Roles for endocytic trafficking and phosphatidylinositol 4-kinase III alpha in hepatitis C virus replication

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Hepatitis C virus (HCV) reorganizes cellular membranes to establish sites of replication. The required host pathways and the mechanism of cellular membrane reorganization are poorly characterized. Therefore, we interrogated a customized small interfering RNA (siRNA) library that targets 140 host membrane-trafficking genes to identify genes required for both HCV subgenomic replication and infectious virus production. We identified 7 host cofactors of viral replication, including Cdc42 and Rock2 (actin polymerization), EEA1 and Rab5A (early endosomes), Rab7L1, and PI3-kinase C2gamma and PI4-kinase IIIalpha (phospholipid metabolism). Studies of drug inhibitors indicate actin polymerization and phospholipid kinase activity are required for HCV replication. We found extensive co-localization of the HCV replicase markers NS5A and double-stranded RNA with Rab5A and partial co-localization with Rab7L1. PI4K-IIIalpha co-localized with NS5A and double-stranded RNA in addition to being present in detergent-resistant membranes containing NS5A. In a comparison of type II and type III PI4-kinases, PI4Ks were not required for HCV entry, and only PI4K-IIIalpha was required for HCV replication. Although PI4K-IIIalpha siRNAs decreased HCV replication and virus production by almost 100%, they had no effect on initial HCV RNA translation, suggesting that PI4K-IIIalpha functions at a posttranslational stage. Electron microscopy identified the presence of membranous webs, which are thought to be the site of HCV replication, in HCV-infected cells. Pretreatment with PI4K-IIIalpha siRNAs greatly reduced the accumulation of these membranous web structures in HCV-infected cells. We propose that PI4K-IIIalpha plays an essential role in membrane alterations leading to the formation of HCV replication complexes.

antivirals | membranous web | PI4K-IIIa | PIK4CA | RNAi

HCV depends on cellular membrane-trafficking pathways at each step of its life cycle, including clathrin-mediated endocytosis, lipid droplet formation for virion assembly, and lipoprotein secretory pathways for egress (1–3). Like all positive-stranded RNA viruses, HCV reorganizes intracellular membranes to establish sites of viral replication (4). HCV proteins are synthesized first at the rough endoplasmic reticulum (ER). Then the nonstructural proteins form viral replication complexes. HCV replication is thought to be intimately associated with modified cellular membranes that have protease- and nuclease-resistant properties (5, 6). Expression of the entire HCV polyprotein or the nonstructural protein NS4B alone results in formation of a unique multi-vesiculated structure termed the "membranous web" thought to be derived, at least in part, from ER membrane (6–8). Studies show that nonstructural proteins and viral RNA synthesis are associated with membranous webs (7, 9). For these reasons they are implicated as the sites of active viral replication, although the precise origin and composition of the web-associated vesicles remains unclear.

Cytosolic membrane rearrangements are a conserved feature of all positive-stranded RNA virus infections. The membrane structures can be derived from a variety of organelles, suggesting potentially divergent mechanisms (4, 10). In the case of poliovirus, a number of mechanisms have been proposed, including the induction of autophagy (11), the recruitment of ADP ribosylating factors (12, 13), and the requirements for fatty acid synthesis and coat protein 1 (COPI) vesicles (14). A number of cellular proteins and pathways have been implicated in the formation of HCV replication complexes: fatty acid and cholesterol synthesis pathways, vesicle-associated proteins (VAP)-A and -B, and early endosomal proteins Rab5, EEA1, rabaptin5, and Rab4 (15–20). These findings seems to suggest the HCV replication complex may consist, at least in part, of components of early endosomes and ER-to-Golgi transport vesicles, although the mechanism of complex formation is unknown.

RNAi analysis is a valuable approach for identifying cellular co-factors necessary for HCV replication (21–26). To gain insight into the mechanism of HCV membrane reorganization, we assembled a small, interfering RNA (siRNA) library targeting 140 cellular membrane-trafficking genes and systematically evaluated their impact on the production of fully infectious HCV and HCV subgenomic replicon replication. The RNAi analysis, in combination with inhibitor, microscopy, and cell fractionation studies, indicates that HCV replication requires cellular genes involved in cytoskeleton regulation, endocytic trafficking, and a critical ER-localized lipid kinase, PI4K-IIIα.

