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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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SUMMARY

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

INTRODUCTION

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

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

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

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

RESULTS

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

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

EGFR Scaffolding Proteins Grb2 and Shc1 Are Relevant for HCV Entry

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

Inhibition of Ras and Upstream MAPK BRaf Decreases HCV Entry

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

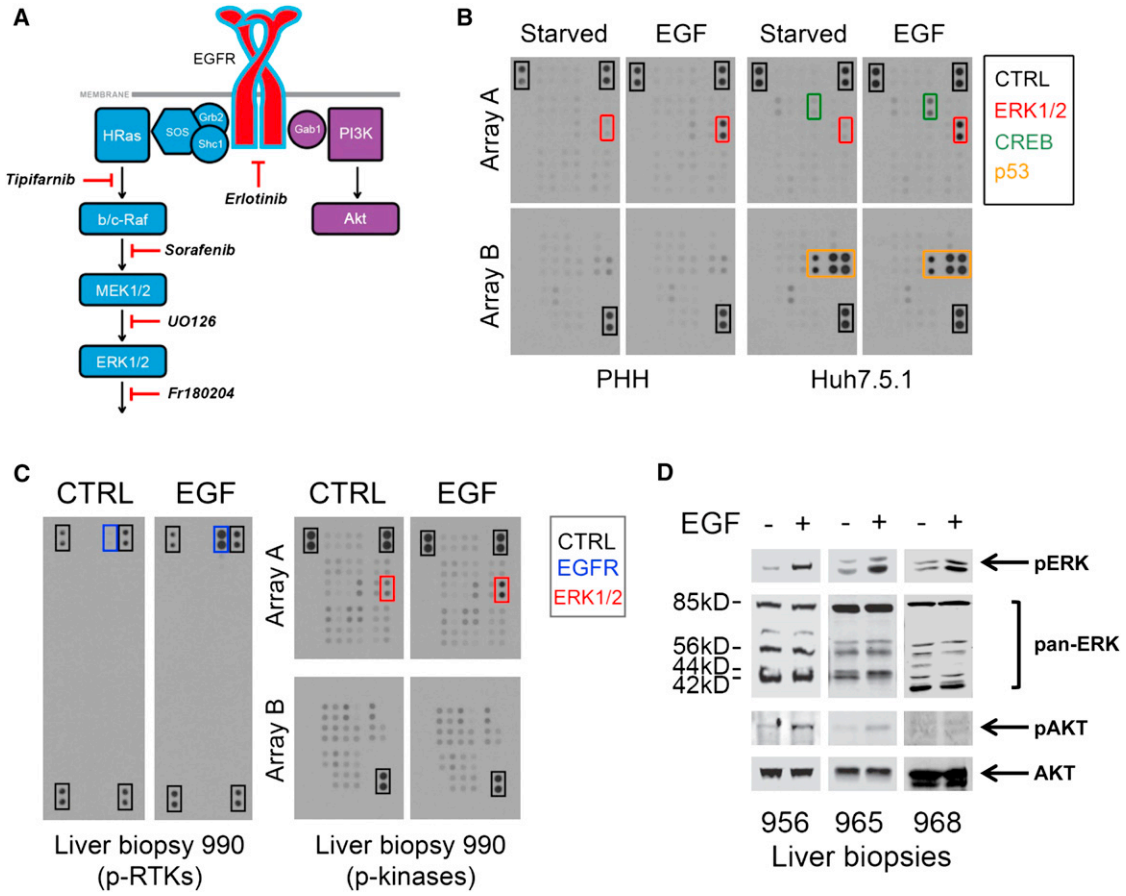

Figure 1. EGFR Signaling Pathways in Human HCV-Permissive Liver Cells, Hepatocytes, and Patient-Derived Liver Tissue

