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The Role of RCK/p54 Enzymatic Activity during Hepatitis C Infection

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The Role of RCK/p54 Enzymatic Activity during Hepatitis C Infection

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ABSTRACT

Hepatitis C virus (HCV) infected 30,500 people in the United States alone in 2014. As a positive-sense single-stranded RNA virus, the HCV genome facilitates translation and replication of the virus within the host cell. During infection, HCV subverts numerous host proteins to aid in viral production. One such protein is DDX6, a DEAD-box helicase that is associated with the decapping complex and microRNA Induced Silencing Complex (miRISC). Previous studies in our lab have shown that DDX6 is required for HCV expression. Specifically, DDX6 modulates the interaction of miR-122 with the HCV 5' UTR and promotes stability and replication of the viral genome. To elucidate which enzymatic activities of DDX6 are required to promote HCV gene expression, we used site-directed mutagenesis to disrupt the enzymatic activities of DDX6. Specifically, we mutated the ATPase, RNA binding and helicase domains within 3x-Flag-DDX6Δsi plasmid DNA. We next used lentivirus transduction to stably express the 3xFlag-tagged DDX6 mutants in Huh7 cells, in which expression of endogenous DDX6 was deleted by gene editing using CRISPR-Cas9. Analysis by immunofluorescence staining and confocal microscopy of uninfected cells, shows wild-type 3xFlag-DDX6 localizing in processing bodies, while the enzymatic mutants show diffuse cytoplasmic staining. DDX6 stable cell lines were infected with the HCV infectious clone JFH-1, and at three-days post-infection cells were harvested and viral and cellular protein and RNA abundance analyzed by western and northern blotting, respectively. Surprisingly, only DDX6 knock-out cells expressing the wild-type 3xFlag-DDX6 were infected with HCV. These data show that the enzymatic activities of DDX6 are critical for HCV infection. Since RNA helicases unwind RNA, it is possible that the helicase and RNA binding activities of RCK/p54 are necessary to remodel the complex secondary structures found on the 5' and 3' untranslated regions of the HCV genome, and may play an important role in the transition between translation and replication.

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Contents

INTRODUCTION

Hepatitis C virus (HCV) infects 71 million people globally. The virus is primarily spread through contaminated blood. Approximately 25% of individuals will develop an acute infection. Most patients however develop a chronic infection, and this chronic infection increases the patient's risk to develop serious liver problems such as cirrhosis of the liver and hepatocellular carcinoma.[1] The early HCV treatments used the dual treatment strategy with interferon-β and ribavirin. While effective, the interferon-β/ribavirin treatment had limited success, as patients frequently became nonresponsive and developed severe side effects.[2] More recently direct-acting antivirals (DAAs), targeting the HCV NS3/4A protease, NS5A phosphoprotein and NS5B RNA-dependent RNA polymerase, have become available.[2] Although these DAAs have significantly improved treatment outcomes, the NS3/4A inhibitors in particular, show lower reactivity across the HCV genotypes and resistance variants are common. [2] Therefore continuing research on HCV may reveal additional effective anti-HCV therapies.

Molecular Biology of HCV

HCV is an enveloped, single-stranded, positive-sense RNA virus in the *Flaviviridae* family of viruses.[3] Other well known viruses in this family include Yellow Fever virus, West Nile virus, Dengue virus and the recently re-emerging Zika virus. The viral RNA contains a single open reading frame, and 5' and 3' untranslated regions (UTRs) (**Figure 1**). HCV uses the viral genome as a template for translation and replication within the host cell. Translation of the HCV genome is directed by the binding of a 40S ribosomal subunit directly to the internal ribosomal entry site in the 5' untranslated region which results in the production a polyprotein that is cleaved into ten viral proteins. The three structural proteins (core, envelope E1 and E2)

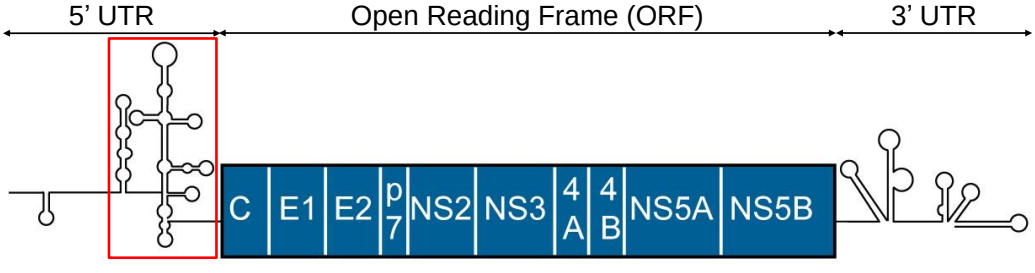

Figure 1. Schematic of the HCV genome. The red box highlights the internal ribosomal entry site.

and seven non-structural (p7, NS2-5B) (**Figure 1**), and facilitate viral RNA replication and assembly into viral particles, and suppression of the innate immune response.[3]

The life cycle of HCV occurs entirely in the cytoplasm of the cell.[4] The virus binds four different receptor and enters the cell by fusing the viral envelope with the cellular endosomal membrane. Viral proteins are immediately following release of the viral RNA into the cytoplasm. The synthesis of the viral proteins such as the HCV NS3/4A protease, NS5A phosphoprotein and NS5B RNA-dependent RNA polymerase facilitate replication of the HCV genome. Replication of the HCV genome occurs using a negative-sense RNA intermediate. The virus assembles on lipid droplets and buds into the ER. New viral particles are released from the cell via the secretory pathway.[4] At each step in the life cycle, HCV interacts with numerous host cell proteins. My research investigate the role of one such host protein namely RCK/p54.