Results

Identification of Membrane-Trafficking Genes That Modulate HCV Replication. We first assembled a customized siRNA library consisting of a commercially available set targeting 122 cellular membrane-trafficking genes (Dharmacon, Inc.) that was supplemented with 18 more genes identified as important for replication and membrane reorganization in other viral systems (supporting information (SI) Table S1). This library was tested systematically for its impact on infectious HCV J6-JFH1 production and subgenomic HCV JFH1 strain replication to identify cellular genes that are important for viral replication (e.g., as opposed to membrane-trafficking genes that may affect HCV entry or egress). For our primary screen, we introduced pools of 4 individual siRNAs into Huh-7.5 cells via electroporation, allowed silencing for 72 h, and then infected these cells with HCV. Cell culture supernatants were collected 2 days after HCV infection. Virus titers were quantified via limiting dilution in at least 2 independent experiments, measured in duplicate, and normalized to at least 4 replicates of cells silenced with an

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The authors declare no conflict of interest.

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Table 1. Membrane-trafficking genes important for HCV replication

Gene	Percent inhibition compared with si-IRR treated cells: HCV replicon	Infectious HCV	Host RNA	Gene function
CDC42	72	78	64	Actin
ROCK2	62	77	77	Actin
RAB7L1	77	81	85	GTPase
EEA1	67	77	57	Endosome
RAB5A	66	72	54	Endosome
PIK3C2G	62	82	58	PI3-kinase
PIK4CA	99	98	78	PI4-kinase

irrelevant siRNA (si-IRR). The siRNAs that inhibited extracellular infectious HCV production by more than 1 SD of si-IRR–treated cells (> 65% inhibition) were classified as "hits" and were characterized further. To limit the possibility of siRNA off-target effects, individual siRNAs then were tested as described earlier. (The individual siRNAs that demonstrated a phenotype consistent with the pooled "hit" siRNAs are reported in the right-most column of Table S1.) We quantified cell viability using a luminescence-based assay that measures cellular ATP levels, following 5 days of silencing to eliminate phenotypes resulting from significantly decreased viability ($P < 0.001$) (Fig. S1). For instance, siRNAs targeting COPI components were not interpretable in our assay because of severely reduced cell viability. Finally, we determined that our siRNAs were functional by quantifying the decrease in cellular target gene RNA relative to 18S levels after 48 h of silencing using real-time RT-PCR assays (Table 1).

The siRNAs identified as affecting the production of infectious virus then were tested in a replication-specific assay using subgenomic JFH1 replicon RNAs encoding a Renilla luciferase reporter gene (sgJFH1-RLuc). Huh-7.5 cells were electroporated with siRNAs, allowed 48 h of silencing, and then transfected with replicon RNAs. Luciferase activity was measured at 4 h and 48 h posttransfection and a 48-h:4-h ratio was calculated. From our RNAi analysis, we identified 7 siRNAs that inhibited HCV subgenomic replication by more than 1 SD of si-IRR treated cells (> 60% inhibition): *CDC42*, *ROCK2*, *RAB7L1*, *EEA1*, *RAB5A*, *PIK3C2G*, and *PIK4CA* (Table 1). The phenotype of silencing of *PIK4CA*, which encodes type III PI4-kinase alpha, PI4K-IIIα, was particularly impressive, because it inhibited HCV replication and virus production by nearly 100%. Inhibition of HCV replicon replication correlated with inhibition of infectious virus production and inhibition of host gene RNA expression (Table 1).

We tested the roles of implicated pathways in HCV-Con1 (genotype 1b replicons) replication with inhibitors. Cytochalasin D (actin polymerization inhibitor) and LY294002 (PI kinase inhibitor) significantly inhibit HCV replication ($P < 0.05$) in a dose-dependent manner (Fig. 1) without affecting cell viability (data not shown). We observed a bimodal response to LY294002 treatment, wherein a small decrease in HCV replication initially occurs at ≈0.5 μM, a dose corresponding to inhibition of PI3Ks; a larger, significant decrease ($P < 0.05$) in HCV replication occurs at ≈30 μM, near ranges known to inhibit type III PI4Ks (Fig. 1*B*) (27, 28). There are 2 isoforms of PI4K-IIIα: the full-length, 230-kDa isoform 2, and the alternatively spliced 97-kDa isoform 1 (27, 28). Only the 230-kDa isoform has type III PI kinase activity that is sensitive to LY294002, suggesting this isoform is the HCV cofactor. We have confirmed that PI4K-IIIα isoform 2 is required for viral replication by testing siRNAs that specifically target the 230-kDa isoform using the HCV replicon replication assay (Fig. S2).

