CONTROL OF THE INTERFERON REGULATORY FACTOR – 3 ANTIVIRAL PATHWAY BY THE HEPATITIS C VIRUS NS3/4A PROTEASE

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DEDICATION

I would like to begin by thanking the science teachers and professors who first exposed me to this exciting and fascinating discipline. They fostered my interest and provided me with the foundation to pursue this dream.

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CONTROL OF THE INTERFERON REGULATORY FACTOR – 3 ANTIVIRAL PATHWAY BY THE HEPATITIS C VIRUS NS3/4A PROTEASE

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CONTROL OF THE INTERFERON REGULATORY FACTOR – 3 ANTIVIRAL PATHWAY BY THE HEPATITIS C VIRUS NS3/4A PROTEASE

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Hepatitis C Virus (HCV) is a major human pathogen that affects 200 million people worldwide. A majority of people exposed to HCV become chronically infected. In order to persist, the virus must encode mechanisms to subvert host immune defenses. We hypothesized that HCV disrupts critical host intracellular antiviral signaling pathways that culminate in activation of the antiviral response.

Using the cell-based replicon system, HCV was found to inhibit the activation of critical intracellular signaling pathways. Our studies identified the NS3/4A protein as an interferon regulatory factor 3 (IRF-3) and NF-κB antagonist and further mapped this activity to the NS3/4A serine protease domain. HCV antagonism of IRF-3 and NF- κB prevents the expression of interferon (IFN) and IFN-stimulated genes required for viral clearance and the generation of a HCV-specific adaptive immune response.

Upstream signaling components of IRF-3 were unknown, therefore, the NS3/4A protease was utilized as a tool to elucidate putative upstream signaling components. Characterization of Toll-like receptor 3 (TLR3) in HCV infection indicated that the NS3/4A protease cleaves TRIF, an essential adaptor protein required for TLR3 signaling. However the TLR3 pathway was not found to be essential for generating the intracellular antiviral response. Furthermore, these studies identified the retinoic acid inducible gene-I (RIG-I) as an intracellular viral double-stranded (ds)RNA sensor through molecular cloning and the characterization of a HCV replication permissive cell line defective in IRF-3 activation. The NS3/4A protease disrupted IRF-3 signaling through both the RIG-I pathway as well as the homologous MDA-5 pathway during viral infection. Lastly, these studies identified novel roles for TBK1, TANK and NEMO as signal transduction intermediates in the intracellular dsRNA response pathways.

The HCV NS3/4A protease is critical for proper viral polyprotein processing, in addition to antagonism of intracellular antiviral signaling pathways. Therefore, the HCV NS3/4A protease is an ideal target for novel therapeutics. Indeed, the HCV replicon could be effectively

eliminated from a persistently infected cell line through the use of an experimental NS3/4A protease inhibitor. These results offer great promise for the effective treatment of chronic HCV infection.

PRIOR PUBLICATIONS

Yoneyama M, Kikuchi M, Matsumoto K, Imaizumi T, Miyagishi M, Taira K, **Foy E**, Loo YM, Gale M Jr., Akira S, Yonehara S, Kato A and Fujita T. *Shared and Unique Functions of the DExD/H-Box Helicases RIG-I, MDA5 and LGP2 in Antiviral Innate Immunity.* Journal of Immunology. September 1, 2005; 175(5); 2851-8.

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ABBREVIATIONS

AP-1	Activator Protein 1
ATF	Activating Transcription Factor
BCL10	B-cell Lymphoma 10
BCR	B Cell Receptor
BMM	Bone Marrow Macrophage
CARD	Caspase Recruitment Domain
CARMA1	CARD, Membrane-associated guanylated kinase (MAGUK) protein 1
CBP	Creb Binding Protein
CTL	Cytotoxic T-lymphocyte
CYLD	Cylindromatosis
DBD	DNA Binding Domain
DNA	Deoxyribonucleic Acid
DSRBM	Double-stranded RNA inding Motif
dsRNA	Double-stranded RNA
EBV	Epstein Barr Virus
eIF	Elongation Initiation Factor
EMCV	Encephalomyocarditis virus
ERK	Extracellular Regulated Kinase
FADD	Fas-Associated Death Domain
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green Fluorescent Protein
HAU	Hemagglutinin Unit
HCMV	Human Cytomegalovirus
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus

HPV	Human Papilloma Virus
IAD	IRF Associated Domain
IFN	Interferon
IκB	Inhibitor of κB
IKK	IκB kinase
IL	Interleukin
IRAK	IL-1 Receptor Associated Kinase
IRES	Internal Ribosome Entry Site
IRF	Interferon Regulatory Factor
ISG	Interferon Stimulated Gene
ISGF3	Interferon Stimulated Gene Factor 3 Complex
IV	Intravenous
Jak1	Janus kinase 1
JNK	c-Jun N-terminal Kinase
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
Luc	Luficerase
Mal	MyD88 Adaptor Like
MALT	Mucosal Associated Lymphoid Tissue
MAP2K or MAPKK	Mitogen-Activated Protein Kinase Kinase
MAP3K or MAPKKK	Mitogen-Activated Protein Kinase Kinase Kinase
MAPK	Mitogen-Activated Protein Kinase
MDA-5	Melanoma Differentiation Associated Gene 5
MEF	Mouse Embryonic Fibroblast
MHC	Major Histocompatability Complex
MIP	Macrophage Inflammatory Protein
MyD88	Myeloid Differentiation 88

NANBH	Non-A, Non-B Hepatitis
NDV	Newcastle Disease Virus
NEMO	NF-κB Essential Modulator
NES	Nuclear Export Signal
NF-κB	Nuclear Factor κB
NIK	NF-κB Inducing Kinase
NLS	Nuclear Localization Signal
NOD	Nucleotide-Binding Oligomerization Domain
NS	Nonstructural
NTP	Nucleotide Triphosphate
NTPase	Nucleotide Triphosphatase
NTR	Non-translated Region
OAS	Oligoadenylate Synthetase
ORF	Open Reading Frame
PAMP	Pathogen-Associated Molecular Pattern
PI3K	Phophoinositide-3 Kinase
pIC	Polyinositol Cytosine
PKC	Protein Kinase C
PKR	Protein Kinase Regulated by RNA
PRD	Positive Regulatory Domain
PRR	Pathogen Recognition Receptor
RANTES	Regulated on Activation Normal T-Cell Expressed and Secreted
RD	Signal Response Domain
RdRp	RNA dependent, RNA polymerase
RIG-I	Retinoic Acid Inducible Gene-I
RIP	Receptor Interacting Protein
RNA	Ribonucleic Acid

RNAi	RNA interference
SCH6	Schering 6 (HCV NS3/4A Protease Inhibitor)
SCID	Severe Combined Immunodeficiency
SenV or SV	Sendai Virus
siRNA	Short interfering RNA
STAT	Signal Transducer and Activator of Transcription
TAK1	Transforming Growth Factor (TGF)-β Activated Kinase 1
TANK	TRAF Family Member Associated NF-κB Activator
TBK1	TANK-Binding Kinase 1
TCR	T-Cell Receptor
TIR	Toll-IL-1 Receptor
TLR	Toll-like Receptor
TNFα	Tumor Necrosis Factor alpha
TNFR	Tumor Necrosis Factor alpha Receptor
TRAF	TNF Receptor Associated Factor
TRAM	TNF-Related Adaptor Molecule
TRIF	TIR Domain-Containing Adaptor Inducing IFNβ
Tyk2	Tyrosine Kinase 2
uPA	Uroplasminogen Activator
VAK	Virus-Activated Kinase

CHAPTER 1: Introduction and Literature Review

Hepatitis C Virus Epidemiology

It is currently estimated that over 200 million people worldwide are infected with Hepatitis C virus (HCV) with approximately 4 million in the United States alone(37). HCV is most commonly transmitted by percutaneous exposure to contaminated blood, most commonly through blood transfusion and needlestick inoculation. Prior to 1991, blood transfusion was the most prevalent transmission mode, however routine screening of blood donors has decreased the incidence to less than 4% of all cases(25,60). Similarly, HCV infection has been associated with intravenous administration of immunoglobulins, clotting factors and organ transplantation from infected donors, however careful screening and decontamination procedures have substantially diminished transmission rates from these sources(200,281). Currently, intravenous drug use accounts for the majority of newly acquired HCV infections. Estimates of individuals acknowledging drug use worldwide have a 50-95% incidence of HCV infection(20,83,192). As a result of these alarming statistics, many larger cities have initiated needle-exchange programs that have been attributed with a decreased incidence of IV drug use related HCV transmission(7). Finally, tattooing and acupuncture have also been

associated with percutaneous transmission although the incidence is relatively rare(137,238). Nonpercutaneous modes of HCV transmission are relatively uncommon but include sexual and perinatal transmission. Interestingly, HIV coinfection increases transmission in both cases(67,272). Incidence of HCV infection is also higher among minorities, people over 40 and in lower socioeconomic status individuals(7). In addition to the need for better therapeutics against HCV infection, these statistics also highlight the important need for public health education efforts about the risks associated with blood-borne HCV infection.

Taxonomy

Hepatitis C Virus (HCV) was identified as the elusive causative agent of post-transfusion, non-A, non-B hepatitis (NANBH) first described in 1974(201). The virus itself was not discovered until 1989 when Michael Houghton's group at Chiron Corporation utilized a lambda phage system generated from high tittered plasma to identify viral genes and subsequently the viral RNA genome(47). The discovery of HCV was a scientific landmark because it was the first virus to be wholly identified using molecular cloning techniques without relying on the use of tissue

culture or a small animal model to propagate the virus. Comparative analysis revealed that HCV had a similar genome organization and protein hydrophobicity profile as other members of the *Flaviviridae* family, which included the pestiviruses and flaviviruses(48). Members of this family include the important human pathogens West Nile virus, Dengue virus and Yellow Fever virus. However HCV was dissimilar enough from the other members of the family that it was designated as its own genus, *hepaciviridae(76)*. Isolates of HCV also reveal marked sequence heterogeneity and based on analysis of sequences of the core, E1 and NS5 regions, has resulted in a classification scheme for the virus into 6 genotypes (designated 1-6). Variability within genotypes has resulted in the further classification of greater than 50 subtypes designated a, b, c, etc(231). In addition to sequence variation, the HCV genotypes also differ in clinical disease course, treatment response rates and somewhat by geographic distribution.

Molecular Virology of HCV

The HCV viral particle consists of an enveloped virion derived from host membranes surrounding a nucleocapsid containing the single stranded, positive sense, 9.6 kilobase viral RNA genome (Fig. 1-1A). Both the 5' and 3' nontranslated regions (NTRs) of the viral genome contain

regions of extensive secondary structure that are among the most highly conserved sequences in the genome. The 5' NTR contains an internal ribosome entry site (IRES) that directs the cap-independent translation of the entire HCV open reading frame which encodes a single polyprotein of approximately 3010 amino acids. The 10 viral proteins are then liberated through co-/post-translational cleavage by both cellular and viral proteases. The core protein is the structural building block of the viral nucleocapsid. It has also been implicated in the regulation/alteration of host cell signaling pathways(170,175). The viral envelope genes E1 and E2 encode the viral glycoproteins that stud the mature viral particle and are responsible for receptor mediated entry into cells through a poorly characterized mechanism. The function of the p7 protein is unknown although it is hypothesized to form an ion channel analogous to that of the influenza virus M2 protein(90,91,193). NS2, together with the N-terminal domain of NS3, forms a zinc-dependent autoprotease that catalyzes the cleavage of the NS2/3 junction(87,100). The NS3 gene encodes a multifunctional enzyme with serine protease, NTPase and helicase activity (Fig. 1-1D). The N-terminal third of NS3 together with the viral NS4A cofactor comprise the viral protease that is responsible for all of the downstream polyprotein processing events(87). Its activity is critical for viral replication/maturation and as such has been a major target for

antiviral drug development. The C-terminal two-thirds of NS3 encode the viral RNA helicase and NTPase activities. Biochemical analysis of the helicase revealed that it unwinds dsRNA or DNA:RNA heteroduplexes in a 3' to 5' direction powered by NTP hydrolysis(133). As mentioned above, the NS4A gene encodes the 54 amino acid peptide cofactor for the NS3 serine protease. NS4A intercalates into the protease and forms a structural component that greatly enhances NS3 protease activity. Additionally, NS4A increases the stability of the NS3 protein most likely by stabilizing the protein in an active conformation and regulates proper intracellular localization(133,154). The NS4B protein is a component of the membrane bound replicase complex however, its specific role is unknown. NS5A is a multiply phosphorylated protein that is also a component of the replicase complex. It has also been shown to antagonize host cell signaling pathways through its interaction with PKR and is likely involved in determining interferon responsiveness(8,80,81). NS5B encodes the viral RNA dependent RNA polymerase (RdRp) and like other RdRp's is highly error prone. The low fidelity of the viral polymerase results in the acquisition of mutations during replication and the generation of a quasispecies population within infected individuals(161). Quasispecies evolution likely provides a selective advantage to variants through increased replication fitness and increased efficiency at immune

evasion. The replicon based tissue culture model has revealed that the structural proteins core, E1, E2, p7 and the nonstructural protein NS2 are dispensable for viral replication which requires only the presence of the NS3 through NS5B proteins which are sufficient for the formation of the membrane associated viral replicase complex and support of viral replication(64,159).