RCK/p54 (DDX6)

RCK/54 is a DEAD-box RNA helicase. The protein contains conserved sequences associated with ATP binding and hydrolysis, helicase and RNA binding activity.[5] The protein is a key component of processing bodies, RNA-protein granules associated with mRNA storage and degradation. RCK however also functions with other proteins to promote decapping of mRNAs and microRNA-mediated gene regulation.[5] Interestingly, RCK/p54 has also been shown to be an oncogene,[6] and is overexpressed in individuals living with chronic HCV infection.[7] Many viruses use RCK, although the function of RCK during infection is largely unknown.[5]

Research from the Pager laboratory has previously shown that knockdown of RCK using target-specific siRNAs decreases the levels of HCV protein and RNA.[8] Interestingly this protein also co-localized with core protein at lipid droplets suggesting a role in the assembly of new HCV particles.[8] More recently they have shown that RCK affects the stability and replication of the HCV RNA, likely by directly the binding of the liver-specific microRNA miR-122 to a particular site in the 5' UTR.[9] We hypothesize that the enzymatic activities of RCK are required to promote HCV gene expression. Therefore, the rationale of this project was to express mutant RCK proteins containing enzymatic deficiencies in Huh 7 cells and investigate which enzymatic properties are important for the HCV life cycle. If any of the enzymatic activities are important for HCV gene expression, we predict a decrease in the levels of viral core protein and viral RNA

when analyzed by western blot and northern Blot analysis respectively, and a decrease in luciferase units for analysis of viral translation and replication using a reporter genome.

MATERIALS AND METHODS

Site-Directed Mutagenesis

Site-directed mutagenesis on the p3xFlag-RCKΔsi plasmid was performed using a Quick Change Lightening Kit (Agilent) and mutagenesis primer with point mutations, as per the manufacturer's instructions. In brief, the following PCR reaction conditions were used: 1 cycle at 95°C for 2 minutes; 18 cycles at 95°C for 20 seconds, 60°C for 10 seconds and 68°C for 2 minute; and 1 cycle at 68°C for 5 minutes. **Table 1** shows the point-mutations introduced into p3xFlag-RCKΔsi. Following the PCR reaction, the DNA wasincubated with 2 µl of Dpn1 and transformed into XL10 gold *E. coli* with 1 µl of β-mercaptoethanol for 30 minutes on ice. The bacteria was heat shocked at 42°C for 30 seconds and then incubated on ice for 2 minutes. SOC media was added after the transformation reaction, and the reaction was shaken at 37°C for one hour. The bacteria hereafter were spread onto an agar plate containing LB agar and carbenicillin (Sigma) and incubated overnight at 37°C. Two bacterial colonies were picked and grown at 225 rpm in a 5ml LB solution containing 0.1 mg/ml ampicillin (Sigma) overnight at 37°C. A miniprep was performed using EZNA plasmid DNA mini kit (OMEGA Bio-Tech). The plasmids were submitted for sequenceanalysis to confirm presence of the mutations. The confirmed plasmids were re-transformed into DH5α bacteria, grown overnight on LB agar and carbenicillin plates at 37°C. Single-colonies were picked to start 100ml LB/ampicillin solution. The bacteria were grown overnight at 37°C at 225rpm. Hereafter, the plasmid was isolated using an EZNA plasmid DNA maxi kit (OMEGA Bio-Tech).

Huh7 and RCK/p54 Huh7 Knockout (KO) Cells

Huh7 cells are a human hepatoma cell line. In our studies we also used RCK/p54 knockout Huh7 cells.[10] These were generated through CRISPR/Cas9 gene editing and were a kind gift from Dr. Jan Carette (Department of Microbiology and Immunology, Stanford University). The cells were maintained at 37°C with 5% CO_2 in DMEM (Life Technologies), 1 mM non-essential amino acids (Life Technologies), 10% fetal bovine serum (FBS; Seradigm) and 1% L-glutamine (Life Technologies). To passage cells, the media was removed and cells were washed two times in 3ml phosphate buffered saline (PS; Life Technologies). Hereafter the

cells were incubated in 1ml trypsin/EDTA solution (Life Technologies) for 5 minutes at 37°C. The trypsin/EDTA solution was inactivated with normal media and cells replated into 100 mm tissue culture plates at 1:3-1:5 ratio. For siRNA and plasmid transfection experiments, Huh7 cells were seeded into 60 mm tissue culture plates at $8x10^5$ cells per plate 4 hours prior to transfection.