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

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

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

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

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

HRas Is a Transducer of EGFR-Mediated HCV Entry

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

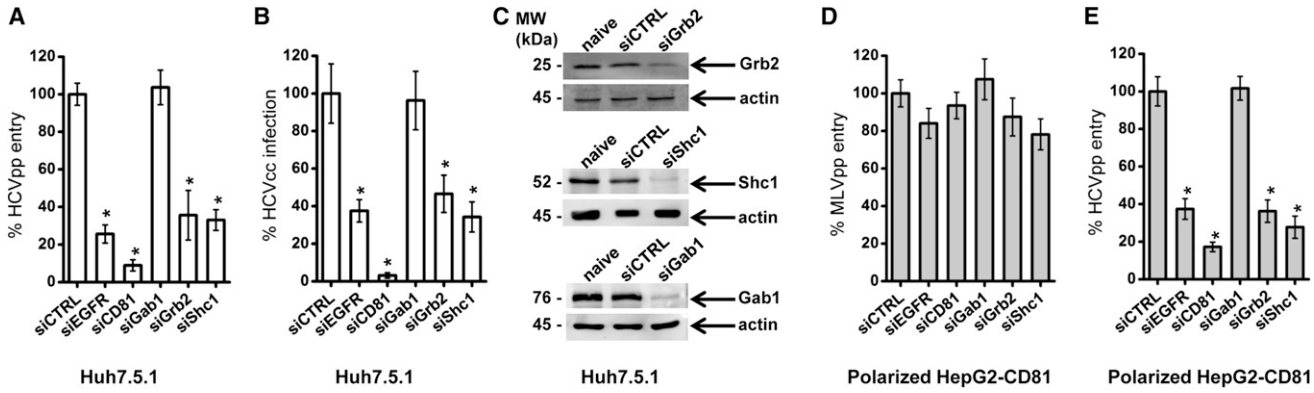

Figure 2. EGFR Adaptors Grb2 and Shc1 Are Relevant for HCV Entry

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

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

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

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

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

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

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

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

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

HRas Associates with HCV Cell Entry Factors CD81 and CLDN1

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

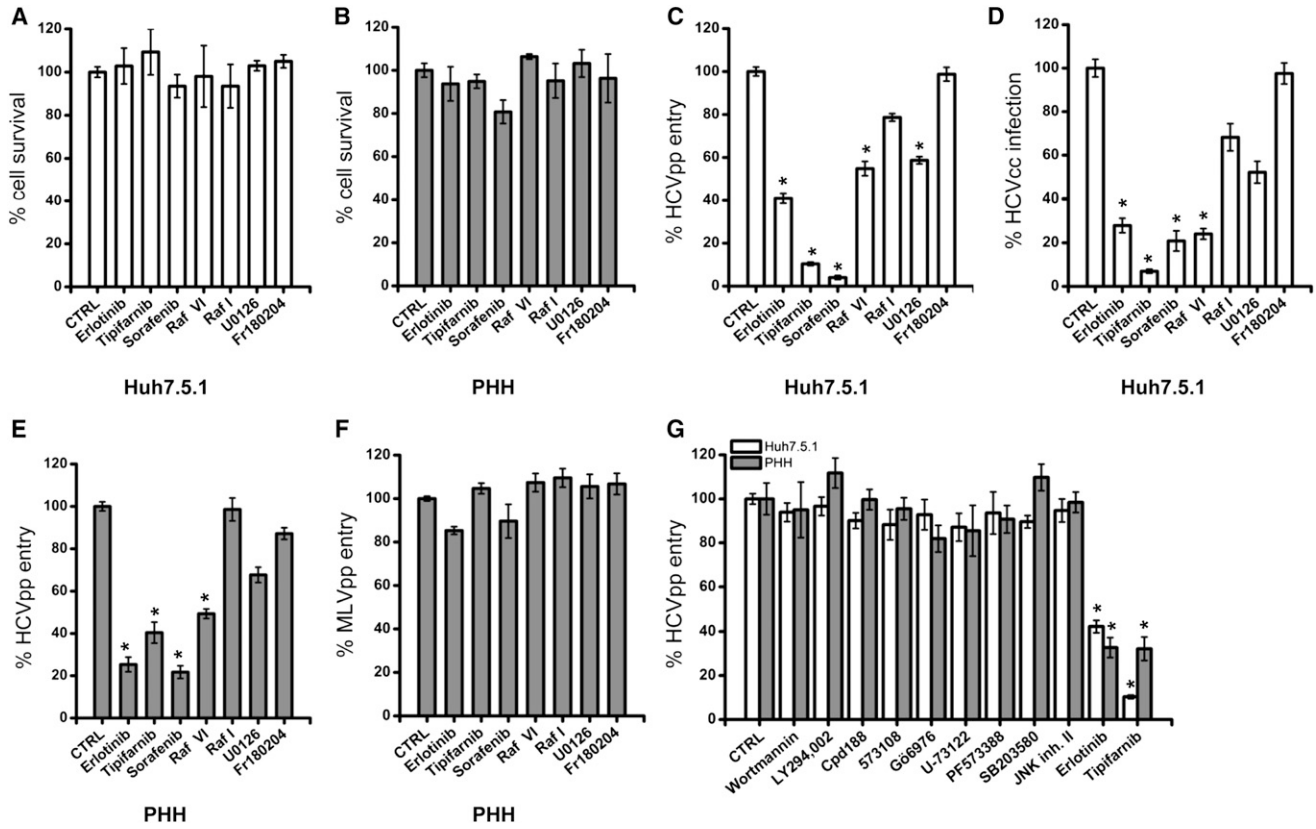

Figure 3. Upstream MAPK Are Relevant for HCV Entry in Hepatoma Cells and Human Hepatocytes