Model Systems to Study HCV Infection

While much has been learned in the past sixteen years about HCV, progress has been hampered by the lack of a robust tissue culture system in which to propagate the virus. However, the development of the replicon-based systems has facilitated molecular studies to ask basic questions about virus host interactions. Two different types of replicons will be used in the studies described herein, the full-length, or genomic replicon, and the subgenomic replicon. The HCV replicon consists of a bicistronic, positive sense RNA encoding the authentic 5' and 3' HCV NTR sequences necessary for initiation of viral translation and replication(159). The genomic replicon consists of a ssRNA encoding the entire HCV ORF whose translation is driven instead by the encephalomyocarditis virus (EMCV) IRES(107) (Fig. 1-1B). The 5' NTR containing the HCV IRES is

followed by the insertion of a neomycin phosphotransferase drug resistance cassette that allows for stable selection of cells harboring the replicon in culture. The subgenomic replicons are identical except that the viral structural genes have been removed and replaced by the neomycin phosphotransferase drug resistance cassette still under control of the HCV IRES followed by the EMCV IRES that drives the expression of the NS3-NS5B polyprotein (Fig. 1-1C). When introduced into cells, the HCV replicon RNA can replicate autonomously in the cytoplasm of cells to high levels analogous to an authentic viral infection. Interestingly, the HCV error prone RdRp results in the evolution of viral quasispecies in cell culture similar to what is seen in infected individuals. The accumulation of different mutations in the HCV coding region imparts differential fitness to the replicon that has facilitated the dissection of virus host interactions within cells(27,237). Groundbreaking work from our lab demonstrated the evolution of interferon resistance in a replicon subjected to innate intracellular pressure(237). This demonstrates the usefulness of the replicon model system to mimic quasispecies diversity, which also occurs in patients, and how the selection of variants that can modulate host processes affects the course of infection.

Surprisingly, Huh7 human hepatoma cells are the only liver cell line capable of supporting the replicon. This has been attributed in part to

potential defects in innate intracellular signaling pathways, emphasizing the importance of these responses in successfully controlling viral infection(132,237). Recently, the possibilities in this field have expanded to include the establishment of alternate cell lines to support the replicon including human HEK 293 and HeLa cells and mouse hepatoma cells(5,127).

Small animal models have also been unavailable for the study of HCV and have dramatically hindered progress to better understand the pathogenesis of HCV infection. While HCV does not directly infect mice, Norman Kneteman's group developed a chimeric liver mouse model in 2001(171) which now provides exciting possibilities for future studies. The chimeric mouse model consists of a SCID background crossed to a transgenic mouse that expresses the uroplasminogen activator (uPA) transgene under the control of an albumin promoter. This transgene directs uPA overexpression in the mouse hepatocytes resulting in a profound hypofibrinogenemic state and hepatocellular death. These mice can then be transplanted with human hepatocytes that effectively repopulate the mouse liver. The human hepatocytes can be targeted for HCV infection which the investigators demonstrated achieved clinically relevant viral titers and importantly could be serially passaged through naïve mice with similar success. This new mouse model provides an

attractive approach to study early events post infection using a defined viral inoculum and should further our understanding of important molecular interactions that facilitate persistent HCV infection.

Chimpanzees (*Pan troglodyte*) are the only primate species shown to be susceptible to HCV infection. It was first demonstrated in chimpanzees that direct injection of HCV RNA is infectious(26,139). This has enabled studies of functionally characterized molecular clones allowing evaluation of viral evolution and quasispecies diversity in the face of host immune responses(26,139). This provides a powerful system to assess the role of mutations that result from the characterization of interesting phenotypes in replicon based studies. The chimpanzee model system has also proven particularly valuable in assessing the early events post infection in a whole animal model as similar studies are difficult in humans as most acute infections are subclinical. The course of acute infection in chimpanzees is remarkably similar to humans for most virologic and clinical parameters. However, a very important distinction is that chimpanzees develop chronic infection at a significantly lower incidence, roughly half the rate as humans(17). This may be the result of a more robust cellular immune response observed in chimps that has been associated with viral clearance(50). Moreover, the same study demonstrated that self-limiting infection correlates with the onset of HCV

specific CD8+ T-cells in the liver of experimentally infected chimps. Perhaps more germane to the topic of this work are the studies demonstrating a robust induction of interferon stimulated genes in the liver of an acutely infected, self-resolving chimpanzee(23,234). Similarly, these chimpanzee studies have also revealed an inverse correlation between ISG induction and chronic infection(23). These studies demonstrate that HCV has the capacity to stimulate a productive immune response and argues that the inhibition of this response is critical in the establishment of persistent infection.

Natural History and Disease Manifestations of HCV

Acute infection with HCV is often subclinical however a minority of patients will present with symptoms of malaise, jaundice, hepatomegaly, hematuria and elevated serum levels of liver enzymes. It is estimated that only 15% of acutely infected individuals will resolve the infection although, interestingly, they will not have sterilizing immunity against reinfection(71,143). Thus, the majority of infected individuals progress to chronic HCV infection and unfortunately, most remain undiagnosed. About one-third of these individuals will develop fibrosis and/or cirrhosis as a result of persistent HCV infection. Up to 3% of these individuals will

subsequently develop hepatocellular carcinoma, a fatal condition(219). As a result of these sequelae, HCV is currently the leading indication for liver transplantation in the United States costing billions of dollars in health care expenditures annually.

Extrahepatic manifestations of HCV infection include autoimmune or immune complex mediated diseases including essential mixed cryoglobulinemia, membranoproliferative glomerulonephritis and porphyria cutanea tarda(94). There is also an association between HCV and lymphoproliferative disorders including non-Hodgkin's B-cell lymphoma(290). These observations may likely be linked to infection and dysregulation of lymphocytes, which although well documented in the literature, has remained controversial. However, it is tempting to speculate that HCV infection of immune effector cells might disrupt normal function through viral processes providing HCV with a decided advantage in maintaining a persistent infection. Alternatively, the lack or inappropriate induction of regulatory cytokines in the HCV infected liver has been associated with defective immune effector cell responses.

Current HCV Therapeutics

The current staple in HCV therapeutics is interferon (IFN) based therapy. Advances over the years have resulted in the generation of pegylated IFN, a polyethylene glycol modification that imparts an increased half-life and improved efficacy(78). Additionally, most regimes include the use of ribiviran, a nucleoside analog that interferes with polymerase activity and HCV replication(165). Unfortunately, in even the best cases, these treatments only result in a 50% clearance rate. Compounding this is the high incidence of adverse side affects associated with long-term IFN therapy including flu-like symptoms, depression and anemia, which results in low patient compliance(164). Additionally, there is a high incidence of IFN resistance in HCV genotype 1, the predominant species affecting the United States(164). The ability of the virus to avoid the actions of IFN a huge advantage for successful chronic infection and as such is the subject of intense study.

It is obvious that better therapeutics are needed to eradicate this virus. Rational drug design aimed at disrupting critical events for viral replication or restoring viral disruption of critical host antiviral processes provide attractive options. This underscores the importance in elucidating virus-host interactions that modulate these processes. With a better understanding of these process in hand, drug development programs will

be better equipped to design the next generation of therapeutics HCV antivirals. Currently, many pharmaceutical companies have HCV NS3 protease inhibitors in development. As mentioned above, the actions of the NS3 protease are absolutely critical for the HCV lifecycle, through processing of the viral polyprotein into mature viral replicase components. Phase I clinical trials with protease inhibitors have demonstrated promise for this therapeutic approach(144). It is even more likely that when used in combination with other therapeutics as a multipronged attack, as in HIV therapies, they may be successful in eradicating the virus. The odds of success for such an approach are more favorable than for HIV because HCV is unable to integrate or remain latent in cells. As an RNA virus, with no DNA intermediate state, it must continually replicate in the cytoplasm of infected cells and is unlikely to mutate rapidly enough to circumvent the actions of multiple therapeutics applied at one time. As the virus is able to develop resistance mechanisms through escape mutations and viral adaptation to single therapeutics, such multicomponent regimes will offer much hope for eradicating this devastating human pathogen.

Adaptive Immune Response to HCV

The role of a functional adaptive immune response is critical for clearance of viral infections. Accordingly, evaluation of the adaptive immune response in patients who successfully clear acute HCV infection reveal a direct correlation with the generation of a multispecific T-cell response(30,93,176). The relative role of antibody mediated immunity in HCV clearance is less well understood. Although anti-HCV antibodies have been detected in responders, reports of viral clearance in the absence of circulating anti-HCV antibodies have also been reported(199,226). Reports abound in the literature documenting impaired and dysfunctional immune effector cell function in HCV chronically infected individuals. Studies have revealed that the generation of an HCV specific T cell response is clearly delayed(50,250). In addition to delayed kinetics of response, quantitative and qualitative defects have also been described for adaptive immune responses from chronic HCV infections. Analysis of T-cells from acutely infected patients revealed impaired production of IFN-γ, deficient expansion capacity, decreased perforin content and impaired killing of target cells which correlates with the development of chronic infection(92,148,271). It has been postulated that the defective generation of an adaptive immune response might be a consequence of the inability of the innate immune response to initially

contain the virus and promote timely and appropriate T-cell priming(21,239). This has been attributed in part to impaired allostimulatory function in dendritic cells from HCV infected individuals(11,123,124,212). It is possible that this occurs through antagonism of their function by products released during viral replication, although impaired dendritic cell function may alternatively be due to direct infection of dendritic cells(147,212). Dendritic cells have an important function as a bridge linking the innate and adaptive immune systems. Triggers from the innate immune system, including the production of IFNα/β, direct the maturation and activation of dendritic cells which are critical in priming the antigen specific adaptive immune response(13). This relationship underscores the importance of proper induction of the innate immune response in generating a productive adaptive immune response.

The Innate Intracellular Response to Viral Infection

Pathogen recognition receptors (PRRs)

Cellular detection of invading pathogens is initiated through the recognition of pathogen associated molecular patterns (PAMPs) present in molecular components of the pathogen(168,169) (Fig 1-2). Host cells rely

on the presence of germ line pathogen recognition receptors (PRRs) to detect the presence of bacterial lipopolysaccharide (LPS), teichoic acid, peptidoglycan, flagellin, lipoprotein, unmethylated CpG containing DNA and viral single-stranded (ss) or double-stranded (ds) RNA and protein products (capsid and glycoproteins)(1,36,84,110,262). There are two types of PRRs, membrane associated and cytoplasmic. These diverse and sometimes overlapping molecules have roles in initiating signal transduction pathways and also as direct antiviral effectors(108,263). They are critical for the early and immediate response to pathogenic threats and play a crucial role in the initiation and priming of the adaptive immune response(2,3,15).

PKR

Protein kinase regulated by RNA (PKR) is a serine/threonine kinase activated upon binding to dsRNA(275) (Fig 1-2). PKR is expressed basally in all cell types and its expression is further induced in response to interferon signaling. PKR recognizes and binds dsRNA through tandem double-stranded RNA binding motifs (DSRBMs) resulting in its dimerization and autophosphorylation(69). Activated PKR is a crucial cellular antiviral effector as it plays an important role in host shutoff translational control programs. PKR achieves this through the

phosphorylation of the alpha subunit and critical component of the multisubunit translation initiation factor complex eIF2(273). Formation of translation initiation complexes with eIF2α is driven by a GTP hydrolysis reaction, which therefore determines the rate of translation initiation(253). Phosphorylation of eIF2α locks the complex in an inactive state preventing its recruitment into the translation initiation machinery effectively shutting down translation and the synthesis of both viral and host proteins(235).

In addition to its role in translational control, PKR is also involved in initiating virus stress response signaling pathways through the activation of the transcription factors IRF-1 and NF-κB(142). Additionally, PKR has been shown to play a role in signaling through TLRs although this function does not appear to require its kinase activity(85,118). Finally, PKR has been implicated as a signaling factor in virally induced apoptosis pathways(53).

2'-5' OAS

The 2'-5' oligoadenylate synthetase (2'-5' OAS) family of proteins also contain a DSRBM which upon binding to a dsRNA substrate, activates the enzyme to catalyze the formation of polyadenosine oligomers(203). Formation of poly(A) oligomers results in the activation of

RNase L, a latent cytoplasmic nuclease capable of catalyzing the degradation of both viral and cellular RNA species, including the 28S ribosomal RNA(112,209). These actions effectively inhibit translation of both host and viral genes and further promote the degradation of viral RNA genomes. RNase L specifically recognizes UU and UA dinucleotide motifs(209). It is interesting and perhaps not surprising that genotypes 1a and 1b of HCV encode much fewer of these substrate recognition motifs suggesting possible viral adaptation and an evasion strategy to this host defense mechanism(95). Furthermore, mice deficient in RNase L are more susceptible to viral infection and have also revealed a role for RNase L in promoting apoptosis demonstrating the important role of this antiviral effector molecule during viral infections *in vivo*(288).

Toll-like receptors

Membrane associated PAMP receptors are exemplified by the Toll-like receptor (TLR) family. To date, ten TLRs have been identified that recognize a diverse set of microbial components and initiate signaling cascades resulting in induction of innate immune response programs and inflammation(1,168). The TLRs are germ line encoded, transmembrane proteins containing an “extracellular” leucine-rich repeat (LRR) domain that mediates recognition of its cognate PAMP(1). The cytoplasmic

portion of the receptor contains a highly conserved Toll/interleukin (IL)-1 receptor (TIR) homology domain that is responsible for recruiting intracellular signaling factors upon receptor stimulation(62,63). All TLRs utilize the adaptor protein MyD88 which recruits IL-1 receptor associated kinases 1 and 4 (IRAK1/4) and TNF receptor associated factor 6 (TRAF6) to signal the downstream activation of IKK and MAPK signaling pathways(190). Additionally, some TLRs utilize alternate TIR containing adaptor proteins for altered specificity of signaling programs. TLR2 and TLR4 can utilize the adaptor proteins Mal and TRAM(73,160,188,279). TLR3 and TLR4 can also utilize the adaptor protein TRIF with imparts IRF-3 activation and IFN-β induction making these two receptors unique in this capacity(189,280).

