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Publication Date
2017

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Elucidating Hepatitis C Virus Core Interactions with the Host Environment, Specifically Phosphorylated IkappaB-alpha

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biology

by

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2017
This Dissertation of Wesley Robert Williams is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

San Diego State University

2017
DEDICATION

I would like to dedicate this work to all my friends and family who supported me throughout the years. Thank you all for being there for me. I hope one day I can repay the favor.
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ACKNOWLEDGEMENTS

First I would like to acknowledge my mentor, Dr. Roland Wolkowicz for supporting me, and guiding me on this journey. It was difficult at times, but his belief in me gave me perseverance and confidence. I have never known a man with as much passion or someone who is willing to give everyone a second chance. I truly hope that some of his enthusiasm has transferred to me.

I would also like to thank the rest of my committee members Dr. David Traver, Dr. Michael David, Dr. Ralph Feuer, and Dr. Tom Huxford. Without their input and guidance, I would not have accomplished so much. I always felt supported, and am honored they are at part of my committee.

Lastly, I would like to thank my friends and family for their support for all these years. It has been difficult, but they have always been there to pick me up when I felt low. My girlfriend, Nicolle has been incredibly supportive and understanding during this whole process. She has been the rock I can rely upon. Thank you all for being a part of the process. I hope I can inspire others as much as I have been.
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PUBLICATIONS


ABSTRACT OF THE DISSERTATION

Elucidating Hepatitis C Virus Core Interactions with the Host Environment, Specifically Phosphorylated IκBα

by

Wesley Robert Williams

Doctor of Philosophy in Biology

University of California, San Diego, 2017
San Diego State University, 2017

Professor Roland Wolkowicz, Chair

Hepatitis C Virus (HCV) is a blood-borne virus found worldwide, though most prevalent in third world countries. Currently, there is no vaccine and chronic infection can lead to hepatocellular carcinoma and liver cirrhosis. Our lab has shown upon HCV infection there is an increase in phosphorylated IκBα (p-IκBα) at five days post infection. This is a surprising as p-IκBα is canonically degraded by the 26S proteasome during activation of the NFκB pathway. In this dissertation, we corroborate that HCV infection does increase p-IκBα at five days post infection, but in addition, discovered p-IκBα locates to the nucleus. This is
interesting as p-IκBα is canonically not found in the nucleus. To elucidate what viral protein is responsible for this phenomenon the mature and immature version of the capsid protein, also known as core, were analyzed due to its ability to interact with host pathways. We discovered not only does the presence of mature version increase the expression of p-IκBα in the nucleus, but it also physically interacts with it. Taken together, these events could be an important part of the viral life cycle, and is a promising possible target for therapeutics.
CHAPTER 1

INTRODUCTION

Background

Hepatitis C Virus (HCV) is found worldwide with roughly 200 million people chronically infected. Central, East Asia, North Africa, and the Middle East have the highest infection rates. It is estimated that roughly 350,000 people die each year from HCV related symptoms\(^1\) with 17,000 new cases of HCV in the United States each year\(^2\). Transmission of the virus is largely through intravenous drug use in developed areas, while blood transfusions in developing countries are the main culprit. Transmission can also occur from mother to child and through sexual intercourse, though the latter is much less likely to occur\(^3\),\(^4\).

Those infected with HCV have a roughly 50-80% of being chronically infected\(^5\). Those chronically infected are at increased risk for developing cirrhosis of the liver and hepatocellular carcinoma\(^6\). While primary associated with the liver, HCV also has off site effects on the kidney such as Type 2 cryoglobulinemic glomerulopathy (CG)\(^7\), it also increases the chance of diabetes\(^8,9\) by affecting the glucose metabolism pathway\(^10\).

Currently there is no known vaccine for HCV infection, though treatment does exist. The classical treatment includes pegylated interferon and antivirals such as Ribavirin (nucleoside inhibitor) and Boceprevir (protease inhibitor) with new drugs, Sofosbuvir and Simeprevir (protease inhibitors) having been approved in 2013. The current approach is to treat the patient with a regiment of
drugs depending on the HCV subtype. This can range from a 24 to a 48 week treatment plan. While new direct acting anti-viral therapies are quite effective, there is still much unknown about the viral life cycle which presents the opportunity of finding other novel and effective treatment solutions. The expensive treatment regiments (with some treatments costing up to hundreds of thousands of dollar per year) coupled with the high mutation rate inherent of RNA viruses, can quickly cause a treatment to become ineffective or impractical. This results in further research needed to combat HCV in the future.

Figure 1.1 HCV Genome. The HCV genome and protein cleavage products from host and viral proteases. The scissors represent host cleavage (A). The viral proteins imbedded into the ER (B).
HCV Virus

HCV is a RNA single stranded, positive sense virus and part of the *Flaviviridae* family. Its genome consists of short non-translated regions (NTRs) found at either end that are important for replication and translation. It also has a 5' internal ribosome entry site (IRES) which allows the virus to translate independently of a 5' cap. HCV enters the cell utilizing various receptors, primarily CD-81 and Claudin-1, though other receptors such as LDL receptor, SR-BI, DC-SIGN, and Occludin also play a role.

Once HCV enters the cell, the genome is decapsidated and translated by the host into a single poly-protein, which is transported and embedded to the endoplasmic reticulum (ER) for further processing into its subsequent viral proteins. In the ER, host peptidases cleave the structural proteins (core, E1, E2, and P7) while the non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) are cleaved by virus protease.

Core is the capsid protein of the virus and forms a dimer in the nucleocapsid. It has also shown to interact with different host pathways, such as TNFα mediated apoptosis, and the ability to amount an immune response via the decreased ability of lymphoid dendritic cells to stimulate allogeneic T cells. E1 and E2 are the two glycoproteins that form a heterodimer and the envelope of the virus. P7 is utilized in viral assembly and involved in releasing viral particles. NS2/3 is the first of two viral proteases of HCV and is responsible for the cleavage of the NS2 and NS3 boundary, though there is still much
unknown about this protease, it is believed to be a cysteine or metalloprotease based on assays involving stimulation with zinc and amino acid motifs 27. The other viral protease is the serine protease NS3/4A, with NS3 as the catalytic unit and NS4A a cofactor, which is responsible for downstream viral protein cleavages 28 29. NS4B associates with the viral polyprotein within the ER 30 and plays important roles in cell signaling pathways, including the induction of ER stress via the transcription factor, ATF6 31. NS5B is the RNA-dependent RNA polymerase needed for replication of the viral genome, though this precise process is still unclear 32, it does interact with other viral proteins including NS3 which plays a part in reducing NS5B RNA synthesis activity 33. Interestingly, there are also alternate reading frame proteins (ARFPs) that are generated during translation, deriving from conserved core regions 34 and generated because of ribosomal frame shift. ARFP’s function is involved in viral persistence 35 and has been linked to repressing interferon responses 36. Following post-translation processes, the virus is assembled with association of lipid droplets 37 and released from the host.

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**Figure 1.2 Mature and Immature core.** Core is first cleaved by host signal peptidase to produce immature core, followed by signal peptide peptidase to produce mature core. Each domain of core is labeled as ‘1’, ‘2’ and ‘3’. Cleavage is performed in the ER by host proteases.
HCV Core

Core is the capsid protein of HCV where it forms a dimer inside of the viral capsid and on the ER \(^{38}\). It is unique as there are two forms, an ‘immature’ form that is made of 191 amino acids, and a ‘mature’ form which is processed from the ‘immature’ form and is estimated to be made up of 177 amino acids \(^{39}\). The HCV polypeptide is embedded in the ER and first cleaved by host signal peptidases (SP) at the boundary of E1 and core to produce an ‘immature’ core. A subsequent cleavage event by host signal peptide peptidases (SPP) cleaves ‘immature’ core into ‘mature’ core and releases it from the ER (Figure 1.2). This ‘mature’ form localizes to lipid droplets where it is involved in packaging of the viral genome \(^{20}\). The mature version of core is the most prominent species during viral infection and processing of immature must happen before generation of the mature version \(^{40}\).