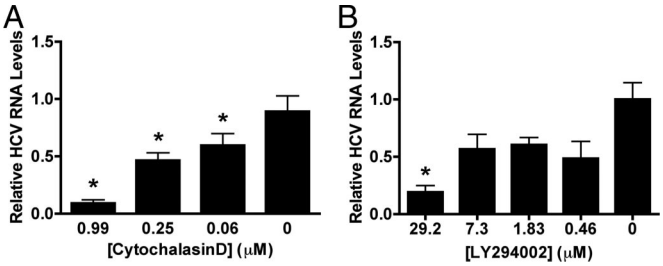

Fig. 1. Effect of drug inhibitors on HCV replication. Huh-7.5 cells containing HCV-Con1 replicons were treated with the indicated concentrations of the inhibitors (*A*) Cytochalasin D or (*B*) LY294002 for 48 h. HCV RNA levels were measured by real-time RT-PCR and normalized to 18S RNA levels. SEM is shown. *, $P < 0.05$, as compared with untreated.

Localization of Endosomal Markers and PI4K-IIIα with the HCV Replicase. We examined the localization of Rab5A, Rab7L1, and PI4K-IIIα with 2 markers of HCV replication, NS5A and dsRNA. NS5A localizes to sites of replication and assembly, whereas an antibody recognizing dsRNA duplexes greater than 40 nucleotides specifically detects replication structures in HCV-infected cells (29) and other viruses with dsRNA replication intermediates (30, 31). We found that HCV replication complexes containing dsRNA substantially co-localize with GFP-Rab5A, GFP-Rab7L1, and PI4KIIIα-GFP and are concentrated near perinuclear regions (Fig. 2*A*). PI4K-IIIα also is present throughout the cytoplasm, as reported (32). Localization of both GFP-Rab5A and PI4KIIIα-GFP also overlaps with NS5A expression (Fig. 2*B*). Although we detected co-localization of dsRNA with GFP-Rab7L1, NS5A seems to localize adjacent to, and not overlapping with, GFP-Rab7L1 (Fig. 2*B*). The significance of this finding is currently unclear.

We examined the localization of endogenous PI4K-IIIα and HCV replication complexes using biochemical fractionation. HCV replication protein complexes associate with detergent-resistant membranes (DRMs), which are thought to be similar to lipid rafts (33). We isolated DRMs from Huh-7.5 cells with or without HCV Con1 replicons and tested for enrichment of HCV NS5A and caveolin-2 (both DRM-positive controls) and absence of calnexin enrichment (negative control) (Fig. 3). Having satisfied these criteria, we probed for PI4K-IIIα and found that a subset of the 230-kDa PI4K-IIIα isoform co-fractionates with HCV replicase marker NS5A in DRMs. This finding provides biochemical evidence that a portion of cellular PI4K-IIIα is present in lipid-rich membranes previously shown to be associated with HCV replication complexes (33) and thus may participate in replication complex formation.

Evaluation of Type II and Type III PI4-Kinases During HCV Infection. There are 4 known cellular PI4-kinases with the same function: phosphorylation of PI to yield PI4P. Their primary distinction is their localization within the cell, resulting in different biological outcomes (28). PI4K-IIIα is localized primarily to the ER, whereas PI4K-IIIβ is localized at the Golgi and regulates Golgi-to-plasma membrane trafficking. The 2 other PI4 kinases, PI4K-IIα and -IIβ, are localized to the plasma membrane, endosomes, and Golgi. They regulate endocytosis and intracellular trafficking of AP-1 adapter molecule cargo (28).

We tested the specificity of PI4K-IIIα function in HCV replication by examining the requirements of all PI4-kinases in either HCV entry or HCV replication assays. The 4 PI4K genes were targeted individually with a pool of 4 siRNAs, and cells were either infected with HCV pseudoparticles (HCVpp) or transfected with subgenomic HCV replicon RNAs. We found that none of the PI4K genes modulate HCVpp entry (Fig. 4*A*), and only PI4K-IIIα is specifically required for HCV replication

