GENOMIC SCREENING IDENTIFIES NOVEL HUMAN KINASES AS SUPPRESSORS OF HCV INFECTION

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GENOMIC SCREENING IDENTIFIES NOVEL HUMAN KINASES AS SUPPRESSORS OF HCV INFECTION

Aram Lee, M.S.

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The Human kinome includes between 500-600 known kinases and open reading frames (ORFs) that play key roles in regulating many cellular processes. Some of these cellular processes include, signal transduction, metabolism, transcription, cell cycle progression, cell movement, apoptosis, differentiation and protein phosphorylation. Various protein and lipid kinases have previously been shown to control viral life cycles. The lipid kinase PI4KIIIA was found to be required for hepatitis C virus (HCV) replication in several genomic screens using small interference RNAs that target human kinases. Although such a loss-of-function approach has led to the identification of host cell factors that are required for HCV replication, no kinases have been found with respect to antiviral activity. Here we adopt a genomic approach to identify human kinases that negatively regulate HCV infection. To this end, a library of activated kinases, which consists of 192 human kinases and kinase-related ORFs, was cloned into a retroviral vector, which adds a myristoylation sequence and flag-epitope tag to each ORF. Using an overexpression screening approach, we identified that several kinases, including Cyclin-dependent kinases regulatory subunit 1 (CKS1B), Mitogen-activated protein kinase kinase 5 (MAP2K5) and Protein kinase C and casein kinase substrate in neurons 1 (PACSIN1), potently suppressed HCV infection. The expression of these active kinases did not activate the nuclear factor-kappaB (NF-κB) pathway or the production of type I interferon and interferon-stimulated

genes (ISGs), instead they acted at different post-entry stages. Specifically, CKS1B significantly inhibited viral RNA replication; MAP2K5 also suppressed virus replication and appeared to exert additional inhibition after replication. PACSIN1, by contrast, inhibited HCV infection via a yet-to-be-defined mechanism.

Public Health significance: Hepatitis C occurs worldwide and is the major cause of liver diseases. Approximately three quarters of people infected with HCV become chronic carriers of the virus and of these chronically infected, majority of the patients develop liver cirrhosis and hepatocellular carcinoma. Current treatments available to patients are costly and cause severe side-effects that result in poor patient compliance. Thus, the inhibitory effects as shown here by targeting kinases, shows the potential of combating HCV infection by activating specific kinase-mediated pathways, which would be an alternative method of treatment of these patients.

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"If I have seen a little further than others,

it is by standing upon the shoulders of Giants"

- Isaac Newton

1.0 INTRODUCTION

1.1 HEPATITIS C VIRUS

1.1.1 HEPATITIS C VIRUS BURDEN AND DISEASE

Hepatitis C virus (HCV) affects approximately 170 million people worldwide and is a major cause of liver diseases. The World Health Organization estimates that the virus infects 3% of the global population and that 3-4 million new infections occur annually [1, 2]. HCV is remarkable in that it has the ability to establish persistent infections and has poor treatment response to prevalent HCV genotypes [3-5]. The majority of HCV infections are chronic with at least 70% of acute infections progressing to persistence, associated with chronic acute hepatitis, cirrhosis, and hepatocellular carcinoma [6, 7]. HCV is primarily transmitted through direct blood contact, although most infections go unrecognized due to mild disease symptoms [3-5].

HCV replicates rapidly in untreated patients, producing about one trillion virus particles per day [8]. HCV is diverse genetically with six major genotypes and each is subcategorized into several subtypes and many strains. Genotype 1 viruses cause the majority of all infections, predominating in North America, South America and Europe, and genotype 2 viruses are less common throughout the world [9]. Genotype 3 viruses predominate in Southeast Asia and Russia, while genotype 4 viruses predominate in the Middle East, Egypt and central Africa. Genotype 5 viruses are responsible for most infections occurring in South Africa and genotype 6 viruses are endemic to Asia [9].

Standard treatment is a combination therapy of polyethylene glycol-conjugated interferon-alpha and ribavirin [10]. Although this combination therapy elicits a strong synergistic inhibitory effect on subgenomic HCV replication, patients are expected to endure severe side effects such as flu-like symptoms, depression and anemia, that often result in poor patient compliance [11, 12]. Also, the high cost of these antiviral drugs prohibit many clinicians from offering treatment to patients who lack the social and financial support necessary for such therapy with strict adherence [11, 12]. The recent approval of two HCV protease inhibitors, Merck's boceprevir and Vertex's teleprevir, in May 2011 by the Food and Drug Administration (FDA) highlighted the success of developing small molecule inhibitors to treat chronic HCV infection [13]. Resistance to these inhibitors, however, is expected to emerge rapidly in clinics. As with HIV treatment, a successful treatment of HCV is expected to involve combination therapy with multiple inhibitors of different target. Currently, neither a prophylactic nor a therapeutic vaccine is available and their development has been hampered by the high variability of the virus.

1.1.2 HCV GENOME

HCV is an enveloped, positive-stranded RNA virus classified in the *hepacivirus* genus within the *Flaviviridae* family [14]. The size of its genome is approximately 9.6 kb and consists of a 5' non-translated region (NTR) that functions as an internal ribosome entry site, a long open reading frame (ORF) encoding a precursor polyprotein of approximately 3000 amino acids and a 3' NTR. The translation of HCV polyprotein is mediated by the internal ribosomal entry site within the 5' NTR. As shown in figure 1, the HCV polyprotein is cleaved by host peptidases and viral proteases, both co- and posttranslationally to yield a total of ten viral proteins, that includes a core protein, E1 and E2 (envelope glycoproteins), p7 (short hydrophobic peptide), which are structural proteins, and a number of nonstructural proteins such as NS2, NS3, NS4A, NS4B, NS5A and NS5B. Studies have shown that NS3 to NS5B proteins are sufficient for HCV RNA replication, which occurs in the membrane-bound replication complex consisting of HCV RNA and proteins [14]. The core and NS5B coding regions also contain *cis*-acting RNA elements important for HCV RNA replication and regulation. Lastly, the newly synthesized HCV proteins and genomic RNA are packaged into progeny virus particles, although the molecular aspects of underlying HCV virion assembly, maturation and egression are not well understood [15].

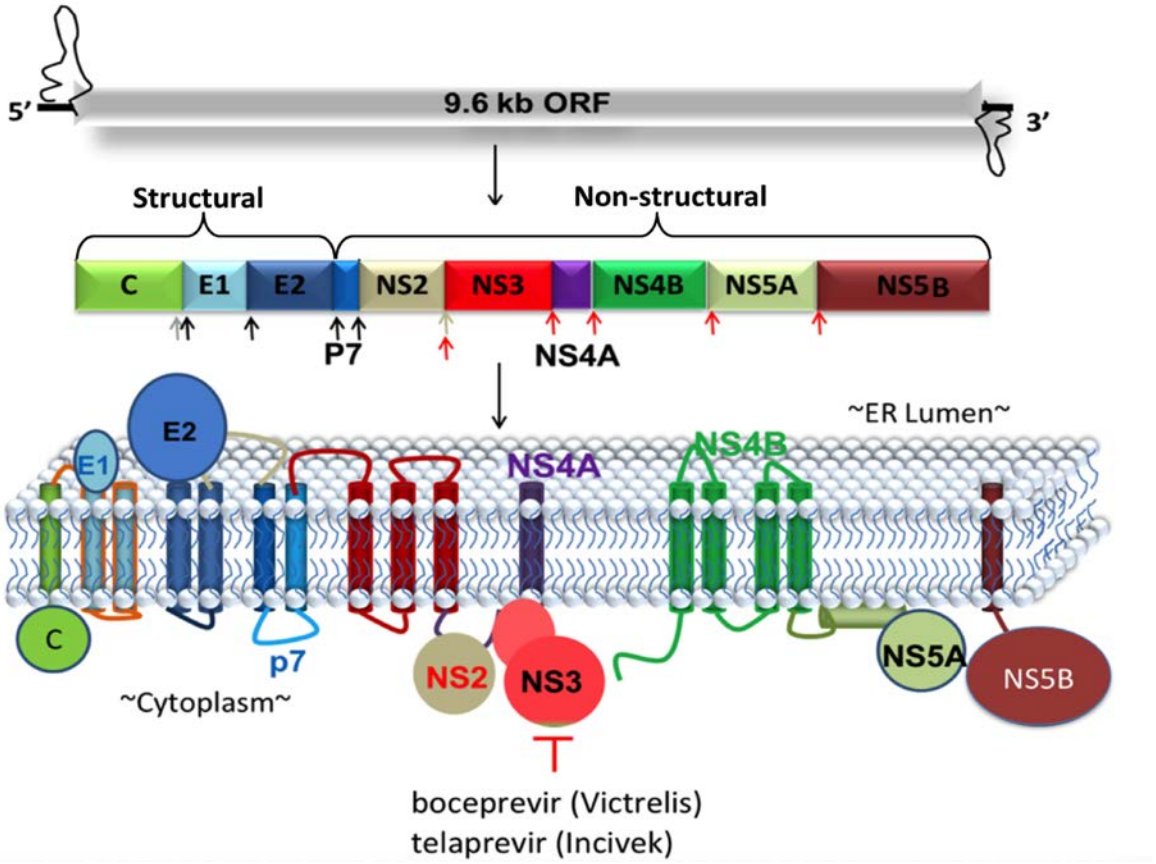


Figure 1. The HCV genome. The HCV genome is approximately 9400bp, which is translated as a single polypeptide that is cleaved to produce 10 mature proteins. Core-E1, E1-E2, E2-p7 and p7-non-structural protein 2 (NS2) junctions are cleaved by cellular peptidases to yield structural proteins. NS3 serine protease cleaves the remainder of the NS polypeptide.

1.1.3 HCV LIFE CYCLE

The positive-sense HCV genome is packaged in an icosahedral scaffold of Core proteins, which are enveloped by phospholipid membranes derived from the host cell endoplasmic reticulum [16, 17]. The viral envelope glycoproteins E1 and E2 mediate fusion to the host cell membrane receptors Tetraspanin (CD81) and Scavenger receptor class B type I (SR-B1) [16, 17]. Additional factors necessary for efficient HCV entry are Claudin 1, Occludin, Low-density lipoprotein receptors and glycosaminoglycans [16-21]. After receptor-mediated entry, the virus

enters cytosol of the host cell by clathrin-mediated endocytosis, and next the fusion to the endosomal membrane is mediated by the acidic endoplasmic environment that lead to the release of the RNA genome into the cytoplasm [17, 22]. HCV genome is then translated by host cell machinery and the structural proteins are inserted into the endoplasmic reticulum lumen and cleaved into functional units by host peptidases [2, 23]. The non-structural proteins are translated on the cytoplasmic side of the endoplasmic reticulum and processed into functional units by viral proteases NS2/3 and NS3/4A. The viral RNA-dependent RNA polymerase, NS5B, initiates HCV replication by synthesizing a single, negative-sense strand from the positive-sense RNA genome [23-25]. Finally, the HCV genomes will enter the endoplasmic reticulum and packaged into virus particles, that are trafficked through the Golgi secretory pathway for extracellular release [23-26].

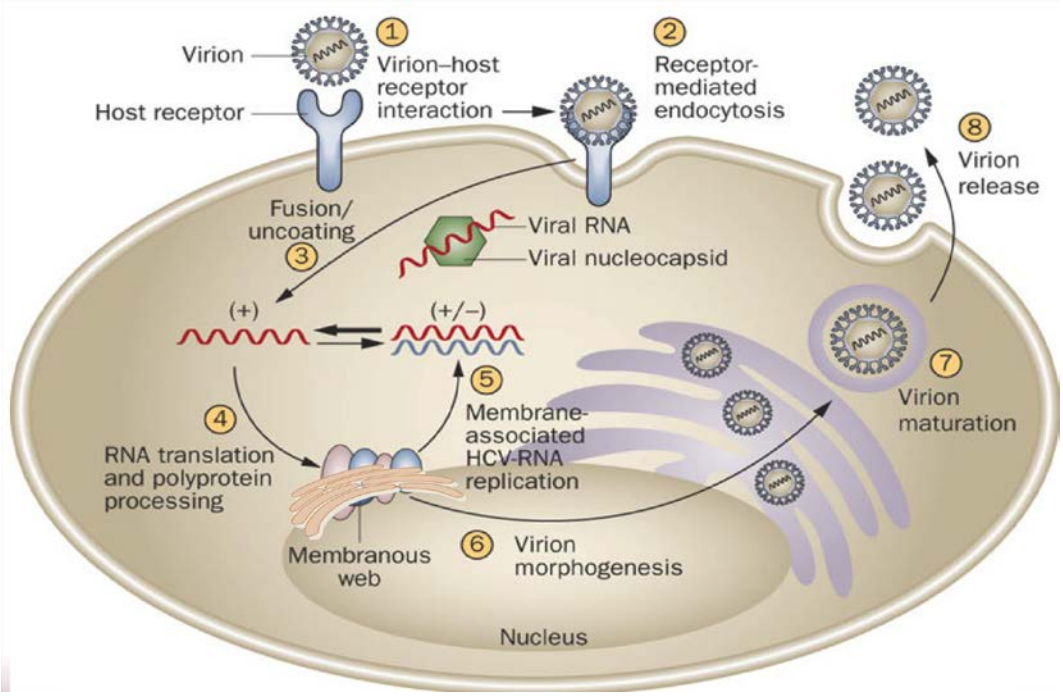


Figure 2. The HCV lifecycle. HCV virions attach to human hepatocytes by binding to various surface molecules including HSPG (heparin sulfate proteoglycans), CD81 and SR-B1 (scavenger receptor class B type 1), and enter by clathrin-mediated endocytosis; virus particles uncoat in the cytosol and host cell machinery translates the genomic RNA along the endoplasmic reticulum membrane; virus replicates and new virions assemble in the endoplasmic reticulum and trafficked through the Golgi secretory pathway for release. Reprinted from Nature Reviews Gastroenterology & Hepatology 6, 403-411 (July 2009), with permission from Nature Publishing Group**.**

1.1.4 HCV ASSEMBLY AND LIPID DROPLETS

HCV particles can be found in low-density fractions of plasma from infected patients. Thomssen and colleagues first demonstrated an association of HCV particles β-lipoproteins [27]. Accumulated evidence now indicate HCV exist as so-called "lipoviroparticles' (LVPs) in vivo, which are rich triglycerides and contain at least apolipoprotein B (ApoB), HCV RNA and the viral core protein at densities of <1.08 g/ml [28, 29]. By contrast, laboratory-grown HCVcc (HCV propagated in cell culture) particles display a slightly higher density (~1.15 g/ml) [30, 31] due to the association with ApoE and ApoCI, and are mostly devoid of ApoB [32-34]. The presence of apolipoproteins significantly influences the viral infectivity as antibodies against ApoE potently neutralized the virus [35]. Moreover, the lipoprotein secretion pathway plays a critical role in the assembly and release of infectious HCV lipoviroparticles [36].

Relevant to my thesis, chronic HCV infection is previously known to induce accumulation of lipids in the liver (steatosis). Recent studies have suggested that cellular lipid droplets (LDs) also play a crucial role in the HCV life cycle (reviewed in [29])[37]. Originated from the ER, LDs are cellular organelles storing lipids. The core of LDs is formed by triglycerides and cholesterol esters, which is then surrounded by a phospholipid monolayer and a host of proteins at their surface. Interactions between HCV core protein and LDs are required for the morphogenesis of infectious HCV. Miyanari et al. elegantly demonstrated that HCV Core recruits nonstructural (NS) proteins and replication complexes to LD-associated membranes, and that this recruitment is critical for producing infectious viruses. In that study, virus particles were observed in close proximity to LDs, indicating that some steps of virus assembly take place

around LDs [38]. It is now postulated that cytosolic LDs serve as the platforms for virus assembly by first recruiting viral Core protein to its surface. The viral NS5A also localizes to LDs, and the colocalization of Core and NS5A is crucial to the assembly of infectious virus via a yet-to-be-defined mechanism.

1.1.5 CELL LINES TO STUDY HCV

The hepatocyte is the primary target cell in HCV infection, although various lymphoid populations such as B cells and dendritic cells have been reported to be capable of being infected at very low levels [39, 40]. In the past, the HCV life cycle and host-virus interactions have been difficult to study due to lack of cell culture and small animal model availability. Thus, the majority of the HCV infection studies in the beginning revolved around infected patients and chimpanzees [41]. This difficulty was overcome by various developments of cell lines and other methods to study HCV life cycle. The development of HCV replicon systems, for example, have helped with the study of HCV translation and RNA replication in human hepatoma-derived Huh-7 cells *in vitro* [42]. By definition, a replicon is a nucleic acid (can be either DNA or RNA) that is capable of autonomous replication. The advantage of using this system is that it allows assessment of HCV replication. The study of HCV entry, on the other hand, became possible by the use of pseudoviruses that mimic HCV entry [43, 44]. It was reported that retroviral construct carrying reporter genes can be pseudotyped with HCV E1 and E2 and produce infectious viral particles [43]. These pseudoviral particles strictly infect primary human hepatocytes or a human liver cell line Huh7 in E1E2-dependent manner. Another major breakthrough in the field came in 2005 by Wakita and colleagues who demonstrated that a HCV isolate named Japanese fulminant hepatitis 1 (JFH1) propagated efficiently in cell culture and produced infectious particles [45].

To date, intragenotypic and intergenotypic constructs carrying reporter genes were developed to allow study of entire HCV life cycle in cell culture system (HCVcc) [46, 47].

1.1.5.1 CELL LINES PERMISSIVE FOR HCV REPLICATION

Productive replication of HCV replicons *in vitro* is extremely cell-type specific, with human hepatoma Huh-7 cells being the most permissive cell line identified so far [46]. The development of these cell lines was made possible after observing that structural proteins are not required for the replication of many positive-sense RNA viruses. The first generation functional HCV replicons were derived from the Con 1 cDNA that was isolated from the liver of a chronically infected patient with genotype 1b strain [48-50]. Efforts to find other permissive cell lines have been devoted in the past and still continue today. The non-hepatic cell lines identified in the past include human cervical cancer-derived HeLa cell line and 293 cells established from human embryonic kidney.

A subclone derived from Huh 7 is the Huh 7.5, with the capacity to support high levels of subgenomic HCV replication in approximately 75% or higher transfected cells [51]. Huh 7.5 cells more readily support RNAs with lower replicative abilities, such as full-length Con1 replicons [51]. This increased permissiveness of Huh 7.5 cells is due to mutational inactivation of the retinoic acid inducible gene-I (RIG-I), a cytoplasmic protein that recognizes structured RNA to induce type I IFN production via activation of transcription factors interferon regulatory factor (IRF)-3 and NF-κB [52].

1.1.5.2 DEVELOPMENT OF HCV REPLICONS

In the past, numerous attempts have been made to propagate HCV in cell culture by infection with virus containing inoculum, but the replication levels were often transient and always so low that HCV RNA synthesis could not be monitored by reverse transcription (RT)-PCR assays, complicating detailed studies of HCV replication [48-50, 53]. For most positive-sense RNA viruses, productive replication can be efficiently made by transfecting permissive cells with the genomic RNAs, allowing to bypass viral entry and uncoating steps. However, transfection of HCV RNAs transcribed from cDNA clones with known infectivity still never effectively established HCV replication [48-50, 53-55]. Thus great efforts were made to generate replicons and it was noted that even the slightest variation in HCV sequence could drastically change the ability of engineered replicons.

For many years, the only HCV replicons able to replicate in cultured Huh-7 cells were derived from Con1 strain. However, this restriction has overcome by the development of replication-competent subgenomic RNAs derived from independent genotype 1b isolates. Unlike the Con1 replicons, HCV genotype 1b does not require cell culture adaptation for efficient replication of subgenomes derived from HCV-N isolate, or for the G418 selection of Huh-7 clones [56, 57].

