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The RCK/p54 Prion-Related Domain and its Influence on the Localization and Function of RCK/p54 During HCV Infection

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"The RCK/p54 Prion-Related Domain and its Influence on the Localization and Function of RCK/p54 During HCV Infection"

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Abstract:

Currently over 180 million people are infected with Hepatitis C virus (HCV) worldwide. HCV infection is a major cause of hepatocellular carcinoma (HCC), liver cirrhosis, and chronic hepatitis. The available antiviral treatment of interferon and ribavirin has limited success, is costly and toxic. Furthermore, there is no vaccine. An increased understanding of how HCV exploits the many cellular pathways and host factors during infection will provide information necessary for the development of novel anti-HCV therapies, which target host proteins rather than the rapidly evolving viral proteins.

During infection, HCV interacts with and manipulates many host mRNA pathways. The Pager lab discovered that RCK, a DEAD-box helicase involved in microRNA gene regulation and mRNA decay, is required for HCV gene expression and virus assembly. RCK contains conserved motifs common to all DEAD-box helicases, as well as an extended N-terminus that contains a 48 amino acid prion-related domain mostly composed of glutamine and glycine residues. I hypothesize that the prion-related domain (PRD) facilitates the localization and function of RCK at HCV assembly sites. To test this hypothesis I used deletion analysis of the PRD to examine the effect of this deletion on RCK protein expression and localization and on HCV gene expression. This study will increase our understanding of HCV infection, as well as the normal cellular role of RCK.

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Introduction:

Hepatitis C virus (HCV) infection is the most common chronic blood borne infection in the United States, for which treatments are costly and not widely available (Hepatitis c information," July). HCV infection can lead to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. Currently, approximately 180 million people are infected with HCV worldwide (Hepatitis c information," July). A protective vaccine is not yet available and the currently available inhibitors of HCV target viral proteins, and resistance to these treatments will most likely arise over time. However, targeting a host factor, rather than a viral protein, may make it more difficult for the virus to develop resistance against the drug. Thus, to develop such a drug a thorough understanding of the virus infection mechanisms and virus-host interactions is desperately.

HCV is an enveloped RNA virus and a member of the genus hepacivirus in the *Flaviviridae* family. This single stranded positive-sense genome consists of conserved 5' and 3' untranslated regions (UTRs) and a single open reading frame that is translated into ten viral proteins: Core, envelope glycoprotein 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (Ashfaq, Javed, Rehman, Nawaz, Riazuddin, 2011). Core, E1, and E2 are the major constituents of the HCV particle, while p7 and NS2 are primarily involved in HCV assembly. NS3 is a helicase with serine-type protease activity in the N-terminal domain that is activated by interaction with NS4A. The formation of membranous vesicular structures can be triggered by NS4B, and these structures can form a 'membranous web' that might serve as a scaffold for the assembly of the viral replication complex. NS5A plays an important role in RNA replication and virion assembly by binding with multiple host cell factors, and the RNA-dependent RNA polymerase is located in NS5B (Bartenschlager, Penin, Lohmann, Andre, 2010).

HCV enters the cell via receptor-mediated endocytosis. Upon uncoating, the viral genome is translated at the rough endoplasmic reticulum (rER) where the polyprotein is cleaved. NS4B mediates the formation of the membranous web where RNA genomes are amplified. The genomes may be encapsulated into new virions and released by the cell in a noncytolytic pathway associated with the VLDL pathway (Bartenschlager, Penin, Lohmann, Andre, 2010).

Additionally, core protein was found in close proximity to cytosolic lipid droplets (cLDs). Lipid droplets are sites of virus assembly, and HCV-Core localizes with several components of lipid droplets such as RCK.

The stability of cellular and viral RNAs can be modified in specific cytoplasmic granular structures, known as processing bodies (P-bodies). P-bodies are discrete cytoplasmic foci where nontranslating mRNAs are either stored or degraded. P-bodies are constitutively present in cells, but their number and size depend on the abundance of RNAs sequestered for storage and turnover (Decker & Parker, 2012). P-bodies are dynamic complexes whose assembly is proportional and dependent to the amount of nontranslating mRNA in cells. P-bodies contain conserved core proteins involved in mRNA decay and translation repression, including a decapping enzyme complex Dcp1/Dcp2, and decapping activators such as Edc3, Lsm1-7 complex and RCK/p54/DDX6 (Decker & Parker, 2012).

RCK, is a member of the DEAD-box helicase family of proteins. DEAD-box helicases are a large group of proteins generally involved in RNP remodeling (Presnyak & Coller, 2013). They are defined by a series of nine conserved sequence motifs, which permit DEAD-box proteins to bind to and hydrolyze ATP, as well as interact with RNA. Members of this protein family function in a variety of roles related to RNA metabolism and RNA processing such as ribosome biogenesis, splicing, and translation (Presnyak & Coller, 2013).

Furthermore RCK enhances miRNA gene regulation, and is required for HCV gene expression and virus assembly at P-bodies (Pager, Schutz, Abraham, Luo & Sarnow, 2013). HCV alters P-body distribution during infections, and RCK, HCV core, and lipid droplets co-localize (Figure 1) (2012). P-bodies themselves do not appear to be required for HCV replication, but depleting P-body proteins such as RCK dramatically reduces HCV gene expression. RCK protein levels were also shown to be elevated in HCV-associated carcinomas (Miyaji et al, 2003).

RCK contains conserved motifs that are common to all DEAD-box RNA helicases, but it also has an extended N-terminus that contains an intrinsically disordered region or prion-related domain (PRD). This domain is composed of 48 amino acids mostly comprised of glutamine and glycine residues (Weston & Sommerville, 2006). P-bodies contain a variety of protein factors, some of which contain Q/N-rich regions (Pop2p, Ccr4p, and Dhh1p). These conserved N-terminal regions have been shown to contribute to efficient accumulation of the proteins in P-bodies under stress conditions. When the Q/N- rich domains were deleted, these proteins showed decreased P-body localization compared to their full-length proteins (Reigns, Alexander, Spiller & Beggs, 2008).

Based on the observations that HCV requires RCK for gene expression and virus assembly, and RCK is localized at lipid droplets, I hypothesized that this prion-related domain facilitates the localization and function of RCK at HCV assembly sites. In order to examine whether RCK still localizes at lipid droplets in the absence of this domain, I examined the effect of a PRD mutant on RCK protein expression and localization, and then on the effects on HCV gene expression.

Through a more comprehensive understanding of HCV's interactions with the host cell, specifically its reliance on host cell proteins, may aid the generation of alternative cellular targets for antiviral treatments. A better understanding of this PRD in RCK, specifically the effects of the deletion on HCV assembly and localization, may provide useful information that can be utilized for future anti-HCV targets.

Materials and Methods:

Cloning Strategy:

Initially, 2ng of plasmid encoding the deleted PRD, pE-mRFP-RCKΔPRD, was transformed into ten μL of DH5α bacteria to grow up more plasmid. The RCKΔPRD sequence within the pE-mRFP-RCKΔPRD plasmid was also PCR amplified to clone the region into the TOPO vector (Life Technologies). Using a forward primer (AAGCTTATGACCACCACTATTAA ACCTG), and a reverse primer (GGATCCTTAAGGTTTCTCATCTTCTACAGG), the PCR reaction was performed as follows: 1 cycle at 94°C for 30 seconds, and then cycling from 94°C for 30 seconds, to 55°C for 20 seconds, and 68°C for one minute twenty seconds, for 35 cycles. Controls included for the PCR reaction were: a positive control with control DNA and a negative control with no DNA. The RCKΔPRD electrophoresed in a 1% agarose/TBE gel, and the PCR product extracted from the gel using the QIAGEN gel extraction kit according to the manufacturer's protocol. To clone the PCR product into pCR2.1-TOPO, the PCR product was combined with the salt solution and the TOPO vector, and was incubated at room temperature for ten minutes. This TOPO mix was then transformed into DH5α bacteria. Colonies were picked and a miniprep was performed to determine the concentration of plasmid. A restriction enzyme digest with BamHI and HindIII were used to confirm that the proper sequence was inserted into the plasmid (Figure 1) . Two samples from the digest were sent for sequencing using the M13 forward (5'-GTAAAACGACGGCCAGT-3') and reverse (5'-AACAGCTATGACCATG-3') primers.

