purchased from Sigma. EWI-2 monoclonal antibody (8A12) was a generous gift from Eric Rubinstein (INSERM U268, Paris, France). Claudin-1 antibody was obtained from Invitrogen. Horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit Ig were purchased from Santa Cruz Biotechnology. Alexa 488 conjugated anti-mouse Ig and Alexa 555 conjugated anti-rabbit Ig was purchased from Invitrogen.

Mutagenesis and transfection

The CD81 constructs with mutations in the juxtamembrane cysteines (position 6, 9, 80, 89, 97, 104, 227, 228) were generated by using fusion PCR, in which the cysteine residues were replaced by alanines. Wild-type CD81 (wtCD81) and CD81 mutants were inserted into pcDNA3.1 (+) vector (Invitrogen), respectively. Each of the resulting plasmids was verified by sequency and transfected into CD81-deficient Huh7 cells by using Lipofecatimane 2000 (Invitrogen).

Incorporation of [^{3}H]-palmitate

For experiments using 2-Bromopalmitate (2-BP), cells were pretreated with 25 μM 2-BP (Sigma) for 24 h. As a negative control for 2-BP, cells were treated with 0.05% DMSO. Then 2-BP and DMSO were removed, and the cells were washed three times with PBS followed by metabolic labeling, immunoprecipitation and lipid raft isolation as described below.

To assess palmitic acid incorporation, cells were serum starved for 3 h and labeled overnight with 0.3 mCi/ml [^{3}H] palmitic acid (PerkinElmer Life Sciences) in DMEM supplemented with 5% fetal bovine serum. Cells were harvested and washed three times with PBS followed by lysis and immunoprecipitation with anti-CD81

antibody 5A6 as described below. The samples were then separated by non-reducing SDS-PAGE, transferred to PVDF membranes, and membranes were exposed to Kodak BioMax MS Film (Eastman Kodak Company, Rochester, NY) for one week at −80 °C.

Immunoprecipitation and immunoblotting

For immunoprecipitation, cells were prepared in lysis buffer containing 1% Brij97 (Sigma), 25 mM HEPES, 150 mM NaCl, 5 mM $MgCl_2$, 20 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. After 1 h at 4 °C, insoluble material was pelleted at 16,000*g* (25 min, 4 °C). The supernatants were treated by incubation with protein G-Sepharose (Roche Molecular Biochemicals, Indianapolis, IN), and then incubated with specific antibodies for 1 h at 4 °C, followed by overnight incubation with protein G-Sepharose. Immune complexes were collected by centrifugation, washed three times in lysis buffer, and then analyzed by SDS-PAGE (12% acrylamide) under non-reducing conditions. The proteins were transferred to PVDF membranes and probed with primary antibody, then following with secondary antibody: HRP-conjugated anti-rabbit or anti-mouse antibodies. Bound antibodies were detected with the ECL Plus Western blotting detection reagents (PerkinElmer Life Sciences). Bands from chemiluminescence were quantitated using AlphaView 3.0 software from NatureGene Corp.

Lipid raft isolation

Cells (5×10^7) were washed twice with ice-cold PBS and lysed on ice for 30 min in 1 ml TNE-buffer (25 mM Tris [pH 7.5], 150 mM NaCl, 5 mM EDTA, and complete protease inhibitor cocktail (Roche)) with 1% Brij97. The cell lysates were homogenized and then centrifuged for 5 min, 1000*g* at 4 °C to remove insoluble materials. The supernatant was mixed with 1 ml 80% sucrose in TNE buffer, placed at the bottoms of ultracentrifuge tubes, overlaid with 7 ml 30% and 3 ml 5% sucrose in TNE buffer. The cell lysates were ultracentrifuged at 4 °C for 18 h at 36,000 rpm in a SW41 rotor (Beckman). After centrifugation, twelve 1-ml fractions were collected from the top to the bottom and analyzed immediately by immunoblotting.

About 20 μl of individual sucrose gradient fractions were resolved by SDS-PAGE (12% acrylamide). Western blotting was performed using caveolin-2, anti-CD71 and CD81 mAbs, with horseradish peroxidase-conjugated secondary antibody. Blots were visualized by chemiluminescence.