Another genotype was derived from clone H77, known as HCV genotype 1a, in which intrahepatic inoculation of H77 RNA results in high viremia in the chimpanzee [58]. However, replicons derived from this infectious H77 clone require at least two adaptive mutations to

productively replicate in cell culture [41, 59]. Transfection of H77 clones with single adaptive mutation S2204I in NS5A into permissive Huh-7 or Huh-7.5 subline allowed for the establishment of the first G418-resistant colonies supporting H77 replication [41, 59, 60].

Finally, genotype 2a is a subgenomic replicon derived from the genotype 2a JFH-1 clone that was isolated from a patient with fulminant hepatitis, and represents the only non-genotype 1 sequence capable of replication in cell culture. The JFH-1 subgenomes replicate with high efficiency in permissive cell lines without any adaptive mutations. So far, the JFH-1 subgenomic RNA is the most efficient replicon tested [61, 62].

1.2 KINOME

Kinome is defined as the protein kinase complement of the human genome [7]. This term was coined in 2002 by Gerard Manning and colleagues when they identified 518 putative human protein kinase genes and the evolution of protein kinases were analyzed [63]. Ever since the discovery that reversible phosphorylation regulates the activity of glycogen phosphorylase, there has been a great interest in the role of protein phosphorylation in regulating protein function [63]. The advancement of DNA cloning and sequencing in the mid-1970s made it possible to confirm the existence of protein kinases. Such near-completion of the human genome sequence now allows the identification of almost all human protein kinases [63]. To date there are approximately 500-600 known kinases and open reading frames (ORFs) that are important in regulating cellular processes. Many studies involving proteomics require cloning and manipulation of these large numbers of protein-encoding sequences, or open reading frames [7].

The ORFeome of an organism corresponds to its complete set of protein-encoding genes, cloned as full-length open reading frames (ORFs) [7]. An open reading frame consists of the entire coding sequence between the initiation and termination codons, excluding the 5' and 3' mRNA untranslated regions [7].

1.2.1 CLASSIFICATION OF THE HUMAN KINOME

The protein kinases are related by their homologous kinase domains, also known as catalytic domains which consists of approximately 200-300 amino acid residues [63, 64]. These kinase domains are what impact their specific kinase activity. There are three key roles that these kinase domains play: 1) binding and orientation of the ATP phosphate donor as a complex with divalent cation (usually Mg^{2+} or Mn^{2+}); 2) binding and orientation of the protein substrate; and 3) transfer of the γ-phosphate from ATP to the acceptor hydroxyl residue (serine, threonine or tyrosine) of the protein substrate [64]. The kinase domains can be further divided into 12 smaller subdomains, defined as regions never interrupted by large amino acid insertions and containing characteristic patterns of conserved residues [64].

Kinases are primarily classified by sequence comparison of their catalytic domains, aided by the knowledge of sequence similarity and domain structure outside of the catalytic domains, and known biological functions [64]. The original classification of kinases is the Hanks and Hunter human kinase classification system that divides the kinases into four broad groups, 44 families and 51 subfamilies [64]. The Hanks and Hunter human kinase classification system basically subdivided the known members of the eukaryotic protein kinase superfamily into distinct families that share basic structural and functional properties, where phylogenetic trees

derived from an alignment of kinase domain amino acid sequences served as the basis for this classification system [64]. The major four groups include: 1) the AGC group, which includes the cyclic-nucleotide-dependent family (PKA and PKG), the protein kinase C (PKC) family, the beta-adrenergic receptor kinase (βARK) family, the ribosomal S6 kinase family, and other close relatives; 2) the CaMK group, which includes the family of protein kinases regulated by calcium/calmodulin, the Snf1/AMPK family, and other close relatives; 3) the CMGC group, which includes the family of cyclin-dependent kinases, the Erk (MAP) kinase family, the glycogen synthase 3 (GSK3) family, the casein kinase II family, the Clk (Cdk-like kinase) family, and other close relatives; and 4) the protein-tyrosine kinase (PTK) group [64].

The AGC group of protein kinases is comprised of mostly basic amino acid-directed enzymes, phosphorylating substrates at Ser/Thr residues. The CaMK group of protein kinases also tends to be basic amino acid-directed and as one can predict the AGC and CaMK groups fall near one another in the phylogenetic tree [64]. Within this group is the Erk (MAP) kinase family, in which for the most part, they are proline-directed enzymes, phosphorylating substrates at sites lying in Pro-rich environments [64]. The protein-tyrosine kinase group consists of a quite large number of enzymes, whose kinase domains specifically phosphorylate on Tyr residues. These major groups are distinct from those kinases that are able to phosphorylate with dual-specificity such as those able to phosphorylate a substrate on Ser/Thr and Tyr, which are members of the MEK family [64].

Since then, Gerard Manning and colleagues have added four new groups, STE group that consists of MAPK cascade families, the CK1 group that consists of CK1, TTBK (tau tubulin

kinase) and VRK (vaccinia-related kinase) families, TKL (tyrosine kinase-like) group that consists of families that resemble both tyrosine and serine-threonine kinases, and finally RGC (receptor guanylate cyclase) group that are similar in domain sequence to tyrosine kinases [63].

1.2.2 KINASES

Protein kinases are one of the largest protein superfamilies identified largely from eukaryotic sources [63, 64]. These enzymes use the γ-phosphate of adenosine triphosphate (ATP) to generate phosphate monoesters using protein alcohol groups on Serine (Ser) and Threonine (Thr) residues, and protein phenolic groups on Tyrosine (Tyr) as phosphate acceptors [64]. The protein kinases comprise a large group of enzymes that mostly catalyze the covalent attachment of a phosphate group to a protein, and in eukaryotic cells, protein kinases mediate most of the signal transduction by modification of substrate activity and control cellular processes including but not limited to metabolism, transcription, cell cycle progression, cell movement, apoptosis, differentiation, and protein phosphorylation [63]. However, most protein kinases act in a network of kinases and other signaling effectors, not just working independently by themselves.

1.2.2.1 MYRISTOYLATION

Myristoylation refers to the irreversible covalent linkage of the 14-carbon saturated fatty acid, myristic acid, to the N-terminalglycine of many eukaryotic and viral proteins through a stable amide bond [65]. This reaction is catalyzed by myristoyl-CoA:protein N-myristoyltransferase (NMT) [65-67]. Historically, N-myristoylation was first described as an unusual blocking group, which prevented the direct use of Edman degradation at the N-terminus of the catalytic subunit

of cyclic AMP-dependent protein kinase and calcineurin B [68, 69]. It was later identified by mass spectrometry that this blocking group is myristic acid. N-myristoylation was first described as a co-translational modification that occurs on a nascent polypeptide following the removal of the initiator methionine residue [70, 71]. Now it is accepted that myristoylation can also occur post-translationally on an internal glycine within a cryptic myristoylation consensus sequence exposed by the action of caspases in apoptotic cells [65, 72-75]. The removal of the initiator methionine is a common modification of many protein, occurring in approximately 80% of total proteins, but only selected proteins are specifically myristoylated due to the selectivity of NMTs [65]. This selectivity is partly explained by the 3D structure of NMT, in which the bent active site cavity is only large enough to selectively accommodate a myristoyl functional group [65, 76]. The general consensus peptide sequence recognized by NMTs is Gly-X_3-X_4-X_5-$(Ser/Thr/Cys)_6$ where X represents most amino acids, except for proline, aromatic or charged residues in position X_3 [65]. It is also noted that Ser, Thr, Cys are preferred at position X_6 although other amino acids can be tolerated occasionally [65].

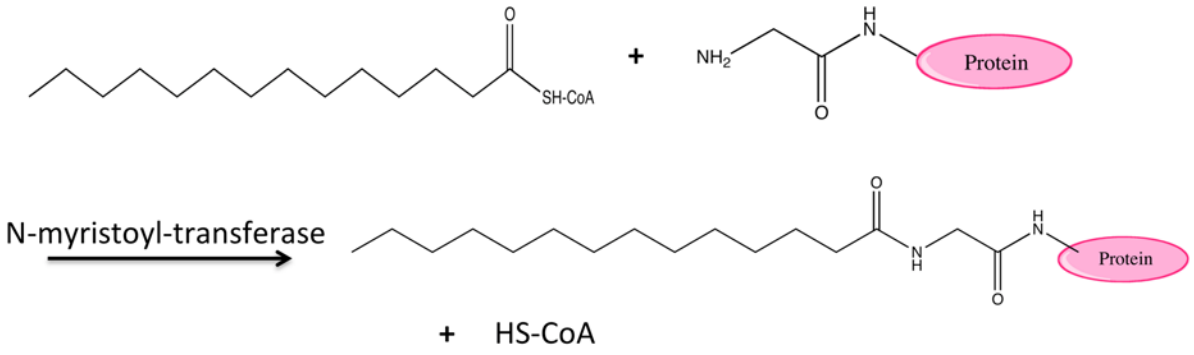


Figure 3. Myristoylation overview. Myristoylation consists of the covalent addition of 14-carbon saturated fatty acid myristate to the N-terminal glycine residue through a stable amide bond. The reaction is catalyzed by the enzyme myristoyl-CoA:protein N-myristoyl-transferase.

Myristoylation has been shown to be important in mediating subcellular targeting, protein-protein and protein-membrane interactions required for the activities of many proteins and is often required for membrane binding [77]. Myristoylation is also involved in differential targeting to membranes and sub-membrane domains known as lipid rafts [65, 78]. Myristoylation is thus important in a variety of cellular functions such as many signaling pathways, oncogenesis and viral replication [65]. The importance of myristoylation in viruses has been studied in the field of HIV-1. Nearly all mammalian retroviral Gag proteins are myristoylated and the Gag protein has been reported to be post-translationally modified with an N-terminal myristate group which targets Gag to lipid rafts, aiding in assembly [79, 80]. In HIV-1, the membrane binding domain maps to the N-terminal region of p17 matrix (MA), and it is known that for the membrane binding domain to function, it has to be covalently modified at the N terminus by myristate and this importance is noted by the fact that almost all mammalian retroviral Gag proteins are myristoylated [79]. This lipid modification of proteins plays many roles inside and outside of the cell including directing proteins to various cellular membranes, sub-membrane domains and promoting protein-protein interactions. Thus, one can view protein lipidation as adding another level of function in many proteins involved in cellular processes. Specifically in relation to kinases, myristoylation is often required for their membrane recruitment and activation. Previous studies for example illustrated this by showing that the addition of a myristoylation sequence activates both the PI3K p110α subunit and AKT [81-86].

1.2.2.2 HEPATITIS C VIRUS AND KINASES

As kinases can be the targets for drugs and a control point for numerous cell behaviors, the kinome has recently been used for large-scale functional genomics with RNAi screens. One

recent report by Reiss and colleagues showed a screening of siRNA library targeting all known and predicted 719 human kinases and identified phosphatidylinositol-4 kinase III alpha (PI4KIIIα) to be important in HCV entry and replication [87]. In this particular study, it was reported that the nonstructural HCV protein NS5A binds to and activates PI4KIIIα, and this kinase seemed to localize primarily at the endoplasmic reticulum (ER), which is thought to be the origin of HCV replication sites [87]. This kinase was previously confirmed by another group, in which they used an siRNA library targeting 140 cellular membrane-trafficking genes and systematically evaluating their impact on the production of infectious HCV and HCV subgenomic replicon replication [88]. HCV infections in particular have been linked to host kinases in the past, and other kinases reported to be important in HCV replication include casein kinases I and II, and choline kinase alpha (CHKA) [89, 90]. Other kinases that have been reported to target NS5A also include AKT, p70s6K, MEK, MKKI and CKII [91]. The casein kinase II (CKII) in particular has been implicated as a NS5A directed kinase important for HCV particle assembly [92].

1.2.2.3 DEVELOPMENT OF THE MYRISTOYLATED HUMAN KINASE LIBRARY

In this thesis project, 192 myristoylated human kinases were purchased from Addgene. Therefore it is important to understand how and why this library was created. In 2007, Boehm and colleagues tried to manipulate normal cells into cancer cells by activating and inactivating different kinase pathways [6]. One could easily do this by predicting which genes are involved and test them, but Boehm and colleagues wanted to do this in the most unbiased way possible to look for novel oncogenes and tumor suppressor genes. Thus, Boehm and colleagues approached this issue by using cDNA libraries, but with such high order of complexity generated by cDNA

libraries, it was difficult to find true candidates in such complex collections of molecules for overexpression. This led to the use of overexpression clones that expresses kinases genes one by one, and allowed this group to identify new breast cancer oncogene [6, 93]. The original Center for Cancer Systems Biology (Dana Farber Cancer Institute)-Broad Human ORF collection (CCSB Broad Kinase Library) from Addgene consists of 559 distinct human kinases and kinase-related protein ORFs in pDONR-223 Gateway Entry vectors, and all clones are clonal isolates that have been end-read sequenced to confirm identity [93]. The kinase ORFs were assembled from various sources: 56% were isolated as single cloned isolates from the ORFeome 5.1 collection; 31% were cloned from normal human tissue RNA (Ambion) by reverse transcription and subsequent PCR amplification adding Gateway sequences; 11% were cloned into Entry vectors from templates obtained from collaborating laboratories, and all ORFs are open (stop codons removed) except for 5 (MST1R, PTK7, JAK3, AXL, TIE1) which are closed (www.addgene.org/human-kinase/).

1.2.2.4 RELEVANCE

Hepatitis C virus continues to be a significant threat to public health worldwide, despite continuous efforts to develop effective treatments and understanding the pathogenesis as well as further enhancing our understanding of the virus itself. The current treatment of HCV infected patients is not highly effective in all cases and produces severe side effects that cause patients to be unwilling to take further drug treatments. The research in kinases in relation to HCV is relatively new and although there have been reports that link between host kinases and HCV infection, anti-viral activity of the kinases to HCV has not been reported nor identified. Thus,

upon identification of such kinase-mediated anti-HCV pathways, these novel kinases could potentially act as novel compounds as means to combat HCV infection.

1.3 SUMMARY

The human kinome includes between 500-600 known kinases and open reading frames (ORFs) that play key roles in regulating many cellular processes. Some of these cellular processes include, signal transduction, metabolism, transcription, cell cycle progression, cell movement, apoptosis, differentiation and protein phosphorylation. Various protein and lipid kinases have previously been shown to control HCV life cycles in several genomic screens using small interference RNAs that target human kinases. In this study, the goal was to identify kinases whose activities limit HCV infection using a gain-of-function approach and to perform initial validation and characterization of kinase-mediated HCV inhibition. The detailed molecular mechanisms will be investigated in depth in the future.

2.0 OBJECTIVE

HCV infection is a leading cause of liver diseases worldwide and due to its persistence, HCV-related pathologies are expected to rise in the future [94]. The combination therapy not only causes severe side effects, but the unavailability of vaccine or alternative treatments calls for attention. In the past, several kinases that are required for HCV infection were identified through siRNA or chemical inhibitor screenings [87, 95-100]. **The objective of this study was to screen for additional human kinases that may suppress or promote HCV infection through a gain-of-function approach. Selected kinases were further characterized in terms of their mechanisms of inhibition.**

2.1 SPECIFIC AIM 1: TO IDENTIFY HUMAN KINASES THAT INHIBIT HCV INFECTION THROUGH GENOMIC SCREENING

Previous studies have identified several kinases involved with HCV entry and replication by siRNA screening or chemical inhibitor based methods, which adopt loss-of-function approach. In my study, an overexpression of the kinases is used for gain-of-function approach to perform primary screening in our 192 human myristoylated kinase library (Table 1) (all plasmids can be found at http://www.addgene.org/MKL/). In this primary screening, the kinases were grouped in pools containing eight kinases to make the screening more manageable in the given time frame. This primary screening led to secondary screening, where the selected pool of kinases considered being significant was individually screened to identify the exact kinase of interest. Validation of the selected kinases was performed to confirm the significance of the identified kinase. This allowed for identifying kinases of interest to be screened effectively.

Table 1. Summary of all kinases included in the cDNA library.

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	CKS1B	TK1	PIM1	CDK9	CHEK1	SGK	DYRK2	PLK1	MAPK7	AKT1	UCK2	CKS2
B	STK17B	CAMKV	CLK3	MPP1	TTK	PIK4CB	PKN2	CKM	STK38L	LCK	MELK	MATK
C	MAP2K5	CDK5	IKBKE	PAPSS1	CSNK1G2	PCTK1	DAK	BMX	TAOK3	PRKCZ	CALM2	CDC2
D	RIOK3	GALK2	PIP5K1A	NEK6	DGKG	CDK2	CDK7	CKMT1A	GCK	CLK2	RPS6KA5	RPS6KA2
E	RET	FASTK	CDK4	MAPK14	LIMK1	BLK	MAP3K7	PKM2	TYK2	MKNK1	PRKRA	MAPK13
F	AURKA	PLAU	PCTK3	OXSR1	MAP3K6	RPS6KB2	VRK2	FGR	RIOK2	PCK2	LIMK2	ACVR1
G	STK3	AMHR2	GK2	ADCK5	CSNK1A1L	MAP2K7	PIP5K3	NEK11	NEK3	GRK6	HIPK1	PMVK
H	PACSIN1	ILK	CLK1	STK33	SYK	ADRBK1	RPS6KL1	HK2	CERK	PDXK	ADRBK2	PIK3R3

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	PCTK2	MAPKAP1	ULK4	ITK	BTK	RPSK6A3	EPHA4	MAP3K8	AKT3	CAMKK1	GK	YES1
B	PRKCI	DLG5	RPS6KA6	CAMK4	HCK	RPS6KB1	MAPK6	TEC	PRKCD	PIK4CA	PIK3CB	VRK3
C	PI4K2B	FRK	PRKAA1	GRK5	PLK2	PFKM	MOBKL2A	AAK1	TSSK1B	MAST1	PDIK1L	NADK
D	DYRK4	MVK	PAK4	TESK1	MAPK12	ITPKB	IHPK2	SPHK2	PIK3CG	STK32C	CKB	ITPK1
E	GAK	PRKACG	STK40	PDPK1	HK1	PIK3R5	PNKP	PRKAG2	NUAK2	SNF1LK	HK3	PIP5K2A
F	PIP5K1B	PBK	STK32B	CMPK	ADPGK	MAP3K14	CSNK1G1	TNK2	NTRK3	DGUOK	MOBKL1A	NME7
G	FGFR1	PFKL	PKN1	PLK4	CKMT2	RIOK1	PLK3	ADCK4	STK4	TIE1	PTK2	SRPK2
H	PRKACB	MAP2K6	PRKAR2A	AXL	PDK1	CAMK1G	TBK1	STK32A	TSSK6	CSNK1E	CAMK2D	CAMK2B

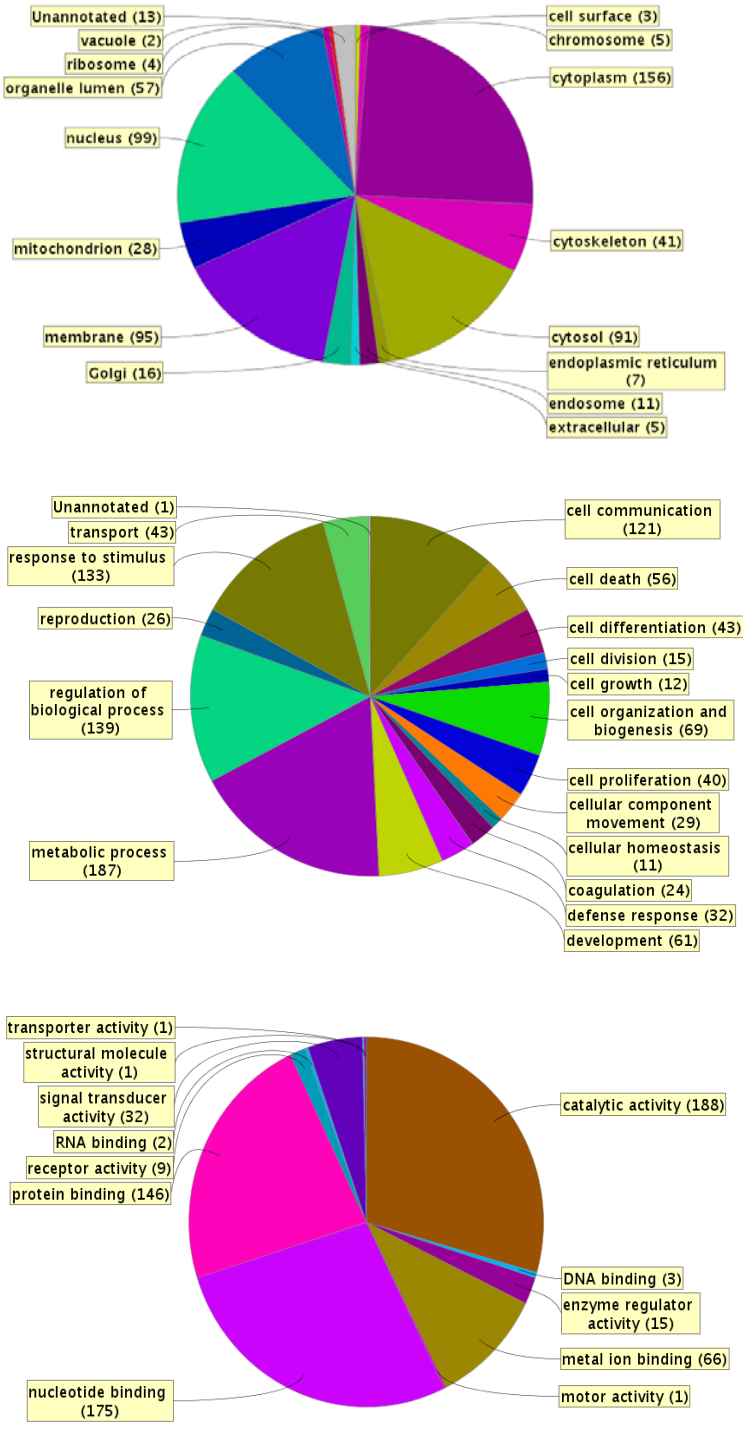


Figure 4. Categorizations of 192 human kinases. 192 human kinases are categorized according to their annotated cellular localizations, involvement in biological processes and molecular functions. The numbers included in the figure indicates the number of kinases that fall into each category. Of note, some kinases are classified into more than one category. The bioinformatics analysis was carried out using Protein center.