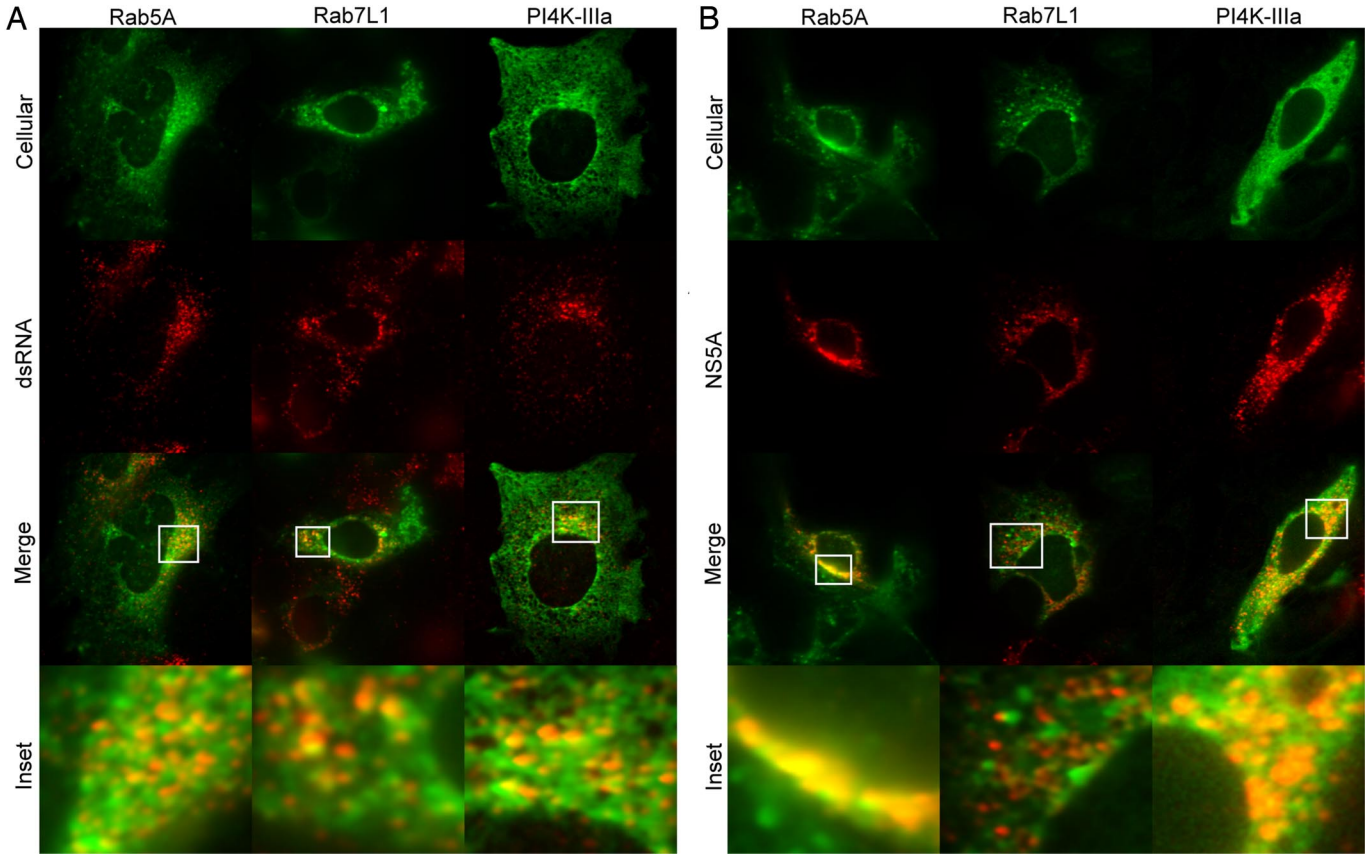

Fig. 2. Localization of HCV replication complexes with Rab5A, Rab7L1, and PI4K-IIIα. Huh-7.5 cells were transfected with GFP-Rab5A, GFP-Rab7L1, or PI4KIIIα-GFP constructs for 24 h, then infected with HCV for 48 h. Cells were fixed and probed with antibodies for (*A*) dsRNA and (*B*) NS5A, detecting HCV replication complexes. *Insets* are zoomed images from boxed areas showing regions of colocalization.

as demonstrated with pooled and individual siRNAs (Fig. 4*B*). The positive controls, si-CD81 for HCVpp and si-HCV for HCV replicons, showed significant inhibition in their respective assays, as expected. Thus, PI4K-IIIα is specifically required for HCV replication.

PI4K-IIIα Expression Is Required for HCV Replicase Formation. We next performed a time course of HCV gene expression following the transfection of HCV RNAs to determine whether the defect was occurring at the stage of translation of HCV RNAs or the subsequent replication step. Huh-7.5 cells were transfected with 4 distinct *PIK4CA* siRNAs or a pool of the siRNAs, maintained for 2 days to establish silencing, then transfected with sgJFH1-RLuc RNAs or with replication-defective sgJFH1-RLuc-GND RNAs that contain a GDD→GND mutation in NS5B, the viral RNA polymerase. Luciferase values were measured at 4, 8, 24, and 48 h after HCV RNA transfection. We observed 2 peaks in luciferase activity in cells treated with si-IRRs and transfected with sgJFH1-RLuc RNAs (Fig. 5*A*). The first small peak at 8 h corresponds to translation of the input RNAs, and the second, larger peak at 48 h corresponds to the translation of newly synthesized RNAs (Fig. 5*A*). In sgJFH1-RLuc cells treated with *PIK4CA* siRNAs, the initial translation peak is unaffected; however, the replication peak was diminished significantly ($P < 0.05$; all individual and pooled *PIK4CA* siRNAs) (Fig. 5*A*). In cells transfected with replication-defective sgJFH1-RLuc-GND RNAs, only the initial translation peak is observed; and *PIK4CA* siRNAs do not alter initial RNA translation significantly in the absence of replication (Fig. 5*B*). We confirmed that the initial peak at 8 h is caused by translation of transfected RNAs by the significant decrease ($P < 0.05$) of luciferase activity in cycloheximide-treated cells (Fig. 5*B*). Thus, despite efficient translation of input HCV RNAs, HCV is unable to establish productive replication in cells in which PI4K-IIIα is silenced.