Production of HCVpp and infection assays

HCVpp were generated by the co-transfection of HEK 293T cells with a HCV envelope protein expression vector and packaged plasmid based on the HIV-1 strain NL4-3 (Invitrogen), as described previously (Tong et al., 2011). Briefly, 293T cells were co-transfected with expression plasmids encoding the HCV envelope glycoproteins, HIV gag/pol (pLP1), HIV rev (pLP2) and pLenti6 encoding luciferase. HCV envelope expression plasmids used here included genotype 1a strain H77 (provided by F.L. Cosset, INSERM U758, Lyon, France), genotype 1b strain Con-1 (provided by C. M.Rice, Rockefeller University, NY, USA) and genotypes 2a (clone UKN2A1.2), 2b (clone UKN2B2.8), 3a (clone UKN3A1.28C), and 4a (clone UKN4.21.16) (provided by J.K. Ball, The University of Nottingham, United Kingdom). Supernatants containing pseudotyped particles were harvested at 48 h post-transfection and filtered through 0.45-μm membranes, and used for infection. Vesicular stomatitis virus glycoprotein (VSV-G) pp was prepared for use as a control. HCVpp and VSV-Gpp were added to Huh-7 cells expressing wtCD81 or CD81 mutants seeded the day before in 24-well plates and incubated for indicated times at 37 °C. The supernatants were then removed, and the cells were incubated in DMEM with 3% FBS at 37 °C. At 72 h post infection, the cells were washed once in PBS then lysed in 50 μl of lysis buffer (Promega). Luciferase activity was quantified using the Bright Glow Luciferase Assay System (Promega).

Production of HCVcc and infection assays

The plasmid pJFH-1, containing the full-length cDNA of JFH-1 isolate and kindly provided by T. Wakita (National Institute of Infectious Diseases, Tokyo, Japan), was used to generate HCVcc as previously described (Wakita et al., 2005; Rouillé et al., 2006). Briefly, the pJFH-1 plasmid was linearized and used as a template for *in vitro* transcription with the MEGAscript kit from Ambion. *In vitro* transcribed RNA was delivered to Huh-7 cells by electroporation, and viral stocks were obtained by harvesting cell culture supernatants 1 week after transfection.

Huh7 cells expressing wtCD81 or CD81 mutants were infected with HCVcc for 5 h at 37 °C. At 72 h postinfection, cells were fixed, permeabilized, and immunostained for NS5A protein using anti-NS5A mAb 9E10 clone (provided by C.M. Rice, Rockefeller University, NY, USA). Infection was determined by enumerating NS5A-positive cells or foci under a fluorescence microscope as previously described (Mee et al., 2009).

E2 binding assays

The soluble E2 (sE2) protein harboring six histidines at its C-terminal was expressed in CHO cells that stably transfected with plasmid containing truncated E2 sequence of H77 strain (364–661 aa in HCV polyprotein), the protein was purified using a nickel-chelating Sepharose resin (Qiagen) from culture supernatants. Binding of soluble E2 glycoprotein, derived from the H77-E2 was performed as previously described (Flint et al., 2000). Huh7 cells (2×10^5) expressing wtCD81 or CD81 mutants were incubated with 5 μg/ml of sE2 for 1 h at room temperature in PBS supplemented with 1% bovine serum albumin and were then washed twice with PBS. Soluble E2 binding to cells was detected by flow cytometry (Beckman Coulter) after labeling with the anti-E2 mAb H53 (provided by J. Dubuisson, Institut Pasteur, Lille, France) followed by an Alexa Flour 488-conjugated anti-mouse IgG.

Flow cytometry

To analyze cell surface expression of CD81, suspended cells expressing wtCD81 or CD81 mutants were treated with anti-CD81 mAb (5A6) for 1 h at 4 °C. The cells were washed with PBS three times, and then treated with an Alexa Flour 488-conjugated anti-mouse IgG. The stained cells were applied to a flow cytometry.

Confocal microscopy

Cells were cultured on eight-well culture slides (Millipore) at 37 °C in a humidified 5% CO_2 atmosphere for 24 h and then subjected to confocal microscopy analysis. Cells were incubated with the mouse anti-CD81 mAb and rabbit anti-caveolin-2 antibody for 1 h at 4 °C, followed by Alexa Flour 488-conjugated anti-mouse and Alexa Flour 555-conjugated anti-rabbit Ig for 1 h at 4 °C.