To subclone RCK/p54ΔPRD into p3xFlag, pTOPO-RCK/p54ΔPRD and p3xFlag were digested with BamHI and HindIII, and p3xFlag further incubated with 1μL of shrimp alkaline phosphatase (New England Biotechnology). Digested fragments were separated on an 1%

agarose/TBE gel, and the RCKΔPRD and linearized p3xFlag plasmid bands were isolated with the Qiagen Gel extraction kit, and ligated using the 5X Rapid Ligation Buffer and T4 DNA Ligase (NEB) (Figure 3B). The ligated sample was then transformed into DH5α bacteria. Individual colonies were grown overnight, a miniprep was used to isolate plasmid DNA according to the manufacturer's instructions (Omega). To identify miniprep DNA containing RCKΔPRD with the p3xFlag plasmid, samples were digested using BamHI and HindIII enzymes and the plasmid DNA containing insert was confirmed by sequence analysis using the M13 forward and reverse primers.

To clone 3xFlag-RCK/p54ΔPRD into pLenti6, 3xFlag-RCK/p54ΔPRD was PCR amplified with a forward primer (5'-CACCATGGACTACAAAGACCATGAC-3'), a reverse primer (5'-TTATTATTTCAGCCCCAGAGCG-3'), 5x Phusion HF Buffer, a dNTP Mix, and Phusion Polymerase (Invitrogen). The PCR samples were run on a gel and the insert was excised using the standard QIAGEN gel extraction procedure. The excised PCR product was cloned into the pLenti vector (Invitrogen) using 2μL of the gel extracted product, 0.5μL of salt solution, and 0.5μL of the pLenti vector. The sample was then transformed into Stbl3 bacteria and colonies were screened by restriction enzyme digestion using XhoI and BamHI, and sent for sequencing using the forward primer C-CMV-24 (5'-TATTAGGACAAGGCTGGTGGGCAC-3') and the reverse N-CMV-30 primer (5'-AATGTCGTAATAACCCCGCCCCGTTGACGC-3').

Cell Culture and Reagents:

Huh7.5 cells were maintained in DMEM media (Life Technologies) with 10% FBS, 1% nonessential amino acids (Life Technologies) and 1% L-glutamine (Life Technologies) at 37°C in 5% CO2. Cells were passaged every three to four days. To passage cells, the media was aspirated, and 1mL of PBS and then 1mL of trypsin (Life Technologies) was added. Cells were

incubated with 1mL of trypsin for approximately two minutes, resuspended in fresh DMEM media and the cell suspension was added to new plates containing fresh DMEM with 5% FBS, 1% glutamine, and 1% nonessential amino acids. 293FT cells were maintained similarly except when passaging the cells were washed twice with 1mL of PBS, before incubating with 1mL of trypsin. Furthermore, 293FT cells were maintained in 10mL media with 50ug/mL G418 (Life Technologies). The Huh7.5 stable cell line expressing RCKΔPRD was also maintained in DMEM media and Blasticidin (4ug/mL; Life Technologies) was added for selection.

Transfection of Plasmid DNA and siRNAs:

The siRNAs that were used to transfect cells were siRCK1 which targets the coding region of RCK at nucleotide position 1210 (sense strand: 5'-GCAGAAACCCUAUGAGAUUUU-3'), siRCK4 which targets the 3'UTR of RCK at nucleotide position 1986-2004 (sense strand: 5'-GGAACUAUGAAGACUUAAAdTdT-3'), and siRCK5 which also targets the 3'UTR of RCK at nucleotide position 2254 (sense strand: 5'-CAGCUGACUCUCGUGCAUUUU-3'). To transfect cells, the cells were seeded $2.5x\ 10^5$cells/6cm plate or $5x\ 10^5$ cells/10 cm plate. The plates were gently rocked to ensure the cells were evenly distributed and then incubated overnight at 37°C. The next day the cells were checked for 80% or more confluency. Two master mixes were created for the transfection reaction. The first mastermix contained 500µL of OptiMEM and the second mastermix consisted of 500µLvOptiMEM and 5µL of Lipofectamine 2000 (Life Technologies). The mixes were incubated at RT for 5min. During this time 10nM siRNAs and 1ug plasmid were added to 10mL conical tubes. After five minutes, 500µL of each mastermix was added to the conical tubes containing plasmid and siRNA. The samples were incubated at RT for 20mins during which time the media was removed from the cells. After the 20min incubation the transfection reagents were

added to the cells. In cases where 10cm plates were used, reaction volumes were doubled. Approximately forty eight hours post-transfection, the cells were harvested for protein and RNA.

Creation of Stable Cell Line:

A Huh7.5 stable cell line that continuously expresses RCKΔPRD was created by transduction of a lentivirus containing 3xFlagRCKΔPRD. To create the lentivirus, lipofectamine 2000 was similarly used to transfect 293FT cells with 3ug of pLenti-3xFlag RCK ΔPRD and 9ug of the packaging mix. The next day the DMEM media was aspirated off and new media was added. Forty-eight hour post-transfection lentivirus was harvested by collecting the media. Aliquots (1ml) of the virus was stored at -80°C until a viral titer was used to determine the concentration of the lentivirus.

To determine the concentration of lentivirus to use for future transduction assays 2 50mL conical tubes were made up, each with 24μL of Polybrene (1μL/mL; Sigma) and 24mL of DMEM. The Polybrene was added to increase the retrovirus gene transfer efficiency. Eight hundred μL of the mix was aliquoted into 10 5mL conical tubes (2 sets of 5 different dilutions ranging from 10^{-2} to 10^{-6}). Into two new 5mL tubes, 900μL of the DMEM and Polybrene mix was added with 100uL of the lentivirus (1:10 dilution). Two hundred uL of this dilution was added into the 800μL of DMEM and Polybrene in the 10^{-2} tube. Then 200uL from the 10^{-2} tube was then taken out and mixed into the 10^{-3} tube. This process was repeated until all of the dilutions were made. This process was also repeated for another set of dilutions, yielding two full sets of dilutions ranging from 10^{-2} to 10^{-6}. The media was aspirated, and 1mL of the DMEM and Polybrene mix was added to each well. One mL of the lentivirus dilutions was added to the Huh7.5 cells. Huh7.5 cells were seeded in 24-well plates 24 hours prior the transduction at a concentration of ??????????. Following transduction with the lentiviruses the cells were then

treated for 12 days with Blasticidin (4μL/10mL; Life Technologies) to select for the cells transduced with the lentivirus. To determine virus titer, cells were washed 2x with PBS and incubated with crystal violet dye (0.1% crystal violent, 20% ethanol), and the surviving cells were counted, and a viral titer was calculated.

To concentrate the lentivirus, the transfection in 293FT cells was repeated with 3ug of pLenti-3xFlag RCKΔPRD and 9ug of the packaging mix following the same procedure as described above, but on the day of harvest the media taken from the cells and filtered through a 0.22μM pore PVDF membrane. Next, 2mL of PEG-it solution (System Biosciences) was added to 8mL of media from the infected cells. The cells were stored at 4°C overnight. The next day the the sample was spun for five minutes at 4°C at 10,000g, and the media was aspirated off. The virus pellet was then resuspended in 100μL of PBS. This created a more concentrated virus that was used for transducing cells.

In order to transduce Huh7.5 cells to create a stable cell line with my mutant 3xFlag-RCKΔPRD, a 10^{-3} dilution of the PEG-it concentrated virus was added to 1mL DMEM (1μL/mL Polybrene). This was then added to cells in a 24 well plate that were previously seeded at 1x 10^5cells/mL the day before. After 24 hours, the media was aspirated off and fresh DMEM was added. The cells were left to grow up for another 24 hours. Blasticidin selection was then used for 2 weeks with 2μg/mL of Blasticidin being added every 2 days. After 2 weeks, the cells were scaled up into larger 10cm plates and the stable cell line stock was frozen down at -80°C. To freeze down the cells, the media was aspirated off of the plates and the cells were trypsinized. The cells were spun down and the media was aspirated off. The pellet was then resuspended in 1mL of freeze down media (90% FBS with 10% dimethylsulfoxide, DMSO).