2.2 SPECIFIC AIM 2: TO DETERMINE THE STAGE OF VIRAL LIFE CYCLE AT WHICH KINASES ACT UPON HCV INFECTION

The identified kinases of interest at this point have been individually selected out from the original kinase library. The selected kinases could be involved in any step of the viral life cycle, such as entry, replication and assembly/release. In order to pinpoint exactly which step of the viral life cycle the kinase is responsible for the inhibition of HCV infection, each step must be investigated. The replication step of the viral life cycle can be determined by RT-PCR. The viral entry can be studied by using pseudoviruses that mimic HCV entry and a cell culture system that supports production of infectious HCV. In this study, HCVpp and VSV-Gpp were used as lentiviral particles pseudotyped with HCV E1 and E2 glycoproteins and VSV-G glycoproteins respectively to study viral entry. To assess the effect of kinases on viral replication, a replicon cell line harboring the full-length genome of the HCV genotype 1b (2-3+) was maintained in cell culture in the presence of various kinases. The viral RNA (vRNA) level was evaluated by real-time PCR.

2.3 SPECIFIC AIM 3: TO INVESTIGATE THE MOLECULAR MECHNISMS BY WHICH KINASES INHIBIT HCV INFECTION

There are several ways for a kinase to inhibit HCV infection: (1) kinases-mediated pathways may activate the production of interferons, which are known inhibitors of viral infection. (2) Kinase expressions may alter cell cycle and HCV replication is prone to the changes of cell cycle. (3) HCV NS5A is a known target for serine-threonine phosphorylation. Although the importance of NS5A phosphorylation remains controversial, there were studies showing that hyperphosphorylation of NS5A decreased virus replication [101]. Hence identified kinases may hyperphosphorylate NS5A. (4) Other possible mechanisms include but are not limited to: disruption of the formation of viral replication complex, interference of viral assembly and release. The first three possibilities were investigated in detail as part of my thesis study and then the possible association of kinases with lipid droplet, the important cellular organelle where virus assembly occurs, was examined by confocal microscopy.

3.0 MATERIALS AND METHODS

3.1 MYRISTOYLATED KINASE LIBRARY

The kinase library consisting of 192 human myristoylated kinases was purchased from Addgene. These kinases were shipped as glycerol stock in two 96-well plates, namely referred to as plates 1 and 2. These kinases contain kinase-related open reading frames (ORFs) cloned into a retroviral vector which adds a myristoylation sequence and flag-epitope tag to each ORF. The plates were stored in -80° C.

3.2 KINASE PLASMIDS

Each individual kinase glycerol stock was inoculated in 3 ml of Luria broth (LB) with ampicillin and incubated overnight at 37° C with vigorous shaking. The DNA was isolated next day using Plasmid Mini Kit I (Omega, bio-tek), and the isolated DNA was subjected to optical density (O.D) reading. DNA isolation was repeated until the concentration and purity ratio were optimal.

3.3 CELL CULTURE

HCV permissive cell line Huh 7.5.1 was used with the passage number between 1-10 at all times. The cells were maintained in complete growth medium DMEM - Dulbecco's modified Eagle medium (Cellgro® Mediatech) supplemented with 10% heat inactivated Fetal Bovine Serum (FBS), 100 U penicillin/0.1 mg/ml streptomycin/2mM glutamine and 0.1 mM nonessential amino acids at 37° C and 5% CO_2.

3.4 LENTIVIRAL PACKAGING SYSTEM

To perform primary screening, lentiviral packaging system was used. This involves three plasmids, eight kinase DNA, MLV-gag-pol and VSV-G packaged in 293T Lenti-X cell line. The kinases were pooled in groups of eight in the primary screening to make the screening manageable in the given time frame. The plasmids were transfected using 1μg/μl of polyethylenimine (PEI) and after 72-hours, the supernatant containing the packaged virus was collected and stored in -80° C freezer. The virus was also harvested twice every 12 hours for a total of three collection times.

In the secondary screening, the same method was applied, except the kinases in each pool were individually packaged.

3.5 VIRUS INFECTION

The virus collected from packaging was used to infect Huh 7.5.1 cells, incubated with the cells in the presence of 4 μg/ml polybrene at 37° C for 3-6 hours prior to removal of virus. The cells were gently washed once with pre-warmed complete media to remove residual virus.

3.6 CELL VIABILITY ASSAY

To rule out the possibility of the contribution of altered cell viability by expressing active kinases, cellular ATP levels was measured. The cellular ATP levels reflect the cell viability and metabolism, made possible by using Cell Titer Glo luciferase kit to be detected in luminometer (Figure 5).

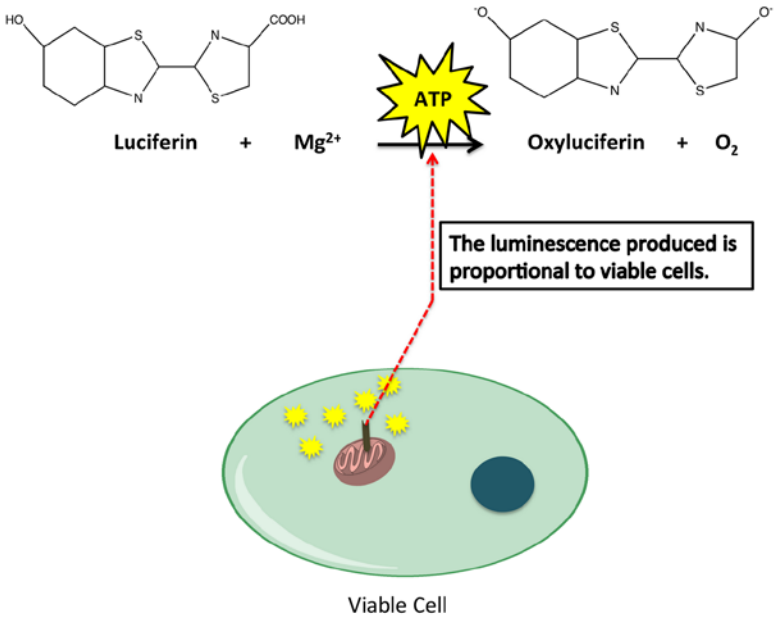


Figure 5. Principle of the cell viability assay. To determine cell viability, cellular ATP levels are measured, in which the luminescence produced by the cells is proportional to the number of viable cells.

3.7 IMMUNOFLUORESCENT STAINING

Huh 7.5.1 cells were seeded at approximately 0.05 x 10^6 cells/well in a 24-well plate covered with collagen-coated coverslips and maintained in complete DMEM medium at 37° C and 5% CO_2. Next day, the kinase plasmids were transfected into Huh 7.5.1 by using Lipofectamine2000 (Invitrogen) and subsequently subjected to JFH-AM2 virus infection for two hours. After 48 hours post infection, samples were washed 3 times in 1X PBS (Invitrogen) for 3 minutes. Cells were then fixed in 2% paraformaldehyde (PFA) for 10 minutes and permeabilized with 0.2% Triton-X 100 (Biomedicals Inc.) for 10 minutes. Then the cells were incubated for 1 hour in a humid chamber with primary antibodies: mouse α-flag (1:1000), human α-HCV E2 (1:200) and/or mouse α-NS5A (1:2000). Cells were next washed in 1X PBS 3 times for 10 minutes each and secondary antibodies fluorescently conjugated to Alexa Fluor 568, Alexa Fluor 488 and/or FITC were used and incubated for another 1 hour in a humid chamber. The coverslips with samples were washed with 1X PBS 3 times for 10 minutes each and Draq 5 (Cell Signaling) was used to stain the nucleus (1:5000) during the second wash. The coverslips were mounted with homemade mounting solution and images were captured using confocal microscopy (Zeiss Meta 510) using 40x objective lens.

3.8 PSEUDOPARTICLE PACKAGING SYSTEM

HCV pseudoparticles (HCVpp) are often used to study viral entry, where the lentiviral particles are pseudotyped by the incorporation of the full-length HCV glycoproteins E1 and E2 onto

lentiviral or retroviral core particles. HCVpp are useful in that the functional conservation of the HCV envelope glycoproteins is maintained.

293T cells were transfected with pTrip-luc, HIV gag-pol and HCV-E1E2 to generate HCVpp or with pTrip-luc, HIV gag-pol and VSV-G for the control VSV-G pseudotype. The virus in the supernatant was harvested after 48 hours and the virus was used to infect Huh 7.5.1 cells, which have been transfected by the kinase of interest. Lysates were made after 48 hours and luciferase readings were obtained with luminometer.

3.9 RNA ISOLATION

Trizol (Life technologies) was used to lyse the cells and the samples were isolated by phenol/chloroform extraction method according to standard protocol and the resulting total RNA were used to perform Real Time RT-PCR.

3.10 REAL-TIME RT-PCR

Cellular RNAs were reverse-transcribed and PCR amplified using the Power SYBR® Green RNA-to-C_T™ 1-Step Kit according to the instructions to users with Step-One Real-time PCR machine (ABI: Applied Biosystems). Cellular RNAs were normalized to a house-keeping gene RPS 11 (40S ribosomal protein 11). The cycling condition was 50° C 10 min (x1), 95° C 5 min

(x1), 94° C 10 sec (x40), 56° C 20sec (x40) and 72° C 30 sec (x40). The detailed protocol has been published by the Wang laboratory [102].

3.11 DETERMINATION OF INTERFERON PRODUCTION

To explore the effects of kinases on the activation of type I interferon, luciferase reporter assay in Huh 7.5.1 cells was used. To this end, an ISRE-luciferase reporter (ISRE-luc, contains 5x ISRE from ISG54 promoter upstream of firefly luciferase) construct and an IFN-β luciferase reporter construct (p125-luc) were transfected into Huh 7.5.1 cells along with the indicated kinase expression plasmid. As a positive control for activation, innate immune signaling plasmid encoding the protein MAVS was included in the study.

3.12 NDV-GFP BIOASSAY

The Huh 7.5.1 cells were transfected with the indicated kinase plasmids and the next day, 50 µl of the Newcastle disease virus expressing the enhanced green fluorescent protein NDV-GFP virus and 50 µl of 1X PBS was added to each sample and the infection lasted for one hour at 37° C. After infection, GFP signal was observed under confocal microscopy (Zeiss Meta 510) using 40x objective lens, 24 hours post infection.

3.13 CELL CYCLE ANALYSIS

To determine the cell cycle progression, a standard propidium iodide (PI) staining of Huh 7.5.1 cells expressing individual kinase was used. Flow cytometry was used for analysis.

3.14 WESTERN BLOTTING

The lysate of kinase transfected Huh 7.5.1 cells were prepared 48 hours post transfection using 1X SDS buffer in PBS. The samples were boiled for 5 minutes at 95 °C and loaded on a 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transferring onto a nitrocellulose membrane. The kinases were determined by Western blotting using mouse anti-flag primary antibody (1:2000). The β-actin protein was used as a loading control.

3.15 CLONING AND SEQUENCING

Using restriction enzymes, flag-tagged kinase sequence was isolated. The purpose of this subcloning was to remove the myristoylation sequence in hopes to express the kinase at higher levels. Then PCR was performed on these kinase sequences to amplify the kinase gene and ligated into an expression vector pQCXIP between the designated restriction enzyme sites (CKS1B: *Bam*HI and *Eco*RI; MAP2K5: *Not*I and *Bam*HI; PACSIN1: *Eco*RI and *Bam*HI). This expression vector is a retroviral vector that is often used to express the target gene in various cell

lines with unique features including: optimization to remove promoter interference, self-activation and the hybrid 5'LTR consists of the cytomegalovirus (CMV) type I enhancer and the mouse sarcoma virus (MSV) promoter [103-107]. The new subcloned kinase plasmids were then isolated and DNA sequenced by the Genomics and Proteomics Core Facility (GPCL) of the University of Pittsburgh. Analysis of the sequence was performed in Vector NTI Advance software.

3.16 PCR

The primers used for the PCR is as follows:

Table 2. Forward and reverse primers of kinases used for PCR

Kinase	Primers
CKS1B	forward: 5'-AGCGGATCCGCCACCATGGACTACAAAGACGATGACGACAAG-3' reverse: 5'-GAGAATTCTCATTTCTTTGGTTTCTTGGGT-3'
MAP2K5	forward: 5'-AGCGCGGCCGCGCCACCATGGACTACAAAGACGATGACGACAAG-3' reverse: 5'-GAGGATCCTCACGGGGGCCCTGCTGGCT-3'
PACSIN1	forward: 5'-AGCGCGGCCGCGCCACCATGGACTACAAAGACGATGACGACAAG-3' reverse: 5'-GAGAATTCCTAGATAGCCTCCACGTAGTT-3'
STK17B	forward: 5'-AGCGGATCCGCCACCATGGACTACAAAGACGATGACGACAAG-3' reverse: 5'- GAGAATTCCTAACAGAGCAAATCTGAAAC -3'

In this PCR, Phusion Hot Start Flex DNA Polymerase Kit was used with the following cycling conditions: 98°C for 30 seconds (35 cycles), 98°C for 10 seconds, 70°C for 30 seconds, 72°C for 30 seconds, 72°C for 5 minutes and 4°C forever.

4.0 RESULTS

4.1 PRIMARY SCREENING OF KINASE LIBRARY

To screen the kinases, primary screening was performed in pools of eight kinases in each group. For example, virus pool group 1 had kinases A1 through H1, and virus pool group 2 had kinases A2 through H2. A group of uninfected Huh7.5.1 cells were used as a negative control and Huh7.5.1 cells infected with HCVcc-luc without expressing any kinases were used as a positive control. Another control used was shCl-1, in which the entry factor of HCV is knocked down, thus we would expect less infection in this group as shown below. The results of luciferase readings showed that virus pool 1 (A1-H1) from plate 1 and virus pool 7 (A7-H7) from plate 2 showed initial regulatory effects on HCVcc infection, which are determined to be statistically significant tested by unpaired t-test. The statistics were analyzed using Prism software.

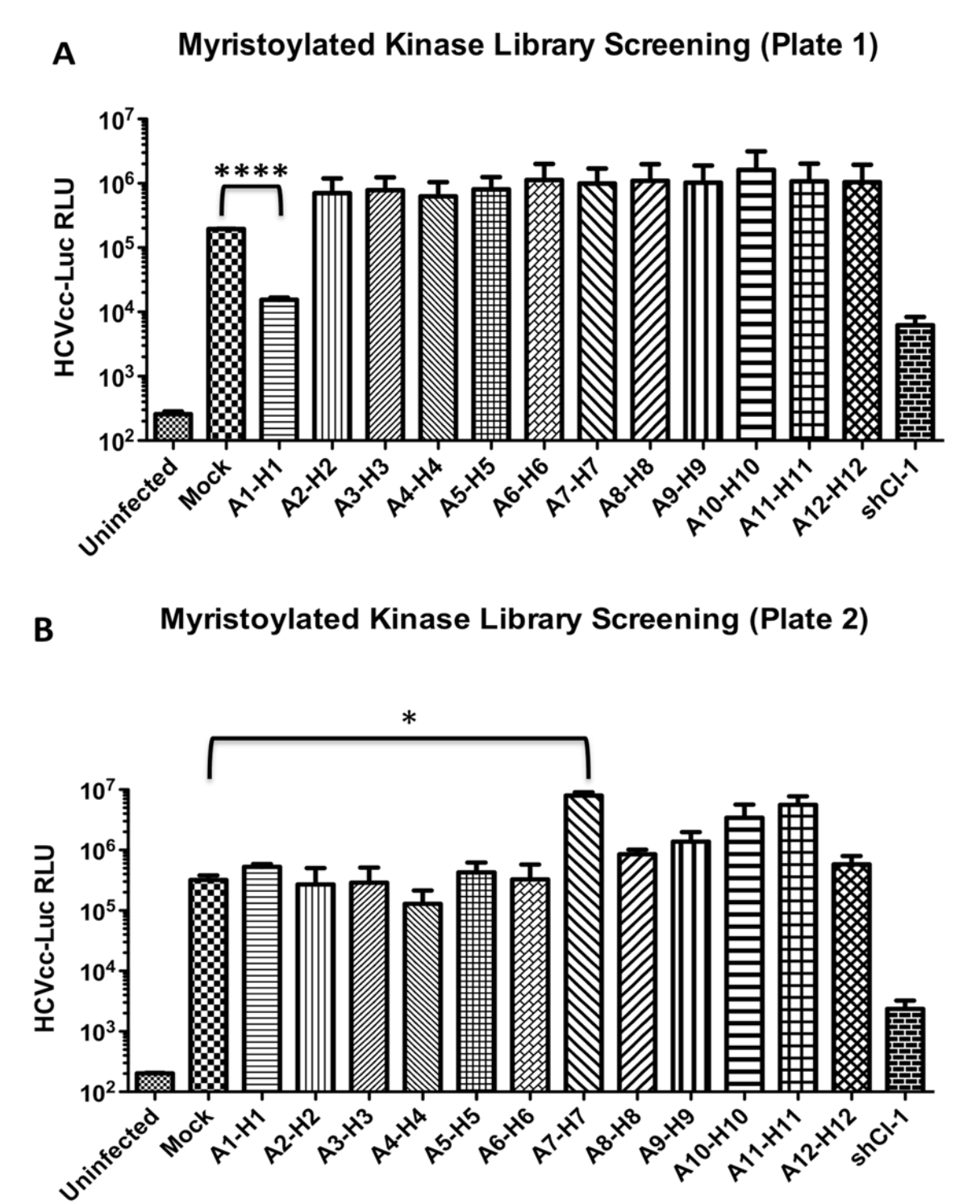
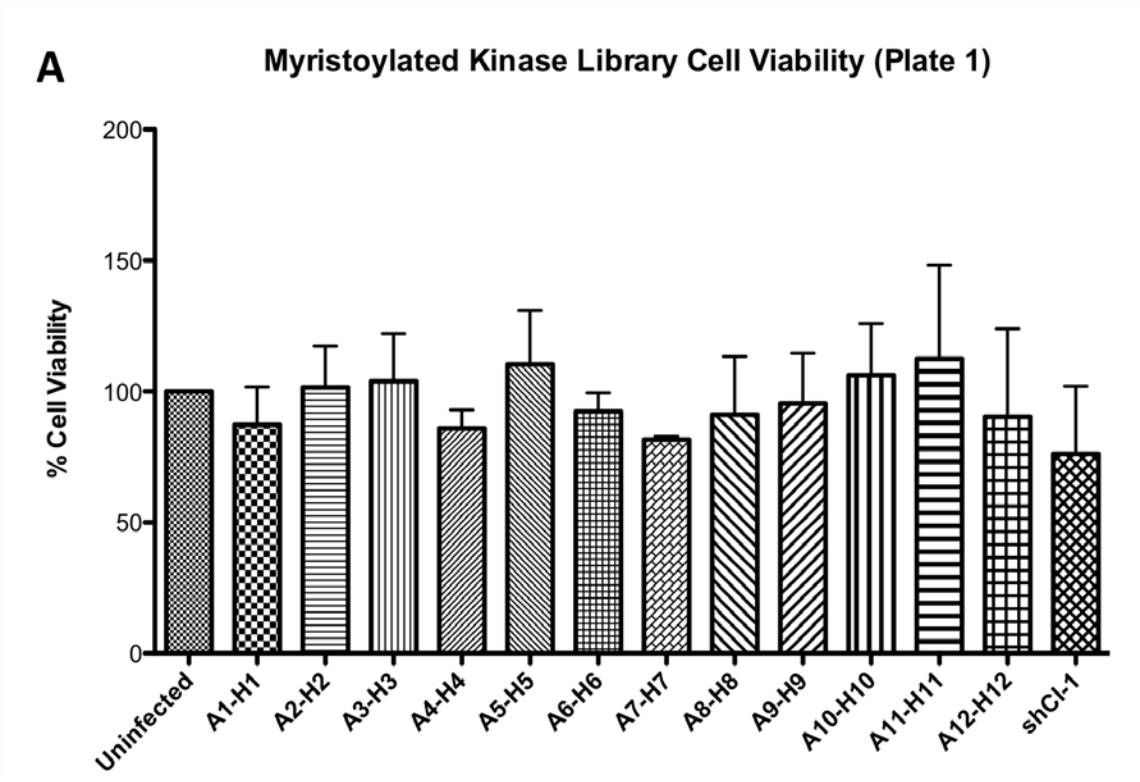