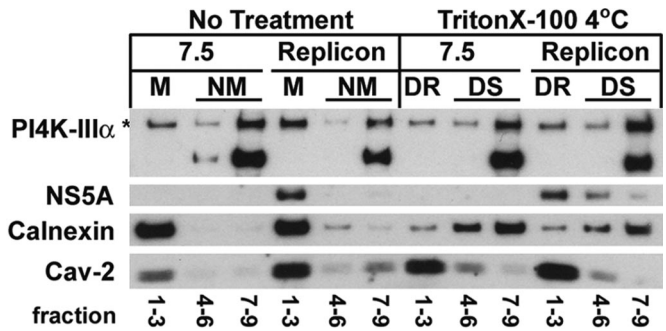

Fig. 3. Co-fractionation of endogenous PI4K-IIIα with HCV replication complexes. Detergent-resistant membranes were prepared from Huh-7.5 cells with and without HCV replicons by TritonX-100 treatment at 4 °C as described (33). Detergent-resistant (DR) membranes remain at the top of the sucrose gradient (fractions 1–3), whereas detergent-sensitive (DS) membranes sediment in the lower fractions (4–9). Protein lysates from pooled fractions were run on SDS/PAGE and probed for PI4K-IIIα, HCV NS5A, calnexin, and caveolin-2 (Cav-2). M, membrane; NM, non-membrane; *, PI4K-IIIα–specific band.

Because PI4K-IIIα is thought to regulate cellular membrane organization and coordinate protein localization at sites in membranes, we reasoned that PI4K-IIIα may be involved in HCV-induced membrane reorganization. To test this possibility,

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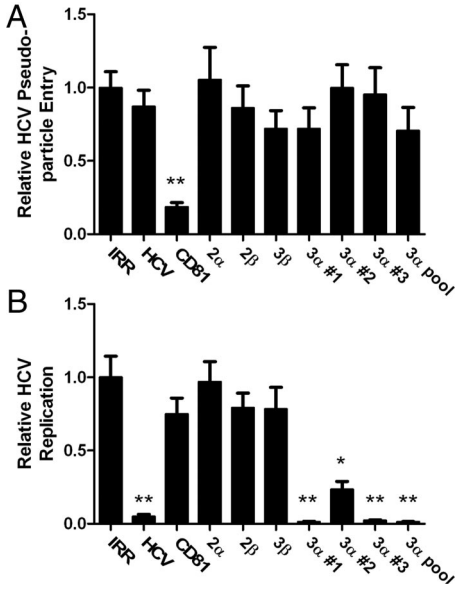

Fig. 4. PI4K-IIIα is specifically required in HCV replication. Type II (α and β) and type III (α and β) PI4Ks were silenced using a pool of 4 individual siRNAs or, in the case of PI4K-IIIα, a pool and individual siRNAs (nos. 1–3). The effects of these siRNAs on (*A*) HCV pseudoparticle entry or (*B*) subgenomic replicon replication were quantified by luciferase activity. si-HCV specifically targets HCV RNA. si-CD81 is a control for HCV entry. Values are relative to irrelevant si-IRR–treated cells. SEM is shown. $n = 10$. **, $P < 0.001$ and *, $P < 0.05$, as compared with IRR.