Plasmids and siRNAs

The control plasmid used in this study were the p3xFlag-Bacterial Alkaline Phosphate (BAP) (Sigma) and p3xFlag-RCKΔsi wild type (WT). Reference 9 describes the cloning of RCK WT into the p3xFlag plasmid, as well as the site-directed mutagenesis PCR perform to mutate the RCK siRNA binding site (Δsi), such that expression of 3xFlag-RCKΔsi would not be affected by the siRNA gene silencing mechanism.[9] The particular mutations to the RCK enzymatic activities are as follows: **1)** a mutation within the DEAD-box motif (E247Q, DEAD>DQAD) to affect ATPase activity; and **2)** two different mutations in motif III (S277A/T279A, SAT>AAA) and motif Va (G397A; RGID>RAID) to affect helicase activity. **3)** Two additional mutations were created to affect RNA binding R174A/R225A and R174E/R225E. In the first mutant, the arginines are mutated to neutral amino acids (alanine), while in the second mutant the arginines are mutated to negatively-charged amino acids (glutamic acid). **Table 1** shows the point-mutations introduced into p3xFlag-RCKΔsi. The small interfering RNAs (siRNAs) used were siGL2 and siRCK/p54: **1)** siGL2 (5'-CGUACGCGGAAUACUUCGAUU-3') was used as a negative contro siRNA. This siRNA targets Gaussian Luciferase 2 (GL2) mRNA, a gene not expressed in Huh7 cells. siGL2 does not affect RCK nor HCV gene expression. **2)** siRCK (5'-GCAGAAACCCUAUGAGAUUUU-3') silences endogenous RCK mRNA expression.

Lipofectamine Transfection

Transfection of Huh7 and RCK KO cells were performed using Lipofectamine 2000 (Invitrogen). For one transfection reaction, a master mix containing 0.5 ml of OptiMEM was added to a 14 ml tube with 10 µl of Lipofectamine 2000. The second master mix containing the

siRNA and plasmid was prepared in a separate 14 ml tube. To this tube 500 µl of OptiMEM was added together with 5 µl of 20 µM siRNA (final concentration of 100 nM) and 1 µg plasmid DNA. After a five-minute incubation period, 500 µl of the Lipofectamine 2000 solution was added to the tubes containing the siRNA and plasmid. The mixtures were further incubated for 20 minutes at room temperature. While this mixture was incubating, the media in the Huh7 plates was aspirated off, and cells were washed with 1 ml of PBS to remove any excess media. Then each separate transfection mix was added at 1 ml to each plate. The plates incubated for 4 hours at 37°C. Hereafter 3 ml of media was added to the cells, and the cells were further incubated overnight at 37°C.

HCV Infection of Huh 7 cells

Twenty-four hours after transfection the Huh 7 cells were infected with the HCV infectious clone JFH-1 (a kind gift from Dr. Takaji Wakita, Tokyo Metropolitan Institute of Neuroscience) at a multiplicity of infection (MOI) of 0.1. Six hours after infection, the cells were trypsinized and re-plated into a 100 mm tissue culture plate. To analyze HCV gene expression, cells were harvested 48 hours post-infection (p.i.). Specifically, the media from the infected cells was aspirated and the cells were washed in approximately 2 ml of PBS. This wash was then aspirated and 1 ml of PBS was added to each plate. The cells were then dislodged using a cell lifter. The suspended cells were either divided into two tubes for protein and RNA analysis, respectively, or processed further for protein analysis. The cells in PBS were centrifuged for 10 seconds at 10,000 rpm. The PBS was aspirated and the cell pellet that was re-suspended in 100 µl of RIPA buffer (100 mM Tris-HCl (pH 7.4), 1% sodium deoxycholic acid, 1% Triton-X100, 0.1% SDS, 150 mM NaCl, and containing the EDTA-free Complete protease inhibitors cocktail (Thermo Fisher Scientific), or 1 ml TRIzol (Life Technologies). Protein lysates were further processed by incubation on ice for 20 minutes, and centrifugation at 14,000 rpm at 4°C for 20 minutes to pellet the cellular debris. The supernatant layer containing the solubilized protein lysate was placed in a separate tube to be used for western blot analysis.

BCA Quantification

To measure the concentration of protein in the cell lysates, a 1:5 dilution of the protein lysate in PBS. The standard BSA concentrations at 200 ng/µl, 400 ng/µl, 600 ng/µl, 800 ng/µl and 900 ng/µl were prepared. A volume of 1.13 µl of reagent A was added to 22.6 µl of reagent S to create an "A" solution. First, 25 µl of this solution was added to each well as well as 5 µl of each standard and sample. To create a color change, 200 µl of reagent B was then added. The absorbance at 562 nm from the BCA protein quantification assay (BioRad) was analyzed using a spectrophotometer (BioTek). The standard samples of BSA used to construct a standard curve which was then used to calculate the concentrations of protein in the cell lysate.