In some experiments, Huh7 cells were incubated with soluble HCV E2 protein (aa 364–661 of H77 strain) expressed by CHO cells in this laboratory at a concentration of 10 μg/ml for the indicated time at 37 °C. Cells were fixed in 3% formaldehyde and then probed with E2 mAb H53 or claudin-1 polyclonal antibody. After the incubation with fluorescence-conjugated secondary antibodies, cells were observed using a confocal fluorescence microscope (Zeiss).

siRNA experiments

The specific siRNAs targeting CD151, siCD151-1 (TCACAGGACTGGCGAGACATT) (Kovalenko et al., 2007) and CD151-2 (CAUGUGGCACCGUUUGCCUTT) (Spoden et al., 2008) were obtained from Invitrogen. As control we used siCD151-1 specific siRNA with two mismatches (TCACCGGACTGACGAGACATT). Huh7 cells were transfected with 30 nM of siRNA using Lipofectamine RNAiMAX according to the manufacturer's instructions. Subsquent experiments were done 72 h after siRNA transfection. Knockdown efficiencies were quantified by flow cytometry.

Conflicts of interest

There are no conflicts of interest to disclose for all authors.

Acknowledgments

We are grateful to C.M. Rice, J. Zhong, F.L.Cosset, E. Rubinstein, J. Dubuisson, T. Wakita and J.K. Ball for gift of research materials, and to You-Heng Wei (Fudan University, Shanghai, China) for excellent technical assistance. This work was funded by research grants from 973 Program of China (2009CB522501, 2009CB522503), Important National Science and Technology Special Projects for Prevention and Treatment of Major Infectious Diseases (2012ZX10002003-004-010), National Natural Science Foundation of China (81071364, 30921006, 31170150 and 30800049), and Shanghai LAD Project (B901).

References

Barth, H., Schnober, E.K., Zhang, F., Linhardt, R.J., Depla, E., Boson, B., Cosset, F.L., Patel, A.H., Blum, H.E., Baumert, T.F., 2006. Viral and cellular determinants of hepatitis C virus envelope–heparan sulfate interaction. J. Virol. 80 (21), 527–535.

Bartosch, B., Dubuisson, J., Cosset, F.L., 2003. Infectious hepatitis C virus pseudoparticles containing functional E1–E2 envelope protein complexes. J. Exp. Med. 197 (5), 633–642.

Berditchevski, F., Odintsova, E., Sawada, S., Gilbert, E., 2002. Expression of the palmitoylation-deficient CD151 weakens the association of $\alpha 3\beta 1$ integrin with the tetraspanin-enriched microdomains and affects integrin-dependent signaling. J. Biol. Chem. 277 (40), 36991–37000.

Bertaux, C., Dragic, T., 2006. Different domains of CD81 mediate distinct stages of hepatitis C virus pseudoparticle entry. J. Virol. 80 (10), 4940–4948.

Boucheix, C., Rubinstein, E., 2001. Tetraspanins. Cell Mol. Life Sci. 58 (9), 1189–1205.

Brazzoli, M., Bianchi, A., Filippini, S., Weiner, A., Zhu, Q., Pizza, M., Crotta, S., 2008. CD81 is a central regulator of cellular events required for hepatitis C virus infection of human hepatocytes. J. Virol. 82 (17), 8316–8329.

Carter, G.C., Bernstone, L., Sangani, D., Bee, J.W., Harder, T., James, W., 2009. HIV entry in macrophages is dependent on intact lipid rafts. Virology 386 (1), 192–202.

Charrin, S., Le Naour, F., Oualid, M., Billard, M., Faure, G., Hanash, S.M., Boucheix, C., Rubinstein, E., 2001. The major CD9 and CD81 molecular partner: identification and characterization of the complexes. J. Biol. Chem. 276 (17), 14329–14337.

Charrin, S., Manie, S., Oualid, M., Billard, M., Boucheix, C., Rubinstein, E., 2002. Differential stability of tetraspanin/tetraspanin interactions: role of palmitoylation. FEBS Lett. 516 (1–3), 139–144.

Charrin, S., Le Naour, F., Labas, V., Billard, M., Le Caer, J.P., Emile, J.F., Petit, M.A., Boucheix, C., Rubinstein, E., 2003. EWI-2 is a new component of the tetraspanin web in hepatocytes and lymphoid cells. Biochem. J. 373 (Part 2), 409–421.