It is composed of three domains, with the first domain having RNA binding capabilities and containing nuclear localization signals (NLS) \(^{41}\). The second domain associates with lipid droplets, and the last domain containing a signal sequence for the viral E1 protein \(^{42}\) used in translocation to the ER (Figure 1.2).

An interesting aspect of core is its ability to interact with different host pathways. This includes lipid genesis, apoptosis, cell signaling \(^{43}\) and localizing within the nucleus \(^{44}\). The multi-interaction aspects of core comes from an intrinsic disordered segment within the first domain where mapped interactions
have taken place utilizing precipitation assays. Due to the multi-functional nature of core with the host, it serves as a target of investigation for insight into therapeutic treatment.

**HCV and Host Signaling**

HCV virus utilizes various host cell machinery and signaling cascades to successfully propagate during its life cycle. An important pathway, and one that will be focused on in this dissertation, is the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) pathway.

Classically, the NFκB pathway can be activated through receptor-mediated interactions via pro-inflammatory cytokines like tumor necrosis factor alpha (TNFα) or interleukin-1 beta (IL-1β). NFκB forms a complex that can be composed as a hetero- or homodimer of RelA (p65), RelB, cRel, p50, and p52 proteins. During activation, IκB kinase (IKK), which is composed of IKKα, IKKβ, and NEMO (IKKγ) is phosphorylated by upstream kinases and in turn phosphorylates IκBα (p-IκBα), the inhibitor of NFκB. Phosphorylation of IκBα is of critical importance for NFκB cascade and is a prerequisite for the subsequent ubiquitination and recognition by the host 26S proteasome degradation machinery (Figure 1.3). It is this degradation that results in the dissociation from NFκB, which exposes the nuclear localization signal, allowing travel to the nucleus to drive expression of NFκB targeted genes.
One of these targeted genes is IκBα; when transcribed and translated, will translocate to the nucleus in an unphosphorylated form. Here it will reassociate with NFκB, masking its nuclear localization signal and export it back into the cytoplasm in an inactive state ready for the next activation event. This reassociation of IκBα allows for quick activation and deactivation to immune responses and creates its own negative feedback loop. It is important to note for this dissertation that phosphorylated IκBα is not found in the nucleus, only unphosphorylated IκBα, as phosphorylated IκBα is slated for degradation as previously mentioned.

Though current studies agree that HCV core does have an effect on the NFκB cascade it is still unclear the exact role, as there is evidence it can both suppress and activate. In addition to the NFκB cascade, core has also been implicit in the activation of the mitogen-activated protein (MAP) kinases p38, JNK, and extracellular signal-regulated kinase (ERK). Along with other viral proteins, such as NS5B, core has also been shown to down regulate STAT1, STAT2, and STAT3 in a viral infection.

In addition to core, NS3 and its cofactor NS4A have shown to influence host cascades as well. Specifically, it has been shown to hinder Toll-like receptor-3 (TLR-3) and retinoic acid-inducible gene 1 (RIG-I) by cleaving the TIR-domain-containing adapter-inducing interferon (TRIF) and mitochondrial antiviral-signaling protein (MAVS). Both NS3 and NS4A also are associated with activating the NFκB cascade.
Expression of NS5A has been to show to interact with PKR, a double strand RNA sensor in the cell, and leads to a decrease of interferon production. It is also known to affect the MAPK pathways and cellular signaling by both pro- and anti-apoptotic mechanisms, and interfering with ROS pathways and phosphatidylinositol 3-kinase signaling pathways\textsuperscript{57}.

In our lab, preliminary data demonstrates that HCV infection increased expression of p-IkBα and localization to the nucleus at five days post infection. This suggests that not only does HCV infection have a part to play in this aspect of the NFκB pathway, but does so in a temporal manner. This is intriguing because this would be the first instance we know of IkBα being found in the nucleus in a phosphorylated state.

Figure 1.3 **Classical NFκB activation.** The classical NFκB activation pathway upon receptor activation showing p-IkBα being degraded upon phosphorylation. This is performed by the host proteasomal machinery\textsuperscript{42}. RelA and p50 are used as an example.
GOALS OF DISSERTATION

This dissertation seeks to understand the phenomena of Hepatitis C virus and its capsid protein, core, in the role of preservation of phosphorylated nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor (p-IkBα) in the nucleus.

We hypothesize that core of HCV infection is ‘rescuing’ p-IkBα from degradation, and subsequently transported to the nucleus. We plan on expanding preliminarily studies looking at p-IkBα expression five days post infection and looking at core as the possible viral protein responsible.

Recently, we have acquired a JFH1-eGFP virus, which contains enhanced green fluorescent protein in the NS5A protein of the virus kindly provided by Dr. Hagedorn’s group. This virus has shown to have similar growth and infection kinetics as wild type virus\textsuperscript{58}, providing a visual method to monitor the virus. This virus will provide a clear and robust method to follow the infection life cycle and events, which is beneficial for a multitude of assays.

We predict that the viral capsid protein, core, is responsible because of its multi-functional nature and its implication in host signaling. We have designed constructs that express the two versions of core (‘immature’ and ‘mature’) with tagged and untagged versions utilizing a hemagglutinin (HA) tag. This will allow for increased efficiency in detection, and possible immunoprecipitation. These constructs will also be designed for expression in an inducible method utilizing the TET on/off system. Briefly, expression is controlled by the addition of
tetracycline (or a derivative, such as doxycycline) and the reverse tetracycline trans activator (rtTA) to drive expression. This gives the flexibility to control the expression of core in a timely fashion which could be crucial because of temporal events that occurs throughout the viral life cycle. These constructs will be expressed into different mammalian cells to analyze cell specificity, and analyze for signaling expression profiles at different time points. To expand upon these studies, a proteasomal inhibitor will also be utilized to determine if core is increasing p-IκBα expression or indeed ‘rescuing’ it from degradation. To help mimic a viral infection’s effect on the NFκB pathway without infection, stimulation using cytokines in conjunction with the expression of core will be performed. This will determine if an activated NFκB pathway is needed for translocation of p-IκBα to the nucleus in the presence of core.

Streptavidin binding peptide (SBP) tagged core constructs for the purpose of Co-immunoprecipitation (Co-IP) have been constructed. A SBP tag is utilized for its ease at precipitation assays. These Co-IP samples will be analyzed for potential binding partners of core, specifically, p-IκBα.
Aim 1: Determine if HCV infection increases expression of p-IκBα five days after infection

1a: Infect Huh7.5.1 cells with JFH1-eGFP virus and analyze p-IκBα expression via western blot and flow cytometry

Aim 2: Confirm location of p-IκBα to the nucleus upon JFH1 infection.

2a: Separate cytosol from nuclear extracts and analyze via western blot

2b: Utilize microscopy to determine localization of p-IκBα after infection.

Aim 3: Express HCV core in vitro to determine expression and location of core and p-IκBα.

3a: Develop constructs that express different tagged versions of core.

3b: Determine localization of core upon transfection.

3c: Look at expression levels and localization of p-IκBα after core transfection.

3d: Utilize a proteasomal inhibitor to increase the potential signal of p-IκBα after infection.

3e: Stimulate cells in conjunction with transfection to stimulate infection.

Aim 4: Utilize Co-immunoprecipitation (Co-IP) with core constructs to determine binding partners

4a: Construct SBP tagged versions of core and develop cell lines.

4b: Stain for binding partners.