SDS-Polyacrylamide Gel Electrophoresis (PAGE) and Western Blot Analysis

Using BCA results, 20 µg cell lysate was prepared in RIPA buffer to a final volume of 20 µl, and 5 µl of 5x SDS-PAGE loading buffer. The proteins were then denatured at 95°C for 5 minutes. The samples (25 µl) were loaded into a 10% SDS-PAGE and electrophoresed at 100 V for two hours in 1x SDS-PAGE running buffer. The electrophoresed proteins were transferred onto a PVDF membrane (that was activated in methanol) at 100 V for one hour at 4°C. After transfer, the membrane was reactivated in methanol, and hydrated in dH_2O and 1x PBS. To examine consistency of protein loading, the membrane was stained in Ponceau S (Sigma). The membrane was then placed in 5% skim "milk" powder in 1x PBS-Tween 20 (PBS-T) for an hour at room temperature to block non-specific binding of antibodies to the membrane. The membrane was incubated at 4°C overnight in primary antibody in 4 ml of 5% milk in PBS-T. The following day, the blot was then washed in PBS-T 3 times for 10 minutes at room temperature before incubation in the secondary antibody. The blot was incubated at room temperature for one hour in secondary antibody before it was washed again three times with PBS-Tween for 10 minutes. The blot was then imaged using a 1:1 mixture of Clarity ECL reagent (BioRad) and H_2O_2. The following primary and secondary antibodies were used: rabbit anti-DDX6 (RCK/p54) (1:5,000; Bethyl Laboratories A300-460A), mouse anti-GAPDH (Calbiochem); and mouse anti-HCV Core C7-50 (1:1,000; Abcam ab2740), and HRP-conjugated donkey anti-mouse and donkey anti-rabbit secondary antibodies (1:10,000; Santa Cruz Biotechnologies).

Clone 3xFlag Genes into pLenti6/V5-D™-TOPO® Vector

3xFlag-BAP and RCK/p54 WT and mutant genes in the p3xFlag plasmid were PCR amplified using prCTP256 (5'-CACCATGGACTACAAAGACCATGAC-3') and prCTP169 (5'-TTATTAAGGTTTCTCATCTTCTACAGG-3'). The following PCR reaction was performed using the high-fidelity Phusion DNA polymerase (Invitrogen): 1 cycle at 98°C for 30 seconds; 35 cycles at 98°C for 10 seconds, 55°C for 30 seconds and 72°C for 1 minute 30 seconds; and 1 cycle at 72°C for 10 minutes. The PCR products were cleaned-up using the QIAquick PCR purification kit (QIAGEN) and eluted in 30 µl distilled H_2O. The PCR product was the incubated with pLenti6/V5-D™-TOPO® vector (ThermoFisher) and a salt solution. The cloning reaction was incubated at room temperature for 30 minutes. The cloned product (2 µl) was then added to 50 µl Stbl3 bacteria (ThermoFisher) and mixed gently. The vial was incubated on ice for 30 minutes before heat shocked for 30 seconds at 42°C. After 2 minutes of incubating on ice the cells were transferred to a 30 ml tube and 1 ml of SOC was added. The tube was shaken at 37°C for an hour before 0.5 ml of the transformation product was plated onto LB agar and carbenicillin and incubated at 37°C overnight. As above single colonies were picked for miniprep extraction and sequence analysis. The plasmids were re-transformed into Stbl3 bacteria. Colonies were picked and shaken with 20 ml LB broth at 37°C overnight. The lentivirus vectors were isolated using a midiprep DNA kit (OMEGA Bio-Tech). The following lentivirus plasmids were used: ATPase mutation (pLenti3xFlag-RCK-DEAD>DQAD), a Δ prion-related domain (PRD) (pLenti3xFLAG-RCKΔPRD), and the helicase mutants (pLenti3xFlag-RCK SAT>AAA and pLenti3xFlag-RCK-RGID>RAID).

Production of Lentivirus and Trnsduction of RCK KO Cells

To produce lentiviruses, 0.75 µg pLenti6/V5-BAP and -RCK plasmids were transfected into 293FT cells with 2.25 µg ViraPower Lentiviral packaging mix using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instuctions. Media from transfected cells was collected 24 hours post-transfection. This media containing lentivirus was stored at 4°C. Fresh media was added to the cells, and again collected at 48 hours post-transfection. Media containing lentivirus was filtered through a 0.45 µm filter. To create RCK stable Huh7 cells, polybrene

(Sigma) was added to the filtered lentivirus, and then added to the RCK KO cells. The RCK KO cells were seeded in 60 mm tissue culture plates 24 hours prior transduction. The media containing lentivirus and polybrene (6 μg/ml; Sigma) were incubated on the KO cells for 24 hours. Hereafter, the media was removed and replaced with fresh media. The cells were further incubated for an additional 24 hours. Forty-eight hours post-transduction, KO cells transduced with 3xFlag-BAP and -RCK were trypsinized and re-plated into 100 mm plates. Cells that were stably transduced were placed under blasticidin selection (5 μg/μl; Invitrogen). These RCK stable Huh7 cells were further maintained in blasticidin.