Infection of Huh7.5 Cells with HCV:

In order to examine the effects of 3xFlag-RCKΔPRD on HCV gene expression, transient transfection was set up in which p3xFlagRCKΔPRD and the siRNA targeting the 3'UTR (siRCK5) were cotransfected in Huh7.5 cells using the method described above. Twenty four hours post-transfection 50μL ($7x10^5$ viral particles) of JFH1 HCV was added to 6cm culture dishes in 1mL of DMEM. Two hours later, the cells were trypsinized and 10^5cells were seeded into an 8-well chamber slide (LAB-TEK®) for confocal imaging. The remaining cells were transferred to a 10cm plate. Twenty-four hours post infection, a second transfection with siRNAs was performed. Forty-eight hours after the second transfection, the cells were harvested for protein and RNA, and media was harvested for viral titers to determine virus concentration intra- and extracellularly. The protein lysates were run out on a SDS-PAGE gel and analyzed via Western blot analysis.

Harvesting:

To harvest Huh7.5 cells for protein and RNA the cells were washed 2x with cold PBS and after the 2nd wash 1mL PBS was added to the plate. A cell lifter was used to scrape the cells from the plate and the suspension was collected in a 1.5mL tube. The cells were spun at RT at 2,000g for 10 minutes, and the PBS was aspirated off. For protein isolation the cell pellet was resuspended in 50-100μL (depending on the size of the pellet) of RIPA (10mM Tris-Cl pH 8.0, 1mM EDTA, 0.1% SDS, NaCL, 1 Triton X- 100, 0.1% sodium deoxycholate) with protease and phosphatase inhibitors (Pierce). The tubes were kept on ice for 20minutes and then spun down at 4°C for 20mins at 12,000g. The supernatant was collected, transferred to a new tube, and stored at -20°C until needed. For RNA isolation the pellet was resuspended in 1mL TRIzol (Invitrogen) and frozen at -20° C until needed. In order to harvest media for viral titers, 72 hours post

infection 500μL of DMEM media containing virus from the infection was harvested using sterile technique and stored at -80°C until needed.

SDS-PAGE and Western Blotting:

Protein concentrations were determined using the Bradford assay (BIO-RAD). Standards of known concentrations of BSA (0ug/mL, 10ug/mL, 20ug/mL, 40ug/mL, 60ug/mL, 80ug/mL, 100ug/mL, 125ug/mL, 150ug/mL, and 200ug/mL) were diluted with PBS, and 10μL of Bradford reagent was added to each standard. The nanospectrometer was used to determine the absorbance values from which a standard curve was generated and used to determine the concentration of the cell lysates. The cell lysate samples were prepared by adding 1μL of lysate to 49μL PBS. An equal volume of Bradford reagent (50μL) was then added and absorbance measured with the nanospectrometer. Twenty μg of protein was used for SDS-polyacrylamide gel electrophoresis.

Once protein concentrations were determined, 20ug of protein was loaded into an SDS-PAGE gel. The lysates were diluted with RIPA buffer to yield a total volume of 25μL. One μL of 5x SDS- PAGE loading dye was then added for every 5μL of sample. The samples were incubated at 95°C for 5 minutes and then placed on ice until the gel was ready to be loaded. A 10% resolving gel was poured with a 5% stacking gel on top. Once the gel was made, the samples were loaded, and the gel was run for approximately 2 ½ hours at 100V. The gel was then transferred to a PVDF membrane in transfer buffer chilled with an ice pack for one hour at 100V. Following transfer, the membrane was activated in methanol and allowed to air-dry. The membrane was then washed in methanol, ddH2O, and PonceauS dye (Sigma) to visualize protein bands.

Following PonceauS staining, the blots were washed in ddH20 and PBS (phosphate buffered saline) containing 0.5% Tween20 (PBS-T) to remove the PonceauS dye and blocked

with 5% milk in PBS-T at RT for 1h. The following primary antibodies were used to detect the protein samples: primary antibody (mouse anti-Flag-HRP, 1:20,000; Sigma, incubation at RT for one hour), mouse anti-GAPDH (1:10,000; Calbiochem, incubation overnight), mouse anti-HCV Core (1:5000; abcam, incubation overnight), mouse anti- NS5A (1:2,000, incubation overnight, Dr. Charles Rice, Rockefeller University), rabbit anti-RCK (C terminal, 1:10,000, Bethyl, incubation overnight). Following incubation with the primary antibodies, the blots were washed 3x for 10min with PBS-Tween. The following secondary antibodies were then applied to the blots if needed and incubated at RT for 1 hour: donkey anti-mouse-HRP secondary antibody (1:10,000; Santa Cruz Biotechnology) and donkey anti-rabbit-HRP secondary antibody (1:10,000; Santa Cruz Biotechnology). The blots were again washed 3x for 5 minutes in PBS-T and incubated with ECL chemilluminescent reagent (Thermo Scientific) for 1 min and exposed to film in a dark room for various exposure times. Following ECL detection and development the blot was treated with sodium azide to remove HRP signals. To strip the blot of HRP signals, 100μL of sodium azide was mixed 10mL of blocking buffer and added to the blot. The blot was treated with sodium azide for one hour at room temperature, followed by three ten minute washed in ddH_20. The blot was washed three additional times for 10 minutes each in 1%. PBS-Tween and the blocked with milk for 30 minutes.

Northern Blotting:

The RNA in TRIzol was first incubated for 15min at RT. Chloroform (0.2mL) was added to each tube and the samples were vortexed. They were then left to incubate for 3mins at RT before they were spun down at 4°C for 15mins at 12,000g. To precipitate the RNA, approximately 430μL of the aqueous phase of the RNA was collected. To the aqueous phase 0.5mL of isopropanol was added to each sample and the samples were inverted to mix. They

were then incubated at RT for 10mins and centrifuged at 4°C for 10mins at 12,000g. Ethanol (75%) was added and the samples were mixed by inverting the tube. The RNA was stored at -20°C overnight, and the next day the samples were spun down at 4°C for 10 minutes at 12,000g, The supernatant was poured off, and the samples were left to air dry at RT for 10 minutes, before the pellets were resuspended in 14μL of ddH20 and the concentrations of RNA were determined on the Nanodrop.

To prep the samples for loading on the 1% agarose gel/ 6.7% formaldehyde gel, the samples were diluted with distilled water and 14μL of loading dye was added. The loading dye consisted of 320μL of formamide, 100μL of 10X MESA, 120μL of formaldehyde, and 2μL of 1% bromophenol blue. After the loading dye was added, the samples were denatured at 65°C for 15mins. The samples were then run at 100V on a 1% agarose gel/ 6.7% formaldehyde gel that was made up of 1g agarose, 80mL of distilled water, 10mL of 10x MESA buffer, and 18mL formaldehyde. The gel was run for approximately an hour and a half in running buffer made of 1X MESA and 6% formaldehyde.

After separating the RNA in the gel, the RNA was transferred from the gel to a Zeta-Probe blotting membrane (Bio-Rad) overnight by capillary transfer with a 20x SSC buffer with 3M NaCl and 0.3M Sodium Citrate. The RNA was UV cross-linked to the membrane, which was then prehydrized in 6mL ExpressHyb (Clontech) for 1 hour at 65°F. An actin probe was generated by digesting 10μg pCR2.1-actin with 2μL EcoRI Buffer, 1uL EcoRI enzyme (NEB-Biolabs) for two hours. An agarose gel was run, and the lower band from the gel was cut out and isolated using a QIAquick Gel Extraction Kit (Qiagen) following manufacturer's protocol. A RadPrime (Invitrogen) kit was used to make the probe following manufactures protocol. To clean the probe a QIAquick Nucleotide Removal Kit (Qiagen) was used following

manufacturer's protocol. Once the probe was generated, 100µL of actin probe was then added into 6mL ExpressHyb Hybridization Solution (Clontech) for one hour and the gel was washed 3x (10mL) of 0.1X SSC/ 0.1% SDS for 15 minutes each. The radioactive probe was then visualized using a Phosphor image screen. A similar protocol was used to generate an HCV probe using the plasmid pHCV-BFPpromoterless and digesting with NcoI restriction enzyme.