4c: Compare to chikungunya capsid
CHAPTER 2
PREFACE

Chapter two focuses on investigating the increased expression and location of p-IκBα at different times post infection with HCV. In order to explore this more fully, I’ve expanded previous experiments to include fractionation assays and fluorescent microscopy to elucidate this event. Using this approach, I demonstrate that HCV is increasing p-IκBα expression and it is found in the nucleus five days post infection. These results raise an interesting phenomenon of how and why the virus is ‘rescuing’ p-IκBα from being degraded and how is it associated with its translocation to the nucleus. In the classical NFκB pathway, p-IκBα will be phosphorylated by IκB kinase (IKK) which will eventually lead to ubiquitination of IκBα and subsequent degradation by the 26S proteasome and releasing NFκB to travel to the nucleus. Here we show that might not be the entire story with HCV infection, and possibly could be utilized by the virus.

Why the virus is preserving p-IκBα from degradation and translocating it to the nucleus is still being investigated, but a possible theory is the virus is trying to sequester or inhibit p-IκBα in the nucleus to keep it from activating downstream its targets. This could prove extremely beneficial for the viral life cycle as a less activate immune response would give the virus an advantage to replicate and propagate.
We have also theorized that core may utilize IκBα as a vehicle to get into the nucleus of the cell, though why is still up to speculation.
RESULTS

_Infection utilizing JFH1-eGFP and its effect on p-IκBα Expression_

We have acquired a JFH1-eGFP virus that expresses eGFP in the NS5 protein of the virus, which was a generous gift by the Dr. Hagedorn group. This allows the direct visualization of infection in tissue culture or other detection assays, and provides a much faster and robust method to visualize viral infection. As previously mentioned, this virus has been shown to have similar infectivity and replication kinetics as wild type JFH1. All infection studies were done with the HCV permissive liver cell line, Huh7.5.1. Briefly, this cell line has a defective RIG-I pathway and possible increase expression of virus (co) receptors on the surface. Huh7.5.1 cells were infected with JFH1-eGFP virus and analyzed at various time points. As can be seen via fluorescent microscopy (Fig. 2.1), five days post infection yields visible eGFP expression by the virus in Huh7.5.1 cells, indicating

![Image of fluorescence microscopy results](image)

Figure 2.1. **Post five day infection of Huh7.5.1 cells with JFH1-eGFP.** (A) Uninfected Huh7.5.1 cells stained with DAPI. (B) Expression of eGFP from JFH1-eGFP five days post infection. (C) Merge of (A) and (B).
that successfully viral replication has taken place. The visual expression of infection is important for our microscopy experiments to accurately examine infected cells and the p-IκBα expression associated with those cells. As can be seen, the GFP shows a perinuclear pattern in the infected which is not surprising considering the ER membrane association of NS5 during the HCV infection cycle.

Flow cytometry was also used to analyze p-IκBα expression (Figure 2.2). In this experiment Huh7.5.1 cells were infected with JFH1-eGFP virus and analyzed via flow cytometry at five days post infection for virus (eGFP), p-IκBα, and total IκBα expression (t-IκBα). It should be noted that the p-IκBα antibody detects only phosphorylated serines at position 32 and 36 (Ser 32/36). HCV is readily expressed at five days post infection (Figure 2.2A) as shown by the increase in eGFP expression compared to a three hour post infection and uninfected cells (naïve). Total IκBα expression (Figure 2.2B) doesn’t appear to have changed, but p-IκBα does show increased expression five days post

Figure 2.2. Flow analysis of JFH1-EGFP infected cells in Huh7.5.1. The orange line are cells five day post infection. The Blue line is three hours post infection, and the red line is uninfected cells. (A) Intracellular staining for core protein. (B) Intracellular staining for t-IκBα. (C) Intracellular staining for p-IκBα.
infection compared to the three hour infection and naïve cells (Figure 2.2C). Though the expression of p-IκBα is slight compared to naive, it does give evidence of p-IκBα expression being elevated later on in infection. This is the first instance we see an increase in p-IκBα at five days post infection.

To elucidate this further, we infected additional Huh7.5.1 and added an earlier time point of 30 minutes based on the quick response rate of NFκB. This time a western blot was performed to investigate expression levels of p-IκBα, and core protein (Figure 2.3). There is also an increase of p-IκBα after five days post infection as has been previously seen in the flow cytometry analysis. There was also lower expression of p-IκBα after four hours infection, which is relatable to the three hours post infection, but predictably there is an increase of pIκBα after 30 minutes infection, which would be consistent with the quick activation of the NFκB pathway. Core is also shown in detectable limits on the fifth day post infection, but not in the other samples. This is consistent with core having robust levels at starting around two to three days post infection and continuing on

![Western blot of different time points post infection of JFH1-eGFP in Huh7.5.1.](image)

Figure 2.3. **Western blot of different time points post infection of JFH1-eGFP in Huh7.5.1.** Each sample was stained for p-IκBα and HCV core. The ‘Stim’ sample are cells stimulated with TNFα (20ng/mL) and IL-1β (20ng/mL). Core staining was used to detect infection. Actin serves as a loading control.
throughout the viral life cycle. Having both flow cytometry and western blot independently confirm that there is an increase of p-IκBα at five days post infection gives support that this event is actually true. To have a clearer understanding of the fold increase of p-IκBα, densitometry analysis was used to evaluate the intensity of each band (Figure 2.4). The samples were normalized compared to the actin control and the naïve control was arbitrarily set to one. As can be seen, there is an increase of over ten-fold in the five day post infection compared to the naïve control, giving evidence in this instance that there is substantial increase in p-IκBα expression. As we are looking at total amounts of p-IκBα in the cell, it is not surprisingly we see a large increase in p-IκBα expression when compared to the fractionation experiments mentioned later on.

The previous experiments indicate that p-IκBα was being expressed more after five days post infection, but we also wanted to confirm that p-IκBα was 

![Figure 2.4](image)

**Figure 2.4.** *Expression fold change of p-IκBα normalized to naïve.* Data shows fold increase compared to the naïve sample. Naïve sample has been normalized to 1.
indeed going to the nucleus. To confirm previous results of p-ΙκBα traveling to the nucleus after JFH1 infection, we expanded the assays utilized to determine this phenomenon by including generation of cytosolic and nuclear fractions to determine localization, and intracellular straining for use in fluorescent microscopy. Confocal microscopy was coupled with JFH1-eGFP infection and intracellular staining to visual localization. Cells were stained with p-IκBα and DAPI (DAPI was used as a nuclear localization control). As shown by the white arrow, it was confirmed that p-IκBα does enter the nucleus (Figure 2.5) after five days post infection, while an earlier time point does not. At this point we had established that p-IκBα levels are increased at five days post infection, but this was the first time that we could detect p-IκBα inside the nucleus. This is

![Fluorescent microscopy of infected Huh7.5.1. by JFH1-eGFP.](image)

Figure 2.5. **Fluorescent microscopy of infected Huh7.5.1. by JFH1-eGFP.** Huh7.5.1 were analyzed one hour post infection (1 hpi) and five day post infection (5 dpi) for localization of p-IκBα.
extremely unusual, as p-IκBα is not normally found in the nucleus. This exciting result forms the foundation of my dissertation.

To corroborate these findings, a fractionation procedure was utilized that separates proteins found in the cytoplasm from those in the nucleus. Briefly, by using different types of detergents, coupled with centrifugation, we were able to robustly and efficiently separate these two fractions and probe them via western blot. This western blot showed that core infection does indeed go into the nucleus upon five days post infection, and shows a slight increase in p-IκBα compared to naïve cells (Figure 2.6). Coupled with the microscopy result, this is the first case of p-IκBα found in the nucleus after viral infection we are aware of. We expect this signal to be low, so it is not surprising the band intensity is light when compared to the naïve nuclear control. As previously mentioned, this is unique...
because p-IκBα is ubiquitinated and eventually degraded by the proteasome. How this event happens will be looked at in the upcoming chapters.