Northern Blot Analysis

RNA was extracted with TRIzol (Invitrogen) per the manufacturer's instruction . Total RNA (10 ug) was resuspended in loading buffer (1% MOPS-EDTA-sodium acetate (MESA), 67% formaldehyde, and 50% formamide). The RNA was denatured at 65°C for 10 minutes and then separated in 1% formaldehyde/agarose gels and ran at 100 V. The RNA was transferred onto a ZetaProbe membrane (BioRad) overnight and then crosslinked using Stratalinker. The membrane was hybridized to examine actin and HCV using ExpressHyb hybridization buffer (Clontech) and dATP-RadPrime DNA labeled (Invitrogen) probes [^{32}P].[8,9]

Luciferase Assay

The subgenomic HCV reporter plasmids sgJFH-1 RLuc replication-competent (WT) and replication-incompetent (GND) were a kind gift from Dr. Glan Randall (University of Chicago).[11] The plasmids were linearized with XbaI, and the linear DNA used as a template for T7 Megascript *in vitro* transcription (Ambion) according to the manufacturer's instructions.[9] RCK KO cells stably expressing 3xFlag-BAP, and RCK WT and mutant genes were seeded into 24 well plates at 5x10^4 cells/well and incubated overnight at 37°C. Cells were transfected with 500 ng sgJFH-1 RLuc RNA using Lipofectamine MessengerMAX transfection reagent (Invitrogen) according to the manufacturer's instructions. Cells were lysed in 1x passive lysis buffer (Promega) 6 hours and 48 hours post-transfection. Three replicates were measured in 96-well plates using the Renilla reagent (Promega) in a luminometer (BioTek). This experiment was

repeated once for replicability.

RESULTS

Knockdown and rescue with wild-type and mutant RCK/p54 protein in Huh7 cells.

The first step in my study was to created mutations in the ATPase and helicase domains of RCK cloned into a 3xFlag-plasmid (**Table 1**). To investigate the role of the enzymatic activities of RCK during HCV infection, we transfected cells with a control siRNA(siGL2) and a RCK target-specific siRNA, such that endogenous RCK expressed in the cells would be depleted. To investigate the specific effects of mutant RCK we co-transfected cells with a control plasmid (p3xFlag-BAP), and p3xFlag-RCK WT and mutants. The p3xFLag-RCK plasmid had been further mutated such that the mRNA expressed from the plasmid would be resistant to siRNA cleavage. Transfected cells were then infected with JFH-1, the HCV infectious clone, and HCV protein abundance examined 3 days p.i. by western blot analysis. Lane 1 in **Figure 2** shows uninfected Huh7 cells. As expected no HCV core protein was detected, and RCK and GAPDH are expressed. Lanes 2-7 show protein from HCV-infected cells. Co-transfection of siGL2 and p3xFlag-BAP had no effect on RCK levels, and HCV core protein is visible (lane 2). In contrast, cells transfected with siRCK and 3x Flag-BAP plasmid resulted in significantly less RCK protein and a decrease in HCV core protein (lane 3). As expected, the depletion of RCK and rescue with

Enzymatic Activity	Domain	aa position	WT Motif	Mutant Motif
ATPase	II	E247Q	DEAD	DQAD
Helicase	III	S277A/T279A	SAT	AAA
Helicase	Va	G397A	RGID	RAID
RNA Binding	Ia-Ic	R174A/R225A	TRE/GRI	TAE/GAI

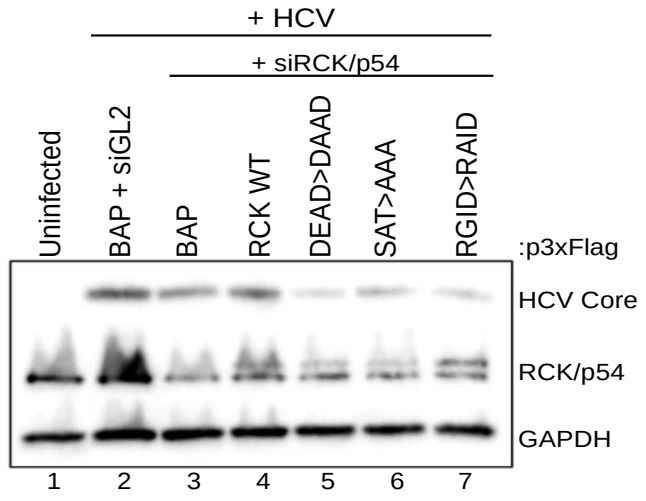

Figure 2. Expression of RCK wild type and mutated RCK/p54 proteins during HCV infection. Huh7 cells were co-transfected with siRNAs and p3xFlag plasmids, and infected with HCV. Protein abundance was analyzed by western blot analysis 3 days p.i.