Figure 6. Initial screening of the myristoylated kinase library. Huh7.5.1 cells were first infected by retrovirus expressing pooled kinases and then by HCVcc-luc (MOI ~0.1). (A) In plate 1, pool of kinases in group 1 (A1-H1) showed inhibitory effects upon HCVcc infection ****$p<0.0001$ and (B) in plate 2, pool of kinases in group 7 (A7-H7) showed enhancing effects on HCVcc infection. Values are expressed as the mean ± SD (n = 2, error bars indicate two independent experiments) tested by unpaired t-test * $p < 0.05$.

To rule out the possible contribution of altered cell viability (confounding factor) by expressing active kinases, the cellular ATP levels in cells expressing kinases was measured, which reflect the cell viability and metabolism, using a Cell Titer Glo luciferase kit. Shown in Figure 7, short-term expression of these active kinases in Huh 7.5.1 cells did not significantly alter cell viability and metabolism.



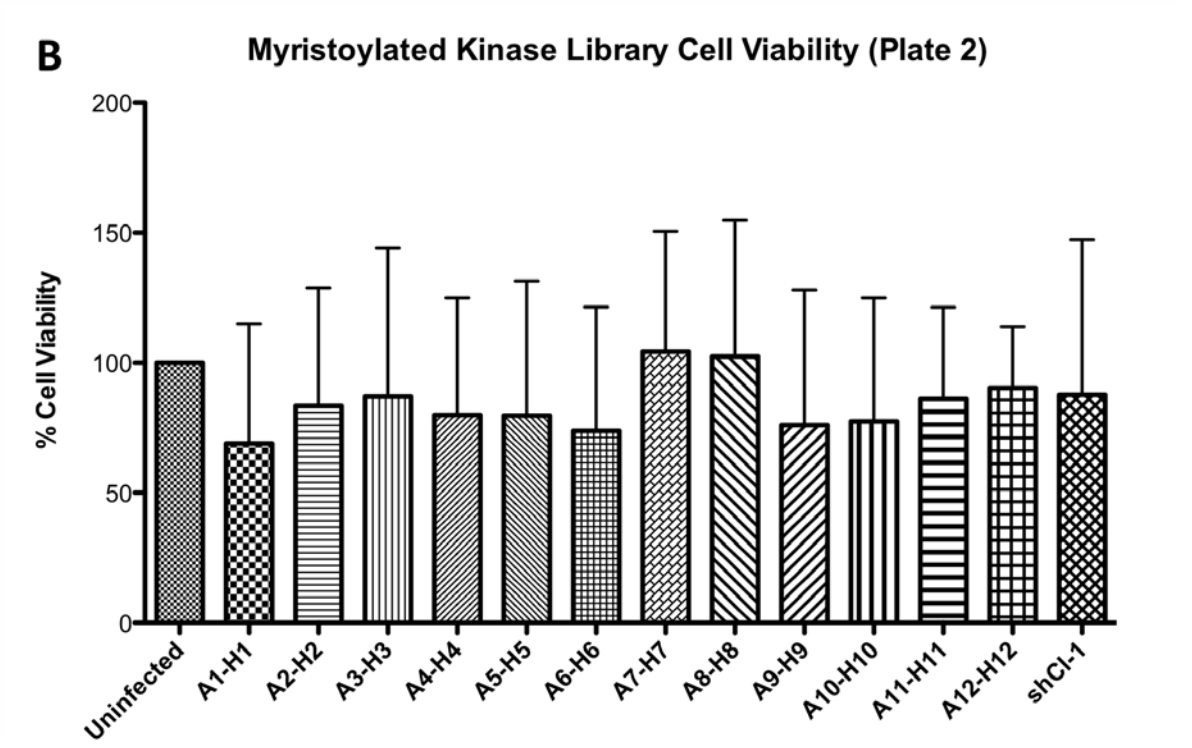
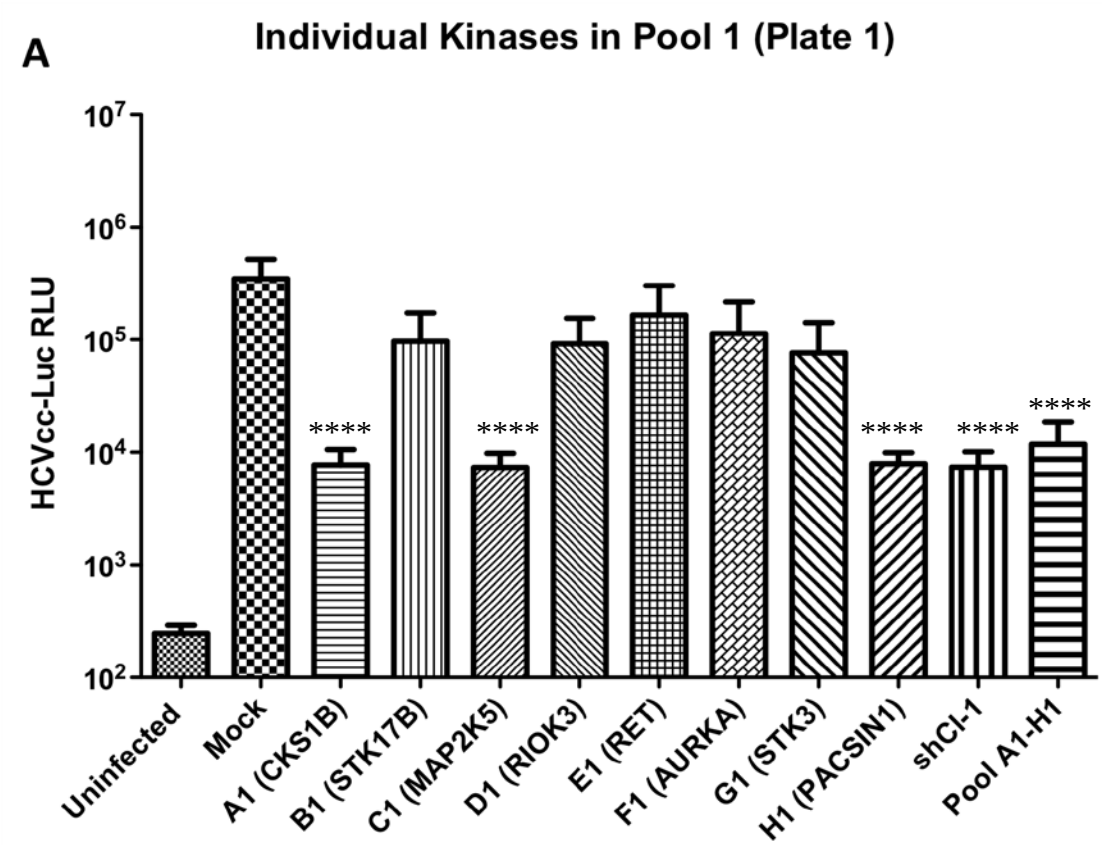


Figure 7. Transient expression of kinases did not alter cell viability. Pools of kinases were transiently expressed in Huh7.5.1 cells for 48 hours to determine cell viability, which was measured as the ATP levels in viable cells. Both plate 1 (A) and plate 2 (B) kinases exhibited no significant cell death observed, when normalized to the uninfected cells. Values are expressed as the mean ± SD (n = 2) tested by unpaired t-test. The error bars indicate two independent experiments.

4.2 SECONDARY SCREENING OF KINASE LIBRARY

To identify the individual kinase from virus pool 1 (A1-H1) from plate 1 and virus pool 7 (A7-H7) from plate 2, these statistically significant pools of kinases identified in primary screening were further screened by individually packaging the kinases using the lentiviral system as described previously. The results represent three independent experiments and statistical significance was tested by unpaired t-test (mean ± SD) using Prism software. It was found that

three kinases CKS1B (A1), MAP2K5 (C1) and PACSIN1 (H1) from plate 1 consistently inhibited HCVcc infection, whereas expression of individual kinase from pool 7 of plate 2 failed to consistently enhance HCVcc infection and hence omitted from subsequent evaluations (Figure 8 and 9).



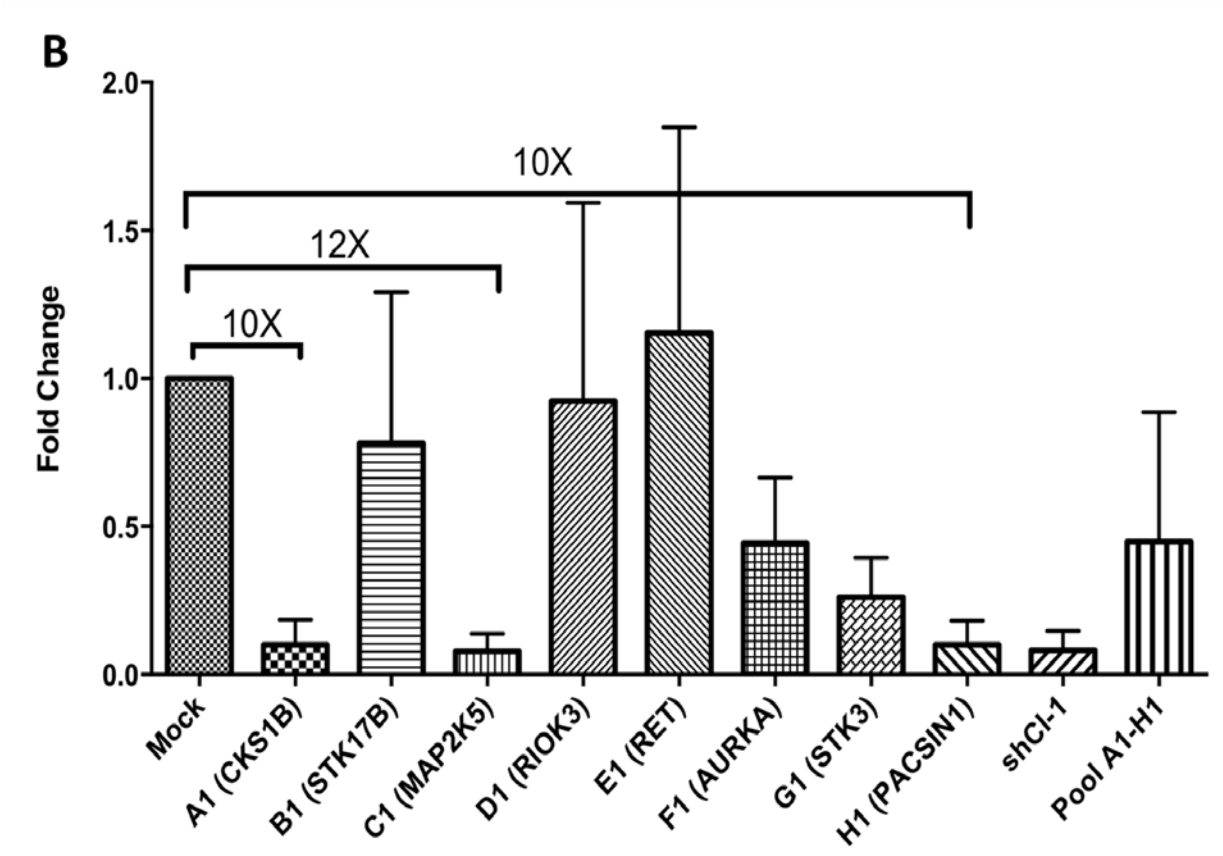


Figure 8. CKS1B, MAP2K5 and PACSIN1 inhibited HCVcc infection. Kinases in group 1 of plate 1 are individually expressed in Huh 7.5.1 cells to determine their effects upon HCVcc-luc infection. (A) Results are plotted as relative light unit. (B) Results are plotted as fold of changes (normalized to the mock infected samples, in which mock represent only HCVcc infected samples). Values are expressed as mean ± SD (n = 3) ****$p<0.0001$. The error bars indicate three independent experiments.

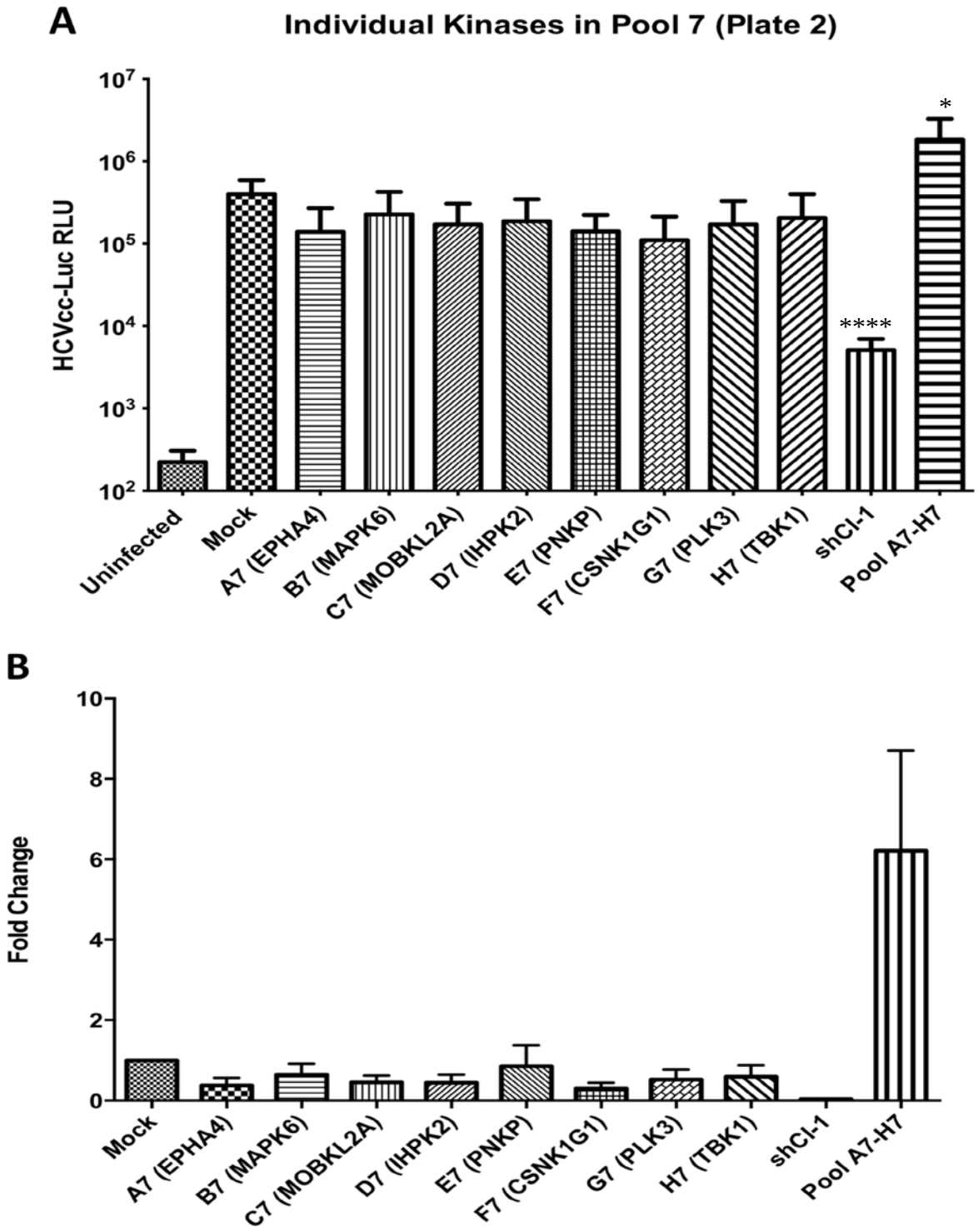


Figure 9. Individual kinases in pool 7 resulted in no significant effects upon HCVcc infection. Kinases in group 7 of plate 2 are individually expressed in Huh7.5.1 cells to determine their individual effects upon HCVcc-luc infection. (A) Results are plotted as relative light unit. (B) Results are plotted as fold of changes (normalized to the mock infected samples). Values are expressed as mean ± SD (n = 3) ****$p<0.0001$ *$p<0.05$. The error bars indicate three independent experiments.

4.3 VALIDATION OF SCREENED KINASES BY IMMUNOSTAINING OF HCV PROTEINS

Three kinases that displayed inhibitory effects upon HCVcc infection in the above study were chosen for further validation. Kinase expressing Huh7.5.1 cells were infected with wild type JFH1 virus (HCVcc) and infected cells were visualized by immunostaining of viral Core and E2 proteins. Shown in Figure 10, the expression of CKS1B (A1), MAP2K5 (C1) and PACSIN1 (H1) significantly reduced HCVcc infection in terms of both the number of infected cells and the size of the foci. By contrast, expressing the kinase STK17B (B1) or CSNK1A1L- as our negative controls, exerted no effect. As a positive control, knocking down HCV entry factor Claudin-1 by shRNA (shRNA Cl-1), abolished HCV infection.