These three different experiments’ findings are very exciting and unique. We are showing that the HCV not only drives an increase of p-IκBα, but that p-IκBα is localized in the nucleus, which is a unique new concept. In the next chapter, we continue to investigate this event and determine what possible viral component(s) is responsible and possible reasons for this occurrence.
CHAPTER 3

PREFACE

In chapter three we look more into what viral component could be causing this increase of p-IκBα during infection. In the previous chapter we saw that HCV infection causes increase expression of p-IκBα and localization of p-IκBα to the nucleus, but what is responsible for this? Here we investigate the viral capsid protein called core, which has been shown to be multi-functional, and translocate to the nucleus.

This hypothesis was elucidated by constructing mammalian expression vectors that express the two versions of core mentioned previously (immature and mature) with an HA tag. This allows for the expression of a single viral protein independently of viral infection while p-IκBα expression levels are analyzed. In these experiments, I have designed core to be expressed in one of two ways. First, in a TET on/off system that allows the control of the temporal expression of core or second, driven by a constitutive promotor designed for transient expression. This gives more control over the timing of core expression, which from chapter two, has shown to be very important.

In these assays, we also look at localization of p-IκBα and core after transfection experiments, with the goal being to detect p-IκBα in the nucleus along with core expression. To expand upon these studies, we will also utilize a proteasomal inhibitor called Bortezomib (BZ) to possibly increase the signal of p-
IkBα if it is indeed being degraded during core expression. This would help clarify if core is increasing the expression of p-IκBα or rescuing p-IκBα from degradation. If core is increasing the expression of p-IκBα, but degradation is still taking place, adding a proteasomal inhibitor should show a large increase of p-IκBα. If core is rescuing p-IκBα, it should show relatively be the same with and without BZ, as it was being ‘rescued’ before degradation. There is also the possibility that the NFκB pathway needs to be stimulated for core to affect p-IκBα. To address this, we added stimulants that compose of TNFα, IL-1β, and peptidoglycan to induce activation of this pathway. The hypothesis being for core to rescue p-IκBα, there has to be an activated NFκB pathway (such as when the virus infects a cell). In this way, it more resembles a viral infection but only have core is present.
RESULTS

Expression of Core Constructs

To help elicit the cause of the rescue of p-IkBα during viral infection, we developed core constructs that will express mature and immature core in mammal cells. Both mature and immature core constructs have been developed.

Figure 3.1 Hemagglutinin tagged mature and immature core. Both were tagged on the N-terminus and constructed in both constitutive and inducible expression systems.

Figure 3.2. Fluorescent microscopy of Huh7.5.1. transfected with core. HA tagged immature core (HIC) and HA tagged mature core (HMC) transfected into HEK 293T cells. HA staining shows successfully expression of core constructs. DAPI is used as a nuclear stain.
in Tet on/off as well as in a constitutive system. All versions of core have also been tagged with a HA tag as noted (Figure 3.1).

**Figure 3.3.** **Inducible HCV core.** HIC and HMC with and without doxycycline (Dox) are shown. Expression of core is only seen in the presence of Dox. Non-transfected cells (Naïve) with and without dox are shown as a control.

**Figure 3.4.** **Fluorescent image of Inducible HCV core.** HIC and HMC with and without doxycycline (Dox) are shown. Expression of HIC and HMC is only seen in the presence of Dox (green). DAPI is used for a nuclear stain.
Constructs expressing HA tagged immature (HIC) and mature (HMC) were transfected into HEK 293T cells and were stained for HA expression two days post transfection. As can be seen (Figure 3.2), there is a robust expression of both core expression constructs as shown by HA staining compared to non-transfected cells. Core is shown to be perinuclear which is consistent with the life cycle of the virus and the association of core to the endoplasmic reticulum and lipid droplets.

We have also designed constructs that will express both HIC and HMC in an inducible manner, utilizing a TET on/off system. Here I show the successful expression of HIC and HMC when doxycycline (derivative of tetracycline) has been added (Figure 3.3 and Figure 3.4), with actin serving as a loading control. This allows us to control the temporal expression of core when doxycycline is added. This is beneficial as it allows more precise control when core is expressed, which can be vital in elucidating its effect on the NFκB pathway.

![Figure 3.5. Fractionation of HIC and HMC with and without stimulation in HEK 293T.](image)

HEK 293T cells were transfected with HIC and HMC and fractionated into cytoplasmic and nuclear fractions. Stimulation occurred two hours before collection.
especially as the increase in expression of p-IκBα is seen at five days post infection. We have also utilized intracellular staining to detect inducible HIC and HMC expression (Figure 3.4). Though not as robust as HIC and HMC expressed via a constitutive promotor when looking at fluorescent images, this is expected because more components are needed to be expressed in the same cell for core expression to occur.

Core constructs in signaling

Utilizing these constructs, I have transfected HEK 293T and Huh7.5.1 to elucidate the role that mature or immature core play independently of viral infection in the increased expression and localization of p-IκBα in the nucleus. HEK 293T cells are used for their ease of transfection and rapid growth, while

![Figure 3.6. Expression fold change of p-IκBα in the nucleus from figure 3.5 normalized to naive. Data shows fold increase compared to the naïve sample. Naïve sample has been normalized to 1.](image-url)
Huh7.5.1 is used more to mimic the natural environment of viral infection. This also gives insight whether this event is cell specific or not.

In the first sets of experiments (Figure 3.5), core was transfected individually and in conjunction with stimulation. It was hypothesized that in order to see the p-IκBα in the nucleus, it might require a stimulated NFκB pathway. This would be closer to a viral infection without having to include the actual virus. In this way, we can demonstrate if HIC and HMC need a stimulated NFκB pathway for the rescue of p-IκBα and compare to transfection of core alone.

We see (Figure 3.5) that there is a presence of p-IκBα in the cytoplasm as well as the nucleus. HIC and HMC transfected samples with stimulation show an increase in p-IκBα in the cytoplasm, which is not surprising considering that we see in increase in p-IκBα with stimulation alone, suggesting that core is not

![Figure 3.7. Fractionation of HIC and HMC with and without stimulation in Huh 7.5.1.](image-url)

Huh7.5.1 cells were transfected with HIC and HMC and fractionated into cytoplasmic and nuclear fractions. Stimulation occurred two hours before collection. Chikungunya virus capsid (ChikV C.) was added as another capsid comparison.
creating an increase in p-\(\text{IkB}\alpha\) in the cytoplasm, but stimulation is. The nuclear fraction shows an increase in p-\(\text{IkB}\alpha\) in the mature core sample, compared to the immature version and the naive control. The expression of HMC in the nucleus is marginally greater than the stimulated sample, which is not surprising considering that we expect only a small amount of p-\(\text{IkB}\alpha\) in the nucleus upon core expression. Surprisingly, we do see a possible decrease of p-\(\text{IkB}\alpha\) with the HIC and HMC samples coupled with stimulation, suggesting that stimulation coupled with core doesn’t preserve p-\(\text{IkB}\alpha\) in the nucleus, but mature core without stimulation does. This gives an indication that the presence of mature core is needed for the increase expression and localization of p-\(\text{IkB}\alpha\) to the nucleus, and is independent of a stimulated NF\(\kappa\)B pathway, in the context of HEK 293T. These results are extremely important, as it gives evidence that core is likely the viral component responsible.

![Bar chart](image)

**Figure 3.8.** Expression fold change of p-\(\text{IkB}\alpha\) in the nucleus of 293T from figure 3.7. Data shows fold increase compared to the naïve sample. Samples were normalized to H3.
To corroborate these findings in a more natural viral infection host environment, Huh7.5.1 cells were transfected in a similar manner as the HEK 293T cells in figure 3.5. Interestingly, we see increased presence of p-IκBα in the nuclear fraction in conjunction with HIC and HMC without stimulation as was seen in the HEK 293T experiment. Though the stimulated HMC sample in this case has a similar expression level to that of core non-stimulated (Figure 3.7), the HIC with stimulation shows a higher expression level (Figure 3.8). This suggest cell type could be a factor in core interactions with p-IκBα In Huh7.5.1 cells in regards to stimulation. This exciting result suggests that there might also be a host factor in Huh7.5.1 cells opposed to HEK 293T cells that could support this translocation and expression. Densitometry analysis was also performed as in the previous experiment (Figure 3.7). Though slight, a small increase in HIC

Figure 3.9. Fractionation of HIC and HMC with and without Bortezomib (BZ). 293T cells were transfected with HIC and HMC and fractionated into cytoplasmic and nuclear fractions. Samples were further dividend into with (+BZ) and without (-BZ) bortezomib.
with stimulation was seen over the stimulation alone, hinting that the immature version of core is important in Huh7.5.1 cells.