TLR3/7/8 are the toll-like receptors capable of recognizing viral single-stranded (ss) and double-stranded (ds) RNA(4,59,98). TLR3 is the best characterized and has been shown to be basally expressed on the cell surface and within endocytic vesicles of macrophages and dendritic cells(163,163). Interestingly, TLR3 is inducible in many other cell types(174,183). Upon binding to its dsRNA ligand, TLR3 recruits the adaptor protein TRIF, also known as TICAM1, which recruits signaling molecules that result in the activation of the transcription factors interferon regulatory factor-3 (IRF-3) and NF-κB (to be discussed in more detail

below). TRIF has been shown to directly recruit the IRF-3 activating kinase TBK1 and additionally to interact with TRAF6 to mediate NF-κB activation(214). TLR7/8 more specifically recognize uridine-rich viral ssRNAs and utilize the classical MyD88 adaptor protein to signal the canonical pathway to activate NF-κB and another, uncharacterized pathway resulting in the activation of IRF-7 which induces the expression of IFN-α(129,130). TLR signaling in dendritic cells has been shown to be critical for priming antiviral CTL responses that are required for effective viral clearance demonstrating the important role that these PRRs serve in regulating the immune response(15,178,218).

RIG-I

Retinoic acid inducible gene-I (RIG-I) has recently been recognized as the cytosolic PRR capable of detecting dsRNA within infected cells (Fig 1-2). Recognition is mediated through binding of dsRNA structures by the C-terminal DExD/H helicase domain (Fig. 1-3A). DsRNA binding triggers an ATP-dependent conformational change exposing the N-terminal, tandem caspase activation and recruitment domain (CARD) for interaction with downstream signaling partners(237,283). The identity of these partners and their signaling intermediates are currently unknown however,

biochemical analysis has revealed that signaling through RIG-I also results in the activation of the transcription factors IRF-3 and NF-κB. Importantly, functional signaling through RIG-I was recently demonstrated by our group to regulate cellular permissiveness to HCV RNA replication in the replicon model(237). Huh7.5 cells had previously been defined as "highly permissive" to HCV RNA replication and characterization of the defect in these cell lines revealed a complete lack of responsiveness to virus infection or the presence of intracellular RNA(28,237). This defect was mapped to a point mutation in the first CARD domain of RIG-I resulting in the substitution of a threonine at codon 55 to an isoleucine. RIG-I T55I is competent to bind to dsRNA yet deficient in signaling the activation of both IRF-3 and NF-κB(237).

MDA5

Melanoma differentiation associated gene 5 (MDA5) is a homolog of RIG-I and is thought to perform a distinct but overlapping role with RIG-I. Like RIG-I, MDA5 encodes a C-terminal DExD/H box helicase and an N-terminal tandem CARD signaling motif (Fig. 1-3B). MDA5 has also been shown to signal the activation of both IRF-3 and NF-κB(282)(our unpublished results). Although MDA5 is also capable of binding dsRNA

structures, it appears to do so with a slightly different specificity(282) (Y-M Loo, unpublished results). This has led to the model that RIG-I and MDA5 exist in the cytosol as dsRNA/viral sensors that through differential and overlapping recognition of viral dsRNA structures allow a broader recognition of the presence of a variety of viruses within cells. Widened specificity and redundancy of PAMP recognition response pathways is a more efficient strategy for maintaining innate defenses and is also seen in the intracellular peptidoglycan PRRs, NOD1 and NOD2.

Constituents of the interferon-β enhanceosome

All of the PRRs discussed above initiate signal transduction pathways that result in the activation and assembly of factors that constitute the interferon-β (IFN-β) enhanceosome (Fig. 1-4A). Induction of IFN-β is the first line of defense in innate antiviral defense (Fig. 1-2). Therefore, it is perhaps not surprising that PRRs would share the common goal of turning on its expression. The IFN-β promoter is composed of four regulatory domains referred to as positive regulatory domains 1-4 (PRDI-IV) (Fig. 1-4A). IRF-1 binds to PRDI, NF-κB recognizes and binds to PRDII, IRF-3 and IRF-7 both bind to PRDIII and a heterodimer of c-Jun and ATF-2 bind to PRDIV(268). The coordinated interaction among these

transcription factors, as an enhanceosome complex, on the IFN-β promoter induces maximal induction of IFN-β expression however, mouse genetic studies have revealed that not all are absolutely essential.

IRF-1

Interferon regulatory factor-1 (IRF-1) was the first identified member of this family of transcription factors. IRF-1 has been implicated in regulating diverse cellular processes through transcriptional regulation of genes involved in cell cycle control, antiviral and stress responses and cytokine signaling(141). Activation of IRF-1, like all IRFs, is achieved through phosphorylation of distinct serine/threonine residues in the C-terminal transactivation domain of the protein. PKR has been shown to be necessary for the activation of IRF-1(142) (Fig. 1-2). Once phosphorylated, IRF-1 translocates to the nucleus where it interacts with other transcriptional activators to induce the expression of target genes containing the PRDI element(141). IRF-1 also regulates its own expression. IRF-1 transactivation is negatively regulated by another family member, IRF-2 which shares the same binding recognition domain and also binds to PRDI elements(96). However, IRF-2 lacks a C-terminal activation domain and thus constitutively occupies PRDI binding sites

shutting off transcription. Activated IRF-1 can displace IRF-2 to induce gene expression however, IRF-1 also induces IRF-2 expression resulting in a negative feedback regulatory mechanism(96).

Although IRF-1 was initially characterized as a component of the IFN-β enhanceosome, it is not strictly required for the expression of IFN-β (Fig. 1-4A). Studies of IRF-1 deficient mice have demonstrated that, with the exception of an increased susceptibility to lethal infection with EMCV, the mice have similar disease courses to other viral infections as their wild-type littermates(136). It is unclear why IRF-1 function is critical during infection with EMCV however, virus infection studies in mice deficient in other IRFs have revealed the specificity for certain viruses to trigger the activation of different IRFs preferentially. Similarly, these studies have also revealed a relative hierarchy for the roles of these important transcription factors in triggering antiviral programs and viral clearance.

IRF-3

An important finding to emerge from the studies described above is the critical function of IRF-3 for the successful induction of IFN-β expression and viral clearance (Fig. 1-2). This is perhaps also reflected by the fact that unlike the other IRFs involved in antiviral response

pathways whose expression is largely inducible, IRF-3 is constitutively expressed in all cell types(216). IRF-3 is a 55kDa transcription factor maintained in a quiescent state in the cytoplasm of cells. It consists of an N-terminal DNA-binding domain and a C-terminal transactivation domain containing a polyproline rich region, IRF association domain and a signal response domain(101,158) (Fig. 1-4B). IRF-3 is phosphorylated in response to a variety of stimuli including stress inducing agents, DNA damage and a variety of PAMPs(135,185,249) (dsRNA, viral nucleocapsid, LPS). Interestingly, activation of IRF-3 through stress inducers or DNA damaging agents results in the N-terminal phosphorylation of IRF-3(126,135,224). The MAP3Ks and DNA-PK have been implicated in this response however the consequence of these phosphorylation events is unclear(126). Bacterial LPS signals the activation of IRF-3 through TLR4 via the adaptor protein TRIF(73,228).

Receptors responsive to viral infection that activate IRF-3 were discussed above and include TLR3, RIG-I and MDA5. The presence of dsRNA or products generated during viral infection, results in the phosphorylation of critical serine/threonine residues in the C-terminal regulatory domain of the protein(114,224). Activation of IRF-3 results in homodimerization and nuclear translocation(114,157). Once in the nucleus activated IRF-3 interacts with the histone acetyltransferase

CBP/p300 coactivators and transactivates target genes, including IFN-β(236,269,285). Identification of the critical phospho-serine residues responsible for the activation of IRF-3 is hotly debated although two important clusters have been identified. The serines in the first cluster, S385 and S386 have been demonstrated to be required for efficient dimerization of IRF-3(113,284,285). Serines in the second cluster, most importantly S396 have been identified as the "minimal phosphoacceptor site" in response to virus infection and intracellular dsRNA stimuli(223). Indeed, phosphomimetic forms of IRF-3 with aspartic acid replacing serines in either S396D or all 5 residues in cluster 2 (S395D, S396D, S402D, T404D, S405D) result in the constitutive activation of IRF-3 and concomitant IFN-β induction. A similar phosphomimetic S385/386D mutant does not similarly activate IRF-3 gene induction(223). However, instead of conflicting, these observations can be seen as complementary with a requirement for sequential phosphorylation at both sites being required for optimal IRF-3 activation. Indeed, the crystal structure was simultaneously solved by two groups and has provided mechanistic insight regarding phosphorylation-induced dimerization(202,244). A model that has emerged from these studies is as follows: quiescent IRF-3 exists in the cytoplasm in an autoinhibited state with regions of the protein occluding the second cluster of serines. However, the S385/S386

residues are more exposed and upon phosphorylation, result in a conformational change that then exposes the second cluster of serines for phosphorylation(179,244). The activity of the kinase directing phosphorylation of these residues might be regulated by viral stimulation and thus could explain how the S385/386D mutant efficiently dimerizes but might still require viral stimulation to be fully active. The second serine cluster can be further subdivided into three distinct clusters based on three-dimensional location and might actually represent targets for distinct kinases(202). It is also tempting to speculate that the first (S385/386) and second (S396) phosphorylation events might also be mediated by two separate kinases however an involvement for the non-canonical IKK family members, TBK1 and IKKε has been demonstrated for phosphorylation of both the S386 and S396 groups. Further studies are obviously required to delineate the role of each serine group and the nature of specific, and perhaps novel, IRF-3 kinases to fully understand the regulatory mechanisms involved in activating this critical antiviral transcription factor.

The two kinases, TBK1 and IKKε, were recently identified as the elusive virus activated kinase(s) responsible for the phosphorylation-induced activation of IRF-3(72,225). The patterns of expression of TBK1 and IKKε differ significantly and actually mirror that of IRF-3 and IRF-7.

Like IRF-3, TBK1 is expressed ubiquitously in all cells(153,252). However, IKKε is expressed basally only in myeloid and lymphoid lineages and inducibly in all others, similar to IRF-7(140,195). Cells from mice deficient in either, or both kinases have revealed much with respect to the *in vivo* function of these kinases. Embryonic fibroblasts from mice with a targeted deletion in TBK1 have a complete defect in virus-inducted activation of IRF-3 and the production of IFN-β(99,167,194). TBK1 deficient MEFs do not express IKKε basally, however, ectopic expression of IKKε in these cells restored virus-induced activation of IRF-3 and IFN-β expression(194). Similarly, bone marrow macrophages (BMMs) isolated from TBK1 deficient mice express IKKε basally and despite the lack of TBK1 retain "levels of viral responsiveness" comparable to wildtype control BMMs(194). Embryonic fibroblasts from IKKε deficient mice demonstrate normal activation of IRF-3 and induction of IFN-β(99). Studies to address signaling through TLR3 and TLR4, which both signal the activation of IRF-3, revealed surprising differences in the requirement for each kinase. Stimulation of TBK1 deficient BMMs with pIC, resulted in a clear defect in IRF-3 activation but a delayed induction of the type I IFN response(194). While it was not directly assessed, this most likely reflects a compensatory role by IKKε and IRF-7 or IRF-5 as IFNα5, an IRF-3 independent target gene, was expressed in these cells. Similarly, ectopic

expression of TRIF, the TLR3 adaptor protein, is able to stimulate IFN-β expression in wildtype MEFs but not in TBK1 deficient MEFs(167) demonstrating a critical role for TBK1 in TRIF/TLR3 dependent signaling. Moreover, stimulation of TBK1 deficient BMMs with the TLR4 ligand LPS resulted in a complete defect for IRF-3 activation and a lack of induction of IFN-β and other ISGs(194). Biochemical studies also revealed that the TLR3/4 adaptor protein TRIF can interact with TBK1, but not IKKε, to direct the activation of IRF-3. Taken together, these studies reveal a crucial role for the IRF-3 kinase TBK1 in TLR3/4 mediated signaling in BMMs and in response to viral stimulation in fibroblasts. Expression of IKKε can compensate for the lack of TBK1 in the antiviral response in both BMMs and when expressed ectopically in fibroblasts but not for TBK1 deficiency in TLR mediated signaling. These data suggest that TBK1 and IKKε have overlapping but also nonredundant roles in the activation of innate antiviral response pathways.

NF-κB

NF-κB is a pleiotropic transcription factor activated in response to a wide variety of cytokine and stress induced pathways. It is also noteworthy in the context of these studies as it is activated in response to

signaling through TLRs and in response to viral infection (Fig. 1-2). The NF-κB family of transcription factors consists of Rel (c-Rel), RelA (p65), RelB, NF-κB1 (p50) and NF-κB2. All members in the family contain a Rel homology domain that facilitates the homo- and heterodimerization and DNA binding activities. NF-κB is held quiescent in the cytoplasm through binding to its inhibitor IκB(19,177). The inhibitory function of IκB is relieved through site-specific phosphorylation of both serine 32 and serine 36 residues by the inhibitory kappa kinase (IKK) complex(33,57,254). The IKK complex consists of the noncatalytic regulatory component NEMO (IKKγ) and the two kinase subunits IKKα and IKKβ and is activated in response to a wide variety of stimuli that are dependent on phosphorylation and/or ubiquitin (K63) dependent modifications(43,58). Phosphorylation of IκB on both Ser32/36 residues targets it for ubiquitination and proteosome mediated degradation thus liberating the NF-κB transcription factors facilitating their interaction and transactivation of NF-κB target genes. As mentioned above, NF-κB binds to the PRDII element in the IFN-β promoter and constitutes an important component of the IFN-β enhanceosome (Fig. 1-4A). Importantly, most cytokines and many ISGs contain NF-κB binding elements in their promoters thus

highlighting the important role for proper activation of NF-κB in innate immune response signal transduction pathways.