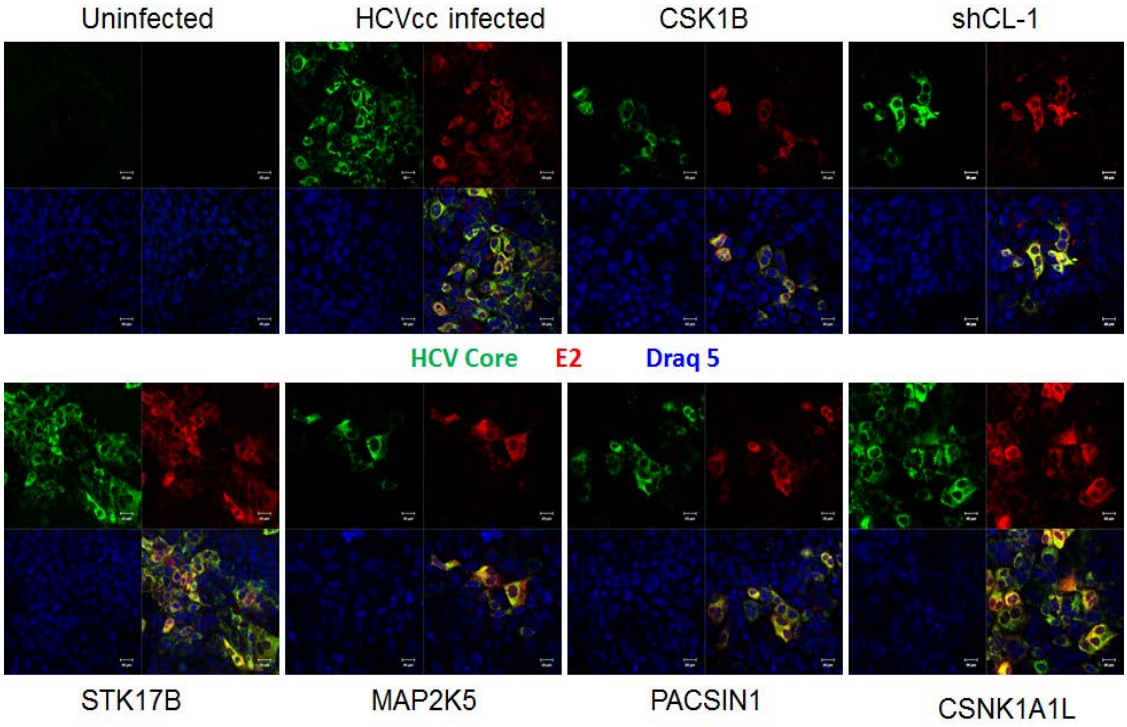


Figure 10. Kinases exhibited significant inhibitory effects against HCVcc infection in Huh 7.5.1 cells. Approximately $4x10^4$ cells were seeded on collagen-coated glass cover slips in 24-well plate and the cells were then infected by JFH1-based HCVcc (MOI ~0.3). 48 hours post-infection, cells were stained for HCV E2 (red), HCV NS5A (green) and nuclei (blue) and imaged under confocal microscope. The images are representatives of at least five independent experiments.

To investigate whether the observed effects are specific for HCV, an evaluation of how these active kinases affect Dengue virus (DENV) infection was performed. Using a prototype DENV2 virus, which was propagated in the laboratory, as demonstrated in Figure 11, it was found that none of the three kinases (CKS1B, MAP2K5 and PACSIN1) exhibited any inhibitory effect upon DENV2 infection (MOI 1 and 0.1 (not shown)).

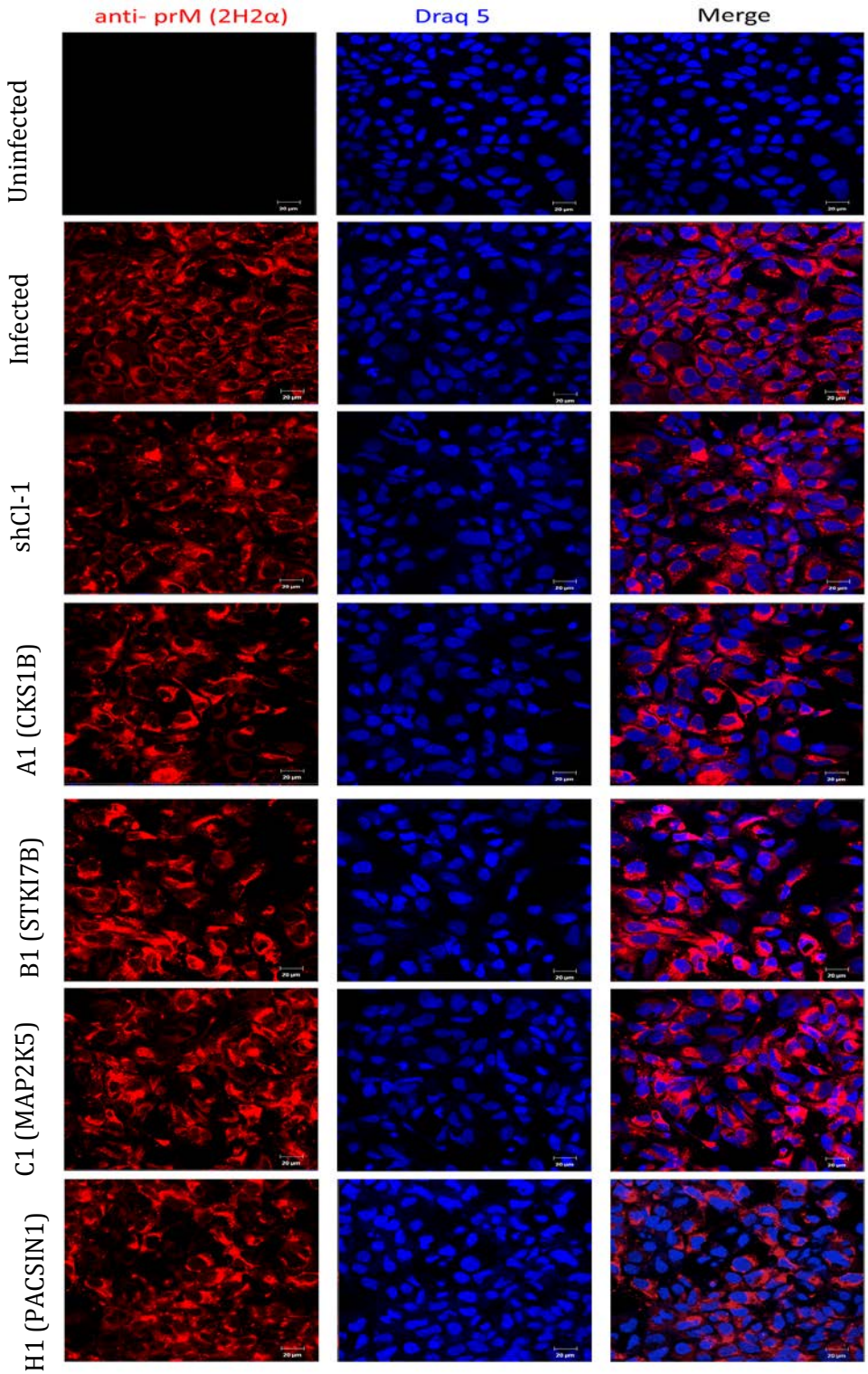


Figure 11. Kinases did not exhibit effects upon Dengue infection. Huh7.5.1 were first infected by retrovirus expressing individual kinase for 24 hours followed by infection with DENV2 (Thailand 16681strain) (MOI = 1). Infected cells were stained for the presence of viral protein prM (2H2 antibody) (red). Nuclei were stained by Draq 5 (blue) and images were taken by using Zeiss Meta LSM 510 confocal microscope. The images are representatives of at least three independent experiments.

4.4 DETERMINATION OF WHETHER KINASE AFFECTS VIRAL ENTRY BY HIV-HCV PSEUDOPARTICLES (HCV_{PP})

To determine at what stage of the viral life cycle that the screened kinases are inhibiting, pseudoparticles were used in this study, as previously described. As shown in figure 12, the kinases did not exhibit any effect on HCV entry upon viral infection. The uninfected Huh7.5.1 cells were used as a negative control and infected Huh7.5.1 cells not expressing kinases were used as a positive control. Additional controls used are shCl-1 and STK17B kinase (kinase in plate 1). The infectivity of Huh 7.5.1 cells in HCVpp infected group was comparable to the positive control VSV-G infected Huh 7.5.1 cells.

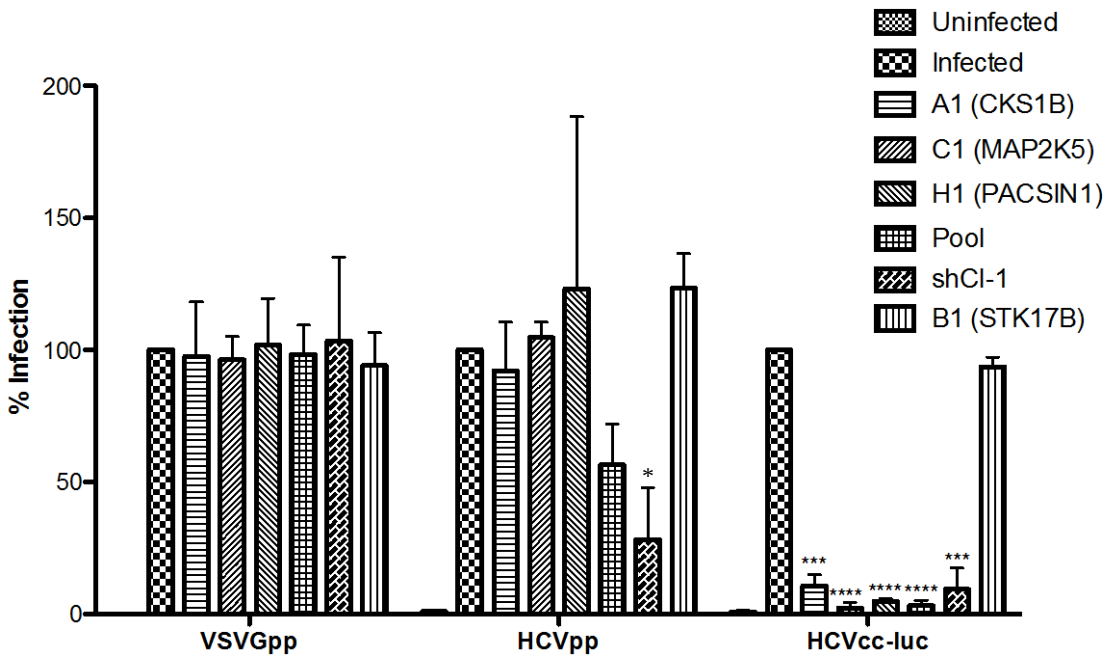


Figure 12. Identified kinases did not inhibit HCV entry. The kinases were transiently expressed in Huh7.5.1 cells and subsequently infected with HCVpp, VSV-Gpp, or HCVcc-luc. The % infection from the mock group (parental cells) was set to 100. Results are presented as mean ± SD (n = 3). * $p<0.05$ *** $p<0.001$ and ****$p<0.0001$. The error bars represent three independent experiments.

4.5 DETERMINATION OF WHETHER KINASE AFFECTS VIRAL REPLICAITON BY RT-PCR

As the kinases did not show any effect on HCV entry, their potential roles in regulating other stages of viral replication were investigated. For this purpose, individual kinase was expressed via retroviral transduction into a HCV full-length replicon cell line named replicon (2-3+), which harbors a full-length genotype 1b HCV genome that actively replicates without secreting infectious virus. Such a system permits direct assessment of the effect of a kinase on stages of viral RNA replication. Shown in Figure 13A, addition of a NS5A inhibitor (DAG114) or recombinant IFN-α both inhibited HCV replication by nearly 100 fold. Expressions of CKS1B and MAP2K5 also significantly suppressed vRNA levels, although the inhibition exerted by MAP2K5 was rather marginal. Lastly, PACSIN1 did not seem to inhibit HCV RNA replication.

Two kinases that showed inhibition on viral replication (CKS1B and MAP2K5) were further evaluated in a dose-dependent experiment for their inhibitory effects. Shown in Figure 13B, there was an inverse correlation between the amount of kinase plasmid that was transfected into replicon cells and the detected vRNA copies. This result corroborated the finding that CKS1B and MAP2K5 inhibit HCV viral RNA replication, although the inhibitory effect of MAP2K5 appears to be much less potent.

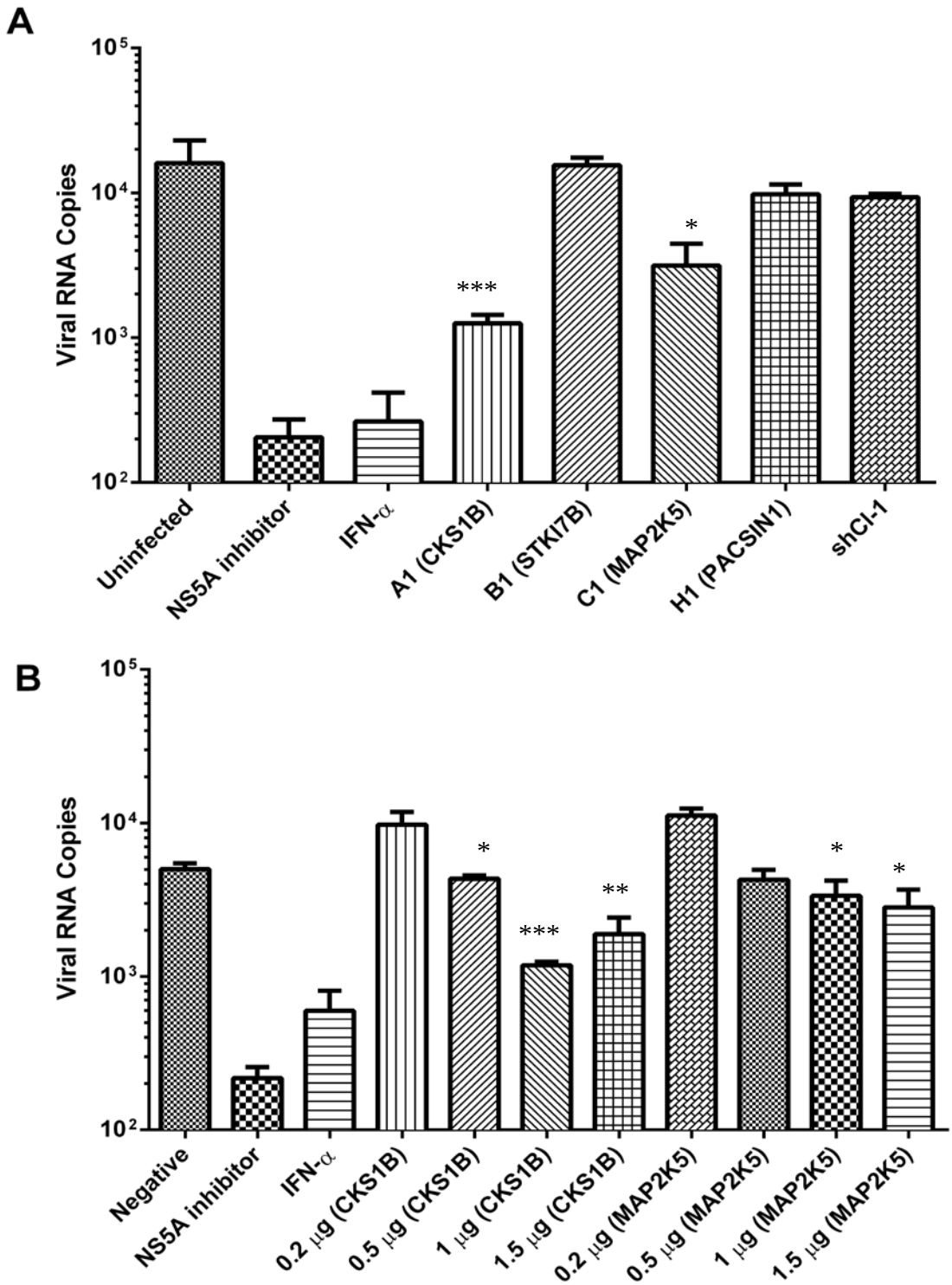
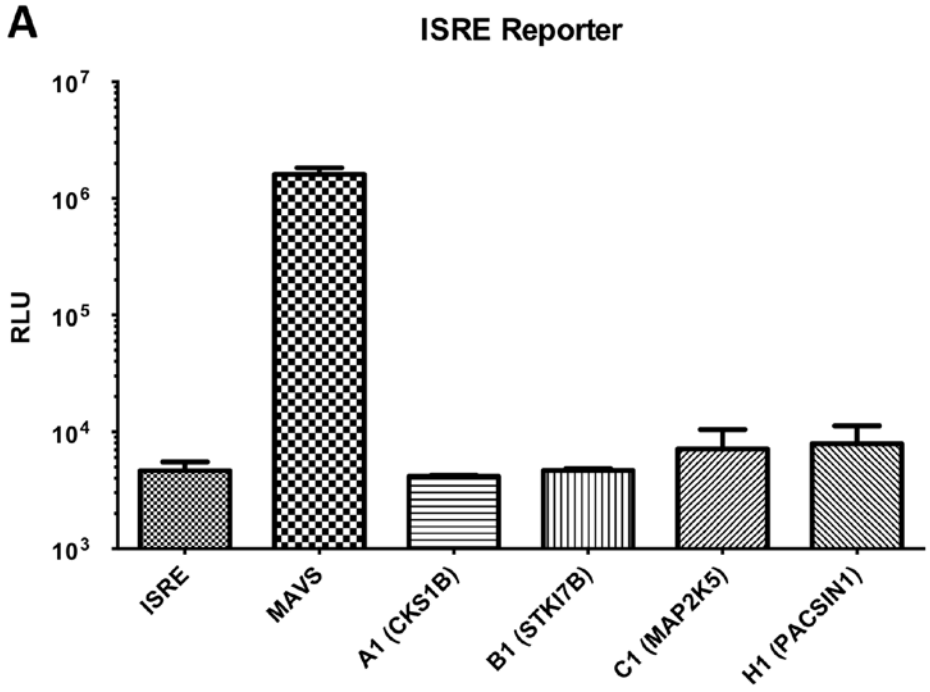


Figure 13. CKS1B and MAP2K5 supressed HCV RNA replication. Total RNA isolated from kinase expressing replicon cells (2-3+) was analyzed by real-time PCR. The positive control treatments added were DAG 114 (a NS5A inhibitor) and IFN-α. (A) Cells were retrovirally transduced to express individual kinases. (B) Cells were transfected with the indicated amount of plasmid DNA shown above panel B, to achieve a dose-dependent effect. In both A and B, viral RNA copies were normalized to the values obtained by measuring a house keeping gene RPS 11 using our published protocol. Results are presented as mean ± SD (n = 2). * $p<0.05$ ** $p<0.01$ and ***$p<0.001$. The error bars indicate two independent experiments.

4.6 DETERMINE THE POTENTIAL ACTIVATION OF INTERFERON PRODUCTION

Kinase-mediated activation of cellular pathways may result in the production of type I interferon, which is known to potently inhibit HCV replication. To explore this possibility, luciferase reporter assays were performed where the interferon response gene (ISG) promoter-driven (IRSE-Luc) and the IFN-beta promoter driven (p125-Luc) luciferase reporter constructs were co-transfected into Huh7.5.1 cells with each kinase construct. Shown in figure 14, whereas MAVS, an important signaling molecule downstream of the RIG-I-mediated antiviral pathway [108], strongly activated both ISRE-luc and p125-luc, none of the kinases activated the transcription of reporter genes, suggesting that signaling from these kinases are not likely to directly induce interferon or ISG production.