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

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

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

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

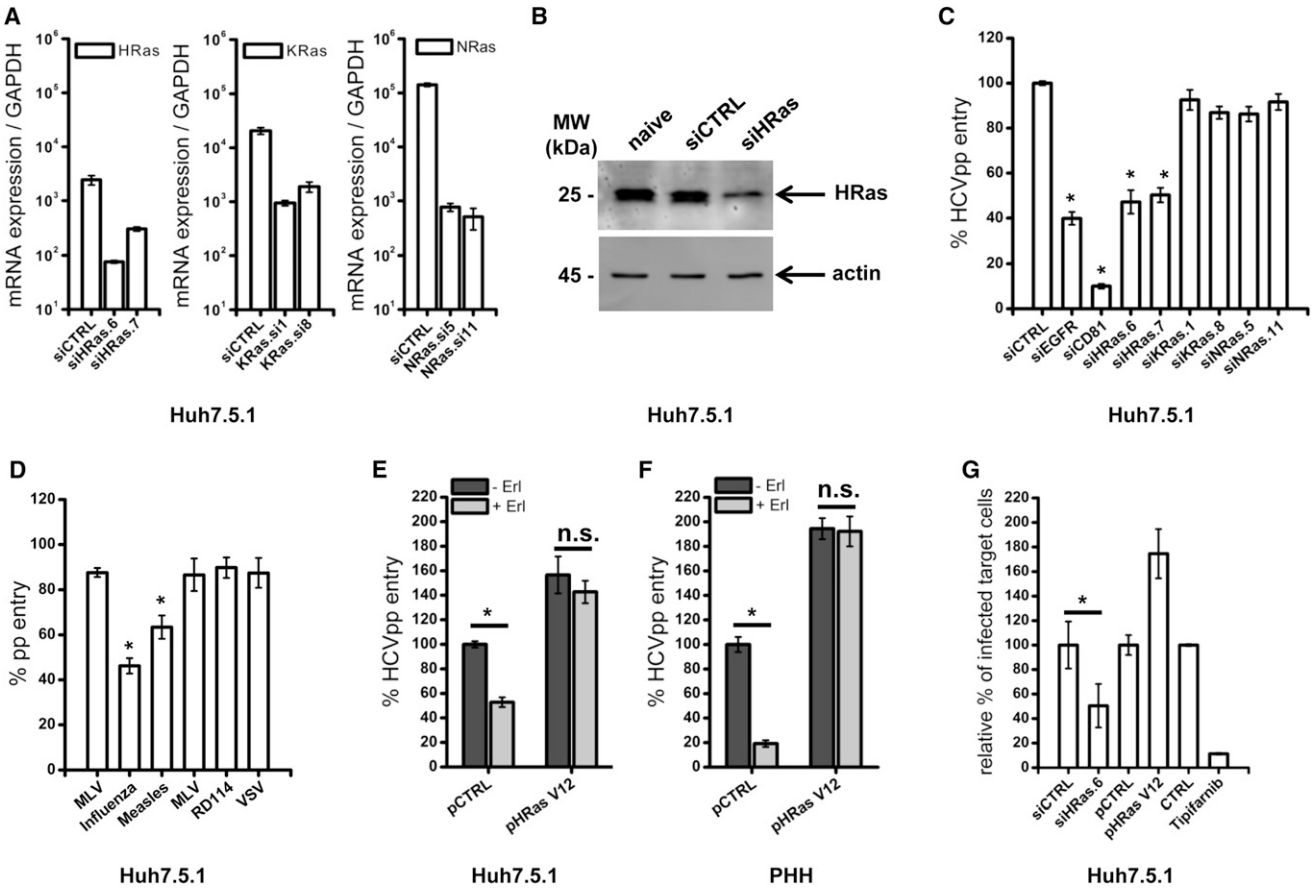

Figure 4. HRas Is a Host Cell Factor for HCV Entry

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

DISCUSSION

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

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

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

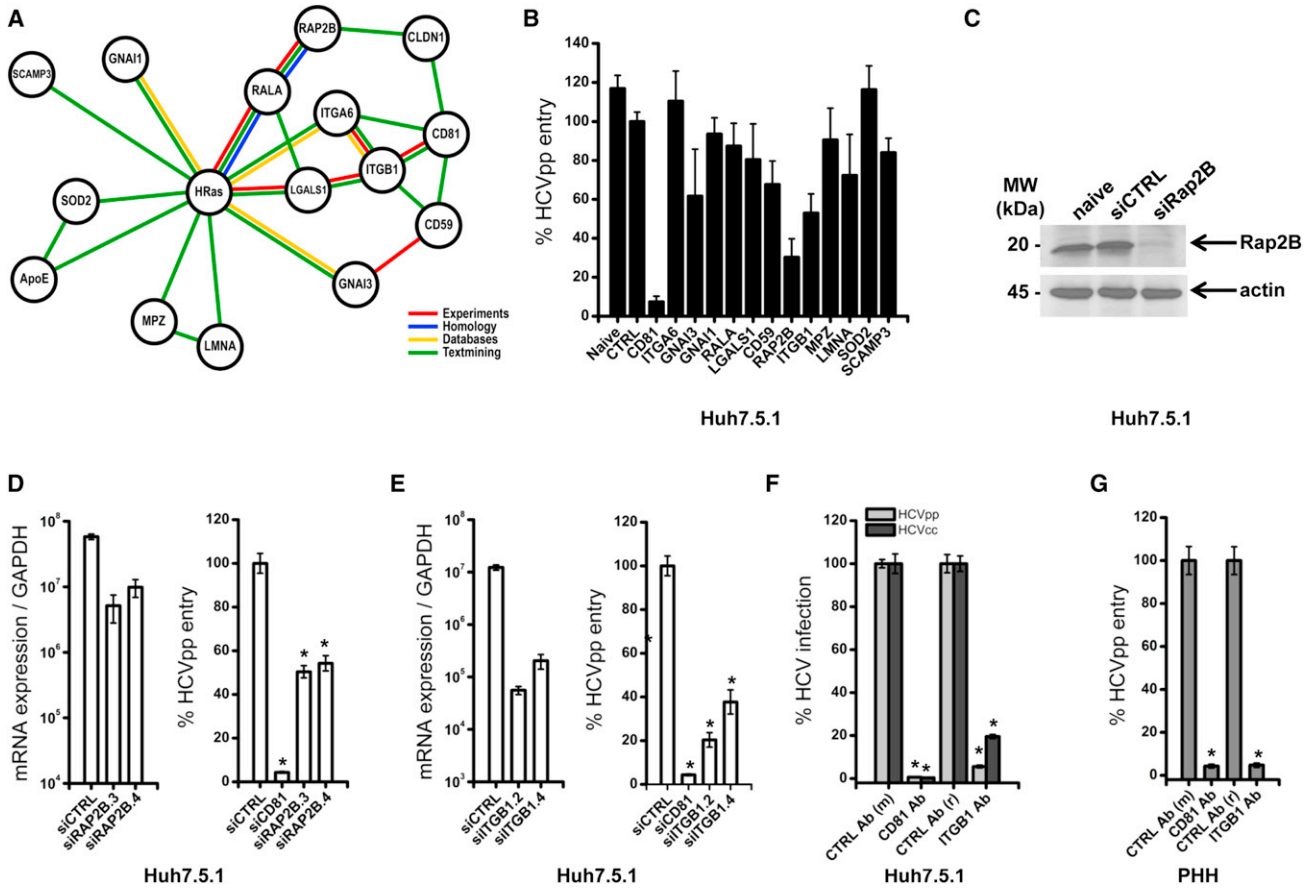

Figure 5. Functional Analysis of HRas-CD81-Associated Proteins Identifies Integrin Beta1 and Rap2B as Previously Undiscovered HCV Entry Factors

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

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

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

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