AP-1

The last component of the IFN-β enhanceosome is actually composed of a heterodimer of c-Jun and ATF-2 that together form the transcription factor activator protein-1 (AP-1)(49) (Fig. 1-4A). The AP-1 family of transcription factors participate in the regulation of a diverse set of cellular processes and are comprised of dimers of basic region-leucine zipper (bZIP) proteins including members of the Jun, Fos, Maf and ATF families(227). AP-1 is activated in response to cytokines, viral and bacterial pathogen recognition, growth factors, hormones, neurotransmitters, cell-matrix associations and a wide range of chemical and physical stress responses(227). Mitogen activated protein kinase (MAPK) signaling cascades are activated in response to this diverse set of stimuli and ultimately mediate the phosphorylation of AP-1 transcriptional components to direct transcriptional activation(39). MAPK signaling proceeds through a well characterized cascade involving a proximal MAP 3 kinase (MAP3K or MAPKKK), which directs the phosphorylation of a MAP 2 kinase (MAP2K or MAPKK). MAP2Ks in turn have the capacity to phosphorylate both threonine and tyrosine residues of MAP kinases

(MAPK) resulting in their activation(120). There are three types of evolutionarily conserved MAPKs in mammalian cells, which include p38, c-Jun N-terminal kinase (JNK) and extracellular regulated kinase (ERK)(120,215). These MAP kinases then activate transcriptional programs through direct phosphorylation of AP-1 constituent proteins. Although it is known that virus infection activates the MAPK, JNK to direct AP-1 transcriptional activity, the underlying signaling mechanisms involved are currently unknown(49).

Interferons

Type I Interferons: Interferon α/β Signaling

Intracellular recognition of viral infection culminates in the production of interferon-β (IFN-β) within infected cells. Secreted IFN-β acts in both an autocrine manner on the cell to amplify the response and, in a paracrine manner to cells in the surrounding tissue to produce an antiviral state within cells aimed at controlling and eliminating the viral infection(233) (Fig. 1-2). In addition to its direct antiviral affects, IFN-β is a pleiotropic cytokine with antiproliferative and immunomodulatory functions. IFN binds to cells through the IFNα/β receptor (IFNAR). Upon engagement, the IFNAR receptor subunits interact causing

transphosphorylation of the constitutively associated tyrosine kinases Jak1 and Tyk2. Once phosphorylated, these kinases recruit and phosphorylate the signal transducers and activators of transcription (STAT) 1/2 proteins. Once phosphorylated, the STATs homo- and heterodimerize and interact with IRF-9 to form the interferon stimulated transcription factor complex ISGF3. Once activated, this complex transactivates the expression of nearly a thousand interferon-stimulated genes (ISGs) with diverse functions involved in creating an antiviral state within cells(220). While the function of most of these genes is currently unknown, many with roles in antagonism of viral replication, translational control, MHCI antigen processing/presentation and immune effector cell modulatory function have been described. Many of the factors involved in either directly sensing, transducing signals or amplifying responses during viral infection (RIG-I, MDA5, TLR3, PKR, 2'-5'OAS, IRF-1, IRF-7, IKKε, IFN-α subtypes, etc) are themselves ISGs which demonstrates the critical role of positive feedback signaling in the induction of productive innate response programs. Indeed the appropriate induction of innate host responses is the first line of defense against viral infection and is critical for the induction and maintenance of the adaptive immune response required for successful viral clearance.

In addition to the classical Jak/STAT signaling pathway, many studies have also implicated a role for alternate factors in IFN signaling. The mitogen activated protein kinases (MAPK) ERK and p38 have also been implicated in IFN signaling although their role in signal transduction remains uncharacterized(258,259). IFN induced activation of PKCδ has also been demonstrated to be required for STAT1 Ser727 phosphorylation, a critical modification for full transactivation function(260). A role for PI3K in IFN signal transduction has also been reported although its exact role in this process is unknown(257,261). While the mechanistic role of these alternate factors in IFN signal transduction remains to be fully elucidated, it is clear that the cell has evolved multiple and elaborate signaling factors to achieve a diverse and comprehensive signaling response to this important innate cytokine. Additionally, these alternate signaling pathways might result in the differential regulation of subsets of ISGs that direct induction of the appropriate stimulus dependent response.

Type II Interferons

The type II interferon, IFNg, plays an important role in both innate and adaptive immunity(29). Unlike type I interferons which can be produced

by all cell types, IFNg is produced by cells of the immune system, primarily natural killer (NK) cells and T cells(24,221). IFNg signals through the IFNg receptor, which is composed of IFNg receptor 1 (IFNgR1) and IFNg receptor 2 (IFNgR2)(9). Associated with the intracellular domains of these receptors are Jak 1 and Jak 2(10,125), which upon binding of IFNg to the receptor, results in dimerization of the receptor and activation of the kinases(10,89). Ultimately, STAT1 is phosphorylated, homodimerizes, and translocates to the nucleus where it binds to GAS elements (gamma activated sequence) to stimulate the expression of IFNg-inducible genes(233). Binding of IFNg to its receptor has many immunoregulatory roles. IFNg can increase the susceptibility of cells to apoptosis by inducing the expression of Fas and Fas ligand(277). Only IFNg can induce the expression of MHC class II proteins thereby promoting CD4+ T cells responses. IFNg also modifies the activity of the proteasome by inducing the expression of LMP2, LMP7, and MECL1. Furthermore, IFNg increases the expression of TAP1 and TAP2 transporter proteins important for transferring peptides generated by the modified proteasome from the cytoplasm to the endoplasmic reticulum to be loaded into the groove of a MHC class I molecule(66,255). Lastly, IFNg plays an important role in determining the balance between Th1 and Th2 immune responses by inhibiting the production of IL-4(79). Taken together, IFNg

plays many important roles in both the innate and adaptive immune response to microbial and viral pathogens.

Viral Antagonism of IRF-3 Function

The critical role for IRF-3 in the induction of IFN-β and antiviral gene induction programs is underscored by the fact that it is a target for regulation by many diverse viruses. Viruses have evolved numerous strategies to impact IRF-3 function at different stages of its activation program. Several viruses encode dsRNA-binding proteins which effectively sequester the viral dsRNA agonist and prevent its recognition by cellular dsRNA PRRs. In addition to preventing the activation of IRF-3, this mechanism also prevents activation of 2'-5' OAS, PKR, IRF-1 and NF-κB. Viruses that employ this strategy include the influenza A virus through the actions of its NS1 protein(246,267), vaccinia virus via the E3L protein(276), herpes simplex virus 1 through the US11 protein and Ebola virus mediated through the VP35 protein(16). The human papilloma virus (HPV16) E6 protein and the rotavirus NSP1 protein bind directly to IRF-3 to inhibit IRF-3 transactivation function(86,207). Human herpes virus 8, the causative agent of Kaposi's sarcoma encodes a viral homolog to IRF-3 called vIRF-1 that is thought to directly bind to IRF-3. Additionally vIRF-1 competes for binding to the IRF-3 transcriptional coactivators CBP/p300

effectively preventing their association with IRF-3(34,155). Similarly, the adenovirus E1A protein(38,121) and the HCMV IE86 protein(248) (R. Taylor, personal communication) compete with IRF-3 for binding to CBP/p300 effectively inhibiting IRF-3 function. Lastly, the Bunyavirus NSs protein has been demonstrated to inhibit the activation of both IRF-3 and NF-κB although the mechanism through which it accomplishes this is unclear(270). Finally, V proteins of paramyxoviruses block phosphorylation of IRF-3 and induction of IFN-β through a variety of mechanisms(97). The common theme to emerge from these studies is that antagonism of the IRF-3 pathway occurs by a diverse group of viruses through a variety of mechanisms and ultimately contributes to viral pathogenesis.

Summary

Hepatitis C virus is an RNA virus that must replicate through a dsRNA intermediate. Persistent infection must therefore associate with the presence of viral dsRNA within infected cells. However, mammalian cells have several mechanisms to detect the presence of invading viral pathogens through dsRNA pathogen recognition receptors, which direct the activation of antiviral response programs. In order to successfully persist within the host, we hypothesize that HCV must somehow avoid the

function of these dsRNA PRRs to prevent the activation of IRF-3 and the induction of antiviral effector genes. The studies presented herein describe our efforts to understand the role of the IRF-3 pathway in controlling HCV infection and to elucidate the mechanism(s) of HCV regulation of the IRF-3 function in order to better understand factors that effect HCV persistence.

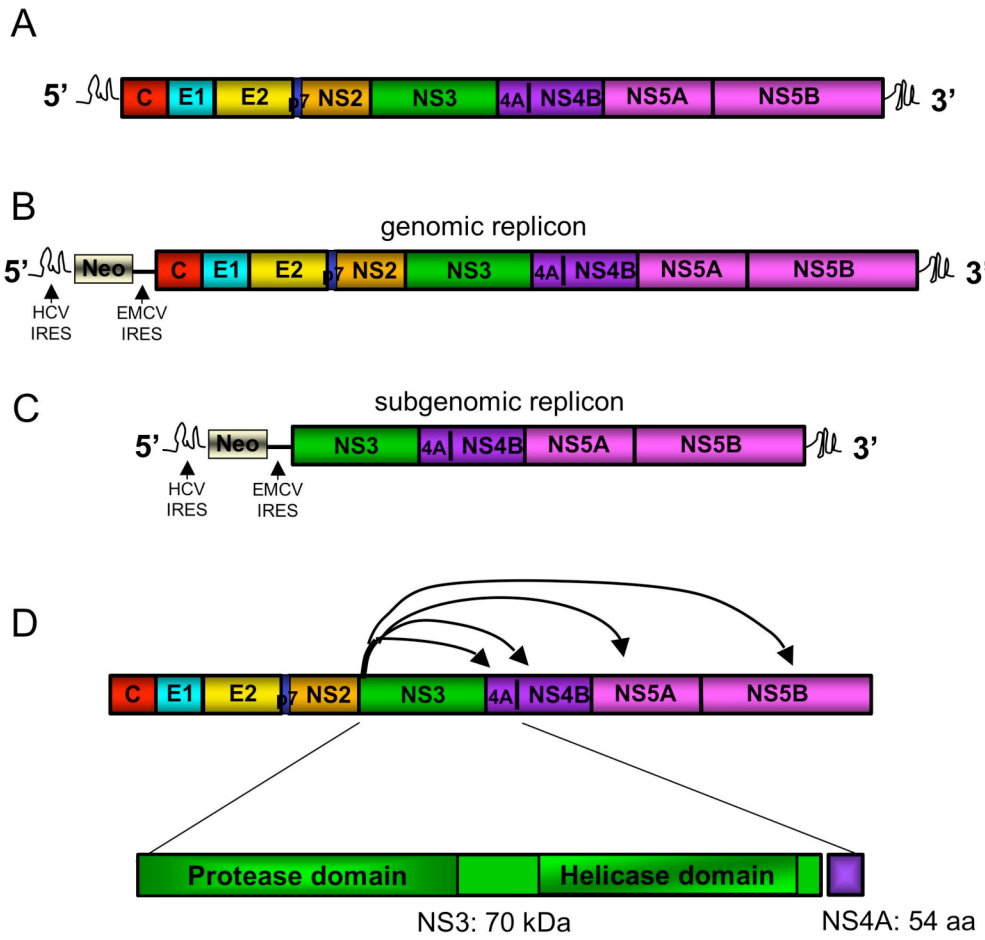


Figure 1-1: HCV genome and replicon schematic.
A. Structural schematic of HCV genome. B. Genome-length and C. subgenomic HCV RNA replicons used in this study. The encode the authentic HCV 5' and 3' NTRs necessary to support viral translation and replication of viral RNAs in culture. Structural representation of RIG-I, N-RIG and C-RIG expression constructs. These autonomously replicating, bicistronic viral RNAs express neomycin phosphotransferase (neo) and are thus selectable. D. Structural schematic of the HCV NS3 and NS4A genes. The amino-terminus of NS3 encodes the viral protease required for the downstream polyprotein processing. The carbyoxyl-terminus of NS3 encodes the viral helicase which is responsible for unwinding viral RNA during genome replication. NS4A functions as an essential cofactor for NS3/4A protease activity.

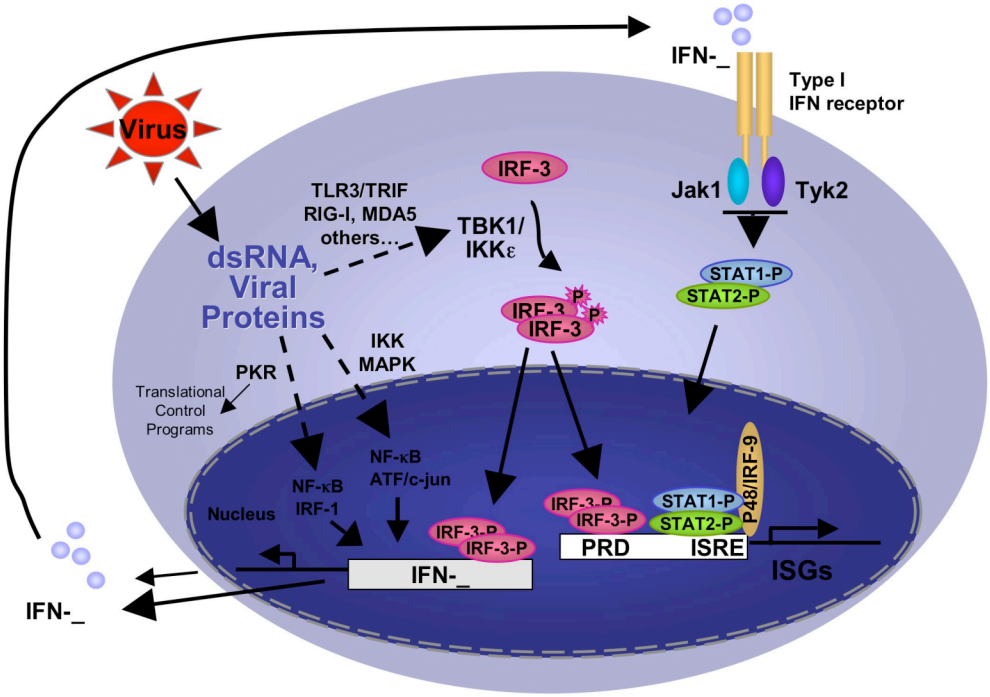


Figure 1-2: Host response to viral infection.
Diagram of the host response to viral infection showing activation of cellular dsRNA PRRs which result in activation of transcriptional programs culminating in IFN-β induction. IFN-β is secreted and signals in an autocrine and paracrine manner through the IFNα/β receptor to activate the JAK/STAT signaling pathway and formation of the ISGF3 transcription factor. The combined actions of these events result in induction of antiviral effector programs to produce an "antiviral state" within the cell.