RCKΔsi WT (lane 4) shows a return to similar viral core level as the siGL2 and BAP plasmid transfection in lane 2. Of note, the overexpressed 3xFlag- RCKΔsi WT and mutant proteins are visible using the rabbit anti-RCK antibody. Because of the additional three Flag epitopes at the N-terminus of RCK, these proteins migrate slower in the gel and are visible as the band above the endogenous RCK band. The rescue with wild type RCK resulted in a double band of RCK. Interestingly expression of the mutated RCK proteins deficient in ATPase and helicase activities resulted in significantly less HCV core expression (lanes 5-7). The GAPDH levels, used as a loading control, were even across all lanes. These data suggest that HCV gene expression requires RCK ATPase and helicase activities.

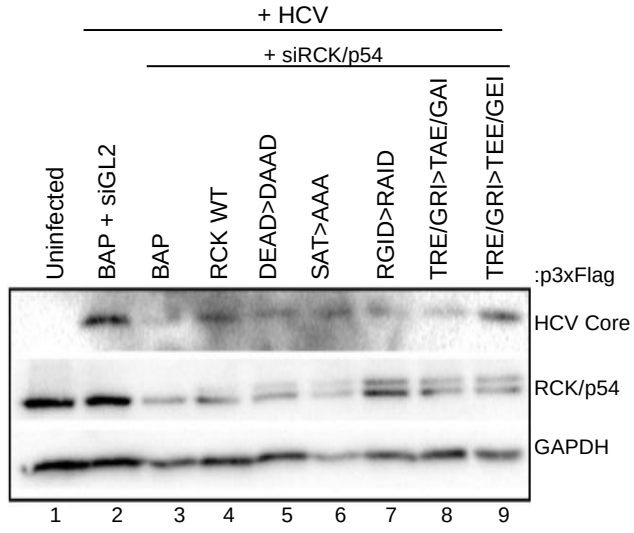

Figure 3. Expression of RCK wild type and RCK/p54 mutant proteins deficient in ATPase, helicase and RNA binding activities during HCV infection. Huh7 cells were co-transfected with siRNAs and p3xFlag plasmids, and infected with HCV. Protein abundance was analyzed by western

The initial analysis of RCK enzymatic activities included the ATPase and helicase motifs, but not the RNA binding motif. We therefore created two additional mutants in motif Ia and Ic. These motifs are predicted to bind RNA and in the RNA structure are opporiste from each other. In RNA binding motifs, arginine (R) is a common amino acids, as the positive charge from the amino acid facilitates the binding to the negatively-charged phosphate backbone of RNA. We therefore created two mutations, the first mutating the arginines to the neutral amino acid alanine (R174A/R225A) and also to the negatively-charged glutamic acid (R174E/R225E). We predict both mutations would disrupt RNA binding. **Figure 3** shows similar results as **Figure 2** on HCV core expression for the control and RCK mutant proteins. The effect of the two mutant RNA-binding RCK proteins on HCV core levels are shown in lanes 8 and 9. Changing the arginine in motifs Ia and Ic to an alanine, decreased levels of HCV core protein. Unexpectedly, the point mutation from arginine to glutamic acid in lane 9, showed similar HCV core expression as the WT rescue. While motifs Ia and Ic are predicted to bind RNA, to our knowledge no biochemical assays have been

undertaken to demonstrate this activity. Therefore, the RCK R174A/R225A mutant shows an affect on HCV, however additional experiments should be performed before concluding that this effect is due to disruption of RNA binding. Performing an immunoprecipitation of RCK WT and R174A/R225A and examining RNA binding would be a first step to investigating this. This assay would also provide insight into why R174E/R225E had no effect on HCV core expression.

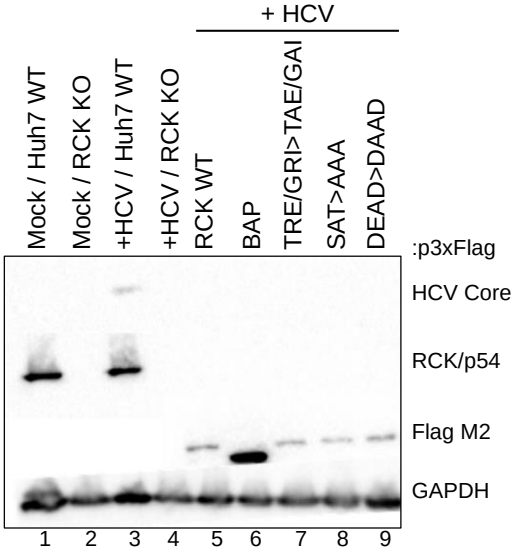

Figure 4. Transient expression of 3xFlag WT and mutant proteins does not rescue HCV gene expression. Huh7 cells and Huh7 RCK KO were transfected with p3xFlag plasmids, and then mock-infected or infected with HCV. Protein abundance was analyzed by western blot analysis 3

Knockdown and rescue with wild-type and mutant RCK/p54 protein in RCK knockout (KO) cells.