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

*$p < 0.0001$.

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

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

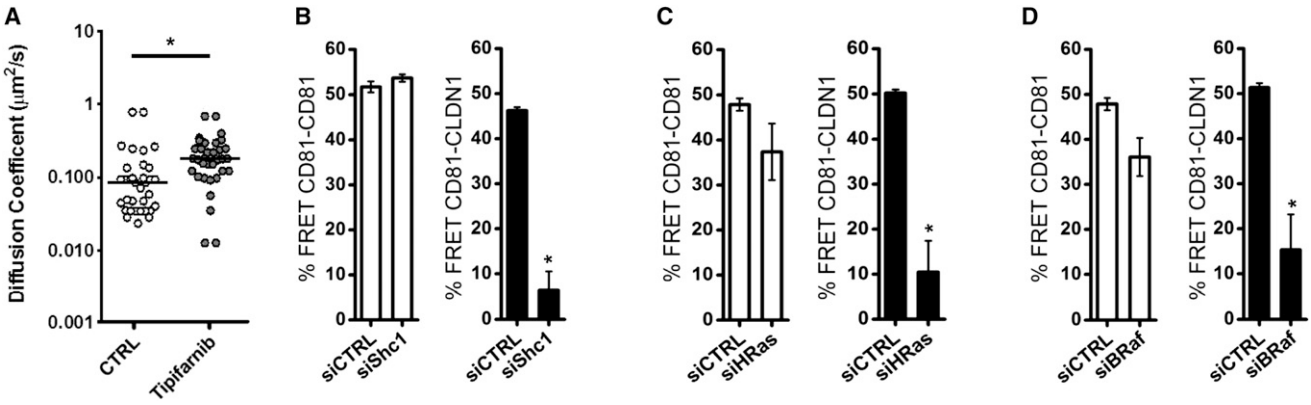

Figure 6. HRas Is Required for Lateral Diffusion of CD81 Promoting CD81-CLDN1 Associations

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

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

*$p < 0.05$.

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

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

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

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

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

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

EXPERIMENTAL PROCEDURES

Cell Lines and Primary Human Hepatocytes

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

Patient-Derived Liver Biopsies

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

Analysis of Cell Signaling with Phosphorylation Arrays

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

Dynamic Phosphoflow Analyses

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

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

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

Functional RNAi HCV Entry Screens

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

Rescue of EGFR Inhibition with a HRas Transcomplementation Assay

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

Proteomic Analyses of Tetraspanin Complexes and Microdomains

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

Imaging Studies

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

Statistical Analysis

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

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

SUPPLEMENTAL INFORMATION

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

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