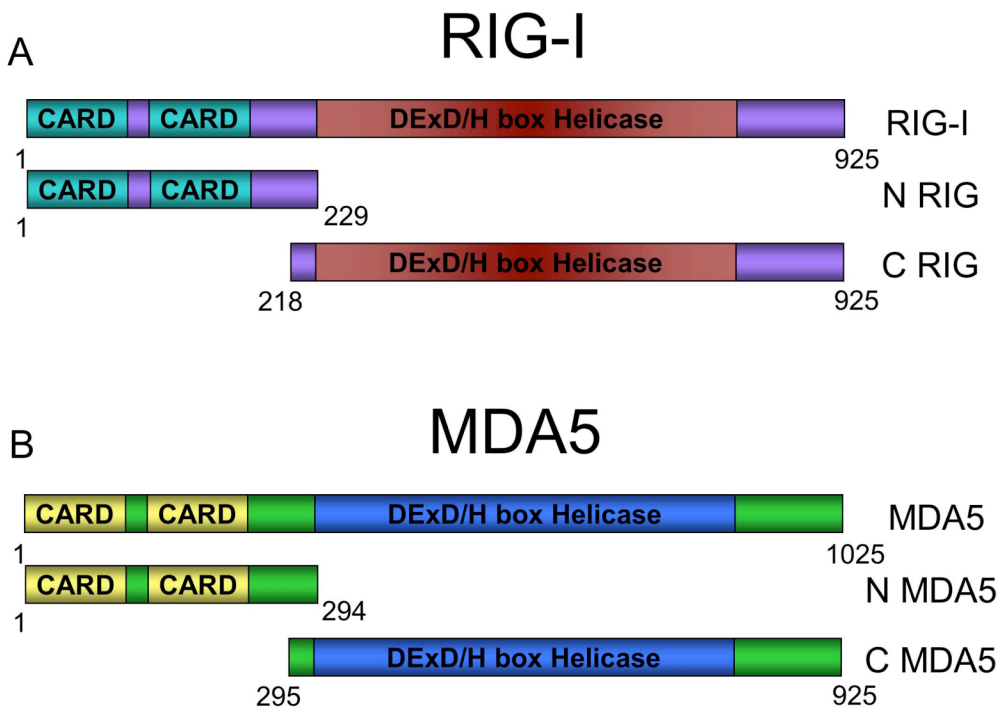


Figure 1-3: Structure of RIG-I and MDA5.
A. Structural representation of RIG-I, N-RIG and C-RIG expression constructs used in this study. The positions of the caspase recruitment domain (CARD), helicase domain and terminal aa are indicated. **B.** Structural representation of MDA5, N MDA5 and C MDA5 expression constructs used in this study. The positions of the caspase recruitment domain (CARD), helicase domain and terminal aa are indicated**.**

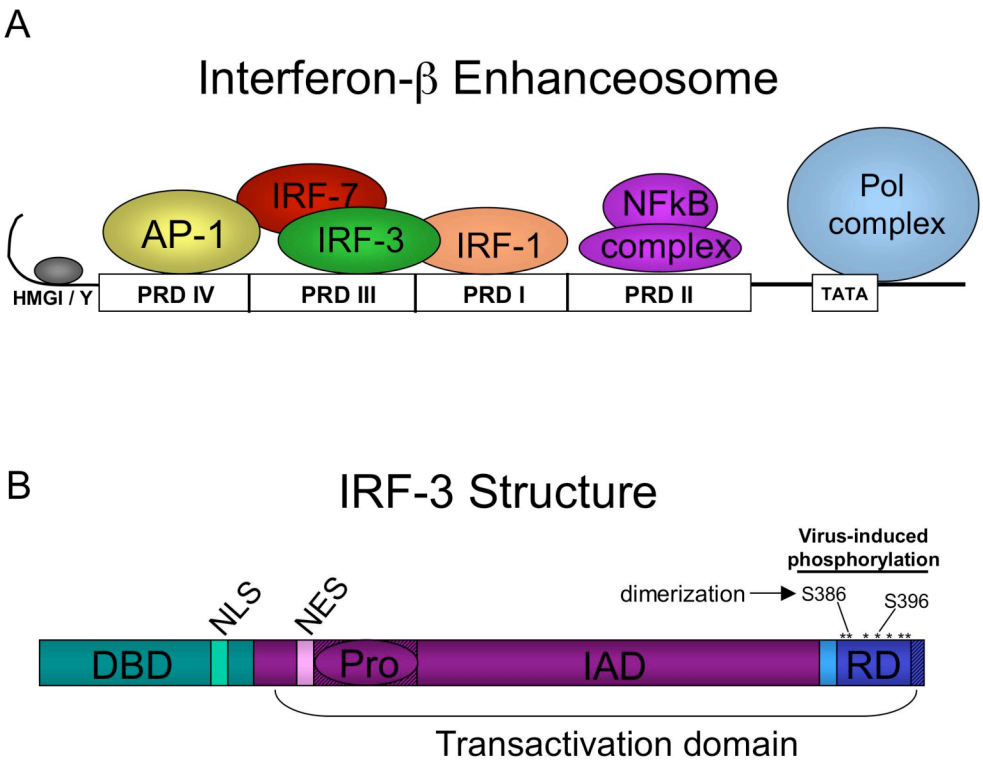


Figure 1-4: Schematic representation of the IFNβ enhanceosome and structure of the IRF-3 protein.
A. Schematic representation of the IFN-β enhanceosome. Shown are the transcription factors AP-1 (ATF2/c-Jun), IRF-3/7, IRF-1 and NF-κB binding to their cognate positive regulatory domains (PRDs). B. Schematic representation of the interferon regulatory factor-3 (IRF-3) transcription factor with the DNA binding domain (DBD), nuclear localization signal (NLS), nuclear export signal (NES), IRF association domain (IAD) and signal response domain (RD) indicated. Serine residues that are phosphorylated in response to viral infection/dsRNA are indicated by asterisks. The critical viral responsive Ser386 and Ser396 are highlighted.

CHAPTER 2: Materials and Methods

Cell Culture and Reagents

Huh7, Huh7.5, A7, 10A, HP, C5B2-3 and C5B 2-3c cells were cultured as described below. All cells were propagated in DMEM supplemented with 10% FBS, 2mM L-glutamine, 1mM sodium pyruvate, antibiotic/antimycotic and MEM non-essential amino acids (cell culture additives from Sigma). Genomic(107) and subgenomic replicons were also cultured in the presence of G418, 800μg/ml and 200μg/ml respectively, for selection. Tetracycline-regulated OS-2 cell lines conditionally expressing HCV proteins were maintained in standard media described above supplemented 400μg/ml G418 and 100μg/ml puromycin and 1μg/ml tetracycline. 293 neo and 293 TLR3 cell lines were a kind gift of Katherine Fitzgerald and cultured in DMEM supplemented with 200μg/ml G418. Wild-type and TRIF null MEFs were a kind gift of Dr. Shizuo Akira. TBK1 null MEFs were generously provided by Dr. Wen-Chen Yeh. For NS3/4A protease inhibition studies, cells were cultured in media containing 10μM or 20μM (as indicated) SCH6, a member of a recently described class of potent experimental peptidic ketoamide inhibitors of the HCV NS3/4A serine protease that was kindly provided by Drs. Michael Murray, Stephany Bogen and Bruce Malcolm of the

Schering-Plough Research Institute, Kenilworth, NJ. For TLR3 signaling, cells were treated with the dsRNA surrogate polyinositol cytosine, pIC, (Amersham Pharmacia) in DMEM at a concentration of 100 μg/ml. For introduction of dsRNA intracellularly, cells were transfected with pIC using Lipofectamine 2000 (Invitrogen). Cells were infected at 100 HAU/ml with Sendai virus (Cantell strain), purchased from Charles River Laboratories. TNFα (Roche) and IL-1β (R&D Systems) were used at 10 ng/ml in DMEM. IFN-α2a (PBL Laboratories was added to DMEM at the concentrations indicated.

Plasmids and Site-directed mutagenesis

Plasmids encoding the HCV core-p7 and NS3/4A proteins were generated through PCR amplification of the appropriate genes from the p90/HCV H77 genotype 1A clone using the primers 5'- AAA GAA TTC ATG AGC ACG AAT CCT AAA CCT CAA AG -3' and 5'- AAA TCT AGA CTA TGC GTA TGC CCG CTG AGG CAA C -3' for core-p7, 5' TATAATCTTATGGCGCCCATCACGGCGTACGCCCAG 3' and 5' TTTGGATCCTTACGTGACGACCTCCAGGTCGGCCGA 3' for NS3, 5' TATAATCTTATGGCGCCCATCACGGCGTACGCCCAG 3' and 5' ATAGGATCCTTAGCACTCTTCCATCTCATCGAACTCCTGG 3' for NS3-4A, and 5' TTTAAGCTTAGCACCTGGGTGCTCGTTGGCGGCGTCC 3'

and 5’ ATAGGATCCTTAGCACTCTTCCATCTCATCGAACTCCTGG 3’ for NS4A. The resultant PCR fragments containing 5’ HindIII and 3’ BamH1 sites were subsequently subcloned into pcDNA3.1(+) from Invitrogen Life Technologies. NS5A was subcloned from the prototype Con1 HCV replicon sequence, pHCV 1bpt using the primers 5'- AAG CTT ATG TCC GGC TCG TGG -3' and 5'- TCT AGA CTA GCA GCA GAC GAC GTC CTC ACT AGC -3'. The resultant PCR fragments containing 5’ HindIII and 3’ Xba1 were subsequently subcloned into pcDNA3.1(+). The pcDNANS3/4A pt plasmid used to generate Northern probes and NS3 point mutants was generated similarly from the prototype Con1 HCV replicon sequence pHCV 1bpt using the primers 5’ AAGCTTATGGCGCCTATTACGGCCTAC 3’ and 5’ TCTAGACTAGCACTCTTCCATCTCATCGAA 3’. The resultant PCR fragments containing 5’ HindIII and 3’ Xba1 were subsequently subcloned into pcDNA3.1(+). The NS5B construct was generated by subcloning the PvuII/NheI fragment of pBI/GFP/NS5B 1A (generous gift of Curt Hagedorn) into the EcoRV/XbaI site of pcDNA3.1(+). NS4AB-His and NS4B-His (HCV strain HC-J4 genotype 1B) were kindly provided by Karla Kirkegaard. Expression constructs encoding the pEF Bos control vector, wild-type, full-length RIG-I, RIG-I N and RIG I C were generously provided by Dr. Takashi Fujita (283). PEF Bos Flag-TBK1, -IKKε and –TRIF

plasmids were a gift from Dr. Tom Maniatis. pCMV-IRF-3 5D and pIRF-3 ΔN encode the phospho-mimetic, constitutively active IRF-3 and dominant-negative mutants of IRF-3, respectively, and were kindly provided by Dr. John Hiscott. The luciferase reporter constructs were kindly provided as follows: pIFN-β and PRDII-luc (Dr. Zhijan Chen, UT Southwestern Medical Center), p561-luc (Dr. Ganes Sen, Cleveland Clinic). pCMV-Renilla was purchased from Promega.

The critical zinc coordinating cysteine residue (C1125A)(51) and active site nucleophilic residue (S1165A) of the protease domain and helicase/RNA binding (W1528A) (243) NS3 mutants were generated by site-directed mutagenesis of the pcDNANS3-4A pt or pFlag NS3/4A construct using the QuikChange™ XL Site-Directed Mutagenesis Kit (Stratagene) as recommended by the manufacturer using the primers 5' CTTGACACCATGCACCGCCGGCAGCTCGGAC 3' and 5' GTCCGAGCTGCCGGCGGTGCATGGTGTCAAG 3' for C1125A, 5'cct aCT TGA AGG GCT CTG CGG GCG GTC CAC TGC tct g 3' (sense) and 5' cag aGC AGT GGA CCG CCC GCA GAG CCC TTC AAG tag g 3' (antisense) for S1165A and 5' CGCGGGCTGTGCTGCGTACGAGCTCACGC 3' and 5' GCGTGAGCTCGTACGCAGCACAGCCCCGCG 3' for W1528A. Mutated sites are underlined. The UT Southwestern Medical Center DNA

sequencing core facility verified the DNA sequence of the resulting expression construct.

Sendai Virus Infection

Cells were infected with Sendai virus (SenV; Charles River Laboratory) for the indicated times using an inoculum of 100 HAU/ml culture media. Sendai virus was prepared at the appropriate dilution in serum-free media from a concentrated virus stock. 0.1 or 0.5 ml of diluted virus were added to cells in 48 or 12-well plates, respectively, and inoculated cultures were placed at 37°C to facilitate virus attachment and infection. After 1hr three volumes of pre-warmed DMEM containing 10% FCS was added to each well of the culture dish, and cells were further cultured for the indicated times prior to harvesting. For each experiment, mock-infected control cultures were similarly incubated in the absence of added virus.