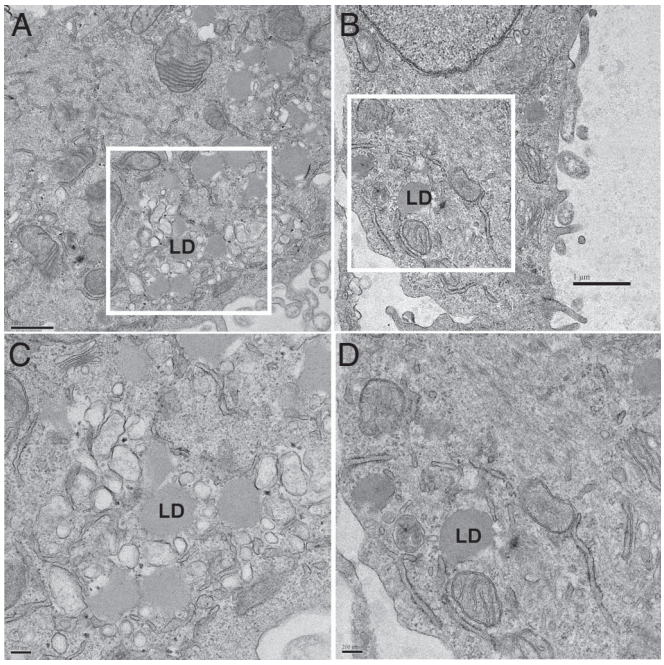

Fig. 6. Silencing PI4K-IIIα inhibits membranous web formation. Huh-7.5 cells were treated with (*A*) irrelevant or (*B*) *PIK4CA* siRNAs for 2 days and then infected with 2 infectious HCV particles per cell for 3 days. Cells were fixed and processed for EM. Magnification is 8,260×. (Scale bar, 1 μm.) *C* and *D* are higher-magnification images of the cell insets in *A* and *B*, respectively. LD, lipid droplet. Magnification is 21,000×. (Scale bar, 200 nm.)

we investigated the effect of silencing PI4K-IIIα expression on HCV-induced membranous web formation. Huh-7.5 cells were silenced with irrelevant or *PIK4CA* siRNAs for 48 h, infected with 2 infectious HCV particles per cell for 3 days, fixed, and processed for electron microscopy. The infected cells were examined randomly for the clustering of heterogeneous vesicles in the cytosol reminiscent of previously described membranous webs (9). An example is shown in Fig. 6*A* and *C*, wherein a membranous web surrounds a cluster of lipid droplets. These structures were not observed in uninfected cells (data not shown). Membranous webs were observed readily in 35% of the infected cells that were treated with si-IRRs (6 of 17 cells contained membranous webs). In contrast, only 1 web was detected in 32 cells (3%) treated with *PIK4CA* siRNAs and infected with HCV, (Fig. 6 *B* and *D*). These data suggest that PI4K-IIIα is required for membranous web formation and the establishment of HCV replication complexes.

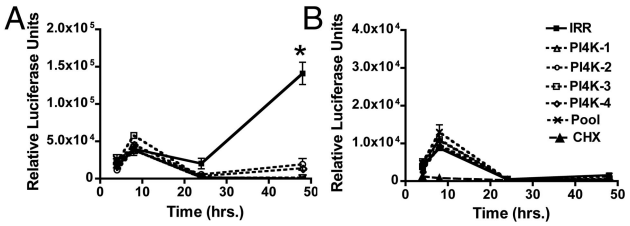

Fig. 5. PI4K-IIIα is required for the replication of HCV RNAs but not for their translation. Huh-7.5 cells were treated for 48 h with either irrelevant (IRR, *solid line*) or individual or pooled *PIK4CA* siRNAs (*dashed lines*). Luciferase values were measured over the indicated time course following transfection of (*A*) sgJFH1-RLuc or (*B*) sgJFH1-RLuc-GND RNAs. Initial translation of the transfected RNAs occurs between 4 and 24 h as confirmed by cycloheximide (CHX) treatment, which significantly ($P < 0.05$) reduced luciferase activity in (*B*). Replication occurs in concert with translation of newly synthesized HCV RNAs between 24 and 48 h. SEM is shown. $n = 3$. Statistically significant differences (*, $P < 0.05$) between IRR and *PIK4CA* siRNA-treated cells occurred only at the 48-h time point in (*A*).

Discussion

Using RNAi to dissect membrane trafficking pathways, followed by studies of drug inhibitors, microscopy, and biochemical assays, we have identified 7 host factors important for HCV replication that regulate (*i*) actin organization, (*ii*) endosomal trafficking, and (*iii*) lipid organization, including a crucial PI4-kinase. The involvement of the actin cytoskeleton confirms a previous report that used several drug inhibitors and demonstrated that both actin and microtubules are necessary for efficient HCV replicon replication (34). Recent evidence shows that NS3 and NS5A bind actin filaments and microtubules, which may function in the movement of viral replication complexes within the cell (35). The requirement of Cdc42 and Rock2 for HCV replication implicates the Rho signaling pathway. Cdc42 is a Rho family GTPase that activates the Arp2/3 complex for nucleation of actin filaments (36). Rock2 is a serine/threonine kinase that acts immediately downstream of Rho, controls reorganization of actin structures, and prevents actin depolymerization (37, 38). The plexstrin homology (PH) domain of Rock2 binds to PI(3,4,5)P_3 and PI(4,5)P_2. Consequently, Rock2 activity may be regulated indirectly by the identified PI3- and/or PI4-kinases. Components of the Rho pathway probably regulate the shuttling of individual viral and host factors or intracellular membranes/vesicles to sites where the HCV replication complex is formed and perhaps the movement of assembled complexes as a whole.