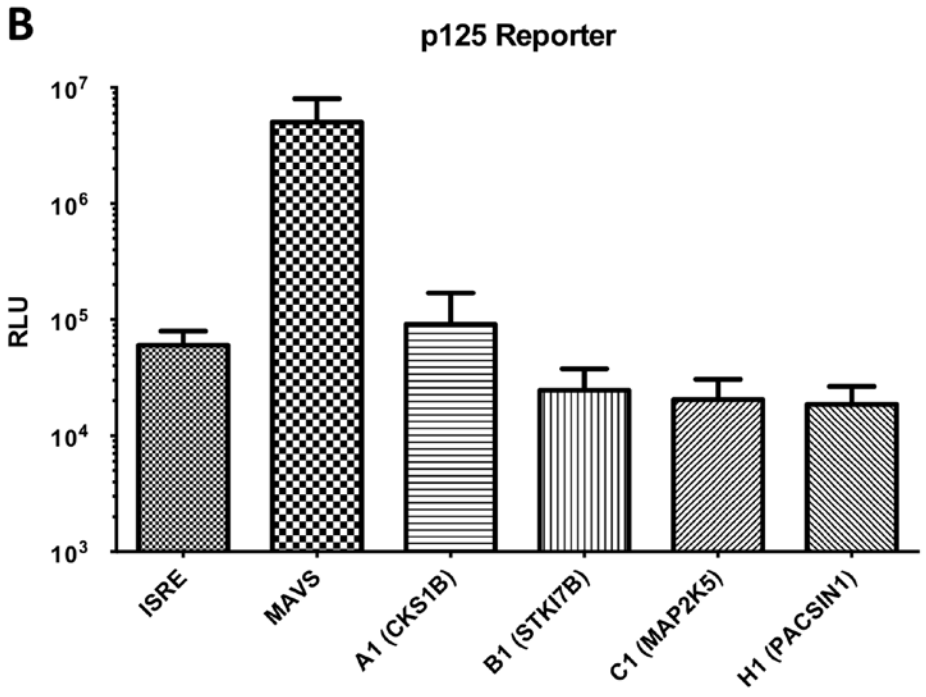


Figure 14. Expression of kinases did not induce transcription from IFN or ISG promoters. Huh 7.5.1 cells were pre-seeded in a 24-well plate. The next day, 0.1 μg of reporter construct was co-transfected with 0.2 μg of indicated kinase expression plasmid using lipofectamine 2000 reagent. 48 hours post-transfection, cells were lysed and luciferase activity was determined (n=2, SD, standard deviation). pISRE-Luc contains 5 copies of ISRE sites from ISG54 promoter upstream of luciferase gene and p125 contains the IFN-α promoter. The error bars represent two independent experiments.

4.7 DETERMINATION OF NDV-GFP BIOASSAY

As shown previously, none of the kinase constructs activated the transcription of either ISRE-luc and p125-luc reporter genes, suggesting that signaling stemming from these kinases are not likely to directly induce interferon or ISG production. However, this was further explored to validate these results, by performing bioassays to measure the production of type I interferon in kinase-expressing Huh 7.5.1 cells using a published protocol that uses a new castle disease virus (NDV) that expresses green fluorescent protein (GFP) [109]. In this bioassay, the presence of type I IFN suppresses the NDV-GFP infections, resulting in diminish of GFP signal. As shown

in Figure 15, whereas addition of recombinant human IFN-α significantly decreased the GFP signal, none of the kinases had any effect on NDV-GFP infection. This result confirmed that overexpression of kinases in Huh7.5.1 cells did not lead to the production of type I interferon.

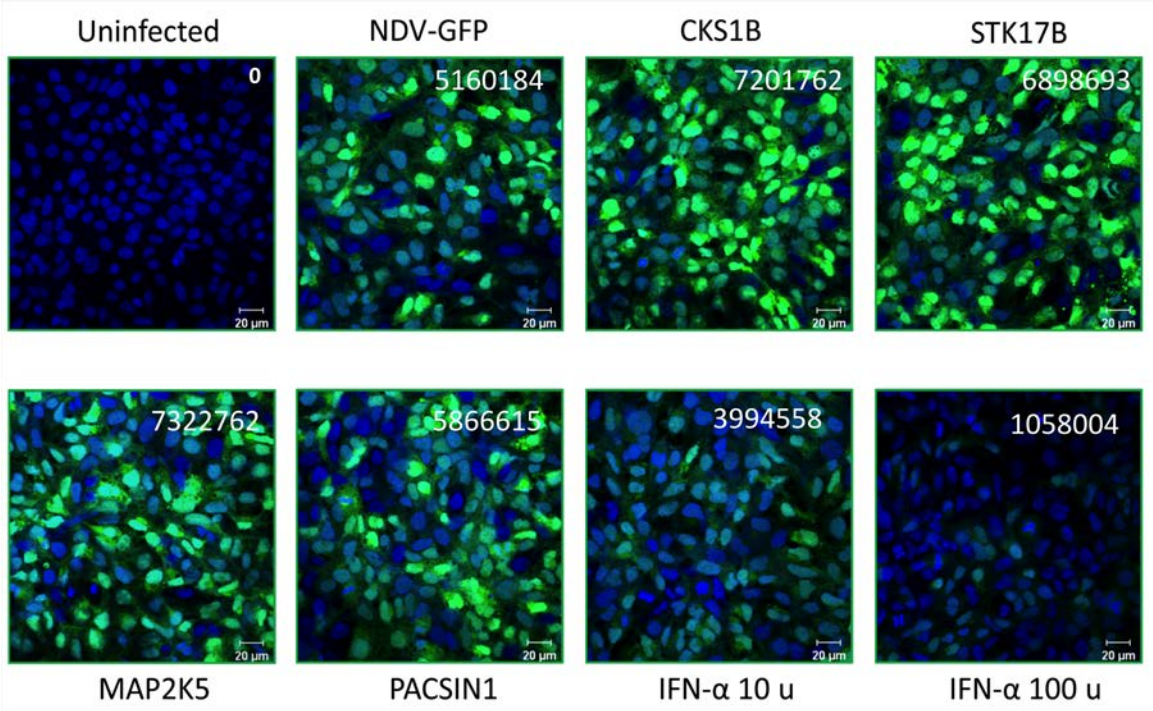
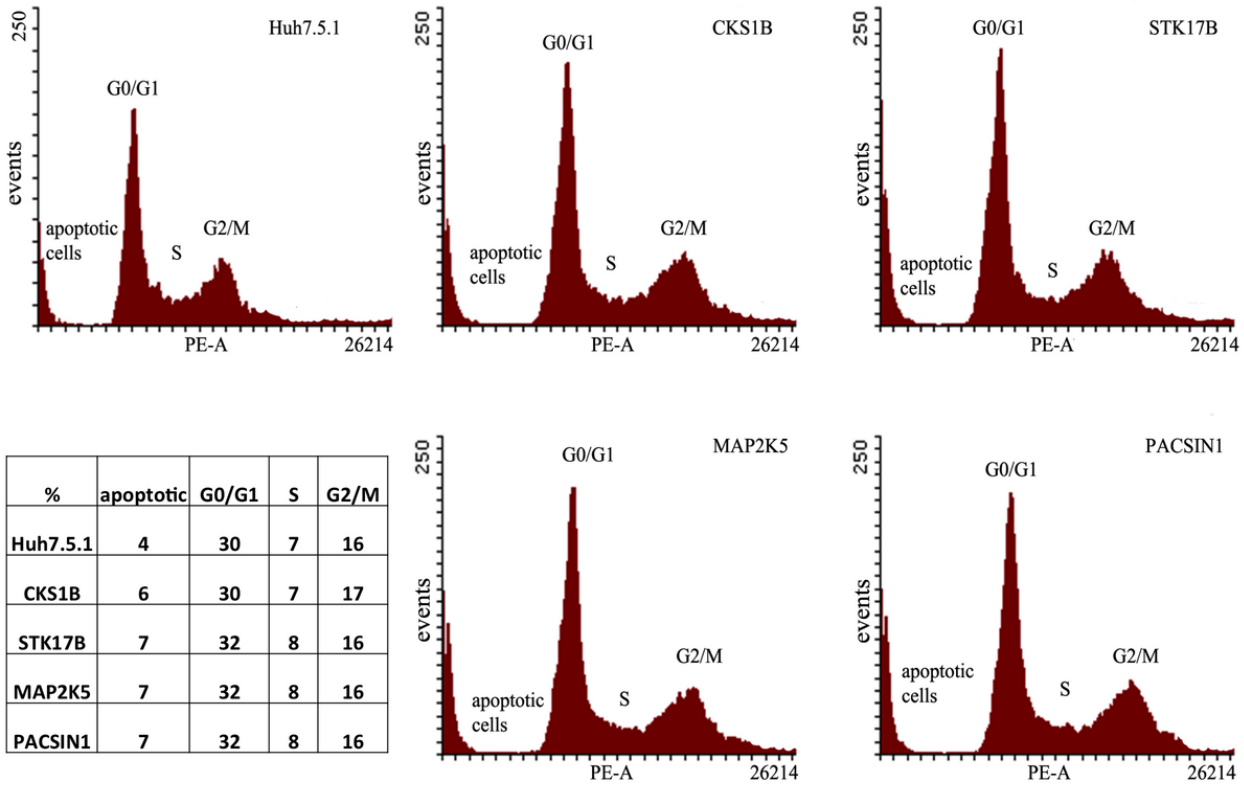


Figure 15. Kinase expression did not suppress NDV infection. Huh7.5.1 cells that express individual kinase were seeded on glass cover slips (24-well plate) and infected by NDV-GFP virus using the protocol described in Methods. 16 hours post-infection, the cells were fixed and imaged for infection (demonstrated as the expression of GFP). Fluorescence was measured by using J software and calculating corrected total cell fluorescence (CTCF). Data shown here are the representatives of three independent experiments.

4.8 DETERMINATION OF WHETHER KINASES ALTER CELL CYCLE

The kinase, CKS1B belongs to the highly conserved cyclin kinase subunit 1 (CKS1) protein family, which interacts with cyclin-dependent kinases (CDKs) and controls cell cycle progression [110, 111]. HCV replication, at least in cell culture, is known to be affected by cell cycle [112]. It has also been reported that HCV infection induces cell cycle arrest at the level of

initiation of mitosis [113]. Thus, it was necessary to investigate the effect of these kinases on cell cycle progression, to rule out the possibility of cell cycle alteration, by the use of standard propidium iodide staining of Huh 7.5.1 cells expressing individual kinase and determine the cell cycle progression (existing protocol in Wang Laboratory). Because it was previously observed that short-term expression of these active kinases in Huh 7.5.1 cells did not significantly alter cell viability/metabolism, it was not highly anticipated that the expression of these kinases would significantly alter the cell cycle progression. As predicted, as shown in figure 16, the activated kinases did not show any alteration of the cell cycle progression.



%	apoptotic	G0/G1	S	G2/M
Huh7.5.1	4	30	7	16
CKS1B	6	30	7	17
STK17B	7	32	8	16
MAP2K5	7	32	8	16
PACSIN1	7	32	8	16

Figure 16. Kinase expression did not alter cell cycle. Kinase expressing Huh7.5.1 cells were fixed in ethanol and stained by propidium iodide (PI). Cell cycle was analyzed by BD Canto flow cytometer. The percentage of cells in each phase is summarized in the table to the left. Results are representative of at least three independent experiments.

4.9 LOCALIZATION PATTERN OF ACTIVATED KINASES

As noted earlier, kinases did not have any effect on viral entry, but CKS1B and MAP2K5 decreased viral RNA when transfected into replicon cells and RT-PCR was performed. Thus it was important to further explain the mechanism of how these activated kinases act to inhibit HCV infection. Previous studies have reported that HCV replication is regulated by at least three mechanisms: (1) presence of IFN, (2) phosphorylation status of viral NS5A and (3) altered cell cycle. However, our interferon production assay and cell cycle analysis did not show any significance in the presence of activated kinases.

To explore the potential effect of kinase activation on the phosphorylation status of HCV NS5A, Huh 7.5.1 cells were infected by vaccinia virus expressing T7 polymerase (MOI 3) for 1 hour followed by transfection of HCVcc-JFH1 construct and individual kinase expression plasmids (data not shown). This procedure has been previously published by Qiu and colleagues as effectively showing the hyper- and hypo-phosphorylated NS5A (p58 and p56) [91]. It has also been reported that hyperphosphorylation of NS5A results in decreased HCV replication [101]. However, conflicting observations have also been reported. The results from this experiment also resulted in conflicting and inconsistent data; therefore the possibility of the activated kinases acting upon NS5A phosphorylation in regulating HCV replication remains inconclusive. It is possible that these kinases may be the cellular kinases that directly phosphorylate HCV NS5A *in vivo*, which can be evaluated in the future (data not shown).

To explore other potential anti-HCV mechanisms, I performed confocal microscopy to observe the localization pattern of the activated kinases. The purpose of this experiment was to

pinpoint where these kinases are located in the cell and to seek clues as to how they may inhibit viral infection. Because there are no commercial antibodies available that give satisfactory staining of endogenous kinases, I subcloned individual kinase gene into the retroviral vector pQCXIP in which a Flag tag was inserted to the N-terminus of each gene. When transfected into 293T cells, all constructs allowed expressions of corresponding kinases as demonstrated by western blotting (Figure 17). Next, kinase-expressing cells were visualized by confocal microscopy. CSK1B displayed a predominantly nuclear localization pattern with a low level of cytoplasmic distribution. STK17B exhibited an exclusive nuclear distribution. Both MAP2K5 and PACSIN1, however, localized to the cytoplasm (Figure 18).

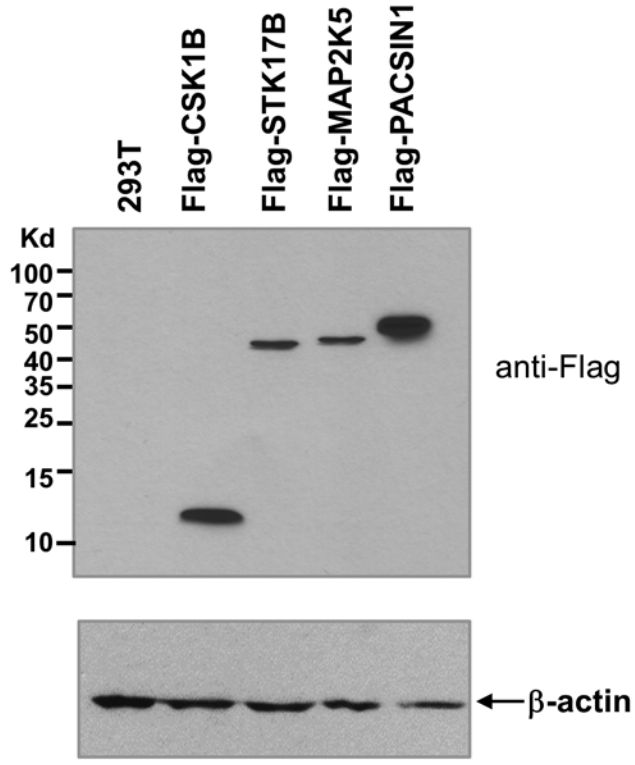


Figure 17. Expression of Flag-tagged kinases in 293T cells. Each kinase was subcloned into retroviral vector pQCXIP that contains a Flag tag at the N-terminus of each gene. 0.5 μg plasmid DNA was transfected into 293T cells. 48 hours post transfection, total cell lysates were prepared and resolved on a 15% SDS-PAGE followed by immunoblotting using the anti-Flag (top panel) and anti-β-actin antibody (bottom panel).

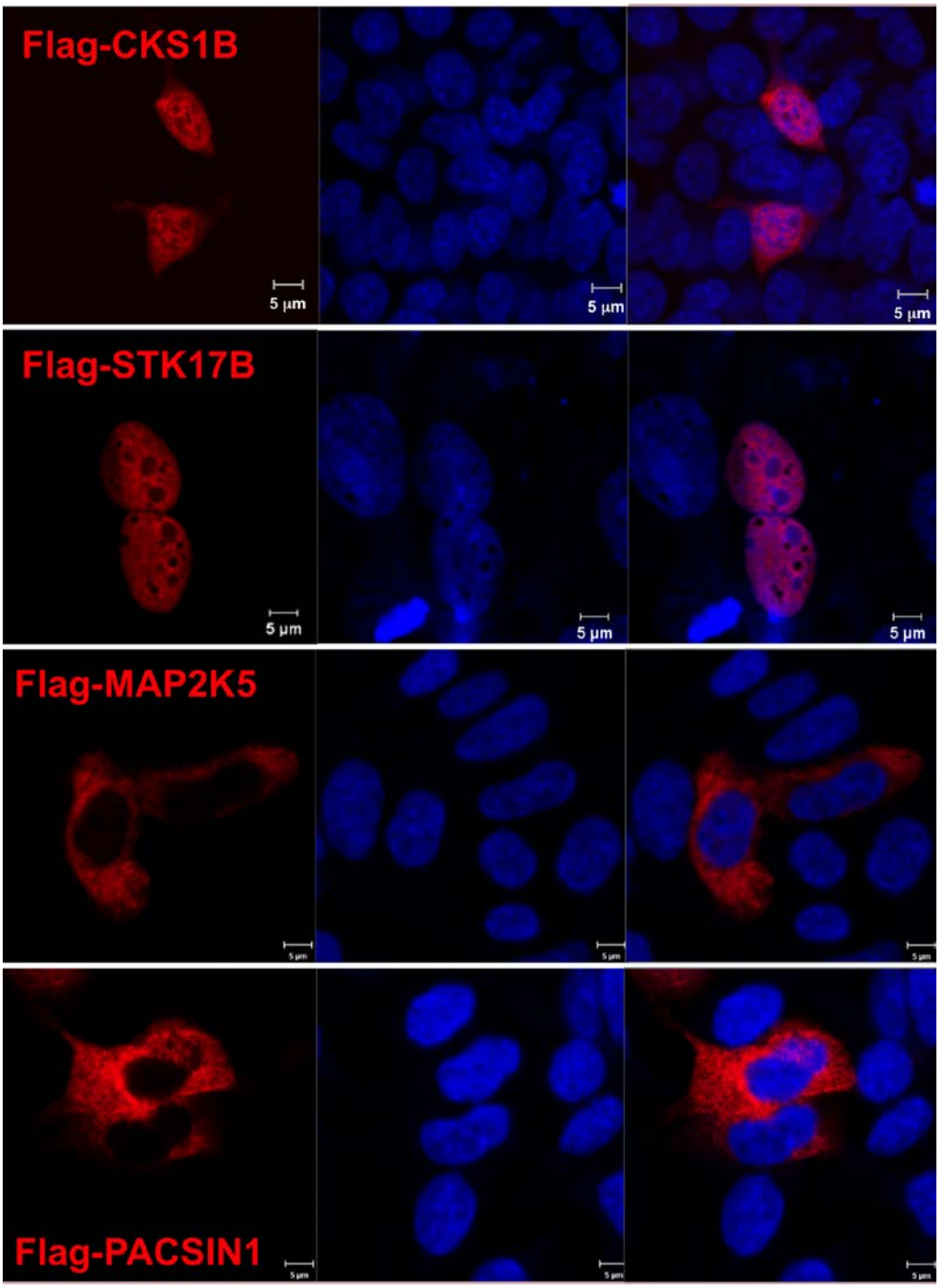


Figure 18. Cellular distribution of kinases. Flag tagged kinases were expressed in Lenti-X 293T cells and immunostained with anti-Flag antibody. Images were taken under a 40X objective using the Carl Zeiss Meta LSM510 laser scanning confocal microscope. Nuclei were stained by Draq 5 (blue). The data is a representation of at least three independent experiments.

4.10 MAP2K5 SURROUNDS LIPID DROPLETS

MAP2K5 showed only marginal inhibition in HCV RNA replication (Figure. 13), raising the possibility that it may have an effect on a post-transcription stage of HCV life cycle. Because of its unique cellular localization pattern, I subsequently explored its association with lipid droplets, the cellular organelle that is known to be important for virus assembly. Using a dye Bodipy (493/503) that specifically stains cellular lipids, I found that MAP2K5 surrounded lipid droplets in a manner very similar to HCV core protein (Figure 19 and 20).