Luciferase Assay

Transfection of plasmid DNA was carried out with FuGENE 6 transfection reagent (Roche Molecular Biochemicals) as recommended by the manufacturer. For each luciferase reporter experiment, cells were

seeded at $2x10^4$ cells in triplicate wells of 48-well plate. For a particular transfection combination, each set of wells was transfected with a transfection master mix containing, on a per well basis, 25 ng of *Renilla* luciferase reporter plasmid pCMV *Renilla*-luc (used to monitor transfection efficiency), 50 ng of the indicated IRF-3-dependent or NF-κB-dependent promoter-firefly luciferase reporter plasmids and 50-200 ng expression construct for a total DNA:FuGENE6 ratio of 1:3 in serum free culture media. For Sendai virus infection (described below), transfected cells were cultured for 24 hr at 37°C prior to virus infection. To evaluate luciferase promoter induction, the Dual-Luciferase^R Reporter Assay System (Promega) was used as recommended by the manufacturer, except that assays were carried out with 20 µl cell lysate and 50 µl of assay reagents. Luciferase activity was measured with a luminometer over a 5 second interval following an initial 2 second delay after the addition of the assay reagent to the cell lysate.

RNA Silencing

For siRNA silencing of gene expression, Huh7 cells were transfected with 80 nM siRNA (Dharmacon) targeting TRIF, RIG-I, or a scrambled negative-control (Ambion) using Oligofectamine (Invitrogen) according to the manufacturer's recommendation. Cells were

cotransfected with pIFN-β-luc and pCMVβgal 24 h later. After an additional 24 h, cells were mock-infected or infected with 100 HAU/ml SenV and harvested 20 h later for luciferase and β-galactosidase assay (Promega). In these experiments luciferase activity was normalized to the β-galactosidase activity.

Microarray analysis

Microarray analyses were carried out within the Molecular Genomics Core Facility of the University of Texas Medical Branch. Huh7 2-3 and Huh7 2-3c cells were grown in 10 cm dishes and were either mock-infected or infected with 100 HAU/ml of SenV and harvested 20 h later for total RNA extraction. 25μg of total RNA was used as template for synthesis of first-strand cDNA, which was used subsequently for second-strand synthesis and production of biotinylated cRNA probes that were hybridized to an Affymetrix human GeneChip® (Hu133A) containing 22,283 oligonucleotide probe sets, according to the manufacturer's protocol (Affymetrix, Santa Clara, CA). GeneChips were scanned using an Affymetrix confocal scanner (Algient), and data analyzed with Microarray GeneSuite software 5.0.

Protein Analysis

Transfection of plasmid DNA was carried out with FuGENE 6 transfection reagent (Roche Molecular Biochemicals) as recommended by the manufacturer. For transient expression of HCV NS3/4A, RIG-I constructs or IRF-3, 10^5 cells were transfected in the well of a 12-well culture dish with a similar mixture that contained 0.25 to 1 μg of the indicated expression construct, and transfected cells were cultured a further 24 prior to SenV infection. To evaluate expression of transfected proteins, Sendai or Hepatitis C viral proteins, ISG56, ISG15 and actin expression, cells were harvested in RIPA buffer (10mM Tris pH 7.5, 150mM NaCl, 0.02% NaN_3, 1% Na-deoxycholate, 1% Triton X-100, 0.1% SDS) supplemented with Sigma protease inhibitor cocktail, Calbiochem phosphatase inhibitor cocktail II and 1μM okadaic acid (Calbiochem). Equivalent amounts (20μg) of extract were subjected to SDS – polyacrylamide gel electrophoresis on either 15% (core, NS4A and ISG15) or 10% (all others) polyacrylamide gels. After electrophoresis, proteins were transferred to NitroPure nitrocellulose transfer membrane (Micron Separations Inc.) at 90V for 1hr. Membranes were blocked with PBS containing 0.1% Tween-20 (TPBS) and 5% dried nonfat milk. We used the following monoclonal (mAb) or polyclonal (pAb) antibody reagents:C anti RIG-I pAb and anti IRF-3 phosphoSer386 pAb (kindly provided by

Takeshi Fujita), anti-IRF3 pAb (kindly provided by Michael David), C7-50 core mAb (kindly provided by Dr. Darius Moradpour), A4 E1 mAb and A52 mAb (kindly provided by Dr. Jean Dubuisson), anti-HCV NS3 mAb (Novacastra Laboratories), anti-NS4A mAb (Virogen, clone 4-F5), anti NS4B pAb (kindly provided by Dr. Ralf Bartenschlager), anti NS5B pAb (kindly provided by Curt Hagedorn), anti-HCV (human patient serum, kindly provided through informed consent by Dr. William Lee), HIS5033, an anti-IRF3 phosphoSer396 rabbit pAb serum(generous gift from John Hiscott), anti-Sendai virus pAb (a kind gift from Dr. Ilkka Julkunen, , anti-P56 rabbit pAb serum (kindly provided by Dr. Ganes Sen), anti-ISG15 rabbit pAb serum (kindly provided by Dr. Arthur Haas), anti IκBα, anti TANK and anti TBK1 rabbit pAb and goat anti-human actin or GAPDH pAb (Santa Cruz), anti TBK1 and IKKε mAbs (Imgenex), anti T7 goat pAb (Novus Biologicals), anti MDA5 goat pAb (Abcam). All primary antibodies were prepared in TPBS containing 10% FBS and 0.02% NaN_3 and incubated either 1hr at room temperature or overnight at 4˚C. Membranes were washed 3X for 5 min with TPBS and probed with peroxidase-conjugated secondary donkey anti-rabbit, donkey anti-mouse, donkey anti-goat or donkey anti-human antibodies (Jackson Immunoresearch) diluted 1/10,000 in TPBS containing 5% dried nonfat milk. Protein bands

were visualized using the ECL+ Western Blotting detection reagents (Amersham Biosciences) followed by exposure of the blot to film.

Immunofluorescence Analysis

Cells were plated 2-5 x 10^4 in chamber slides and transfected/infected as described above. After appropriate time period (16hr for viral infection/24-48hr for transfection), slides were washed with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. Cells were permeablized with 0.2% Triton X 100 for 15 min and then blocked with 10% normal goat serum. Cells were incubated for 1 hr with 1/500 dilution of rabbit anti-IRF-3 pAb serum (generously provided by Dr. Michael David), 1/X dilution of rabbit anti-RIG-I pAb (generously provided by Dr. Takashi Fujita), 1/500 M2 anti-Flag mAb (Sigma) or 1/200 anti-NS3 mAb (Novocastro Laboratories). Slides were washed three times followed by 1 hr incubation with 1/1000 dilution of goat anti-rabbit IgG-FITC or goat anti-mouse Rhodamine X conjugated secondary antibody. Nuclei were visualized using a DAPI counterstain. Cells were washed three times, the chamber removed and the slide allowed to dry. The coverslip was mounted with Vectashield, sealed and examined for fluorescence.

Native Gel

For dimerization assay studies, cells were cultured and treated as described above. Cell pellets were harvested in nondenaturing lysis buffer (50mM Tris-Cl pH 8.0, 1% NP40, 150mM NaCl) and supplemented with the protease and phosphatase inhibitors described above. The extracts were vortexed and centrifuged to remove the insoluble fractions. READY GELS J (Bio-Rad) are pre-run at a constant current of 40mA for 30 min with upper chamber buffer (25mM Tris-Cl pH 8.4, 192mM glycine and 0.2% deoxycholate) and lower chamber buffer (25mM Tris-Cl pH 8.4 and 192mM glycine). 10μg sample was then added and the samples electrophoresed for 50 min at 25mA. The gel was then soaked in SDS running buffer (25mM Tris pH 8.3, 250mM glycine, 0.1% SDS) for 30 min at room temperature to remove residual deoxycholate. Gels were transferred and probed as described above for standard SDS PAGE gels.

Electrophoretic Mobility Shift Assay

For gel-shift analysis, cells were plated in 10 cm dishes and cultured as described above. Cells were harvested by scraping into ice cold PBS and pelleted at 1850 g for 10 min at 4°C. The supernatent was removed and the packed cell volume measured. Cells were incubated on ice in lysis buffer (50mM Tris-HCl pH 8.0, 60mM KCl, 1mM EDTA, 2mM

DTT and 0.15% NP40) to facilitate swelling. Lysates were centrifuged at 3300 g for 15 min to separate cytosol and nuclei. Nuclei were lysed in 1 volume lysis buffer (20mM Tris-Cl pH 8.0, 400mM NaCl, 1.5mM $MgCl_2$, 200μM EDTA and 25% glycerol) and incubated on ice for 2 min. Added NaCl to nuclear extracts for a final concentration of 400mM to strip proteins from DNA and incubated on ice for 10 min. Samples were vortexed for 15 sec and centrifuged at maximum speed for 15 minutes to collect nuclear extract. Probe labeling was performed by incubating 5 pmol dsDNA template, ^{32}Pγ-ATP, and T4 Kinase (GibcoBRL) for 30 min at 37°C. The probe was purified on a spin column to remove unincorporated nucleotide (BioRad). Binding reactions were carried out with 5 μg RNA, 50,000 cpm radiolabeled probe in EMSA binding buffer (60mM HEPES pH 7.9, 50mM NaCl, 1mM EDTA, 0.5mM DTT, 0.3 mg/ml BSA, 52μg/ml salmon sperm DNA and 10% glycerol) for 30 min at 30°C. Samples were separated on a 6% TBE gel (prerun 3hr at 200V) for 90 min at 275V at 4°C. Gels were fixed (50% methanol, 10% acetic acid) for 30 minutes and dried for 90 min at 80°C on a vacuum dryer. The blots were subjected to autoradiography.

Real time RT-PCR

RNA was extracted from cells using Trizol Reagent as recommended by the manufacturer (Invitrogen Life Technologies, Inc.). 5 µg of purified total cellular RNA was treated with Turbo DNAse (Ambion) to remove any contaminating genomic DNA for 1hr at 37°C. Enzyme activity was neutralized with DNAse inactivation reagent for 2hr at room temperature (Ambion). To reverse transcribe the RNA, 2 µg and **?**µM oligo dT were heated to 50°C for 2.5 min followed by the addition of a mastermix containing RNAsIN, 0.5 mM dNTPs and Omniscript reverse transcriptase (Qiagen) and incubated for 1hr at 37°C. 50 ng of the resultant cDNA was used for real time PCR analysis using the primers 5' GACTGGACGTGGCAAAACAA 3' (sense) and 5'TTGAATGCATCCAATATACACTTCTG 3' (antisense), SYBR green PCR mix and the ABI 5700 real time PCR machine with all reactions conducted in triplicate. Data was analyzed using the $\Delta\Delta C_T$ method for determining relative RNA amounts.

Northern Blot Analysis

RNA was extracted from cells using Trizol Reagent as recommended by the manufacturer (Invitrogen Life Technologies, Inc.). Purified RNA was resuspended in water, quantified by spectrometry, and

10 μg was mixed with RNA loading buffer (Ambion). After heating at 50°C for 10 min, RNA samples were separated through a 1% agarose gel containing 2.2 M formaldehyde, 20 mM MOPS (pH 7.0), 8 mM NaOAc and 1 mM EDTA (pH 8.0). For transfer, the gel was soaked in water for 1 hr with gentle agitation, changing the water three times to remove formaldehyde followed by incubation in 20X SSC for 15 min. RNA transfer onto Nytran membrane was carried out using the Schleicher & Schuell Turboblotter downward transfer system as recommended by the manufacturer. After transfer, RNA was UV crosslinked to the nylon membrane. Random priming was used to generate DNA probes specific for the HCV NS3 coding region, ISG15, ISG56, OASI, 6-16, and human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using Klenow DNA polymerase and mixed nonomer random primers in a reaction that contained α^{32}P-dCTP. Hybridization was performed using the ULTRAhyb™ reagent (Ambion) and 10^6 cpm/ml of radiolabeled probe at 48°C for 16 hr. Blots were rinsed twice for 5 min. each with preheated 2X SSC/0.1% SDS wash buffer, followed by two 15 min washes with preheated 0.1X SSC/0.1% SDS wash buffer. Blots were subjected to autoradiography. GAPDH was used as a loading control.

Protein stability analysis

For metabolic labeling studies, 4 x 10^5 cells were plated in 6 cm dishes and cultured and transfected as described above. Cells were washed twice and starved in methionine deficient media (Cellgro) for 1 hr prior to radiolabeled pulse. The culture media was removed and replaced with methionine deficient media supplemented to 200μCi/ml ^{35}S-methionine Easy Tag EXPRESS protein labeling mix (Perkin Elmer) for 30 min to 1 hr. The label media was removed, the cells washed to remove excess radiolabeled methionine and complete media was added for the duration of the chase. Cells were harvested by scraping and lysed in RIPA buffer as described above. The protein of interest was immunoprecipitated as described below. The pellet fractions were eluted in SDS sample buffer and separated on 10% SDS PAGE gels. Gels were fixed (50% methanol, 10% acetic acid solution), soaked in Amplify fluorogenic reagent (Amersham) and dried for 90 min at 80°C on a vacuum dryer. The blots were exposed to film and subjected to autoradiography.