We find that HCV relies on endosomal proteins for replication. A role for early endosomes in HCV infection has been suggested previously in data showing that pan-*RAB5* siRNAs inhibit HCV replicons, Rab5A co-immunoprecipitates with HCV NS4B, and Rab5B and EEA1 co-localize with NS4B (19). EEA1 is recruited by PI(3)P lipids and Rab5, binding both at

early endosomal membranes dependent on PI3-kinase activity (39, 40). It is possible that the PI3-kinase identified in this screen, PI3K-C2γ, is important for this activity.

The function of Rab7L1 is poorly understood. Although it is 35% identical to the late endosomal protein Rab7A, we find that Rab7L1 does not co-localize with late endosomal markers but instead localizes partially with Golgi (data not shown), as was reported previously (41). There is precedent for the association of viral and cellular replicase proteins with the Golgi apparatus. For example, VAP-B was found to be co-localized with NS5A at the ER and Golgi (18). Rab7L1 may be involved in budding or fusion events at the Golgi that are necessary for viral replicase formation.

We identified 2 PI kinases, PI3K-C2γ and PI4K-IIIα, as modulators of HCV replication. PI3K-C2γ is a poorly characterized class II PI3-kinase with a narrow expression profile, being reported only in liver, breast, and prostate tissue. Interestingly, PI3K-C2γ expression increases during liver regeneration (42). PI3K-C2γ has substrate specificity for PI and PI(4)P, generating PI(3)P and PI(3,4)P_2, and is thought to regulate endocytic trafficking (43). PI4K-IIIα localizes mainly to the ER and produces pools of PI(4)P. We found co-localization of GFP-tagged PI4K-IIIα with NS5A and also co-fractionation of endogenous PI4K-IIIα and NS5A in lipid rafts. Inhibition of membranous web formation by *PIK4CA* siRNAs (Fig. 6) correlates with the dramatic decrease in viral replication and infectious virus production (Table 1). These data indicate a role for PI4K-IIIα in establishing sites of viral replication. PI4K-IIIα (230 kDa) was shown previously via 2-hybrid analysis to bind HCV NS5A, with unknown consequences (44). In our current model, PI4K-IIIα may nucleate the formation of membranous webs and HCV replication complexes via generation of PI(4)P and/or direct protein–protein interactions. These complexes are hypothesized to include VAP-A and -B, EEA1, Rab5A, and Rab7L1.

RNA viruses have evolved diverse ways to manipulate membrane trafficking pathways. Our analysis suggests that mechanisms of HCV membrane reorganization are distinct from those of picornaviruses, and the involvement of multiple endocytic vesicles in HCV replication is reminiscent of coronaviruses and the alphaviruses, such as Semliki Forest virus (45, 46). Numerous questions remain concerning the mechanism of HCV replicase formation. Not yet understood is the interface of NS4B, which can drive membranous web formation, and PI4K-IIIα; also, the cellular and/or viral factors that depend on PI4K-IIIα for replicase formation have not yet been identified. Finally, characterization of the role of PI-kinases and other essential host factors in HCV infection may contribute to the development of alternative therapeutics of HCV and related viruses.

Materials and Methods

Cells, Virus, and Reagents. Huh-7.5 cells are a subline derived from Huh-7 hepatoma cells that are highly permissive for HCV replication (47, 48). Huh-7.5 cells containing genotype 1b HCV-Con1 replicon, Con1/Fl-neo(S2204I), were used for studies of drug inhibitors (47, 48). Cells were maintained in DMEM-high glucose supplemented with 0.1 mM non-essential amino acids, 10% FBS, and 1% penicillin-streptomycin (Invitrogen). The Con1-replicon cell medium also contained 0.5 mg/ml G418. The HCV genotype 2a infectious clone pJFHxJ6-CNS2C3 replaces core through the first transmembrane domain of NS2 of a JFH-1 backbone with J6 genotype sequence (49). Drug inhibitors used were cytochalasin D (Sigma) and LY294002 (Calbiochem) diluted in DMSO. Cycloheximide (Sigma) was diluted in DMEM and used at final concentration of 50 μg/ml.