Because of variable knockdown efficiencies using siRNAs, these experiments were repeated in Huh7 cells in which CRISPR-Cas9 gene editing technology had been used to knockout RCK.[10] In **Figure 4**, lanes 1 and 3 protein lysates from Huh7 WT cells. In these lanes endogenous RCK is detected by western blot analysis. In contrast, but as expected, no endogenous RCK is visible in the RCK KO cells (Lanes 2 and 4). When HCV infection was examined in Huh7 WT and RCK KO cells, core protein was only detected in Huh7 WT cells (lane 3). These data show that RCK is indeed required for HCV gene expression.

We next examined HCV gene expression in RCK KO cells transiently expressing 3xFlag-BAP, RCK WT and three enzymatic mutants of RCK (lanes 5-9). While immunoblotting for Flag revealed transient expression of the 3xFlag proteins, these proteins were not detected using the anti-RCK antibody (**Figure 4**). Moreover, HCV core protein was not detected (lanes 5-9), not even when RCK WT expression was rescued in the RCK KO cells (lane 5). These data indicate that transient expression of 3xFlag proteins is unable to rescue HCV gene expression.

HCV gene expression in RCK KO stably expression 3xFlag BAP and RCK proteins.

Because transient expression of RCK WT and mutant proteins did not rescue HCV gene expression we next sought to stably express these proteins in the RCK KO cells. To this end we sub-cloned the 3xFlag-BAP, RCK WT and RCK mutant genes into the pLenti vector. Lentiviruses expressing the genes were produced in 293FT cells, and subsequently transduced into the RCK KO. Stable gene expression was achieved by blasticidin selection.

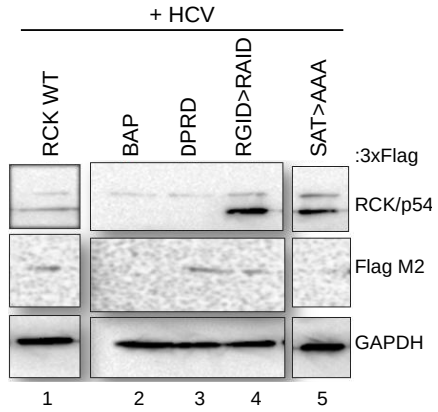

Figure 5. HCV gene expression in RCK KO cells stably expressing 3xFlag proteins. Huh7 RCK KO stably expressing 3xFlag proteins were infected with HCV , and protein abundance was analyzed by western blot analysis 3 days p.i.

Note: The data presented in this figure are from the same blot. The image however was rearranged for

Using these stable cells expressing RCK WT and mutant proteins we next invested the effect on HCV gene expression. Specifically, the cells were infected with HCV and cell lysates harvested 3 days p.i. We first investigated protein expression by western blot analysis. We detected RCK expression in cells stably expressing RCK WT, RGID>RAID and SAT>AAA proteins (**Figure 5**, lanes 1, and 4-5). We did not detect RCK expression in cells stably expressing a mutant RCK protein in which the prion-related domain (PRD) had been deleted (lane 3). The anti-RCK antibody used in our studies targets the N-terminus and with the deletion of the PRD domain, the antibody recognition site was also deleted. However, the ΔPRD RCK protein was detected using the anti-Flag antibody (lane 3). Disappointingly, we did not detect HCV protein.

Because we were unable to detect HCV core protein in **Figure 5**, we next investigated HCV RNA abundance. RNA from the same experiment was extracted and analyzed by northern blot (**Figure 6**). Northern blot analysis confirmed low levels of HCV RNA expression in BAP, DDX6-E247Q and DDX6-SAT-AAA stable cell lines. HCV RNA was observed in KO cells expressing RCK WT and RCKΔPRD (lanes 1 and 5). Consistent with our earlier data we did not detect HCV RNA in cells stably expressing 3xFlag-BAP, or RCK DEAD>DAAD and SAT>AAA (lanes 2-4). **Figure 5** and **Figure 6** together show that HCV can infect RCK KO cells stably expressing RCK WT and RCKΔPRD, but not the mutant RCK proteins. Moreover,

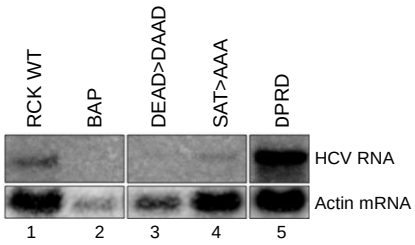

Figure 5. HCV RNA abundance in RCK KO cells stably expressing 3xFlag proteins. Huh7 RCK KO stably expressing 3xFlag proteins were infected with HCV, and RNA abundance was analyzed by norther blot analysis 3 days p.i.