Charrin, S., Yalaoui, S., Bartosch, B., Cocquerel, L., Franetich, J.F., Boucheix, C., Mazier, D., Rubinstein, E., Silvie, O., 2009. The Ig domain protein CD9P-1 down-regulates CD81 ability to support *Plasmodium yoelii* infection. J. Biol. Chem. 284 (46), 31572–31578.

Delandre, C., Penabaz., T.R., Passarelli, A.L., Chapes, S.K., Clem, R.J., 2009. Mutation of juxtamembrane cysteines in the tetraspanin CD81 affects palmitoylation and alters interaction with other proteins at the cell surface. Exp. Cell Res. 315 (11), 1953–1963.

Dunphy, J.T., Linder, M.E., 1998. Signaling functions of protein palmitoylation. Biochim. Biophys. Acta 1436 (1–2), 245–261.

Flint, M., Dubuisson, J., Maidens, C., Harrop, R., Guile, G.R., Borrow, P., McKeating, J.A., 2000. Functional characterization of intracellular and secreted forms of a truncated hepatitis C virus E2 glycoprotein. J. Virol. 74 (2), 702–709.

Flint, M., von Hahn, T., Zhang, J., Farquhar, M., Jones, C.T., Balfe, P., Rice, C.M., McKeating, J.A., 2006. Diverse CD81 proteins support hepatitis C virus infection. J. Virol. 80 (22), 11331–11342.

Gordon-Alonso, M., Yanez-Mo, M., Barreiro, O., Alvarez, S., Munoz- Fernandez, M.A., Valenzuela-Fernandez, A., Sanchez-Madrid, F., 2006. Tetraspanins CD9 and CD81 modulate HIV-1-induced membrane fusion. J. Immunol. 177 (8), e5129–5137.

Harris, H.J., Davis, C., Mullins, J.G.L., Hu, K., Goodall, M., Farquhar, M.J., Mee, C.J., McCaffrey, K., Young, S., Drummer, H., Balfe, P., McKeating, J.A., 2010. Claudin association with CD81 defines hepatitis C virus entry. J. Biol. Chem. 285 (27), 21092–21102.

Hemler, M.E., 2001. Specific tetraspanin functions. J. Cell Biol. 155 (7), 1103–1107.

Hemler, M.E., 2008. Targeting of tetraspanin proteins—potential benefits and strategies. Nat. Rev. Drug Disc. 7 (9), 747–758.

Israels, S.J., McMillan-Ward, E.M., 2010. Palmitoylation supports the association of tetraspanin CD63 with CD9 and integrin αIIbβ3 in activated platelets. Thromb. Res. 125 (2), 152–158.

Kapadia, S., Barth, H., Baumert, T., McKeating, J.A., Chisari, F.V., 2007. Initiation of hepatitis C virus infection is dependent on cholesterol and cooperativity between CD81 and scavenger receptor B type I. J. Virol. 81 (1), 374–383.

Kovalenko, O.V., Yang, X., Kolesnikova, T.V., Hemler, M.E., 2004. Evidence for specific tetraspanin homodimers: inhibition of palmitoylation makes cysteine residues available for cross-linking. Biochem. J. 377 (Part 2), 407–417.

Kovalenko, O.V., Yang, X.H., Hemler, M.E., 2007. A novel cysteine cross-linking method reveals a direct association between claudin-1 and tetraspanin CD9. Mol. Cell Proteomics 6 (11), 1855–1867.

Krieger, S.E., Zeisel, M.B., Davis, C., Thumann, C., Harris, H.J., Schnober, E.K., Mee, C., Soulier, E., Royer, C., Lambotin, M., Grunert, F., Dao Thi, V.L., Dreux, M., Cosset, F.L., McKeating, J.A., Schuster, C., Baumert, T.F., 2010. Inhibition of hepatitis C virus infection by anti-claudin-1 antibodies is mediated by neutralization of E2–CD81–claudin-1 associations. Hepatology 51 (4), 1144–1157.

Lauer, G.M., Walker, B.D., 2001. Hepatitis C virus infection. N. Engl. J. Med. 345 (1), 41–52.

Linder, M.E., Deschenes, R.J., 2003. New insights into the mechanisms of protein palmitoylation. Biochemistry 42 (15), 4311–4320.

Levy, S., Shoham, T., 2005. Protein–protein interactions in the tetraspanin web. Physiology 20, 218–224.

Lozahic, S., Christiansen, D., Manie, S., Gerlier, D., Billard, M., Boucheix, C., Rubinstein, E., 2000. CD46 (membrane cofactor protein) associates with multiple beta1 integrins and tetraspans. Eur. J. Immunol. 30 (3), 900–907.