RNAi. Membrane-trafficking genes were targeted by gene-specific siRNAs from the siARRAY Human Membrane Trafficking siRNA library (122 targets) and 18 additional siGENOME SMARTpools (Dharmacon-Thermo Fisher Scientific; Table S1). siIRRs and HCV-specific siRNAs sequences are described elsewhere (24) and were used as controls. RNAi assays were performed as described previously (21, 24). Briefly, 1×10^6 Huh-7.5 cells in 0.05 ml of cold 1X PBS, pH 7.4, were electroporated with 1 nanomole of siRNA using an ECM 830 electroporator (BTX Genetronics) with a 96-well attachment. Cells (≈8,000) were plated in 96 wells. Cell viability following siRNA treatment was determined using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) (see *SI Methods*).

Infectious Virus Production Assay. Cells were infected 72 h after siRNA electroporation with 0.5 infectious HCV particles (JFHxJ6-CNS2C3) per cell for 6 h and maintained for 2 days at 37 °C. Cell culture supernatants were titered by limiting dilution analysis via immunohistochemistry using monoclonal anti-NS5A antibody (9E10; gift of C. M. Rice, The Rockefeller University, New York) as described (25).

HCV Replication Assay. Subgenomic JFH1-RLuc or JFH1-RLuc-GND replicon RNAs (see *SI Methods* for construction) were transcribed in vitro as described (50). Cells were transfected 48 h after siRNA-electroporation with 0.1 μg of replicon RNA in TransMessenger reagent (Qiagen) for 1 h, and then the medium was changed as recommended by the manufacturer. Cell lysates were collected at various time points, and Renilla luciferase (Promega) activity was measured using a 96-well luminometer (Centro LB 960, Berthold Technologies).

HCV Entry Assay. HCVpp were generated as described previously (51–53) (see *SI Methods*). Cells were infected 72 h after siRNA electroporation with 100 μl of HCVpp with 8 μg/ml Polybrene (Sigma) for 4 h. Cell lysates were collected at 48 h after HCVpp infection, and firefly luciferase (Promega) activity was measured using a 96-well luminometer (Centro LB 960, Berthold Technologies).

Statistical Analysis. SEM was calculated from at least 3 replicates. A 2-tailed pairwise *t* test was performed to assess significant differences between samples.

Real-Time RT-PCR. Total RNA was harvested from cells using an RNeasy 96 kit (Qiagen) for analysis of host gene expression or HCV RNA levels. Cellular RNAs were reverse-transcribed and PCR amplified using the SuperScript™ III Platinum One-Step qRT-PCR System with Platinum Taq (Invitrogen) and TaqMan Gene Expression Assays (Applied Biosystems) (Table S2). Genotype 1b HCV replicon RNA was quantified using the Platinum qRT-PCR ThermoScript™ One-Step System (Applied Biosystems). Cellular RNAs were normalized to GAPDH levels and HCV RNAs to 18S levels. PCR conditions are described in *SI Methods*. Data were analyzed relative to si-IRR–treated controls. All assays were performed on an ABI 7300 system and analyzed with SDS 1.3 software (Applied Biosystems).

Immunofluorescence Assay. Huh-7.5 cells on glass coverslips were infected with HCV and/or transfected with constructs expressing GFP-tagged cellular proteins using Lipofectamine2000 (Invitrogen). For construction of GFP fusions see *SI Methods*. Cells were processed and imaged as described in *SI Methods*.

Cellular Fractionation and Membrane Floatation Analysis. Fractionation of HCV-Con1 replicon cells and Huh-7.5 cells was performed as described (33). Pooled gradient fractions consisted of 400 μl of individual fractions. Pooled fractions were concentrated to 150 μl using YM-10 centricons (Millipore).

Western Blot Analysis. Protein samples were separated on 4%–20% SDS/PAGE gels (Lonza, Inc.) and transferred to nitrocellulose. Membranes were probed with the following antibodies: anti-NS5A (9E10; gift of C. M. Rice); anti-PI4K-IIIα (#4902; Cell Signaling Technology); anti-calnexin (Stressgen); and caveolin-2 (BD Biosciences). Proteins were visualized by HRP-conjugated secondary antibodies (Pierce), SuperSignal-Femto chemiluminescent substrate (Pierce), and exposed to film.

Electron Microscopy. Cells were processed for electron microscopy as described in *SI Methods*. Images were taken at 300 kV with an FEI Tecnai F30 electron microscope equipped with a high-performance Gatan CCD camera.

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