Immunoprecipiations

For immunoprecipitation studies, 4 x 10^5 cells were plated in 6 cm dishes and cultured and transfected as described above. At 24 hr post

transfection, cells were either harvested for analysis or in some cases infected with Sendai virus as described above for the indicated times. Cell pellets were resuspended in Buffer A (25mM Tris pH 7.2, 150mM NaCl, 1% NP40) supplemented with phosphotase and protease inhibitors as described above. Cells were disrupted through a 22G syringe and rocked for 30 min at 4°C. Cells were pelleted at maximum speed for 15 min and the supernatent collected. Protein concentration was determined by Bradford assay and equivalent amounts of extract were applied to M2 anti-Flag conjugated agarose beads (Sigma) and incubated with rocking overnight at 4°C. Supernatent fractions were collected and the beads were washed 3X with Buffer A. Samples were boiled in SDS sample loading buffer and separated by SDS PAGE and analyzed by immunoblot assay.

CHAPTER 3: Control of interferon regulatory factor-3 by the hepatitis C virus serine protease

Introduction

HCV infection is a leading cause of liver related morbidity and mortality worldwide. This is underscored by the fact that most individuals exposed to this virus develop persistent infection that is frequently refractory to current interferon (IFN)-based therapies(78,166). HCV persistence is due in part to an ability of the virus to incorporate adaptive mutations and replicate as a population of genetically distinct quasispecies(68,74). However, persistence is also likely to reflect specific disruption of host immune responses by HCV proteins(81,247,289).

Cellular control of virus infection is mediated through a variety of processes that impact various stages of the viral lifecycle(128), and the interferon regulatory factors (IRFs) are key transcription factors that initiate this cellular antiviral state(14). IRF-3 is a latent cytoplasmic factor that is activated through phosphorylation by an undefined virus-activated kinase (VAK)(157,285). VAK induction is stimulated by double-stranded RNA (dsRNA) intermediates and other products of virus replication that accumulate during infection(232,285). Phosphorylation of IRF-3 leads to its dimerization, nuclear translocation and subsequent induction of type I

IFNs and a variety of other antiviral effectors(157,205). HCV RNA replication has the capacity to activate IRF-3 and to stimulate the cellular antiviral response(77), suggesting that control of IRF-3 could be an important determinant of viral persistence. This notion is underscored by the lack of resistance to virus infection in IRF-3-null mice(213), and the fact that many viruses encode proteins that perturb IRF-3 function(14,232). The goal of this study was to examine the impact of HCV on the host IRF-3 pathway and conversely, to assess the ability of the IRF-3 pathway to control HCV infection.

Results

HCV imposes a blockade to IRF-3 activation

Since there are no fully permissive cell culture models to propagate HCV(204), we utilized Huh7 human hepatoma cell lines supporting replication of genome-length HCV genotype 1b RNA (C5B2-3) (19) to study the effect of HCV replication on IRF-3 function (Intro Fig. 1-1B). Sendai virus infection of control hepatoma (Huh7) cells induced the activation and nuclear translocation of IRF-3, demonstrating that these cells harbor an intact IRF-3 pathway (Fig. 3-1A upper panel). However, when we examined the subcellular localization of IRF-3 in C5B2-3 cells

there was no active, nuclear IRF-3 despite the presence of endogenous replicating HCV RNA and protein expression (Fig. 3-1A left middle panel). Furthermore, Sendai virus infection of C5B2-3 cells similarly did not result in the activation and nuclear accumulation of IRF-3 suggesting a general blockade in viral activation of IRF-3 in these cells (Fig. 3-1A right middle panel). Prior treatment of C5B2-3 cells with high concentrations of IFN-α2b (109) effectively eliminated both HCV RNA and proteins to generate a cured cell line C5B2-3c. Challenge of the cured cell line with Sendai virus infection revealed a restoration of the IRF-3 response (Fig. 3-1A lower panel). These data demonstrate that the block in virus-activation of IRF-3 in C5B2-3 replicon cells was mediated through HCV-specific disruption. Similarly, inhibition of Sendai virus induced IRF-3 activation was also observed in UHCV11 osteosarcoma cells which conditionally express the entire genotype 1a HCV polyprotein under control of a tetracycline-regulated promoter (Fig. 3-1B). Importantly, these cells do not harbor replicating HCV RNA nor the structured 5' and 3' NTRs of the viral genome. Thus, IRF-3 inhibition occurs independently of HCV RNA replication, in different cell types and with multiple HCV genotypes.

To determine whether HCV proteins similarly influence IRF-3-dependent gene expression, we assessed the activity of promoters from the IFN-β, ISG56, ISG15, and RANTES genes in C5B2-3 cells supporting

HCV RNA replication, and in the IFN-α2b-cured counterparts (Fig. 3-1C). Each of these promoters requires activated IRF-3 for induction(88), and each could be efficiently induced in control Huh7 cells upon Sendai virus infection. In contrast, the induction of promoter activity by Sendai virus was blocked in C5B 2-3 cells supporting HCV replicons, but was fully restored in the cured cells correlating with the absence or presence of IRF-3 in the nuclei of these cells observed above. Thus, one or more HCV proteins specifically disrupt viral activation of the IRF-3 pathway, thereby blocking downstream expression of IRF-3 target genes.

Inhibition of IRF-3 is mediated by the HCV NS3/4A holoenzyme complex

To identify which HCV protein(s) mediate the IRF-3 blockade, we examined IRF-3-dependent promoter activity in transfected Huh7 cells expressing the HCV structural proteins or individual NS proteins. Transient expression of the structural proteins (core, E1, E2, and p7) or the nonstructural proteins, NS4B, NS5A or NS5B did not significantly affect Sendai virus induction of the IRF-3-dependent ISG56 promoter (Fig. 3-2A). In contrast, expression of the NS3/4A protein complex strongly inhibited ISG56 promoter induction. Since the stability, subcellular localization and function of the NS3 protease is dependent upon

noncovalent association with NS4A(52), we also tested the activity of these proteins individually in blocking IRF-3 activation. Neither NS3 nor NS4A alone, nor the expression of an NS4A-4B protein significantly impaired Sendai virus-induction of the ISG56 promoter (Fig. 3-2A). Thus, the HCV NS3/4A complex is responsible for blocking virus induction of the IRF-3 pathway.

To determine how NS3/4A disrupts IRF-3 function, we characterized the IRF-3 present in cell lines that conditionally express either NS3/4A or the complete genotype 1a polyprotein, or, as controls, the HCV core or NS5A proteins. In the absence of HCV proteins, Sendai virus infection triggered the accumulation of the high mass, hyperphosphorylated isoform of IRF-3, its dimerization and subsequent nuclear translocation resulting from viral induction of VAK activity (Fig. 3-2B and 3-2C)(224). Expression of the HCV core and NS5A proteins had no effect on Sendai virus activation of IRF-3, but expression of either NS3/4A or the entire HCV polyprotein blocked Sendai virus-induced accumulation of the hyperphosphorylated IRF-3 isoform, thereby precluding IRF-3 activation, dimerization (Fig. 3-2B) and nuclear translocation (Fig. 3-2C). Thus, the HCV NS3/4A complex disrupts viral activation of the IRF-3 pathway by perturbing critical events that signal

IRF-3 phosphorylation effectively preventing its downstream effector functions.

NS3/4A mediated inhibition of IRF-3 function requires intact protease activity

Like many viral proteins, the HCV NS3/4A protein complex encodes several functions. The amino terminus of the protein encodes the viral protease responsible for the downstream cleavage and liberation of the viral nonstructural proteins. The carboxyl terminus of the protein encodes the viral helicase which functions to unwind viral dsRNA during replication. As a helicase, the protein contains the ability to bind dsRNA. The helicase also encodes an NTPase activity which it utilizes to drive the helicase unwinding function of the protein. To determine if either the enzymatic activities or RNA-binding properties of NS3/4A are required for disruption of IRF-3 activation, we introduced single amino acid substitutions into NS3 that eliminate either the protein's RNA-binding and helicase functions (W1528A)(243) or its serine protease activity (C1125A)(52). We confirmed that the NS3/4A complex encoding the C1125A mutation lacked protease activity when expressed in Huh7 cells. We then assessed the ability of each mutant to block Sendai virus induction of IRF-3 activation when expressed in Huh7 cells as NS3/4A complexes. The W1528A

mutant, but not the C1125A mutant, retained the ability to inhibit Sendai virus-induced activation of the IRF-3-dependent ISG56 promoter (Fig. 3-3A). We also mutated the protease active site serine to an alanine (S1165A) to more directly eliminate protease function and minimize structural changes in the protein. This protease mutant similarly lost the ability to inhibit Sendai virus induced IRF-3 activation. These results suggest that it is the serine protease activity and not the RNA-binding/helicase function of NS3 that is responsible for perturbation of the IRF-3 pathway.

To test this hypothesis more directly, we assessed the ability of the wild-type NS3/4A complex to block Sendai virus activation of IRF-3 in the presence of SCH6, one of a class of recently described specific peptidiomimetic ketoamide inhibitors of the NS3/4A serine protease (Schering Plough). Consistent with the ability of SCH6 to inhibit the NS3/4A protease, SCH6 effectively blocked cis-active cleavage of the NS3/4A precursor protein in osteosarcoma cells that conditionally express this segment of the polyprotein (Fig. 3-3B). Importantly, SCH6 treatment of cells also rescued Sendai virus-induced IRF-3 hyperphosphorylation and IRF-3 nuclear translocation (Figs. 3-3B and 3-3C). Since SCH6 is a specific inhibitor of the NS3/4A protease, these data provide strong evidence that the serine protease activity of the NS3/4A complex is

required for blockade of the IRF-3 pathway. These results are thus consistent with the failure of the C1125A and S1165A mutants to block IRF-3-dependent promoter induction (Fig. 3-3A). Importantly, these data suggest that antiviral inhibitors of protease activity can reverse the IRF-3 blockade imposed by the NS3/4A protease.

Regulation of IRF-3 function directly correlates with HCV fitness

To further define the relationship between HCV replication and IRF-3 function, we characterized the IRF-3 activation status and HCV viral RNA replication in cells containing HCV subgenomic replicons. Replicons constructed from the wild-type Con1, genotype 1b HCV sequence replicate poorly in Huh7 cells, but accumulate adaptive mutations in the NS proteins that enhance their replication capacity(27,159). We identified replicon-bearing cell lines, termed HP and A7, in which HCV RNA abundance inversely correlated with the host cell activation state of IRF-3. Moreover, in all other replicons that we examined, the overall replicon fitness inversely correlated with the level of IRF-3 activation detected in the respective replicon host cell. The HP replicon, containing two adaptive mutations within NS3 (P1115L and K1609E), exhibits robust replication and blocked both basal and Sendai virus-induced IRF-3 phosphorylation, nuclear translocation and IRF-3 dependent promoter activity (Fig. 3-4A, 3-

4B, 3-4D). We confirmed that this inhibition was not due a global defect in nuclear transport because the phospho-mimetic IRF-3 5D mutant(158) efficiently localized to the nucleus when introduced into HP cells (Fig. 3-4C). In contrast, the A7 replicon, containing the adaptive mutations A1113P and L1701F within NS3/4A, replicates to approximately 1/10th the level on a per cell basis as the HP replicon. A7 replicon cells exhibit a low basal level of nuclear localized, hyperphosphorylated IRF-3 and induce IRF-3 dependent promoter activity that is further responsive to Sendai virus challenge (Fig. 3-4A, 3-4B and 3-4D). Thus, HCV replication has the potential to both activate and block the IRF-3 pathway and this directly correlates with overall viral fitness.

Induction of IRF-3 effector programs control HCV RNA replication

We next examined the direct influence of IRF-3 activation on HCV RNA replication. The basal activation of IRF-3 in cells harboring the A7 HCV replicon observed above correlated with low viral RNA levels and basal activation of the IRF-3 dependent genes ISG56 and ISG15 as demonstrated by both Northern and Western blot analysis (Fig. 3-5A and 3-5B). Expression of the constitutively active IRF-3 5D mutant(158) in A7 HCV replicon cells further increased the expression of ISG15 and ISG56 and associated with a modest reduction in viral RNA levels. In contrast,

expression of the dominant-negative IRF-3 ΔN mutant(158) resulted in a greater than 500% increase in HCV RNA levels and a parallel reduction in IRF-3 target gene expression. These data clearly demonstrate that abrogation of the IRF-3 pathway enhances HCV RNA replication. Consistent with this, the efficient NS3/4A mediated disruption of the IRF-3 pathway mediated by the HP replicon was associated with a much higher basal abundance of HCV RNA and lack of induction of IRF-3 target genes (Fig. 3-5B, lower panel, compare lanes 6 and 11). However, expression of IRF-3 5D in HP cells, which effectively bypasses the block imposed by NS3/4A, induced IRF-3 target gene expression and concomitantly reduced HCV RNA abundance. However, expression of the dominant negative IRF-3 ΔN mutant had no further effect on replicon RNA abundance, consistent with a preexisting block in the IRF-3 pathway imposed by NS3/4A. These observations identify IRF-3 as an important factor in regulating the cellular antiviral response to HCV and demonstrate that artificial modulation of this pathway has the capacity to control HCV replication.

Inhibition of NS3/4A protease activity enables host recognition of HCV PAMPs and restores the antiviral response program

Our results with the SCH6 protease inhibitor described above revealed that it has the potential to relieve protease-mediated inhibition of IRF-3 function. We wished to extend these observations and determine if inhibition of NS3/4A protease function could restore the ability of the host cell to respond to HCV RNA replication. To address this, we attempted to unmask the NS3/4A-mediated blockade of IRF-3 function in HP replicon cells by treatment with the SCH6 NS3/4A protease inhibitor. We chose this cell line because the HP replicon is so efficient in abrogating host-signaling responses leading to the activation of IRF-3 and we wanted to test the efficacy of a potential therapeutic inhibitor in restoring this response. Sendai virus infection of control Huh7 cells induced a host response marked by IRF-3 hyperphosphorylation and subsequent IRF-3 dependent ISG56 gene expression (Fig. 3-5C, left panel). Within 24 hrs of the addition of SCH6 to the culture medium, a similar host response was induced in the Huh7-HP cells, in the absence of Sendai virus infection (Fig. 3-5C, right panel). Identical SCH6 treatment did not evoke these responses in control Huh7 cells (Fig. 3-5C, left panel). These data suggest that in addition to directly inhibiting the critical role of the protease in the viral replication cycle, protease inhibitors also have the potential to

disable NS3/4A mediated inhibition of IRF-3 function. This reprieve allows the host cell to regain the ability to respond to endogenous HCV PAMP stimuli and effectively induce response pathways that control infection. Thus, HCV RNA replication has an inherent capacity to signal an IRF-3 dependent host response, thereby triggering an IFN amplification loop that would otherwise limit viral replication in the absence of an effective NS3/4A blockade. This provides an extremely attractive and promising therapeutic target that should demonstrate enhanced efficacy in the clinical setting.