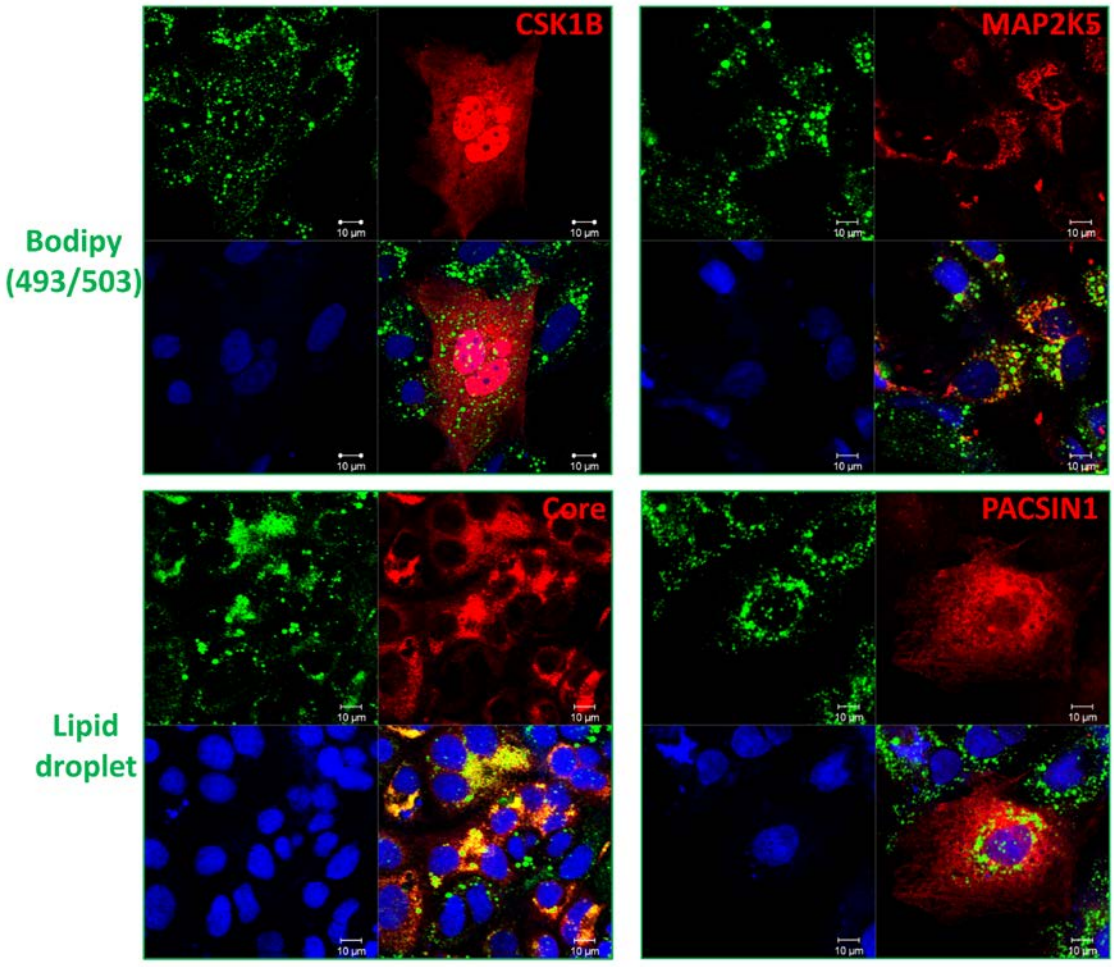


Figure 19. Localization of kinases with respect to lipid droplets. Flag-tagged CSK1B, MAP2K5 and PACSIN1 plasmid was transfected into Huh7.5.1 cells. Cells were fixed and stained for each kinase (red) and then lipid droplet (green). For reference, HCV-infected Huh7.5.1 cells were stained for Core and lipid droplet. The data represent two independent experiments.

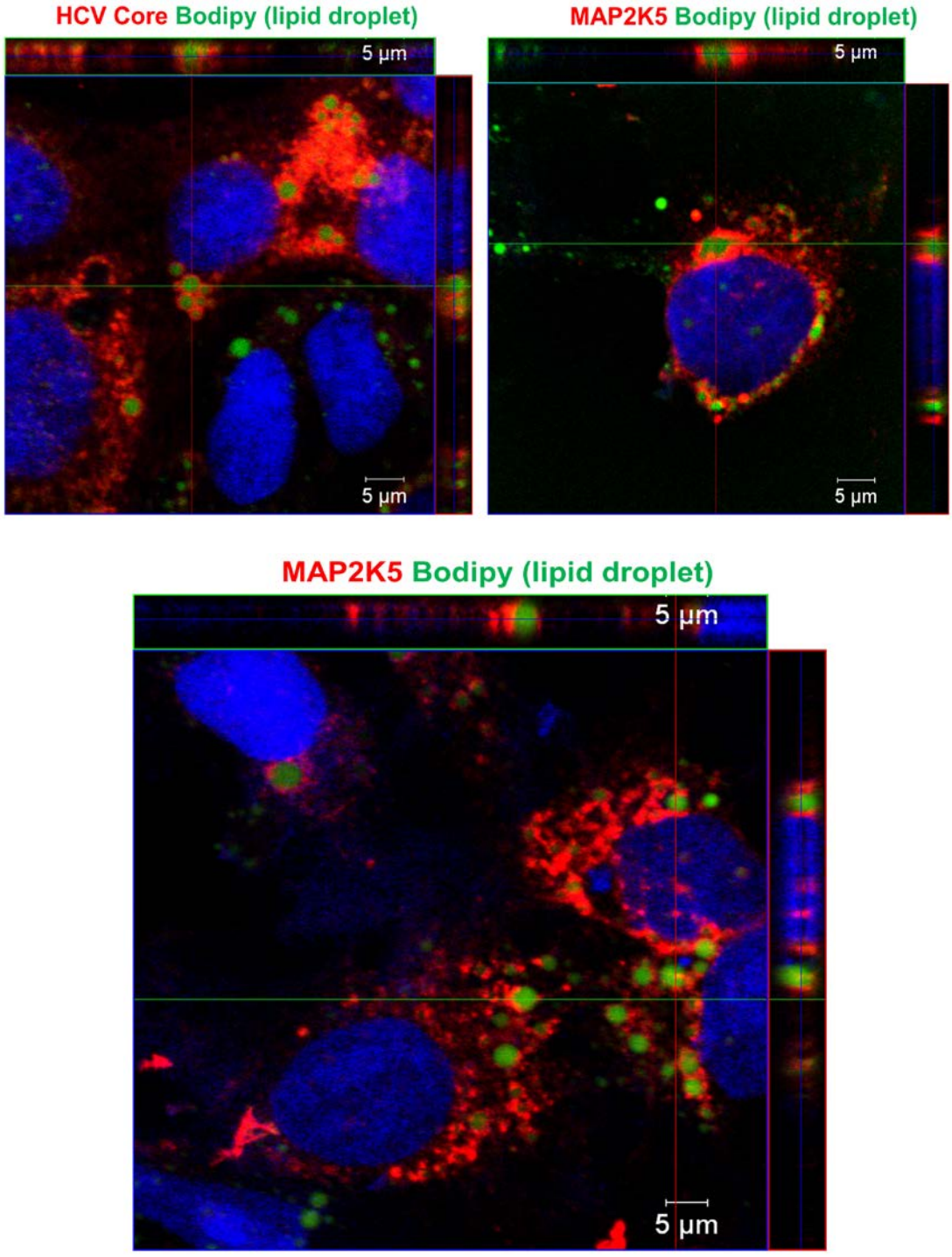


Figure 20. MAP2K5 surrounds lipid droplets. 3D scanning of MAP2K5 expressing Huh7.5.1 cells was performed. Diagonally projected images (XY, XZ and YZ) are shown here. Consistent with what has been reported, HCV core surrounded lipid droplets where the virus assembly occurs. Similarly, MAP2K5 appeared to cover the lipid droplets although this does not mean a direct interaction between them. The data represent two independent experiments.

4.11 PACSIN1 DECREASED THE LEVEL OF P7

Currently, specific functions to HCV viral proteins have been well documented except for the role of the small membrane polypeptide p7. While recent in vivo experiments indicate that p7 is essential for infection, subgenomic HCV replicons do not contain p7, illustrating that p7 is not necessary for RNA replication [114, 115]. Ectopically expressed, p7 is primarily localized in the endoplasmic reticulum as an integral membrane protein and display a topology with both N- and C- termini pointing towards the lumen of endoplasmic reticulum [114-116]. NMR experiments and molecular dynamics (MD) simulations have recently identified the secondary structure elements of p7, and to the construction of a three-dimentional model of the monomer in a phospholipid bilayer [114, 117, 118]. In vitro studies have also reported that p7 oligomerizes and functions to conduct ions across in artificial membrane systems [114, 118-120]. Experimentally, Steinmann and colleagues in 2007 reported that p7 was shown to be critical for virus assembly and release, but not essential for its infectivity [114]. Thus, while the basic structural features of p7 are becoming gradually known, the exact mechanism of p7 in viral assembly and release, and the conditions that lead to the assembly of a functional channel still remains to be better understood.

To investigate the possible effect of kinase expressions in viral protein production, I performed co-transfection experiment followed by Western blot analysis. Since PACSIN1 did not show any significant effect on viral replication nor did it co-localize with lipid droplets in previous experiments, we co-transfected PACSIN1 plasmid with the viral protein plasmids in 293T cells and see whether that alters the viral protein expression. All ten HCV viral proteins were co-transfected with PACSIN1 initially, although only p7 and NS4B are shown in figure 21. It was found that the presence of PACSIN1 decreased the expression of HCV p7 in a dose-

dependent manner. As a loading control β-actin was used and NS4B is included in the figure below to show that this effect was specific to p7 but no other viral proteins.

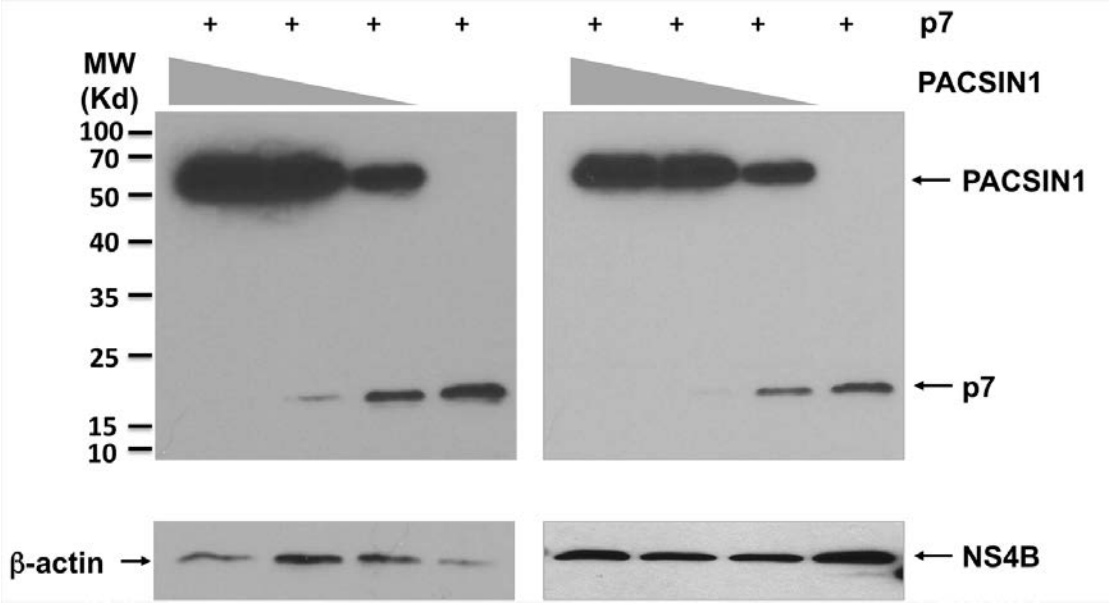


Figure 21. PACSIN1 decreased the level of p7. PACSIN1 and p7 were co-transfected in 293T cells, followed by Western blotting. As PACSIN1 expression decreased (0.5μg, 0.3μg and 0.2μg), p7 viral protein expression increased as shown above indicating inverse relationship. NS4B is shown as a control and β-actin is shown was a loading control. The Western blot on the left is an over-exposure to clearly illustrate the trend in p7 viral protein expression.

5.0 DISCUSSION

The discovery of reversible phosphorylation regulating the activity of glycogen phosphorylase has led a great interest in the role of protein phosphorylation in regulating protein function [63]. Since then, the near-completion of the human genome sequence has allowed for the identification of almost all human protein kinases [63]. To date there are approximately 500-600 known kinases and open reading frames (ORFs) that are important in regulating cellular processes. The protein kinases comprise a large group of enzymes that mostly catalyze the covalent attachment of a phosphate group to a protein, and in eukaryotic cells, protein kinases mediate most of the signal transduction by modification of substrate activity and control cellular processes including but not limited to metabolism, transcription, cell cycle progression, cell movement, apoptosis, differentiation, and protein phosphorylation [63].

HCV infections in particular have been linked to host kinases in the past, and here we postulated that these kinases could be the target to control HCV infections. The objective of this study was to screen for novel human kinases that may suppress HCV infection through a gain-of-function approach. This approach is different from the screens in the past, which were siRNA screens or chemical-inhibitor based screens (loss-of-function approach).

To screen for the kinases of interest, primary screening was performed in pools of eight kinases in each group. One may argue that packaging eight kinases instead of packaging individually may have brought issues in terms of plasmids competing with each other or not being packaged properly, however, it has been observed through previous reports and methods set in Wang's Laboratory, that it is possible to package eight kinases to produce a homogenous population of the virus expressing all eight kinases [121]. Also, the infection system we have described here is a set protocol known to work in our laboratory, which effectively infects naïve Huh 7.5.1 cells to express the kinase.

It was a concern that just because the kinases were packaged and delivered to naïve cells, it does not mean that the kinases are automatically activated. However, in this particular study, I assume that the kinases are activated because all kinase plasmids have a myristoylation sequence and as mentioned earlier, this myristoylation is required for almost all kinases to be activated and important for membrane association and recruitment of necessary factors. Also, the fact that I observed a significant inhibition of HCV infection from the primary screening, and downstream experiments to prove this, I am confident that these kinases are indeed activated. Note that in order for a definitive kinase assay however, whether the kinases are being activated or not, substrates of the kinases can be used to confirm this, but in our study, the three kinases CKS1B, MAP2K5 and PACSIN1 did not have any known substrates available. Another way of confirming the activation of kinases would have been to create kinase mutants, and create a mutation in the kinase domains, but in this study, we felt that this was beyond the scope of this thesis project. Yet another approach to confirm the kinase activation would have been to utilize kinase inhibitors, but to my knowledge there are no available inhibitors of CKS1B and

PACSIN1, while MAP2K5 inhibitor is rather non-specific. According to the original design, the kinases are activated in my study due to presence of the myristoylation sequence. As shown by the immunofluorescent stainings, I also demonstrated that these kinases suppress HCV infection, but not Dengue virus.

Further functional characterization revealed that the three kinases did not inhibit viral entry. However, CKS1B and MAP2K5 both suppressed viral RNA replication although to varied degrees. Moreover, MAP2K5 and PACSIN1 may have additional effects on a post-replication stage, which will be explored in the future study. At this point, I conclude that at least CSK1B primarily inhibits HCV by suppressing viral replication.

Experiments were also performed to explain the mechanism of how these activated kinases act to inhibit HCV infection. Since I observed the kinases regulating viral replication, previously reported relationship between HCV replication and kinases were investigated and these reports have indicated three mechanisms: (1) presence of IFN, (2) phosphorylation status of viral NS5A and (3) altered cell cycle. The interferon production assay and cell cycle analysis did not show any significant changes in the presence of activated kinases and although data are not shown, the potential effect of kinase activation on the phosphorylation status of HCV NS5A was also investigated. Previous reports have shown conflicting results and our study also showed conflicting and inconsistent data; therefore we ruled the possibility of the activated kinases acting upon NS5A phosphorylation in regulating HCV replication as inconclusive. It is possible that these kinases may be the cellular kinases that directly phosphorylate HCV NS5A *in vivo*, which can be evaluated in the future.

Because I failed to observe any significance in the aforementioned three main mechanisms that have been reported to regulate HCV replication, other possibilities were subsequently explored. Through confocal microscopy studies, I determined where these kinases are distributed in the cell and attempted to whether they co-localize with viral proteins. Although I have not seen co-localization of kinases with viral protein NS5A (data not shown), the immunofluorescent staining revealed that MAP2K5 localized to the cytoplasm and surrounded lipid droplets, which are known to be the sites of HCV assembly. This result suggests that MAP2K5 may have an effect on the viral assembly. Again, it is insufficient to conclude a direct impact on virus assembly by MAP2K5 at this stage, a bold and intriguing hypothesis, however, will be: MAP2K5 disrupts the virus assembly by phosphorylating a viral target or a host protein.

In regards to PACSIN1, our study shows that the overexpression of this kinase decreases p7 viral protein. The dose-dependent response clearly indicated that as PACSIN1 expression increases, p7 viral protein expression significantly decreased. Other viral protein also co-transfected in this experiment did not show any changes in response to PACSIN1. There are many recent reports indicating that p7 functions to conduct ions in artificial membrane systems and was experimentally shown to be critical for viral assembly and release. However, the exact role that p7 participates in viral assembly and release remains unclear at this time. Thus, we speculate that PACSIN1 may modulate HCV infection by down-regulating p7 through unknown mechanism.

6.0 FUTURE DIRECTIONS

In this project, kinases were thoroughly screened and studied for the mechanism behind how the kinases suppress HCV infection. Future experiments could include creating kinase mutants to confirm that the kinases used in this study are indeed activated. Also, mechanisms of how these kinases are inhibiting HCV infection can be further investigated. In this study, several experiments were performed in attempts to investigate kinase mechanism, such as interferon production, cell cycle analysis, NS5A phosphorylation and co-localization studies. However, there are other possible mechanisms of how kinases suppress HCV infection and these other possibilities may be further explored. For example, Immunoprecipitation could be done to investigate whether there is a direct interaction between the kinases and HCV viral proteins. Specific to MAP2K5, we showed that this kinase might disrupt the virus assembly by localizing to the outer surface of lipid droplets. This could be taken further to investigate its effect on virus assembly in relation to lipid droplets.

7.0 PUBLIC HEALTH RELEVANCE

HCV is a global concern and the treatments available to patients often times result in poor response due to severe side effects and poor patient compliance. Thus, the findings of novel kinases suppressing HCV infection reveal much potentials. One of these potentials of course is the recognition of previously unidentified inhibitory effects of HCV infection by kinases, but this also highlights the potential of combating HCV infection by activating specific kinase-mediated pathways. It is also my prediction that the kinase-mediated inhibition of HCV infection would be more specific as a treatment or target, compared to the treatments available today.

BIBLIOGRAPHY

1. Brown, R.S., *Hepatitis C and liver transplantation.* Nature, 2005. **436**(7053): p. 973-8.
2. Major, M.E. and S.M. Feinstone, *The molecular virology of hepatitis C.* Hepatology, 1997. **25**(6): p. 1527-38.
3. Sharma, S.D., *Hepatitis C virus: molecular biology & current therapeutic options.* Indian J Med Res, 2010. **131**: p. 17-34.
4. Seeff, L.B., *Natural history of chronic hepatitis C.* Hepatology, 2002. **36**(5 Suppl 1): p. S35-46.
5. Strader, D.B., et al., *Diagnosis, management, and treatment of hepatitis C.* Hepatology, 2004. **39**(4): p. 1147-71.
6. Boehm, J.S., et al., *Integrative genomic approaches identify IKBKE as a breast cancer oncogene.* Cell, 2007. **129**(6): p. 1065-79.
7. Rual, J.F., et al., *Human ORFeome version 1.1: a platform for reverse proteomics.* Genome Res, 2004. **14**(10B): p. 2128-35.
8. Herrmann, E., et al., *Hepatitis C virus kinetics.* Antivir Ther, 2000. **5**(2): p. 85-90.
9. Mondelli, M.U. and E. Silini, *Clinical significance of hepatitis C virus genotypes.* J Hepatol, 1999. **31 Suppl 1**: p. 65-70.
10. Schaefer, M., et al., *Hepatitis C infection, antiviral treatment and Mental Health: A European Expert Consensus Statement.* J Hepatol, 2012.
11. Butt, A.A., et al., *Reasons for non-treatment of hepatitis C in veterans in care.* J Viral Hepat, 2005. **12**(1): p. 81-5.
12. Manns, M.P., et al., *Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial.* Lancet, 2001. **358**(9286): p. 958-65.
13. Assis, D.N. and J.K. Lim, *New Pharmacotherapy for Hepatitis C.* Clin Pharmacol Ther, 2012.
14. Lindenbach, B.D. and C.M. Rice, *Molecular biology of flaviviruses.* Adv Virus Res, 2003. **59**: p. 23-61.
15. Murray, C.L., C.T. Jones, and C.M. Rice, *Architects of assembly: roles of Flaviviridae non-structural proteins in virion morphogenesis.* Nat Rev Microbiol, 2008. **6**(9): p. 699-708.
16. Scarselli, E., et al., *The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus.* EMBO J, 2002. **21**(19): p. 5017-25.
17. Blanchard, E., et al., *Hepatitis C virus entry depends on clathrin-mediated endocytosis.* J Virol, 2006. **80**(14): p. 6964-72.