Lu, Y., Liu, D.X., Tama, J.P., 2008. Lipid rafts are involved in SARS-CoV entry into Vero E6 cells. Biochem. Biophys. Res. Commun. 369 (2), 344–349.

Medigeshi, G.R., Hirsch, A.J., Streblow, D.N., Nikolich-Zugich, J., Nelson., J.A., 2008. West Nile virus entry requires cholesterol-rich membrane microdomains and is independent of alphavbeta3 integrin. J. Virol. 82 (11), 5212–5219.

Mee, C.J., Harris, H.J., Farquhar, M.J., Wilson, G., Reynolds, G., Davis, C., van, I.S.C., Balfe, P., McKeating, J.A., 2009. Polarization restricts hepatitis C virus entry into HepG2 hepatoma cells. J. Virol. 83 (12), 6211–6221.

Meuleman, P., Hesselgesser, J., Paulson, M., Vanwolleghem, T., Desombere, I., Reiser, H., Leroux-Roels, G., 2008. Anti-CD81 antibodies can prevent a hepatitis C virus infection *in vivo*. Hepatology 48 (6), 1761–1768.

Molina, S., Castet, V., Pichard-Garcia, L., Wychowski, C., Meurs, E., Pascussi, J.M., Sureau, C., Fabre, J.M., Sacunha, A., Larrey, D., Dubuisson, J., Coste, J., McKeating, J., Maurel, P., Fournier-Wirth, C., 2008. Serum-derived hepatitis C virus infection of primary human hepatocytes is tetraspanin CD81 dependent. J. Virol. 82 (1), 569–574.

Montpellier, C., Tews, B.A., Poitrimole, J., Rocha-Perugini, V., D'Arienzo, V., Potel, J., Zhang, X.A., Rubinstein, E., Dubuisson, J., Cocquerel, L., 2011. Interacting regions of CD81 and two of its partners, EWI-2 and EWI-2wint and their effect on hepatitis C virus infection. J. Biol. Chem. 286 (16), 13954–13965.

Pique, C., Lagaudriere-Gesbert, C., Delamarre, L., Rosenberg, A.R., Conjeaud, H., Dokhelar, M.C., 2000. Interaction of CD82 tetraspanin proteins with HTLV-1 envelope glycoproteins inhibits cell-to-cell fusion and virus transmission. Virology 276 (2), 455–465.

Ploss, A., Evans, M.J., Gaysinskaya, V.A., Panis, M., You, H., de Jong, Y.P., Rice, C.M., 2009. Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. Nature 457 (7231), 882–886.

Resh, M.D., 1999. Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. Biochim. Biophys. Acta 1451 (1), 1–16.

Rocha-Perugini, V., Montpellier, C., Delgrange, D., Wychowski, C., Helle, F., Pillez, A., Drobecq, H., Le Naour, F., Charrin, S., Levy, S., Rubinstein, E., Dubuisson, J., Cocquerel, L., 2008. The CD81 partner EWI-2wint inhibits hepatitis C virus entry. PLoS One 3 (4), e1866.

Rocha-Perugini, V., Lavie, M., Delgrange, D., Canton, J., Pillez, A., Potel, J., Lecoeur, C., Rubinstein, E., Dubuisson, J., Wychowski, C., Cocquerel, L., 2009. The association of CD81 with tetraspanin-enriched microdomains is not essential for hepatitis C virus entry. BMC Microbiol. 9, 111.

Rouillé, Y., Helle, F., Delgrange, D., Roingeard, P., Voisset, C., Blanchard, E., Belouzard, S., McKeating, J., Patel, A.H., Maertens, G., Wakita, T., Wychowski, C., Dubuisson, J., 2006. Subcellular localization of hepatitis C virus structural proteins in a cell culture system that efficiently replicates the virus. J. Virol. 80 (6), 2832–2841.

Sala-Valdes, M., Ursa, A., Charrin, S., Rubinstein, E., Hemler, M.E., Sanchez-Madrid, F., Yanez-Mo, M., 2006. EWI-2 and EWI-F link the tetraspanin web to the actin cytoskeleton through their direct association with ezrin–radixin–moesin proteins. J. Biol. Chem. 281 (28), 19665–19675.