Chikungunya virus capsid was used as another capsid to act as a comparison. Chikungunya is part of the *alphavirus* genus from the *togaviridae* family and its capsid protein is known to travel to the nucleus. We were curious if a capsid from another viral family could be showing similar effects to core protein in relation to $p\text{-}I\kappa B\alpha$ localization and expression. This would give insight as to whether the rescue and translocation of $p\text{-}I\kappa B\alpha$ was limited to core or could include other viruses. In this experiment, we don’t see an increase of $p\text{-}I\kappa B\alpha$ in the cytoplasm, but are currently expanding these studies to better elucidate the role of chikungunya could play.

Since $p\text{-}I\kappa B\alpha$ is classically degraded via the proteasome during activation, it is possible the levels of $p\text{-}I\kappa B\alpha$ in the presence of core could be

![Figure 3.10. Expression fold change of $p\text{-}I\kappa B\alpha$ in the nucleus of 293T from figure 3.9. Data shows fold increase compared to the naïve sample. Samples were normalized to H3.](image-url)
enhanced with the addition of a proteasomal inhibitor. In our case, we utilized bortezomib (BZ) which binds to the active site of the 26S proteasome and inhibits it. This effectively blocks degradation of proteins that have been ubiquitinated.

Figure 3.11. **Fluorescent image of HIC and HMC transfected in 293T.** (A) Transfection of HIC and HMC and stained for DAPI, HA, and p-IκBα as shown. (B) Enlarged HIC image. White arrows indicate an example of the localization of p-IκBα in nucleus of HIC transfected cell.
and destined for degradation. This allows for a more robust picture of the amounts of p-IκBα that might be rescued in order to increase the presence of p-IκBα, which from previous experiments has shown to be small. It would also help elucidate if core is activity rescuing p-IκBα from degradation or possibly increasing expression to a point where it can’t be degraded fast enough. If core is rescuing p-IκBα from degradation, it would be expected there would be little change in the amount of p-IκBα with or without a proteasomal inhibitor because p-IκBα was being rescued before it had a chance to reach the degradation pathway. If core was increasing the expression of p-IκBα, then an increase in the amount of p-IκBα should be seen, as the p-IκBα that is usually degraded will be preserved.

Surprisingly, there was lower expression seen in the samples that were treated with BZ (Figure 3.9, Figure 3.10) except for the HMC sample. In the non-treated BZ samples mature core again appears to be increasing expression of p-IκBα as was seen in figure 3.6, and corroborating past results. Surprisingly, HMC with BZ showed elevated expression compared to all other samples treated with BZ. This could result from IκBα binding to HMC instead of NFκB, and thus allowing translocation to the nucleus. Though when in the NFκB cycle this event happens still needs to be determined. As for the other samples treated with BZ, this low expression could be explained because BZ inhibits all degradation performed by the proteasome. When the NFκB pathway is stimulated and released from p-IκBα, it travels to the nucleus to activate the expression of its
targeted genes. One of those genes being IκBα, which will then go on to repress NFκB in the nucleus by binding it and transporting it back out into the cytoplasm. If IκBα can never be released from NFκB because of lack of degradation, it won’t translocation to the nucleus.

To corroborate these findings utilizing another method, fluorescent microscopy was used to visualize the presence of HIC and HMC and its effect on p-IκBα expression and localization. As in the western blots, HIC and HMC was transfected into HEK 293T cells and analyzed for the presence of p-IκBα and core (HA) forty-eight hours later.

As shown (Figure 3.11), there is robust core expression in both HIC and HMC samples by evidence of HA staining. This staining shows the expected localization of core in the cytoplasm and perinuclear areas. It is difficult to determine if there is core in the nucleus, though this could be the result of low amounts of protein expression in the nucleus, or inefficient antibody binding. When stained for p-IκBα in the same samples, we found detection of p-IκBα in the nucleus (figure 3.10B) as well as in the cytoplasm. Coupled with western blot data shown in this chapter, this supports the hypothesis that HCV core protein is a viral protein that is rescuing p-IκBα and possibly aiding its translocation to the nucleus.
CHAPTER 4
PREFACE

From the previous two chapters, I have shown that hepatitis C virus and subsequently, core, could cause p-IκBα to translocate to the nucleus instead of degrading via the proteasomal pathway. In the previous chapter, I show evidence that core independently has an effect on p-IκBα increased expression and translocation to the nucleus but is this chapter I investigate if core is directly acting on p-IκBα.

In this chapter I look to identifying a physical interaction between p-IκBα and core. While core has an effect on p-IκBα expression and location, there could be many factors at play, both host and viral. Here, I have constructed core constructs expressing a streptavidin binding peptide (SBP) tag that has been successfully used in pulldown experiments in the past. Additionally, these constructs were engineered to have a fluorescent marker expressed independently of core and be to utilized in retro-viral technology to create stable expressing transduced cell lines. These fluorescent markers allow the use of fluorescent-activated cell sorting (FACS) to sort for clonal cell populations that have been successfully transduced. It is important to have a population of cells that are all expressing SBP tagged core in order to increase the signal of potential binding partners. In this way, we maximize the detection of binding partners in preparation that minimal amounts of p-IκBα or other factors bind to core. This is important because of data in the previous chapters hint that there could be small
amounts of p-IκBα found in the nucleus upon core expression. In addition, these cell lines continuously express SBP tagged core allowing for ease of use and robustness when compared to transfections. Utilizing these tagged core proteins with streptavidin magnetic beads, I can pulldown core and potential binding partners. I also utilized A/G agarose beads as an alternate way to pulldown core (and other proteins) to test the robustness of binding interactions.

Pulldowns in context with stimulation were also performed as it in previous chapters. This will add another dimension to the study of this interaction, linking stimulation with the expression of core to the localization of p-IκBα in the nucleus.

In this way, I plan on exploring the binding of core and p-IκBα, which will lead to a better understanding of the viral life cycle.
RESULTS

A streptavidin binding protein was added to the n-terminus end of both mature and immature core (Figure 4.1). This allows the use of streptavidin magnetic beads to pull down these core constructs and any interacting binding partners. These constructs were engineered to be expressed in mammalian cells and have a fluorescent marker for future identification purposes.

These SBP tagged core constructs were transduced utilizing retro-viral technology, and sorted into stable expressing populations in HEK 293T cells. Though there is still SBP tagged mature core (SMC) and immature core (SIC) not bound to the streptavidin beads as shown by the lysate control, there was successful binding of SMC and SIC as shown by the pulldown samples (figure 4.2). The lower expression of SIC in regards to SMC can be explained by different insertion locations during the retro-viral infection process, which can amount to different expression profiles.

The tumor protein p53 is a known binding partner of core was also analyzed for binding of both versions of core as a control for assay robustness.

Figure 4.1. Streptavidin binding peptide (SBP) tagged immature and mature core diagram. SBP was tagged on the N-terminus of immature core (SIC) and mature core (SMC).
and successful binding. As expected, there was significant expression of p53 found in the lysate control, but there was also p53 found in the SMC pulldown sample. This is showing that not only did SMC bind to the beads, but p53 was also bound to core and could be detected. This provides the ‘proof of principle’ that we can not only pull down core, but binding partners as well. While SMC pulled down p53, SIC didn’t show detected amounts of p53. This could be explained by the lower amounts of SIC compared to the SMC cell line, or that p53 needs the mature form of core for interaction. These results demonstrate that not only have we successfully created two stable producing cell lines that expressed SMC and SIC, but we were able to pull down a known binding partner.