HCV Titers:

In order to determine the concentration of intracellular and extracellular virus, 500µL of DMEM media and 1000µL of cells suspended in PBS were harvested from cells infected with JFH1 HCV. Following the harvest, 4 sets 6 of tubes were made with dilutions of 10^{-1}, $10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}$, and 10^{-6}. To make these dilutions 100µL of the cell suspension was added to 900µL of DMEM in a 1.5mL tube yielding a 10^{-1} dilution. From that tube 100µL of the cell suspension and DMEM mixture were taken out and put into another 900µL of DMEM media to create a 10^{-2} dilution. This process of diluting the cell suspension was repeated to create the dilutions up to 10^{-6}, and another set of the six dilutions was made. This process was then repeated to create two sets of the same six dilutions. Once the dilutions were made, the 400µL samples were added to Huh7.5 cells that were grown up to 80% confluency in a 48 well plate. The dilution samples were added to the cells. These cells were then incubated for 72 hours at 37C. Post 72 hours, the media was aspirated off and the cells were washed 2x in PBS. The cells were then fixed in cold methanol for 20 minutes. The methanol was poured off and the wells left to air dry for 10 minutes. Once dry, the wells were washed 3x for 10 minutes each in 1% fish gelatin (Thermo Scientific) in PBS . Next, 100µL of the primary antibody, mouse anti-HCV Core was added 1:2,000 in PBS/1% fish gelatin and left to incubate over night. The next day the cells were washed extensively in PBS at RT and stained with a AlexaFluor 488 goat anti-

mouse IGg (1:200, Life Technologies). The plates were washed again in PBS, and stained with Hoechest in PBS/1% fish gelatin at 1:10,000 at RT for 5 minutes. The cells were washed in PBS for 5 minutes, and 500µL of PBS was added to each well. The fluorescent foci were counted the same day to determine the viral titer.

Confocal Microscopy:

To visualize the localization of 3xFlag-RCKΔPRD in Huh7.5 cells, confocal microscopy was used. Cells that were cotransfected with siRCK5 3xFlagRCKΔPRD and then infected 24 hours later, as described above, and seeded into an 8-well chamber slide (LAB-TEK®) in 500µL of DMEM. Ninety six hours later, the media was removed and the wells were washed 2x in PBS. The cells were fixed in 4% PFA/PBS for 20 minutes. After that they were washed 2x in PBS and they were permeabilized using 0.5% TX-100/1% fish gelatine in PBS. They were blocked 3x for 10 minutes in 1% fish gelatin/PBS. Next, 100µL of primary antibody solution, mouse anti-core 1B (1:1,000) and rabbit anti-C terminal RCK (1:1,000) was added and incubated overnight. The wells were then washed 2x for 10 minutes each in 1% fish gelatin/PBS. 100µL of secondary antibody solution was added, donkey anti-rabbit 647 (1:200) and donkey anti-mouse 488 (1:200), to the plate and incubated for one hour at room temperature in the dark. The cells were then washed 2x for 5 minutes in 1% fish gelatin/PBS. 100µL donkey anti-Flag-CY3 (Sigma) in 1%FG/PBS (1:200) was added to each well and incubated overnight in 4°C. The next day the wells were washed 2x for 5 minutes in 1% fish gelatin/PBS and incubated for 10 minutes in 100µL of Hoechst stain (1:10,000) in 1%fish gelatin/PBS. The wells were washed 2x for 5 minutes in 1% fish gelatin/PBS and 5µL per well of fluoromount (SouthernBiotech) was added before coverslip was applied. Slides were imaged on a Zeiss LSM 710 using a 63x oil immersion objective, and images were processed in ImageJ.

Results:

Cloning RCKΔPRD into Plasmid Vectors:

In order to examine the expression of RCKΔPRD in Huh7.5 cells a p3xFlagRCKΔPRD construct was created (Figure 2). To accomplish this the RCKΔPRD coding region was first cloned from pE-mRFP-RCKΔPRD into a pCR2.1-TOPO vector (Figure 3). From the pCR2.1-TOPO plasmid, RCKΔPRD was subcloned into a 3xFlag vector for expression in Huh7.5 cells (Figure 4). The 3xFlagRCKΔPRD region was also subcloned into a pLenti6/V5-D-TOPO vector in order to generate lentivirus for the creation of a Huh7.5 RCKΔPRD stable cell line (Figure 5).

Expression of RCKΔPRD in Huh7.5 Cells:

To analyze the expression of RCKΔPRD the 3xFlagRCKΔPRD plasmid was transfected into Huh7.5 cells. The cells were harvested and protein expression was analyzed via western blot analysis (Figure 6). Figure 6 shows control expression of 3xFlagBAP (Bacterial Alkaline Phosphatase) and 3xFlagRCKΔPRD in Huh7.5 cells. Once I was able to show that 3xFlagRCKΔPRD could be expressed in Huh7.5 cells, I created a pLenti3xFlagRCKΔPRD (Figure 5) in order to make a lentivirus to create a Huh7.5 RCKΔPRD stable cell line. Prior to creating the stable cell line, I tested the expression of pLenti3xFlagRCKΔPRD by transfecting Huh7.5 cells and analyzing protein expression via western blot analysis (Figure 7). As seen in Figure 7, 3xFlagRCKΔPRD from the pLenti plasmid was expressed in Huh7.5 cells.

Knockdown of exogenous RCK with siRNAs and rescue with RCKΔPRD:

In order to test the affects of mutant RCK, it was necessary to knockdown endogenous RCK. SiRNAs targeting the coding region and the 3'UTR of RCK mRNA were tested to determine which siRNAs provided the best knockdown of endogenous RCK. Huh7.5 cells were transfected with transfection reagent (lipofectamine), control siRNA (siMVP) and RCK-specific

siRNAs (siRCK1, siRCK4, and siRCK5), and the protein from the cells was harvested and analyzed via Western blot. Through analysis of the abundance of RCK we determined that siRCK1 (targeting the coding region) and siRCK5 (targeting the 3'UTR) (Fig 8, lanes 3 and 5) efficiently knocked down RCK. Furthermore examination of GAPDH levels showed that our samples were equally loaded (Figure 8?).

After determining that siRCK1 and siRCK5 efficiently depleted endogenous RCK, we next examined the expression of exogenous RCKΔPRD while simultaneously knocking down endogenous RCK with siRNAs. Huh7.5 cells were transfected with lipofectamine, siMVP, siRCK1, and siRCK5 with or without p3xFlagRCKΔPRD. Protein was harvested and abundance was analyzed via western blot analysis (Figure 9). As seen in Figure 9, detection of the Flag epitope showed decreased expression of 3xFlagRCKΔPRD with the siRNA (siRCK1) targeting both endogenous and exogenous RCK, but that 3xFlagRCKΔPRD was expressed when cotransfected with siRCK5 (Fig 9, lanes 8 and 11). Therefore siRCK 5 targeting the 3'UTR should be used for future experimentation so that endogenous RCK can be knocked down in Huh7.5 cells and RCK expression is rescued with 3xFlagRCKΔPRD.

After confirming that p3xFlagRCKΔPRD could be expressed in Huh7.5 cells while simultaneously knocking down endogenous RCK with siRCK5, targeting the 3'UTR of RCK, a time course transfection was undertaken to determine if this expression of 3xFlagRCKΔPRD could be maintained for 96 hours, the time required for HCV viral infection. A transient transfection was set up using a lipofectamine control and pLenti3xFlagΔPRD, p3xFlagBAP, and p3xFlagRCK with and without siRCK5, targeting the 3'UTR (Figure 10). Cells were harvested from this transfection after 48 (Fig 10A), 72 (Fig 10B), and 96 (Fig 10C) hours, and protein expression was examined using Western blotting analysis. Detection of the Flag protein

3xFlagRCKΔPRD was expressed up to retained expression for 96 hours. GAPDH signal however was not detected, so this experiment will be repeated for further analysis.

HCV Infection with RCKΔPRD Mutant:

In order to examine the effects of RCKΔPRD on HCV protein expression, Huh7.5 cells were transfected with p3xFlagRCK and siGL2, p3xFlagRCK and siRCK5, and p3xFlagRCKΔPRD and siRCK5. Twenty four post-transfection the cells were then infected with 50μL of JFH1 HCV ($8x10^5$ viral particles/mL), and a repeat transfection with the siRNAs was done 24 hours post infection. Seventy two hours post infection, protein was harvested and abundance analyzed via western blot analysis (Figure 13). When p3xFlagRCK was cotransfected with a control siRNA, siGL2, there was an overexpression of RCK from presence of the exogenous and endogenous RCK in the cells as well as HCV NS5A expression. When the cells were cotransfected with p3xFlagRCK and siRCK5 there was knockdown of endogenous RCK, but RCK expression was rescued with p3xFlagRCK. There was also a rescue in HCV NS5A expression. Additionally, when the cells were cotransfected with 3xFlagRCKΔPRD and siRCK5, there was rescue of RCK expression from the mutant, but there was a depletion in HCV NS5A expression. It can also be seen from this western blot that the p3xFlag constructs were expressed in the Huh7.5 cells as seen with Flag signal as well as even protein loading as shown with the GAPDH signal (Figure 13).