Sanz-Cameno, P., Martín-Vílchez, S., Rodriguez-Munoz, Y., Moreno-Otero, R., 2008. Implication of tetraspanin-enriched microdomains (TEMs) in hepatitis C virus. Hepatology 48 (1), 346–348.
Scheiffele, P., Roth, M., Simons, K., 1997. Interaction of influenza virus haemagglutinin with sphingolipid–cholesterol membrane domains via its transmembrane domain. EMBO J. 16 (18), 5501–5508.
Seigneuret, M., 2006. Complete predicted three-dimensional structure of the facilitator transmembrane protein and hepatitis C virus receptor CD81: conserved and variable structural domains in the tetraspanin superfamily. Biophys. J. 90 (1), 212–227.
Shi, W., Fan, H., Shum, L., Derynck, R., 2000. The tetraspanin CD9 associates with transmembrane TGF-alpha and regulates TGF-alpha-induced EGF receptor activation and cell proliferation. J. Cell Biol. 148 (3), 591–602.
Shoham, T., Rajapaksa, R., Kuo, C.C., Haimovich, J., Levy, S., 2006. Building of the tetraspanin web: distinct structural domains of CD81 function in different cellular compartments. Mol. Cell Biol. 26 (4), 1373–1385.
Silvie, O., Charrin, S., Billard, M., Franetich, J.F., Clark, K.L., van Gemert, G.J., Sauerwein, R.W., Dautry, F., Boucheix, C., Mazier, D., Rubinstein, E., 2006. Cholesterol contributes to the organization of tetraspanin-enriched microdomains and to CD81-dependent infection by malaria sporozoites. J. Cell Sci. 119 (Part 10), 1992–2002.
Spoden, G., Freitag, K., Husmann, M., Boller, K., Sapp, M., Lambert, C., Florin, L., 2008. Clathrin- and caveolin-independent entry of human papillomavirus type 16e involvement of tetraspanin-enriched microdomains (TEMs). PloS One 3 (10), e3313.
Stamataki, Z., Grove, J., Balfe, P., McKeating, J.A., 2008. Hepatitis C virus entry and neutralization. Clin. Liver Dis. 12 (3), 693–712.
Stipp, C.S., Kolesnikova, T.V., Hemler, M.E., 2001. EWI-2 is a major CD9 and CD81 partner and member of a novel Ig protein subfamily. J. Biol. Chem. 276 (44), 40545–40554.
Tong, Y., Zhu, Y., Xia, X., Liu, Y., Feng, Y., Hua, X., Chen, Z., Ding, H., Gao, L., Wang, Y., Feitelson, M.A., Zhao, P., Qi, Z.T., 2011. Tupaia CD81, SR-BI, claudin-1, and occludin support hepatitis C virus infection. J. Virol. 85 (6), 2793–2802.
Uittenbogaard, A., Smart, E.J., 2000. Palmitoylation of caveolin-1 is required for cholesterol binding, chaperone complex formation, and rapid transport of cholesterol to caveolae. J. Biol. Chem. 275 (33), 25595–25599.
Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H.G., Mizokami, M., Bartenschlager, R., Liang, T.J., 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. 11 (7), 791–796.
Yauch, R.L., Kazarov, A.R., Desai, B., Lee, R.T., Hemler, M.E., 2000. Direct extracellular contact between integrin alpha(3)beta(1) and TM4SF protein CD151. J. Biol. Chem. 275 (13), 9230–9238.
Yunta, M., Lazo, P.A., 2003. Tetraspanin proteins as organizers of membrane microdomains and signaling complexes. Cell Signal. 15 (6), 559–564.
Zeisel, M.B., Koutsoudakis, G., Schnober, E.K., Haberstroh, A., Blum, H.E., Cosset, F.L., Wakita, T., Jaeck, D., Doffoel, M., Royer, C., Soulier, E., Schvoerer, E., Schuster, C., Stoll-Keller, F., Bartenschlager, R., Pietschmann, T., Barth, H., Baumert, T.F., 2007. Scavenger receptor BI is a key host factor for hepatitis C virus infection required for an entry step closely linked to CD81. Hepatology 46 (6), 1722–1731.
Zhong, J., Gastaminza, P., Chung, J., Stamataki, Z., Isogawa, M., Cheng, G., McKeating, J.A., Chisari, F.V., 2006. Persistent hepatitis C virus infection *in vitro*: coevolution of virus and host. J. Virol. 80 (22), 11082–11093.