In order to expand upon previous results, stimulation was added to one set of immunoprecipitation experiments. In previous experiments, stimulation occurred two hours before collection, but in these pulldown experiments, we

![Figure 4.2. Co-Immunoprecipitation of core construct and p53. SBP tagged mature core (SMC) and SBP tagged immature core (SIC) pulled down utilizing streptavidin binding beads. Non-bound extracts are represented by the ‘lysate’ samples and successfully pull downed proteins are represented by ‘Pull Down’ samples. p53 is used as a known binding control for core](image-url)
stimulated twenty-four hours before collection. The hypothesis being, more time might be needed for core to associate with p-IκBα. The initial infection experiments in chapter 2 show increased and translocation of p-IκBα to the nucleus at five days post infection, suggesting the NFκB pathway has also been active for that time. In order to better mimic infection, temporal stimulation of the NFκB pathway was extended.

For this experiment, instead of utilizing magnetic beads, A/G agarose beads were used to immuno-precipitate core from HCV and from the related virus, chikungunya. This was done because chikungunya core is not SBP tagged, and for comparability reasons, wanted both cores to be precipitated identically. The SIC and SMC cell lines and chikungunya capsid were immunoprecipitated utilizing anti-capsid antibodies for HCV and chikungunya respectively.

We see in the stimulated samples of SMC and SIC the presence of p-IκBα when immunoprecipitated utilizing anti-core antibody. This gives supporting

![Figure 4.3](image)

**Figure 4.3.** Co-Pull down of core construct and p-IκBα in stimulated cells and non-stimulated cells. SBP tagged mature core (SMC) and SBP tagged immature core (SIC) pulled down utilizing A/G agarose beads and HCV core antibody.
evidence that core does interact with p-IκBα, but needs stimulation (Figure 4.3), and supports the hypothesis that mature core might need an activated NFκB pathway for association of p-IκBα. This is more evident as we fail to see p-IκBα with core in the non-stimulated sample set. Interestingly, this could point to the fact that both immature and mature core can bind to p-IκBα in a stimulated state, such as viral infection, but the mature version is what is responsible for the translocation to the nucleus based on the previous experiments. This exciting result is the first time that we are aware of that core directly interacts with p-IκBα, and requires an activated NFκB pathway. Thus, we have shown that not only does core affect the expression of p-IκBα and localization, but directly interacts with it. The ‘smear’ found in the naïve, non-stimulated samples are speculated to be a degradation product.

Chikungunya viral capsid was also analyzed via pulldowns in a similar manner as SIC and SMC. There is expression of the capsid in both stimulated and non-stimulated samples (figure 4.4), though only stimulated chikungunya

Figure 4.4. Co-Immunoprecipitation of chikungunya core construct and p-IκBα in stimulated cells and non-stimulated cells. Chikungunya capsid (ChC) and chikungunya capsid with stimulation (ChC+S) pulled down utilizing A/G agarose beads and chikungunya capsid antibody.
capsid effectively immunoprecipitated p-\(\kappa\)B\(\alpha\), while non-stimulated did not. As found in the previous SIC and SMC experiment, the ‘smear’ found the naïve, are speculated to be a degradation product.

This is another exciting result, as it demonstrates that HCV core might not be the only viral capsid protein this event occurs with, and other capsid proteins from other viruses might be utilizing p-\(\kappa\)B\(\alpha\).

Taken together, these results indicate that there is an association of HCV mature and immature core with p-\(\kappa\)B\(\alpha\) in the stimulated fraction. Interestingly, there also appears to be an interaction of p-\(\kappa\)B\(\alpha\) with stimulated chikungunya capsid suggesting that a stimulated pathway is necessary for the interaction of capsid and p-\(\kappa\)B\(\alpha\) with these two viruses.
CHAPTER 5

DISCUSSION

Even though there has been much progress in the understanding of HCV and its treatments, there are many unknown processes and components that are involved with the HCV life cycle. While recent discoveries have made HCV more manageable for the population at large; costs, availability to treatment, and understanding of the virus still remains a problem. This creates unique opportunities for the study of the virus in order to better understand its life cycle and to create new therapeutic approaches. It is our goal to better understand these processes in order to create more efficient treatments.

In this dissertation, we look at HCV and its role on signaling, specifically p-IκBα in the NFκB pathway. The NFκB pathway is an important mechanism for the host to fight various infections or pathogens. NFκB is a fast-acting responsive element, that is held in an inactive state by the IκB family. Once classically activated, IκB is targeted by ubiquitin and degraded by the 26S proteasome. This releases NFκB and exposes the nuclear localization signal (NLS), thus allowing travel to the nucleus. There, NFκB targets the expression of genes that code for immune factors to help the host combat pathogens. One of these genes is IκB, which once expressed, will enter the nucleus and reassociate with NFκB and export it out the nucleus. In this way NFκB creates its own negative feedback loop.
Previous preliminary results have hinted that HCV infection could cause an increase of p-IkBα at around five days post infection. In chapter two, we look at the infection aspect of HCV and p-IkBα expression. Here we show that HCV infection does increase expression of p-IkBα five at days post infection, and p-IkBα can be found in the nucleus by utilizing fractionation assays. This is very surprising considering that canonically, p-IkBα is rapidly degraded by the proteasome. To elucidate these phenomena, we used fluorescent microscopy, western blots, and flow cytometry to confirm that this increase expression for p-IkBα was indeed happening. Interestingly, it seems to be happening at five days post infection. We also performed fractionation on infection experiments to localize where exactly this increase of p-IkBα was occurring. Taking these results into consideration, it shows that HCV infection seems to increase expression of p-IkBα, and possibly be involved in translocation to the nucleus. This event raises the question of why and how the virus was accomplishing this.

The capsid protein of HCV, also known as core exists as two forms: immature and mature. Core is first cleaved from the rest of the viral proteome by signal peptidase to create the immature version. This is quickly followed by a second cleavage event by signal peptide peptidase to create the mature form. Capsid’s main role is two encapsulate the viral genome, but there have been many reported instances where capsid is involved with other processes or proteins.
It is for this reason in chapter three we focus on the possible connection of HCV core and its role in p-IkBα expression. Here I express immature and mature version of core independently of viral infection. This allows us to help answer the question of what viral factor(s) could be responsible for the results in the infection studies. While, it is possible that there could be other viral factors that I am not accounting for in this chapter, the nature and localization of core make it a likely player, and is the reason why this avenue was pursued.

Expression and cellular fractionation of the two versions of core was performed, and expression of p-IkBα was analyzed as previously in chapter 2. Interestingly, when expressing mature core individually, we see an increase of p-IkBα. This isn’t too surprising as the majority of core found in virally infected cells is the mature version, and would likely be the version to associate with p-IkBα. To elucidate these findings further, we added stimulation of the NFκB pathways with expression of core. The reasoning being core might require an active NFκB pathway in order for this effect on p-IkBα to occur, such as seen in viral infection. While the initial experiment showed a decrease of p-IkBα with stimulation in HEK 293T, the next stimulation experiment shows a higher p-IkBα increase compared to the naive control, and on par with core without stimulation in Huh7.5.1. There are also the increased amounts of the immature version of core with stimulation compared to stimulation alone. This could be explained by the timing of stimulation in conjunction with the expression of core or due to cell type. Thus showing, at least in this case, that cell type and stimulation time are important.
I have also added a proteasomal inhibitor, Bortezomib (BZ) that inhibits protein degradation via the 26S proteasome. The 26S proteasome degrades proteins that have been tagged with ubiquitin chains, such as p-IκBα. The reasoning for adding this inhibitor is to identify if core is ‘rescuing’ p-IκBα from degradation, or increase the expression of p-IκBα, or perhaps both.