The cells from this experiment were also harvested for RNA for a Northern blot to examine HCV RNA expression. Unfortunately, the radioactivity that was used to detect the RNA was a month old, and no HCV or actin RNA was detected. Furthermore, viral titers were performed, however the cells were unexpectedly washed away during fixation of the cells, and

therefore viral concentrations could not be determined. This experiment will be repeated in order to examine RNA expression and viral concentrations.

In order to examine the effects of RCKΔPRD on localization with HCV Core protein, cells from the previous experiment were analyzed via confocal microscopy (Figure 14). However, as seen in Figure 14, there wasn't sufficient knockdown of endogenous RCK. The localization of 3xFlagRCK and 3xFlagRCKΔPRD with HCV Core were compared, and both RCK and RCKΔPRD showed similar localization with HCV Core protein at lipid droplets as indicated by the small brightly stained foci dispersed throughout the cytoplasm of the cells. This experiment will be repeated with higher concentrations of siRCK5 to knockdown endogenous RCK. The true effects of RCKΔPRD on RCK and HCV localization can then be determined.

Creation of Huh7.5 RCKΔPRD Stable Cell Line:

In order to transduce cells to create a stable cell line with RCKΔPRD being continuously expressed, the 3xFlagRCKΔPRD coding region was subcloned into pLenti6/V5-D-TOPO vector and expression was examined in Huh7.5 cells (Figure 7). Once it was confirmed that 3xFlagRCKΔPRD could be expressed in Huh7.5 cells, lentivirus was created using pLenti3xFlagRCKΔPRD and a pLenti packaging plasmid. The lentivirus was collected and a viral titer was determined by infecting Huh7.5 cells with serial dilutions of the virus. A viral titer was determined after two weeks of blasticidin selection by staining the cells with crystal violet and counting foci. This titer was calculated to be $9.5\ x\ 10^4$ transduction units/mL. Because this calculated viral titer was lower than anticipated, this experiment was repeated where the virus was be concentrated. The same procedure was repeated to create the virus, but on the day of harvest the media taken from the cells and run through PVDF membrane. PEG-it solution was added to media from the infected cells and the virus precipitated out of solution overnight. The

viral pellet was resuspended in PBS to create a more concentrated virus that was used to transduce Huh7.5 cells to create the stable cell line.

Once the stable cell line was created, an experiment was set up to see if 3xFlagRCKΔPRD could be expressed in those cells while endogenous RCK was knocked down.. To do this, Huh7.5 cells, Huh7.5 3xFlagBAP, and Huh7.5 3xFlagRCK, and Huh7.5 3xFlagRCKΔPRD were transfected with a lipofectamine control, siGL2, siRCK1, siRCK4, and siRCK5 (Figure 12). This experiment was also repeated two times, as the first time that this experiment was done, RCK expression could not be seen. Figure 12 shows a repeat of this experiment, and here it is possible to see that RCKΔPRD expression in the stable cell line (Figure 12, lane 20). However, because GAPDH signal was not detected, this experiment will need to be repeated again to determine if RCKΔPRD retains expression with endogenous RCK knockdown. Although the Huh7.5 RCKΔPRD stable cell line was created, and expression tested, these cells did not survive after being frozen down at -80°C, and another stable cell line was created. Future experimentation is needed with this stable cell line to analyze RCKΔPRD expression and the effects on HCV gene expression.

Conclusion:

RCKΔPRD was successfully cloned into both the 3xFlag vector and the pLenti vector, and the plasmids could be expressed in Huh7.5 cells. Additionally, because the PRD is deleted from RCK in the mutant plasmid, the mutant has a lower molecular weight than wild-type RCK, the mutant should run farther down the SDS-PAGE gel. In the western analysis following the transfection of p3xFlagRCKΔPRD and pLenti3xFlagRCKΔPRD, when comparing RCK and RCKΔPRD expression, RCKΔPRD comparatively runs lower on the SDS-PAGE gel. This provides sufficient evidence that RCKΔPRD is being expressed in Huh7.5 cells. Furthermore, siRCK5 targeting the 3'UTR of RCK could be used to knockdown endogenous RCK and that RCKΔPRD could rescue RCK expression.

When infecting Huh7.5 cells with cells depleted of endogenous RCK but expressing RCKΔPRD, there was a reduction in HCV NS5A expression. This suggests that RCKΔPRD has a deleterious effect on the HCV virus and what. Furthermore, in infected Huh7.5 cells with endogenous RCK, RCKΔPRD colocalized with HCV Core. This localization was similar in cells with wild-type RCK being expressed. This experiment should be repeated with the knockdown of endogenous RCK, to examine the effects of RCKΔPRD on RCK localization with HCV Core. This would provide further insight into the function of the PRD of RCK in Huh7.5 cells.

Last, the Huh7.5 RCKΔPRD stable cell line created expressed RCKΔPRD. Further studies will need to be done with this stable cell line to examine if there are any differences in those infected cells in comparison to the data that was collected from the transient transfection of p3xFlagRCKΔPRD and siRCK5.

Overall, these data suggest that the PRD is important for HCV protein expression, as in its absence HCV NS5A expression was depleted. However, the reasoning behind this depletion

of HCV NS5A protein expression remains unclear. Future studies should be undertaken to examine the effects of the PRD on RCK localization and association with the virus intracellularly. If this region is in fact important for HCV gene expression, it would be interesting to examine how the structure of the region affects HCV-RCK interaction. It would also be interesting to examine truncations of the PRD to determine which region(s) of the PRD is/are necessary for HCV gene expression. Future examination of the effects of RCK ΔPRD may provide insight about the localization and assembly of HCV.

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Figures:

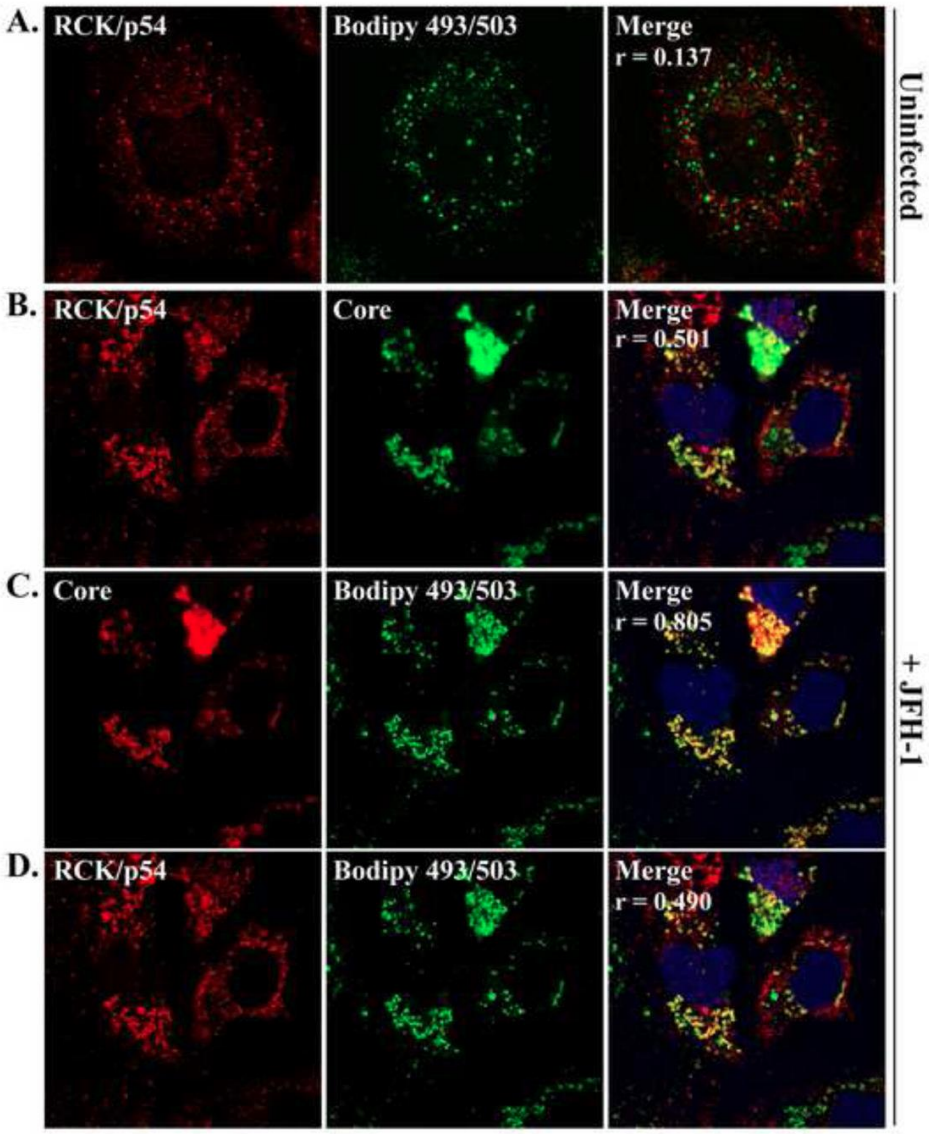


Figure 1: Localization of RCK HCV core, and lipid droplets during JFH-1 infection. (A) Localization of RCK and lipid droplets in uninfected Huh7 cells. (B-D) Localization of RCK, HCV core, and lipid droplets in JFH-1 infected Huh7 cells 3 days post infection.

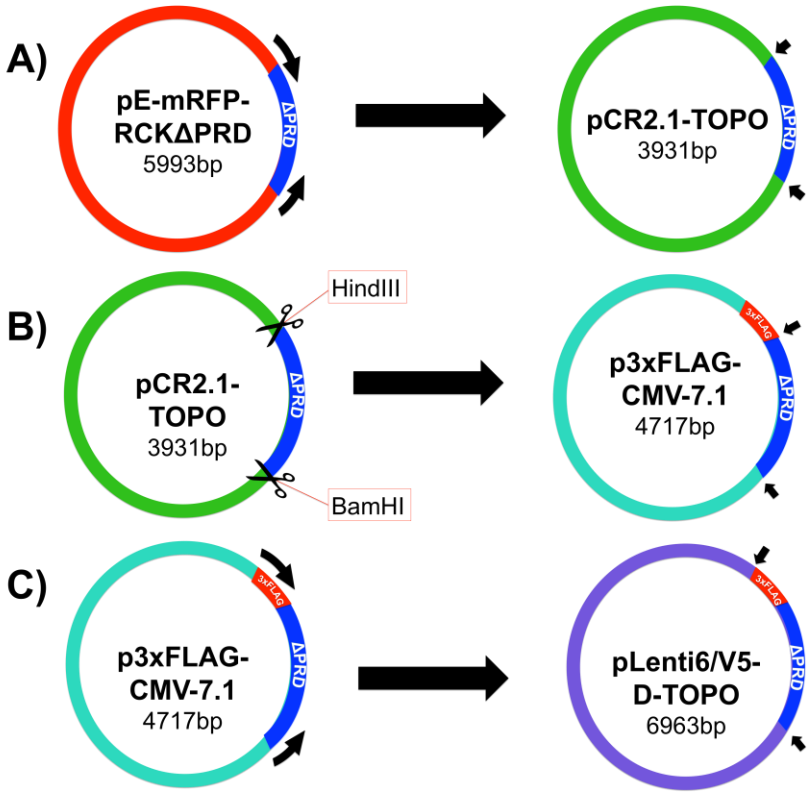
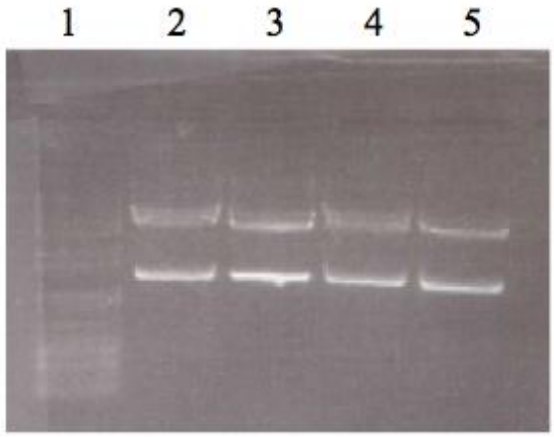
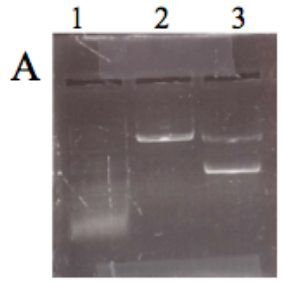


Figure 2: Cloning Strategy Used to Create pLenti3xFlagΔ Construct. (A) The RCK/p54 gene containing a deleted (Δ) PRD was PCR amplified using specific primers and inserted into a TOPO vector. (B) Restriction enzyme digest using HindIII and BamHI was used to sub-clone ΔPRD into p3xFlag. (C) 3xFlag-tagged ΔPRD was PCR amplified using specific primers and inserted into the pLenti vector. Arrows indicate the region inserted into plasmid.

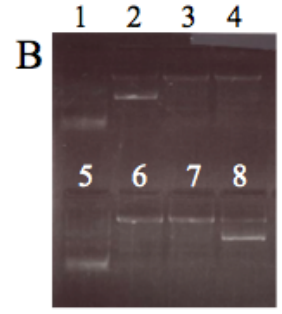


Lane #	Sample
1	Ladder
2-5	Colonies from Transformation of TOPO RCKΔPRD

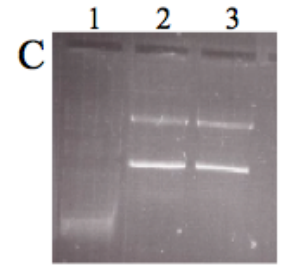
Figure 3: Screen of DH5α for TOPORCKΔPRD. Colonies of DH5z transformed with TOPORCKΔPRD were selected for by ampicillin resistance and screened using HindIII and BamHI digest of miniprep DNA.



Lane #	Sample
1	Ladder
2	p3xFlagCMV-7.1
3	pCR2.1-TOPO-RCKΔPRD

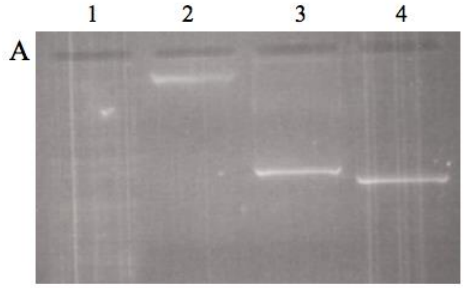


Lane #	Sample
1	Ladder
2-4	Colonies from Transformation of 3xFlagRCKΔPRD
5	Ladder
6-8	Colonies from Transformation of 3xFlagRCKΔPRD

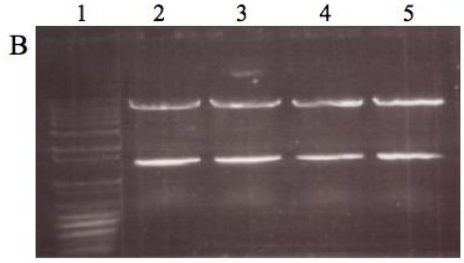


Lane #	Sample
1	Ladder
2	Mini Prep of 3xFlagRCKΔPRD
3	Maxi Prep of 3xFlagRCKΔPRD

Figure 4: Creation of 3FlagRCKΔPRD. (A) The RCKΔPRD was cloned into p3xFlagCMV-7.1 using HindIII and BamHI. (B) Screening clones of 3xFlagRCKΔPRD for RCKΔPRD. (C) Confirmation of RCKΔPRD in mini and maxiprep samples.