Note: The data presented in this figure are from the same blot. The image however was rearranged for this figure presentation.

Note: These data are from Gaston Bonenfant, and used in this Thesis

the HCV infection in these cells is not as robust as in Huh7 cells.

HCV translation and replication in RCK KO stably expression 3xFlag BAP and RCK proteins.

To determine if RCK affects HCV translation or replication we used a subgenomic JFH-1 genome assay containing the *Renilla* luciferase.[11] This reporter genome contains the HCV 5' UTR followed by the Renilla luciferase gene (RLuc). The RLuc is separated from the HCV nonstructural proteins by an EMCV IRES. The 3' UTR flanks the ORF containing the HCV NS2-NS5B proteins. The sgJFH-1 genome was in vitro transcribed and transfected into the RCK KO cells stably expressing 3xFlag proteins. Because the HCV genome is singl-stranded positive-sense, the RNA is translated at early time points post-transfection (e.g. 6 hours). However over time and the accumulation of the HCV nonstructural proteins necessary for replication, replication the sgJFH-1 genome, and with the increase in HCV genomes luciferase activity at 48 hours post-transfection may be used as a proxy for replication. We therefore measured luciferase expression 6 and 48 hours post-transfection (Figure 6), while the luciferase measurement 48h post transfection served as a proxy for replication. For the RCK WT we observed modest luciferase activity at 6 hours, which increased at 48 hours. In contrast we observed low luciferase activity in cells stably expressing the RCK the helicase mutant (SAT>AAA).While these data are consistent with earlier data we did observed modest luciferase activity at 48 hours post-infection, suggesting that the helicase activity of RCK may be important for HCV translation. For the RCK ΔPRD mutant we observed luciferase activity similar to RCK WT at 6 hours post-

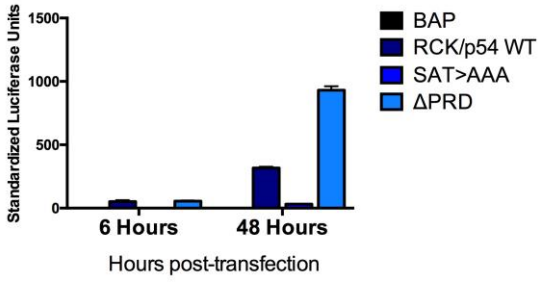

Figure 6. HCV translation and replication in RCK KO cells stably expressing 3xFlag proteins. Huh7 RCK KO stably expressing 3xFlag proteins were transfected with in vitro transcribed sgJFH-1 RLuc RNA, and harvested and Renilla luminescence measured

transfection. However, at 48 hours RCK ΔPRD dramatically increased luciferase data, suggesting that PRD may limit HCV.

DISCUSSION

The goal of this project was to investigate the role of RCK/p54 enzymatic activities on HCV infection. To this end, 3xFlag-tagged RCK/p54 plasmids containing mutations in the enzymatic domains (e.g. sites involved in ATPase, helicase, and RNA binding) were expressed in Huh7 cells depleted of endogenous RCK/p54 and infected with HCV. The results shown in **Figure 2** and **Figure 3** demonstrate successful knockdown of endogenous RCK and a corresponding decrease in expression of HCV core following expression of the mutant RCK proteins. The reduction of HCV core protein following expression of RCK/p54 containing mutations inhibiting helicase (SAT>AAA; RGID>RAID), and ATPase (DEAD>DQAD) activities suggest these enzymatic activities are critical for the function of RCK/p54 in promoting HCV gene expression.

RCK is a DEAD-box RNA helicases. Other proteins in this family have been shown to unwind RNA, or RNA-protein interactions. Because ablation of the ATPase and helicase activities affected HCV gene expression it is possible that these activities of RCK/p54 are necessary to remodel the complex secondary structures found on the 5' and 3' untranslated regions of the HCV genome, and/or remodel RNA-proteins to promote the transition between translation and replication.

Mutating the putative RNA binding domain (R174/225A) also decreases of HCV core levels. Unexpectedly, the R174/225E mutant did not have the same effect on HCV core protein. This difference in RNA binding phenotypes may be a consequence of a possible conformation change in the RCK protein or alternate mechanism to bind HCV RNA. While motifs Ia and Ic are putative RNA binding domains, it is possible that these regions do not bind RNA, and rather have a completely different function.

The observed decrease in HCV expression suggests that each of these mutations interfere with the HCV infection. We verified these results in Huh7 cells lacking RCK. However, we did not observe rescue of HCV gene expression when RCK was transiently expression, likely due to inefficient transfection. To address this, we created RCK KO cells that stably express the 3xFlag-

tagged proteins. In cells stably expressing RCK WT and ΔPRD we were able to detect HCV RNA but not HCV core protein. However, using a sgJFH-1 RLuc reporter genome we determined that the helicase mutant SAT>AAA likely affects translation, while the ΔPRD promotes replication. Together this data indicates that HCV might subvert RCK to facilitate viral gene expression at different steps in the infectious cycle.

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