18. Pileri, P., et al., *Binding of hepatitis C virus to CD81.* Science, 1998. **282**(5390): p. 938-41.
19. Owen, D.M., et al., *Apolipoprotein E on hepatitis C virion facilitates infection through interaction with low-density lipoprotein receptor.* Virology, 2009. **394**(1): p. 99-108.
20. Barth, H., et al., *Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate.* J Biol Chem, 2003. **278**(42): p. 41003-12.
21. Bartosch, B., et al., *Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor.* J Biol Chem, 2003. **278**(43): p. 41624-30.
22. Tscherne, D.M., et al., *Time- and temperature-dependent activation of hepatitis C virus for low-pH-triggered entry.* J Virol, 2006. **80**(4): p. 1734-41.
23. Moradpour, D., F. Penin, and C.M. Rice, *Replication of hepatitis C virus.* Nat Rev Microbiol, 2007. **5**(6): p. 453-63.
24. Egger, D., et al., *Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex.* J Virol, 2002. **76**(12): p. 5974-84.
25. Chang, M., et al., *Dynamics of hepatitis C virus replication in human liver.* Am J Pathol, 2003. **163**(2): p. 433-44.
26. Quinkert, D., R. Bartenschlager, and V. Lohmann, *Quantitative analysis of the hepatitis C virus replication complex.* J Virol, 2005. **79**(21): p. 13594-605.
27. Thomssen, R., et al., *Association of hepatitis C virus in human sera with beta-lipoprotein.* Med Microbiol Immunol, 1992. **181**(5): p. 293-300.
28. Herker, E. and M. Ott, *Emerging role of lipid droplets in host/pathogen interactions.* J Biol Chem, 2012. **287**(4): p. 2280-7.
29. Herker, E. and M. Ott, *Unique ties between hepatitis C virus replication and intracellular lipids.* Trends Endocrinol Metab, 2011. **22**(6): p. 241-8.
30. Gastaminza, P., S.B. Kapadia, and F.V. Chisari, *Differential biophysical properties of infectious intracellular and secreted hepatitis C virus particles.* J Virol, 2006. **80**(22): p. 11074-81.
31. Lindenbach, B.D., et al., *Cell culture-grown hepatitis C virus is infectious in vivo and can be recultured in vitro.* Proc Natl Acad Sci U S A, 2006. **103**(10): p. 3805-9.
32. Merz, A., et al., *Biochemical and morphological properties of hepatitis C virus particles and determination of their lipidome.* J Biol Chem, 2011. **286**(4): p. 3018-32.
33. Gastaminza, P., et al., *Ultrastructural and biophysical characterization of hepatitis C virus particles produced in cell culture.* J Virol, 2010. **84**(21): p. 10999-1009.
34. Meunier, J.C., et al., *Apolipoprotein c1 association with hepatitis C virus.* J Virol, 2008. **82**(19): p. 9647-56.
35. Chang, K.S., et al., *Human apolipoprotein e is required for infectivity and production of hepatitis C virus in cell culture.* J Virol, 2007. **81**(24): p. 13783-93.
36. Ye, J., *Reliance of host cholesterol metabolic pathways for the life cycle of hepatitis C virus.* PLoS Pathog, 2007. **3**(8): p. e108.
37. Fukasawa, M., *Cellular lipid droplets and hepatitis C virus life cycle.* Biol Pharm Bull, 2010. **33**(3): p. 355-9.
38. Miyanari, Y., et al., *The lipid droplet is an important organelle for hepatitis C virus production.* Nat Cell Biol, 2007. **9**(9): p. 1089-97.

39. Lerat, H., et al., *In vivo tropism of hepatitis C virus genomic sequences in hematopoietic cells: influence of viral load, viral genotype, and cell phenotype.* Blood, 1998. **91**(10): p. 3841-9.
40. Zignego, A.L. and C. Brechot, *Extrahepatic manifestations of HCV infection: facts and controversies.* J Hepatol, 1999. **31**(2): p. 369-76.
41. Kolykhalov, A.A., et al., *Transmission of hepatitis C by intrahepatic inoculation with transcribed RNA.* Science, 1997. **277**(5325): p. 570-4.
42. Lohmann, V., et al., *Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line.* Science, 1999. **285**(5424): p. 110-3.
43. Bartosch, B., J. Dubuisson, and F.L. Cosset, *Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes.* J Exp Med, 2003. **197**(5): p. 633-42.
44. Hsu, M., et al., *Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles.* Proc Natl Acad Sci U S A, 2003. **100**(12): p. 7271-6.
45. Wakita, T., et al., *Production of infectious hepatitis C virus in tissue culture from a cloned viral genome.* Nat Med, 2005. **11**(7): p. 791-6.
46. Zhong, J., et al., *Robust hepatitis C virus infection in vitro.* Proc Natl Acad Sci U S A, 2005. **102**(26): p. 9294-9.
47. Lindenbach, B.D., et al., *Complete replication of hepatitis C virus in cell culture.* Science, 2005. **309**(5734): p. 623-6.
48. Bartenschlager, R. and V. Lohmann, *Novel cell culture systems for the hepatitis C virus.* Antiviral Res, 2001. **52**(1): p. 1-17.
49. Krieger, N., V. Lohmann, and R. Bartenschlager, *Enhancement of hepatitis C virus RNA replication by cell culture-adaptive mutations.* J Virol, 2001. **75**(10): p. 4614-24.
50. Lohmann, V., et al., *Mutations in hepatitis C virus RNAs conferring cell culture adaptation.* J Virol, 2001. **75**(3): p. 1437-49.
51. Blight, K.J., J.A. McKeating, and C.M. Rice, *Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication.* J Virol, 2002. **76**(24): p. 13001-14.
52. Foy, E., et al., *Control of antiviral defenses through hepatitis C virus disruption of retinoic acid-inducible gene-I signaling.* Proc Natl Acad Sci U S A, 2005. **102**(8): p. 2986-91.
53. Pietschmann, T., et al., *Characterization of cell lines carrying self-replicating hepatitis C virus RNAs.* J Virol, 2001. **75**(3): p. 1252-64.
54. Pietschmann, T. and R. Bartenschlager, *The hepatitis C virus replicon system and its application to molecular studies.* Curr Opin Drug Discov Devel, 2001. **4**(5): p. 657-64.
55. Frese, M., et al., *Interferon-alpha inhibits hepatitis C virus subgenomic RNA replication by an MxA-independent pathway.* J Gen Virol, 2001. **82**(Pt 4): p. 723-33.
56. Guo, J.T., V.V. Bichko, and C. Seeger, *Effect of alpha interferon on the hepatitis C virus replicon.* J Virol, 2001. **75**(18): p. 8516-23.
57. Ikeda, M., et al., *Selectable subgenomic and genome-length dicistronic RNAs derived from an infectious molecular clone of the HCV-N strain of hepatitis C virus replicate efficiently in cultured Huh7 cells.* J Virol, 2002. **76**(6): p. 2997-3006.
58. Ait-Goughoulte, M., et al., *Hepatitis C virus genotype 1a growth and induction of autophagy.* J Virol, 2008. **82**(5): p. 2241-9.

59. Yanagi, M., et al., *Transcripts from a single full-length cDNA clone of hepatitis C virus are infectious when directly transfected into the liver of a chimpanzee.* Proc Natl Acad Sci U S A, 1997. **94**(16): p. 8738-43.
60. Blight, K.J., et al., *Efficient replication of hepatitis C virus genotype 1a RNAs in cell culture.* J Virol, 2003. **77**(5): p. 3181-90.
61. Date, T., et al., *Genotype 2a hepatitis C virus subgenomic replicon can replicate in HepG2 and IMY-N9 cells.* J Biol Chem, 2004. **279**(21): p. 22371-6.
62. Kato, T., et al., *Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon.* Gastroenterology, 2003. **125**(6): p. 1808-17.
63. Manning, G., et al., *The protein kinase complement of the human genome.* Science, 2002. **298**(5600): p. 1912-34.
64. Hanks, S.K. and T. Hunter, *Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification.* FASEB J, 1995. **9**(8): p. 576-96.
65. Martin, D.D., E. Beauchamp, and L.G. Berthiaume, *Post-translational myristoylation: Fat matters in cellular life and death.* Biochimie, 2011. **93**(1): p. 18-31.
66. Boutin, J.A., *Myristoylation.* Cell Signal, 1997. **9**(1): p. 15-35.
67. Farazi, T.A., G. Waksman, and J.I. Gordon, *The biology and enzymology of protein N-myristoylation.* J Biol Chem, 2001. **276**(43): p. 39501-4.
68. Carr, S.A., et al., *n-Tetradecanoyl is the NH2-terminal blocking group of the catalytic subunit of cyclic AMP-dependent protein kinase from bovine cardiac muscle.* Proc Natl Acad Sci U S A, 1982. **79**(20): p. 6128-31.
69. Aitken, A., et al., *Identification of the NH2-terminal blocking group of calcineurin B as myristic acid.* FEBS Lett, 1982. **150**(2): p. 314-8.
70. Wilcox, C., J.S. Hu, and E.N. Olson, *Acylation of proteins with myristic acid occurs cotranslationally.* Science, 1987. **238**(4831): p. 1275-8.
71. Deichaite, I., et al., *In vitro synthesis of pp60v-src: myristylation in a cell-free system.* Mol Cell Biol, 1988. **8**(10): p. 4295-301.
72. Martin, D.D., et al., *Rapid detection, discovery, and identification of post-translationally myristoylated proteins during apoptosis using a bio-orthogonal azidomyristate analog.* FASEB J, 2008. **22**(3): p. 797-806.
73. Sakurai, N., et al., *Detection of co- and posttranslational protein N-myristoylation by metabolic labeling in an insect cell-free protein synthesis system.* Anal Biochem, 2007. **362**(2): p. 236-44.
74. Sakurai, N. and T. Utsumi, *Posttranslational N-myristoylation is required for the anti-apoptotic activity of human tGelsolin, the C-terminal caspase cleavage product of human gelsolin.* J Biol Chem, 2006. **281**(20): p. 14288-95.
75. Yap, M.C., et al., *Rapid and selective detection of fatty acylated proteins using omega-alkynyl-fatty acids and click chemistry.* J Lipid Res, 2010. **51**(6): p. 1566-80.
76. Bhatnagar, R.S., et al., *The structure of myristoyl-CoA:protein N-myristoyltransferase.* Biochim Biophys Acta, 1999. **1441**(2-3): p. 162-72.
77. Johnson, D.R., et al., *Genetic and biochemical studies of protein N-myristoylation.* Annu Rev Biochem, 1994. **63**: p. 869-914.
78. McCabe, J.B. and L.G. Berthiaume, *Functional roles for fatty acylated amino-terminal domains in subcellular localization.* Mol Biol Cell, 1999. **10**(11): p. 3771-86.
79. Resh, M.D., *A myristoyl switch regulates membrane binding of HIV-1 Gag.* Proc Natl Acad Sci U S A, 2004. **101**(2): p. 417-8.

80. Provitera, P., R. El-Maghrabi, and S. Scarlata, *The effect of HIV-1 Gag myristoylation on membrane binding.* Biophys Chem, 2006. **119**(1): p. 23-32.
81. Martin, S.S., et al., *Phosphatidylinositol 3-kinase is necessary and sufficient for insulin-stimulated stress fiber breakdown.* Endocrinology, 1996. **137**(11): p. 5045-54.
82. Tanti, J.F., et al., *Overexpression of a constitutively active form of phosphatidylinositol 3-kinase is sufficient to promote Glut 4 translocation in adipocytes.* J Biol Chem, 1996. **271**(41): p. 25227-32.
83. Klippel, A., et al., *Membrane localization of phosphatidylinositol 3-kinase is sufficient to activate multiple signal-transducing kinase pathways.* Mol Cell Biol, 1996. **16**(8): p. 4117-27.
84. Martin, S.S., et al., *Activated phosphatidylinositol 3-kinase is sufficient to mediate actin rearrangement and GLUT4 translocation in 3T3-L1 adipocytes.* J Biol Chem, 1996. **271**(30): p. 17605-8.
85. Kohn, A.D., et al., *Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation.* J Biol Chem, 1996. **271**(49): p. 31372-8.
86. Kohn, A.D., F. Takeuchi, and R.A. Roth, *Akt, a pleckstrin homology domain containing kinase, is activated primarily by phosphorylation.* J Biol Chem, 1996. **271**(36): p. 21920-6.
87. Reiss, S., et al., *Recruitment and activation of a lipid kinase by hepatitis C virus NS5A is essential for integrity of the membranous replication compartment.* Cell Host Microbe, 2011. **9**(1): p. 32-45.
88. Berger, K.L., et al., *Roles for endocytic trafficking and phosphatidylinositol 4-kinase III alpha in hepatitis C virus replication.* Proc Natl Acad Sci U S A, 2009. **106**(18): p. 7577-82.
89. Foy, E., et al., *Regulation of interferon regulatory factor-3 by the hepatitis C virus serine protease.* Science, 2003. **300**(5622): p. 1145-8.
90. Tellinghuisen, T.L., K.L. Foss, and J. Treadaway, *Regulation of hepatitis C virion production via phosphorylation of the NS5A protein.* PLoS Pathog, 2008. **4**(3): p. e1000032.
91. Qiu, D., et al., *The effects of NS5A inhibitors on NS5A phosphorylation, polyprotein processing and localization.* J Gen Virol, 2011. **92**(Pt 11): p. 2502-11.
92. Quintavalle, M., et al., *Hepatitis C virus NS5A is a direct substrate of casein kinase I-alpha, a cellular kinase identified by inhibitor affinity chromatography using specific NS5A hyperphosphorylation inhibitors.* J Biol Chem, 2007. **282**(8): p. 5536-44.
93. Utermark, T., et al., *The p110alpha isoform of phosphatidylinositol 3-kinase is essential for polyomavirus middle T antigen-mediated transformation.* J Virol, 2007. **81**(13): p. 7069-76.
94. Cabibbo, G., et al., *Causes of and prevention strategies for hepatocellular carcinoma.* Semin Oncol, 2012. **39**(4): p. 374-83.
95. Tai, A.W., et al., *A functional genomic screen identifies cellular cofactors of hepatitis C virus replication.* Cell Host Microbe, 2009. **5**(3): p. 298-307.
96. Vaillancourt, F.H., et al., *Identification of a lipid kinase as a host factor involved in hepatitis C virus RNA replication.* Virology, 2009. **387**(1): p. 5-10.
97. Borawski, J., et al., *Class III phosphatidylinositol 4-kinase alpha and beta are novel host factor regulators of hepatitis C virus replication.* J Virol, 2009. **83**(19): p. 10058-74.

98. Coller, K.E., et al., *RNA interference and single particle tracking analysis of hepatitis C virus endocytosis.* PLoS Pathog, 2009. **5**(12): p. e1000702.
99. Li, Q., et al., *A genome-wide genetic screen for host factors required for hepatitis C virus propagation.* Proc Natl Acad Sci U S A, 2009. **106**(38): p. 16410-5.
100. Hsu, N.Y., et al., *Viral reorganization of the secretory pathway generates distinct organelles for RNA replication.* Cell, 2010. **141**(5): p. 799-811.
101. Evans, M.J., C.M. Rice, and S.P. Goff, *Phosphorylation of hepatitis C virus nonstructural protein 5A modulates its protein interactions and viral RNA replication.* Proc Natl Acad Sci U S A, 2004. **101**(35): p. 13038-43.
102. Liu, S., et al., *Human apolipoprotein E peptides inhibit hepatitis C virus entry by blocking virus binding.* Hepatology, 2012. **56**(2): p. 484-91.
103. Adam, M.A., et al., *Internal initiation of translation in retroviral vectors carrying picornavirus 5' nontranslated regions.* J Virol, 1991. **65**(9): p. 4985-90.
104. Griffiths, J.C., et al., *Induction of high-titer neutralizing antibodies, using hybrid human immunodeficiency virus V3-Ty viruslike particles in a clinically relevant adjuvant.* J Virol, 1991. **65**(1): p. 450-6.
105. Choate, K.A., et al., *Transglutaminase 1 delivery to lamellar ichthyosis keratinocytes.* Hum Gene Ther, 1996. **7**(18): p. 2247-53.
106. Kinsella, T.M. and G.P. Nolan, *Episomal vectors rapidly and stably produce high-titer recombinant retrovirus.* Hum Gene Ther, 1996. **7**(12): p. 1405-13.
107. Ory, D.S., B.A. Neugeboren, and R.C. Mulligan, *A stable human-derived packaging cell line for production of high titer retrovirus/vesicular stomatitis virus G pseudotypes.* Proc Natl Acad Sci U S A, 1996. **93**(21): p. 11400-6.
108. Seth, R.B., et al., *Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3.* Cell, 2005. **122**(5): p. 669-82.
109. Biswas, N., et al., *The ubiquitin-like protein PLIC-1 or ubiquilin 1 inhibits TLR3-Trif signaling.* PLoS One, 2011. **6**(6): p. e21153.
110. Zhan, F., et al., *CKS1B, overexpressed in aggressive disease, regulates multiple myeloma growth and survival through SKP2- and p27Kip1-dependent and -independent mechanisms.* Blood, 2007. **109**(11): p. 4995-5001.
111. Martin-Ezquerra, G., et al., *CDC28 protein kinase regulatory subunit 1B (CKS1B) expression and genetic status analysis in oral squamous cell carcinoma.* Histol Histopathol, 2011. **26**(1): p. 71-7.
112. Scholle, F., et al., *Virus-host cell interactions during hepatitis C virus RNA replication: impact of polyprotein expression on the cellular transcriptome and cell cycle association with viral RNA synthesis.* J Virol, 2004. **78**(3): p. 1513-24.
113. Kannan, R.P., et al., *Hepatitis C virus infection causes cell cycle arrest at the level of initiation of mitosis.* J Virol, 2011. **85**(16): p. 7989-8001.
114. Steinmann, E., et al., *Hepatitis C virus p7 protein is crucial for assembly and release of infectious virions.* PLoS Pathog, 2007. **3**(7): p. e103.
115. Griffin, S.D., et al., *A conserved basic loop in hepatitis C virus p7 protein is required for amantadine-sensitive ion channel activity in mammalian cells but is dispensable for localization to mitochondria.* J Gen Virol, 2004. **85**(Pt 2): p. 451-61.
116. Carrere-Kremer, S., et al., *Subcellular localization and topology of the p7 polypeptide of hepatitis C virus.* J Virol, 2002. **76**(8): p. 3720-30.

117. Cook, G.A. and S.J. Opella, *NMR studies of p7 protein from hepatitis C virus.* Eur Biophys J, 2010. **39**(7): p. 1097-104.
118. Montserret, R., et al., *NMR structure and ion channel activity of the p7 protein from hepatitis C virus.* J Biol Chem, 2010. **285**(41): p. 31446-61.
119. Griffin, S.D., et al., *The p7 protein of hepatitis C virus forms an ion channel that is blocked by the antiviral drug, Amantadine.* FEBS Lett, 2003. **535**(1-3): p. 34-8.
120. Haqshenas, G., et al., *Hepatitis C virus p7 protein is localized in the endoplasmic reticulum when it is encoded by a replication-competent genome.* J Gen Virol, 2007. **88**(Pt 1): p. 134-42.
121. Hutchinson, E.C., et al., *Genome packaging in influenza A virus.* J Gen Virol, 2010. **91**(Pt 2): p. 313-28.