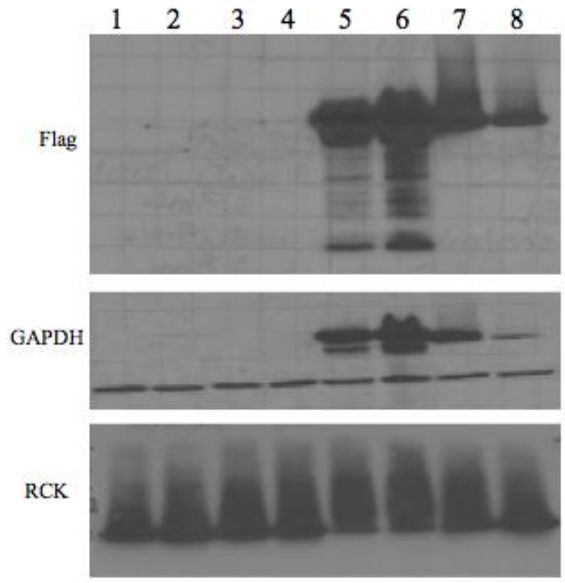


Lane #	Sample
1	Ladder
2	Negative Control
3	p3xFlagCMV-7.1-RCK
4	p3xFlagCMV-7.1-RCKΔPRD



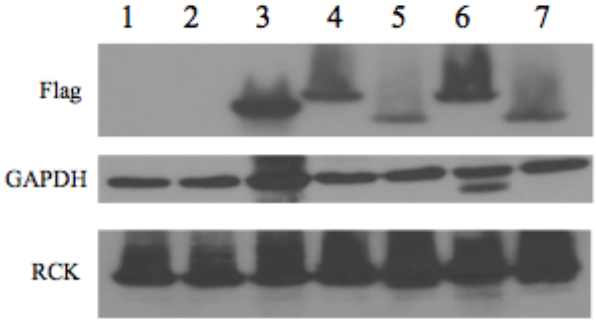
Lane #	Sample
1	Ladder
2-5	Colonies from Stbl3 Bacteria with pLenti6/V5-D-TOPO-3xFlagRCKΔPRD

Figure 5: Creation of pLenti3xFlagRCKΔPRD (A) PCR amplification of 3xFlagRCKΔPRD coding region. (B) Screen of RCKΔPRD using XhoI and BamHI digest of pLenti6/V5-D-TOPO-3xFlagRCKΔPRD in Stbl3 bacteria.



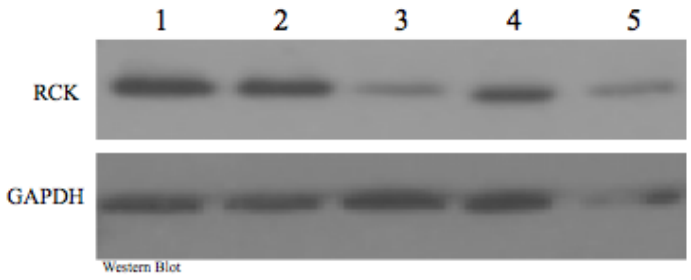
Lane #	Sample
1-2	Lipofectamine
3-4	3xFlag
5-6	3xFlagBAP
7-8	3xFlagRCKΔPRD

Figure 6: Expression of 3xFlagRCKΔPRD in Huh7.5 Cells. Huh7.5 cells were transfected with p3xFlagCMV-7.1, p3xFlagBAP, and p3xFlagRCKΔPRD and protein harvested and analyzed via western blot.



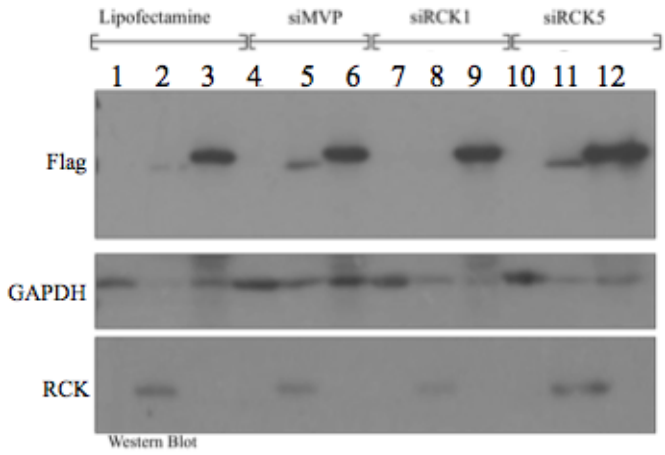
Lane #	Sample
1	Lipofectamine
2	3xFlag
3	3xFlagBAP
4	3xFlagRCK
5	3xFlagRCKΔPRD
6	pLenti3xFlagRCK
7	pLenti3xFlagRCKΔPRD

Figure 7: Expression of pLenti3xFlagRCKΔPRD in Huh7.5 Cells. Huh7.5 cells were transfected with control plasmids and pLenti3xFlagRCKΔPRD and protein harvested and analyzed via western blot.



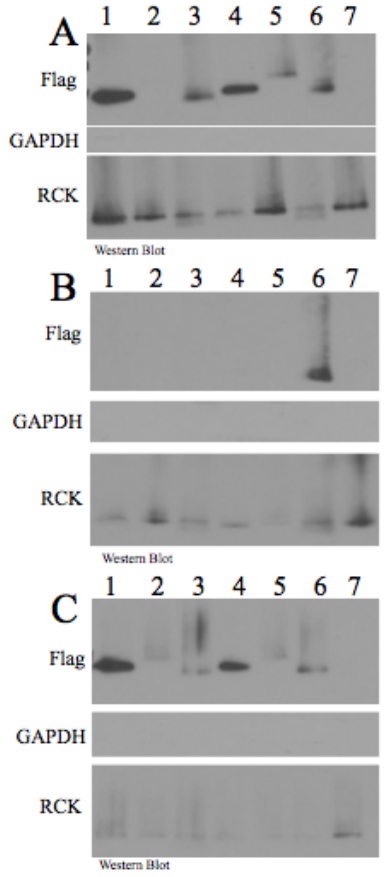
Lane #	Sample
1	Lipofectamine
2	siMVP (Control)
3	siRCK1 (targeting coding region)
4	siRCK4 (targeting 3'UTR)
5	siRCK5 (targeting 3'UTR)

Figure 8: Knockdown of endogenous RCK with siRNA. Huh7.5 cells were transfected with Lipofectamine and siMVP controls. SiRCK1 targets nucleotide position 1210 in the RCK the coding region. SiRCK4 targets nucleotide position 1986-2004 of RCK 3'UTR and siRCK5 targets nucleotide position 2254 of RCK 3'UTR. Protein was harvested and analyzed via western blot.



Lane #	Sample
1	Lipo
2	Lipo + 3xFlagRCKΔPRD
3	Lipo + 3xFlagBAP
4	siMVP
5	siMVP + 3xFlagRCKΔPRD
6	siMVP + 3xFlagBAP
7	siRCK1
8	siRCK1 + 3xFlagRCKΔPRD
9	siRCK1 + 3xFlagBAP
10	siRCK5
11	siRCK5 + 3xFlagRCKΔPRD
12	siRCK5 + 3xFlagBAP

Figure 9: SiRNA knockdown of endogenous RCK and rescue with RCKΔPRD. SiMVP, siRCK1, and siRCK5 were cotransfected with p3xFlagBAP and p3xFlagRCKΔPRD in Huh7.5 cells. Protein was harvested and analyzed via western blot.



Lane #	Sample
1	3xFlagBAP
2	3xFlagRCK
3	pLenti3xFlagRCKΔPRD
4	3xFlagBAP + siRCK5
5	3xFlagRCK + siRCK5
6	pLenti3xFlagRCKΔPRD+ siRCK5
7	Lipofectamine

Figure 10: siRNA knockdown of endogenous RCK and rescue with RCKΔPRD after 96 hours. SiRCK5 was cotransfected with p3xFlagBAP, p3xFlagRCK, and pLenti3xFlagRCKΔPRD. Protein was harvested after (A) 48 hours, (B) 72 hours, and (C) 96 hours and analyzed via western blot.