The addition of BZ actually shows a decrease of p-IκBα in the nuclear fraction except for HMC, which could be explained by HMC binding to IκBα and thus not being bound to the cytoplasm by NFκB. The decrease in the other samples could be explained by the fact that degradation of p-IκBα is needed to release NFκB to travel to the nucleus and drive the expression of immune response proteins, including IκB, its own inhibitor. Without this release, no new IκB will be translated and a decrease in expression would be seen. In the non-treated BZ samples, we observed increased p-IκBα in the HMC sampled compared to naïve as has been observed in previous experiments.

In the last chapter, we look at establishing a physical connection between core and p-IκBα. We have shown that core does have an effect on the expression of p-IκBα and translocation, but investigate if there is association. To test this, cell lines were created that had been transduced with retro-virus that contain SBP tagged immature and mature core versions. These stable expressing cells were utilized in conjunction with streptavidin beads and A/G agarose beads to successfully co-precipitate binding partners of core. In order to successfully prove that I can pull down binding partners utilizing my
methodology, we first looked at p53, a known binding partner of core. After successfully accomplishing this, we immunoprecipitated mature and immature core along with the chikungunya capsid which served as another viral capsid control. Stimulation and non-stimulation of these capsid samples was also performed in order to determine if stimulation was a prerequisite for binding. We found that p-IκBα does co-precipitate with mature and immature core in the presence of stimulation, as well as chikungunya capsid, though not when non-stimulated. This was a little surprising, as previous results seem to indicate that stimulation might not be needed, though this can be explained by different stimulation times.

Given the collection of these results, it can be concluded that HCV virus does effect p-IκBα expression five days post infection and surprisingly, p-IκBα is found in the nucleus instead of following the canonical degradation pathway. This would be the first time to our knowledge that p-IκBα is found in the nucleus. It is this translocation event which prompted us to elucidate what factor of HCV could be responsible. Following fractionation experiments with western blots, and coupled with microscopy, it was concluded that the viral capsid has an important role in this event. Not only is core found in the nucleus along with p-IκBα, but it appears the cell type is important regarding which version of core is more effective.

To find if core is actually interacting with p-IκBα, an immunoprecipitation experiment was performed with the immature and mature versions of core along
with chikungunya capsid to serve as another viral capsid control. It was found p-IκBα is co-precipitated along with both versions of core along with chikungunya capsid, but only when stimulated.

Overall, this is the first instance where p-IκBα was found in the nucleus upon HCV infection, and core appears to be a component in this event. As to why the virus is rescuing p-IκBα, we hypothesize the p-IκBα in the nucleus could bind to NFκB and inhibit the expression of certain NFκB associated genes that could be detrimental to the viral life cycle.
CHAPTER 6

MATERIALS AND METHODS

HEK 293T

The human embryonic kidney 293T (HEK 293T) were generously provided by Dr. Christopher Glembotski (San Diego State University, San Diego). Cells were maintained in complete Dulbecco’s Modified Eagle’s media (DMEM) media supplemented with 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA), glutamine (2mM), penicillin G (100 units/mL), and streptomycin (100 μg/mL). These cells were passaged when confluency was 70%

PHOENIX GP

Human epithelial kidney cells used as a second-generation retroviral producer cell line were generously provided by Dr. Gary Nolan (Stanford University, CA). Cells were maintained in Dulbecco’s Modified Eagle’s media (DMEM) supplemented with 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA), glutamine (2mM), penicillin G (100 units/mL), and streptomycin (100 μg/mL). These cells were passaged when confluency was 70%

HUH7.5.1

Huh7.5.1 cells were generously provided by Dr. Frank Chisari (Scripps Research Institute, La Jolla, CA). Cells were maintained in Dulbecco’s Modified Eagle’s media (DMEM) supplemented with 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA), glutamine (2mM), penicillin G (100 units/mL),
and streptomycin (100 μg/mL). These cells were passaged when confluency was 70%.

**PLASMIDS**

The retroviral transfer vector pBMN.HIC.i.GFP (HA-tagged immature core) was constructed by amplifying Hepatitis C virus capsid from Ad-JFH1-DV3-EGFP genome (generously provided by Dr. Curt H. Hagedorn’s group (University of Utah, Salt Lake City, UT) using the forward primer, 5’-ATATGGATCCACCATGTACCCATACGATGTTCCAGATTACGCTAGCACAAA TCCTAAACC-3’ and reverse primer, 5’-ATATGCTAGCTTTAAGCAGAGACCAGGAACGTGATGC-3’. This product was cut with BamHi and Nhei and ligated into the cloning vector, pJET to utilize a Xhoi site needed for downstream ligation. pJET.IMC was digested with BamHi and Xhoi and ligated into pBMN.i.GFP cut with BamHi and Xhoi. The retroviral transfer vector pBMN.HMC.i.GFP (HA-tagged mature core) was constructed by amplifying Hepatitis C virus capsid from Ad-JFH1-DV3-EGFP genome (generously provided by Dr. Curt H. Hagedorn’s group (University of Utah, Salt Lake City, UT) using the forward primer, 5’-ATATGGATCCACCATGTACCCATACGATGTTCCAGATTACGCTAGCACAAA TCCTAAACC and reverse primer, 5’-ATATGCTAGCTTTAAGCAGAGAAGGGGAACC-3’. This product was cut with BamHi and Nhei and ligated into the cloning vector, pJET to utilize a Xhoi site needed for downstream ligation. pJET.IMC was digested with BamHi and
Xhoi and ligated into pBMN.i.GFP cut with BamHi and Xhoi. The inducible lentiviral plasmid pHTRE.HIC.i.neptune was constructed by amplifying Hepatitis C virus capsid from Ad-JFH1-DV3-EGFP utilizing forward primer, 5'-ATATGGATCCCCACCAGGCAACAATCCTAAACC-3' and the reverse primer, 5'-ATATGCTAGCTTAAAGCAGAGACCGGAACGATGC-3'. This product was cut with BamHi and NheI. The vector, pHTRE.G4.i.neptune, was cut with BamHi and NheI to remove G4 (GAL 4) and ligated to the insert.

pHTRE.HMC.i.neptune was constructed by amplifying Hepatitis C virus capsid from Ad-JFH1-DV3-EGFP utilizing forward primer, 5'-ATATGGATCCCCACCAGGCAACAATCCTAAACC-3' and the reverse primer, 5'-ATATGCTAGCTTAAAGCAGAGACCGGAACGATGC-3'. This product was cut with BamHi and NheI. The vector, pHTRE.G4.i.neptune, was cut with BamHi and NheI to remove G4 (GAL 4) and ligated to the insert. The retroviral plasmid pBMN.SIC.i.mCherry (Streptavidin binding protein tagged to immature core) plasmid was constructed by amplifying Hepatitis C virus capsid from Ad-JFH1-DV3-EGFP genome (generously provided by Dr. Curt H. Hagedorn’s group (University of Utah, Salt Lake City, UT) using the forward primer, 5'-ATATGGATCCAGGACAAATCCTAAACC-3' and reverse primer, 5'-ATATCTCGAGTTAAGCAGGACGGAACGATGC-3'. This product was digested with BamHi and Xhoi. This digested core insert was then ligated into pBMN.SBP.CSN1.i.mCherry that had been digested with BamHi and Xhoi to remove CSN1. This resulted in pBMN.SIC.i.GFP. The same process was used
for the SBP tagged mature core, with the expectation being the reverse primer used was 5’-ATATCTCGAGTTATTAGAAGATAGAAAAGGGAAACC-3’ when constructing the core insert.

**WESTERN BLOT**

HEK 293T cells were collected and pelleted and then washed with PBS before being incubated with modified Radioimmunoprecipitation assay (RIPA) buffer with the serine protease cocktail phenylmethane sulfonyl fluoride (PMSF) for 30 minutes on ice. Laemmli buffer with 5% beta-mercaptoethanol (BME) was added to each sample and samples were boiled at 100° C for 5 minutes. The samples were loaded onto 12-15% SDS-PAGE Tris-Glycine gels and run at 60mA for 1 hour. Proteins were then transferred to a polyvinylidene fluoride PVDF membrane (Genesee Scientific Corporation, San Diego, CA) at 350mA for 1 hour and 30 minutes. The membranes were then blocked using 5% milk (Saco Foods, Middleton, WI) in TBST for 1-2 hours at room temperature. Following blocking, membranes were incubated in a 5% milk mixture with either rabbit anti-HA primary antibody (Santa Cruz Biotechnology, Dallas, TX) at 1:2000 dilution, mouse anti- p-IκBα (Cell Signaling, Danvers, MA) at 1:1000 dilution, mouse anti-Rho (Santa Cruz Biotechnology, Dallas, TX) at 1:1000, rabbit anti-H3 antibody (San Diego, CA) at 1:1000, and mouse anti-core antibody (BioFront Technologies, Tallahassee, FL) at 4°C overnight. The following day membranes were washed three times with TBST. Next, membranes were incubated with either anti-rabbit or anti-mouse HRP conjugated secondary antibodies (Cell
Signaling, Beverly, Ma) at 1:2000 dilution in 5% milk for 1-2 hours at room temperature. The membranes were then again washed three times with TBST. Protein detection was done using the ECL Start Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK) and Blue Devil autoradiography film (Genesee Scientific Corporation, San Diego, CA). All samples were quantified using a Pierce™ BCA protein assay kit (Thermo Fisher Scientific Waltham, MA.) Control samples were stimulated with a cocktail of TNFα (20ng/mL), IL-1β (20ng/mL), and peptidoglycan (100ng/mL) 30 minutes before collection or 24 hours before collection. Bortezomib (1mM) was added to cells 24 hours before collection.

**INTRACELLULAR STAINING FOR PHOSPHOFLOW ANALYSIS**

Huh7.5.1 cells used for phosphoflow analysis were collected in 0.5% trypsin EDTA for 5 minutes, directly added to paraformaldehyde (final concentration of 4%), and incubated at room temperature for 10 minutes. Cells were washed twice in ice cold PBS and permeabilized in 90% ice cold methanol. Samples were stained using anti- p-IkBα at 1:200 dilution, and anti-core at 1:200 dilution (antibody information found in western blot section above). Secondary antibodies (Cell Signaling, Danvers, MA) were used at a final concentration of 1:500. All samples were analyzed using a BD FACSCanto Flow cytometer and FlowJo software.
FRACINATION FOR WESTERN BLOTS

The REAP protocol was used for fractionation of samples. Briefly, samples were centrifuged and resuspended in 0.1% NP-40. An aliquot was taken from this, and used as the ‘whole homogenate’ control. The samples were centrifuged again, and the supernatant was collect as the cytosolic fraction. The remaining pellet was washed twice in 0.1% NP-40 and then resuspended in 1X Laemmli buffer. Laemmli buffer was also added to whole homogenate and cytosolic fractions and all fractions were boiled for 5 minutes. All samples were quantified using a Pierce BCA protein assay kit (Thermo Fisher Scientific Waltham, MA.)

VIRUS PRODUCTION AND TRANSDUCTIONS

For the production of the MLV (pBMN constructs) based virus, a 10cm² plate of Phoenix GP cells at 60% confluence was transfected with 3ug of packaging vector (pBMN.HMC.i.GFP for example) and 3ug of a vector expressing the envelope glycoprotein of the Vesicular Stomatitis Virus (pCI-VSVg) by mixing the plasmids in a 125uL of FCS-free DMEM and 48uL of 7.5mM PEI MAX (Polysciences, Warrington, PA). Media was replaced 24 hours post-transfection and viral supernatant was collected 48 hours after transfection and filtered with 0.45 micron PTFE filters (Pall Corporation). The supernatant was used to spin-infect naïve HEK 293T cells in a six-well plate at 1500 x g, 32°C for 90 minutes in a hanging bucket rotor centrifuge (Becton Dickinson). 24 hours post-infection, fresh media was added to the cells.
FLOW CYTOMETRY AND SORTING

Flow cytometry and sorting were performed on a BD FACS Aria with 405nm, 488nm, and 633nm lasers at the San Diego State University FACS core facility. Data was collected on FACSDiva 6.1.1.

PULLDOWNS

Cells were centrifuged and resuspended in modified NP-40 buffer (150mM NaCl, Tris-Cl 50mM, 10% Glycerol, 0.25% NP-40) or RIPA buffer (50mM Tris HCl pH8, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) with Complete Protease Inhibitor cocktail (Roche) at a concentration of \(10^7\) cells/mL. After incubation on ice for 30 minutes, cell lysates were centrifuged at 14,000 x g for 15 minutes at 4°C and supernatants were transferred to new tubes. The lysates were incubated for 1 hour with 50 uL of Dynabeads® MyOne Streptavidin T1 beads (Invitrogen, Carlsbad, CA) on a rotating rack at 4°C. The beads were separated from the sample with a magnetic rack and the supernatant was retained for lysate control. The beads were washed five times with 1mL lysis buffer and the bound proteins were eluted and then boiled with Laemmli buffer. Samples were then analyzed via western blot. All samples were quantified using a Pierce™ BCA protein assay kit (Thermo Fisher Scientific Waltham, MA.)

CONFOCAL

For confocal microscopy experiments, cover slips were prepared in a 24-well plate by first sterilizing the slips in 95% Ethanol, washing them once with
phosphate buffer saline (PBS), then treating them with 200μL of 0.1% gelatin in PBS for 30 minutes at 37° C. After 30 minutes the excess gelatin was removed from each well and the plate was allowed to dry. for 1-2 hours at room temperature. 1.0 x 10⁵ HEK 293T cells were then plated to each well and allowed to incubate for 24 hours before being transfected using the protocol listed above. 48 hours post-transfection cells were washed once with PBS and fixed using a 4% formaldehyde solution at room temperature for 20 minutes. Cells were then washed with PBS and appropriate wells were permeabilized with 90% methanol for 10 minutes. Following permeabilization cells were washed with PBS and blocked in a 1% bovine serum albumin (BSA) solution for one hour at room temperature. Following BSA treatment cells were stained with primary antibody at a final dilution of 1:200 overnight at 4° C. The next day cells were washed three times with PBS and incubated with a fluorescently conjugated secondary antibody at a final dilution of 1:500 for 1-2 hours at room temperature. Cells were then washed three times with PBS and stained with DAPI, for nuclear staining, at 1:5000 dilution. Slides were then prepared by washing cells once with PBS then removing cover slips from the wells and mounting on a microscope slide using ProLong Diamond Antifade Mountant (Life Technologies, Eugene, Or) and allowed to dry overnight. Slides were then analyzed using the Zeiss 710 Confocal Microscope and data was collected using ZEN digital imaging software.
HCV VIRUS

The Ad-JFH1-DV3-EGFP virus was a generous gift by Dr. Curt Hagedorn’s group (University of Utah, Salt Lake City, UT). To propagate the virus, 10cm plates of Huh7.5.1 cells were seeded and allowed to grow to 50% confluency. A virus aliquot was added to the cells and infectively was checked 72 hours post infection via fluorescent microscopy. Huh7.5.1 were passaged when reached 80% confluency, with 5mL of media collected from the previous plate added to the new passage plate, along with 5mL of fresh DMEM. This increases the amount of virus the cells are in contact with. Titer of virus was determined as described by Kato et al. Briefly, naïve Huh7.5.1 cells were incubated with virus containing media for 4 hours, then replaced with fresh DMEM. Cells were then incubated for 72 hours. The presence of fluorescent puncta was used to generate fluorescent focus units per mL (FFU/mL).
REFERENCES