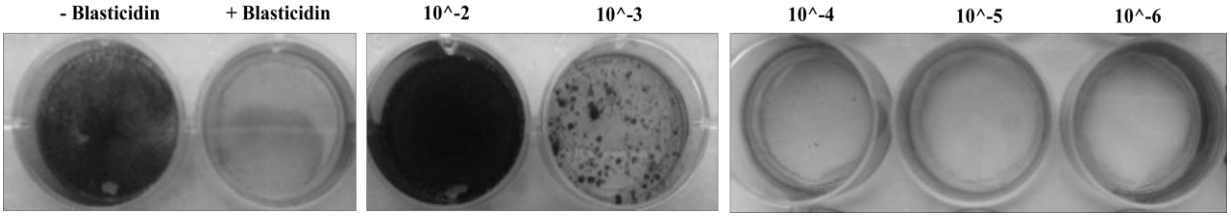
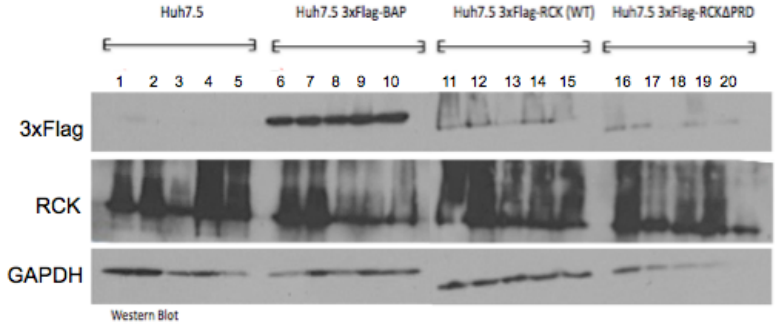
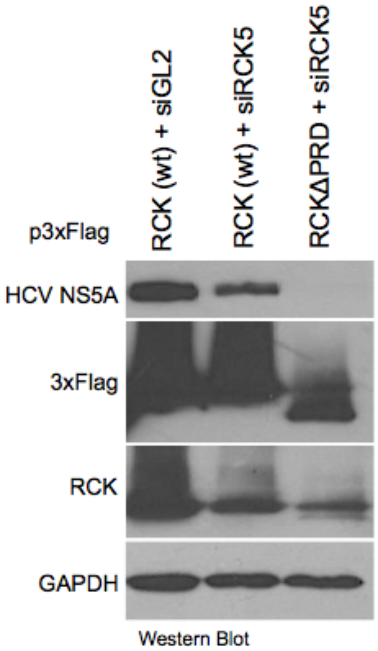


Figure 11: Viral tittering of p3xFlagRCKΔPRD lentivirus. Huh7.5 cells under blasticidin selection were transduced with serial dilutions of p3xFlagRCKΔPRD lentivirus. Viral titers were determined after two weeks by staining with crystal violet and counting foci.



Lane #	Sample
1	Lipofectamine
2	siGL2
3	siRCK1
4	siRCK4
5	siRCK5
6	Lipofectamine
7	siGL2
8	siRCK1
9	siRCK4
10	siRCK5
11	Lipofectamine
12	siGL2
13	siRCK1
14	siRCK4
15	siRCK5
16	Lipofectamine
17	siGL2
18	siRCK1
19	siRCK4
20	siRCK5

Figure 12: Knockdown of endogenous RCK using siRNA targeting 3'UTR of RCK and expression of RCKΔPRD from Huh7.5 stable cell line. Huh7.5, Huh7.5 3xFlag BAP, Huh7.5 3xFlagRCK, and Huh7.5 3xFlagRCKΔPRD stable cell lines were transfected with a control siRNA and siRCK1, siRCK4, and siRCK5. Protein was harvested and analyzed via western blot analysis.



Lane #	Sample
1	3xFlagRCK (wt)+ GL2
2	3xFlagRCK (wt)+ siRCK5
3	3xFlagRCKΔPRD + siRCK5

Figure 13: Effects of RCKΔPRD on HCV NS5A expression in Huh7.5 cells infected with JFH1 HCV. Huh7.5 cells were transfected with p3xFlagRCK and siGL2, p3xFlagRCK and siRCK5, and p3xFlagRCKΔPRD and siRCK5. Twenty four hours post transfection the cells were infected with JFH1 HCV and 24 hours post infection a subsequent siRNA transfection was repeated. Protein was harvested 48 hours later and analyzed via western blot analysis.

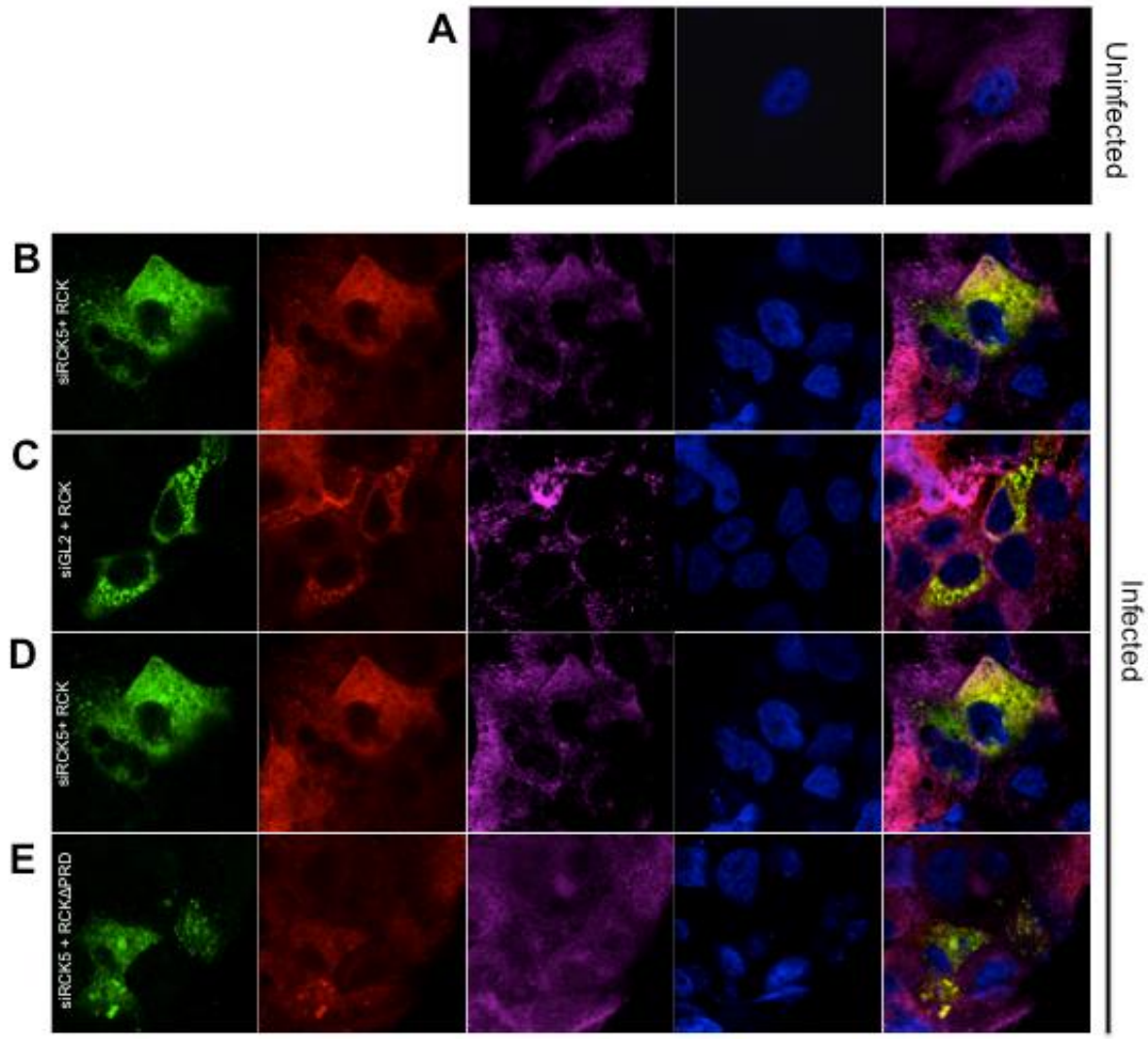


Figure 14: Localization of RCKΔPRD in Huh7.5 cells during HCV infection. (A) Uninfected Huh7.5 cells. (B) Infected Huh7.5 cells transfected with siRCK5. (C) Infected Huh7.5 cells transfected with siGL2 and 3xFlagRCK. (D) Infected Huh7.5 cells transfected with siRCK5 and 3xFlagRCK. (E) Infected Huh7.5 cells transfected with siRCK5 and 3xFlagRCKΔPRD.