Identification of the HCV 5' NTR as a viral dsRNA PAMP

In order to determine what the HCV stimulus was that led to the activation of IRF-3 and induction of host antiviral programs (Fig 3-4 and 3-5) we assessed the ability of viral products to trigger this response. As demonstrated above, HCV viral protein products do not trigger the activation of IRF-3 or induction of antiviral effector genes (Fig. 3-2). However, virally encoded dsRNAs have been demonstrated to be a potent trigger of the IRF-3 pathway(269). We reasoned that conserved regions of HCV dsRNA structure encoded in the 5' NTR might serve as an agonist for the activation of IRF-3. To test this hypothesis, we generated RNAs from either the HCV 5' NTR or a polyadenylated ssRNA as a control.

Sendai virus infection or transfection of the HCV 5' NTR into U2OS cells resulted in a robust nuclear accumulation of IRF-3 however, transfection of cells with the poly(A) construct did not (Fig. 3-6). NS3/4A blocked the activation of IRF-3 signaled by both Sendai virus and the HCV 5' NTR (Fig. 3-6). These data describe the HCV 5' NTR as a potent dsRNA PAMP capable of activating the host IRF-3 response pathway. Importantly however, the virus encodes a mechanism through the actions of the NS3/4A protease to abrogate this response.

Discussion

Our results show that HCV interacts directly with host pathway(s) that signal IRF-3 activation. Studies in the A7 subgenomic replicon and involving overexpression of a constitutively active IRF-3 mutant reveal that activated IRF-3 function suppresses HCV RNA replication. Similarly replicon studies also reveal that HCV RNA abundance is increased upon disruption of IRF-3 function, either through the actions of the NS3/4A protease or a dominant negative IRF-3 mutant. Thus, the activation status of IRF-3 is an important factor in the virus-host cell interaction and determines the outcome of infection. The ability of the NS3/4A protease to effectively inhibit IRF-3 demonstrates that HCV, like other viruses(220), has evolved a specific mechanism that allows it to circumvent a major arm

of the host response to infection. Indeed, examination of HCV chronically infected human liver biopsies has shown a clear blockade of nuclear localized IRF-3 in HCV infected hepatocytes (P. Fish, unpublished observations). This data correlates with absent and/or decreased levels of transcripts of IRF-3 target and other ISGs isolated from the liver of chronically infected HCV patients(115). Taken together, data derived from HCV infected individuals correlates with our studies suggesting that NS3/4A control of IRF-3 signaling imparts a selective advantage to HCV persistence by preventing the induction of antiviral response pathways that would otherwise culminate in elimination of the virus from infected cells.

IRF-3 induces the expression of a variety of cellular genes including the type I IFNs(184,285), which contribute to and further amplify the antiviral response by inducing hundreds of interferon stimulated genes (ISGs)(128,220). Importantly, work from our group has demonstrated that the direct action of P56, the product of the ISG56 gene, plays an important role in controlling translation initiation of the HCV IRES(266). It is therefore in the virus's best interest to avoid turning on the expression of this known antiviral effector and doubtless many other uncharacterized effectors that have roles in directly inhibiting HCV replication. Thus, regulation of IRF-3 function by the HCV NS3/4A protease imposes a

significant limitation upon the host's ability to respond to HCV infection. It may promote viral persistence following initial infection, while also serving to reduce the effectiveness of IFN therapy for chronic hepatitis C, since many ISGs contain IRF-3 target sites within their respective promoter/enhancer regions(88,184). Candidate anti-HCV therapeutics that target the viral NS3/4A protease, while interfering with processing of the viral polyprotein and thus inhibiting viral replication, may also function to restore the IRF-3 pathway. Indeed, our results demonstrate the likelihood of such dual therapeutic mechanisms, since the peptidomimetic ketoamide SCH6 protease inhibitor compound both restored the host IRF-3 pathway and inhibited viral polyprotein processing resulting in the elimination of the HCV replicon from Huh7 cells. Such "dual efficacy" should be considered in the overall approach to HCV protease inhibitors destined for future clinical evaluation. Phase I clinical trials with a protease inhibitor developed by Boehringer have demonstrated enhanced effectiveness in patients resulting in a significant drop in viral load(144). It is likely that when used in combination with other antivirals to combat the emergence of resistant quasispecies, HCV viral clearance may indeed become a reality.

The exact mechanism of NS3/4A mediated IRF-3 pathway inhibition still remains to be elucidated. The studies described above

however have revealed that NS3/4A activity likely involves perturbation of cellular processes that catalyze or otherwise trigger IRF-3 phosphorylation, dimerization and nuclear translocation(158). The VAK responsible for phosphorylation of IRF-3 in this context has yet to be identified, and upstream steps in this signaling pathway remain poorly defined. However, our results suggest that one or more signaling components in the IRF-3 activation pathway, including possibly the VAK, could be specifically targeted for proteolysis by NS3/4A. The requirement for the serine protease activity of NS3/4A identifies a unique mechanism of IRF-3 regulation. No other virus to date has utilized an enzymatic mechanism to specifically disrupt IRF-3 function. While this offers exciting therapeutic opportunities as discussed above, it also provides us with a powerful tool to probe signaling pathways with the hope of further elucidating factors involved in the poorly characterized antiviral/dsRNA sensing pathway.

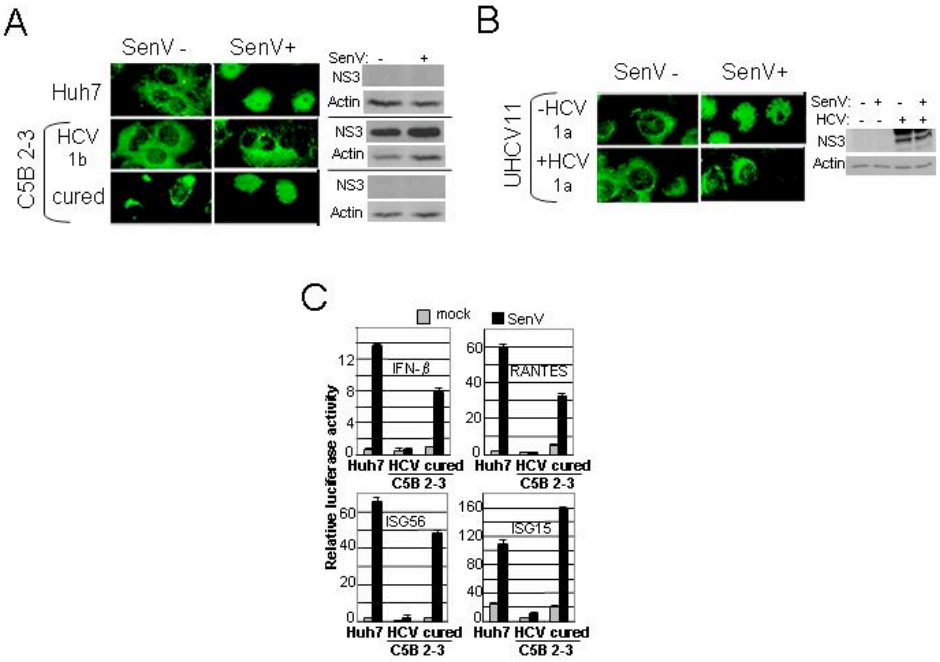


Figure 3-1: HCV imposes a blockade to IRF-3 function.
A. Cells were immunostained for IRF-3 after a 16 hr. mock infection (left panels) or infection with SenV (right panels). Panel sets, from top to bottom, show IRF-3 subcellular localization in parental Huh7 cells, Huh7-derived C5B 2-3 cells harboring replicating genome-length HCV 1b RNA (HCV 1b) or their IFN-α2b cured counterparts (cured). **B.** IRF-3 subcellular localization in UHCV11 cells cultured to repress (-HCV 1a) or induce expression of the HCV 1a polyprotein (+HCV 1a). At the right are shown immunoblots of extracts from these cell cultures stained with antiserum against NS3 or actin (control). **C.** Huh7 cells, C5B 2-3 cells containing replicating HCV RNA (HCV) or cured C5B 2-3 cells were transfected with the indicated promoter-luciferase reporter constructs and then infected with SenV (black bars) or mock infected (gray bars). Luciferase activities were determined from cell extracts. Values shown are an average of three experiments.

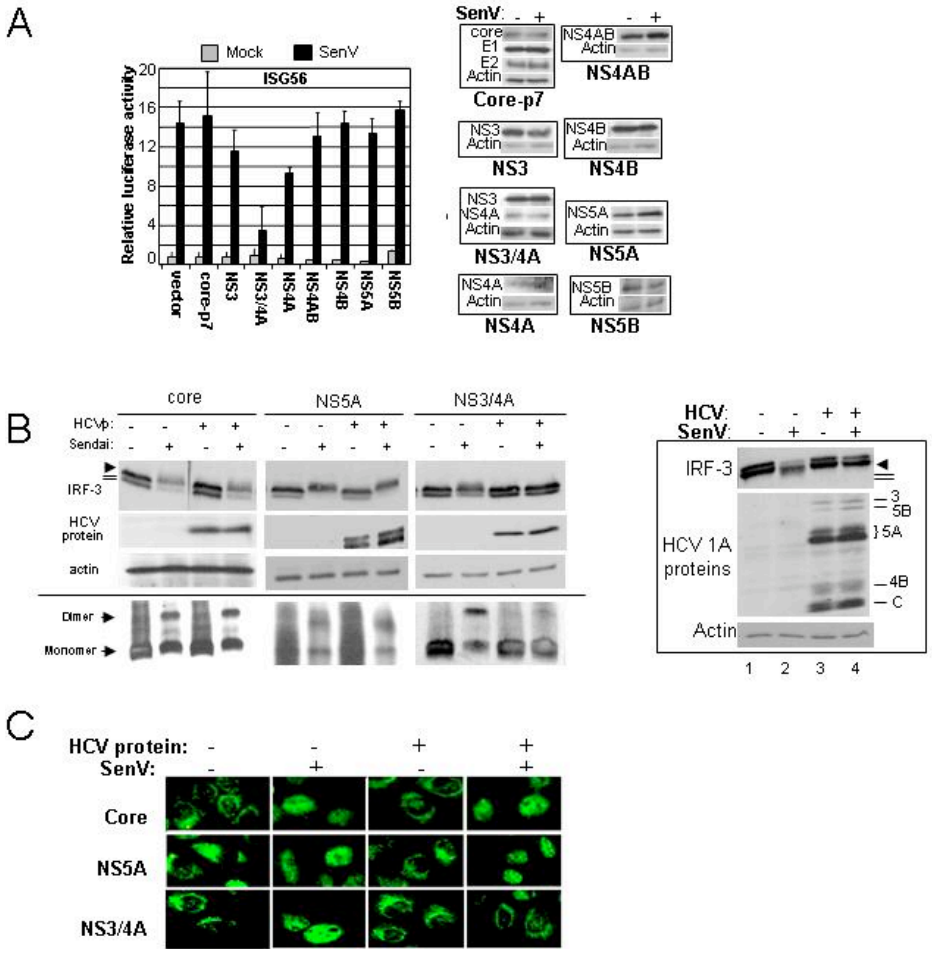


Figure 3-2: Inhibition of IRF-3 is mediated by the HCV NS3/4A holoenzyme complex.

A. The left panel shows luciferase activities (an average from 3 experiments) in extracts derived from Huh7 cells that were co-transfected with the ISG56 promoter-luc construct and (from left to right) vector alone or HCV protein expression plasmids encoding the core-p7 cassette, NS3, NS3-4A, NS4A, NS4AB, NS4B, NS5A, or NS5B. Cells were either infected (black bars) or not infected (gray bars) with SenV. When compared to cells expressing NS3/4A, the SenV-induced increase in luciferase activity within each culture was significant (Student's *t* test; $p \leq 0.008$). On the right are shown immunoblots of HCV protein and actin in the corresponding cell cultures. **B**. Stable osteosarcoma cell lines that conditionally express the HCV core, NS5A or NS3-4A proteins were cultured to repress (-) or induce (+) HCV protein expression, and either infected or not infected with SenV. IRF-3, HCV, and actin protein

expression were profiled by immunoblotting (upper panel sets). The right panel set shows a similar analysis of UHCV11 cells cultured to repress or induce the expression of the HCV 1a polyprotein. The arrowhead and hash marks next to the IRF-3 blots denote the high-mass hyperphosphorylated (virus-activated) and basally phosphorylated (inactive) IRF-3 isoforms, respectively. The hash marks next to the UHCV 11 blot indicate the positions of the HCV core protein (C) and the nonstructural proteins 3, 5B, 5A, and 4B. The HCV proteins were detected using a well-characterized anti-HCV patient serum(77). Left ower panels set shows IRF-3 dimerization assay profiled using nondenaturing immunoblot analysis with the monomeric and dimeric forms of IRF-3 indicated. **C**. IRF-3 subcellular localization was determined by immunofluorescence in cells expressing core, NS5A or NS3/4A in the presence or absence of SenV infection.

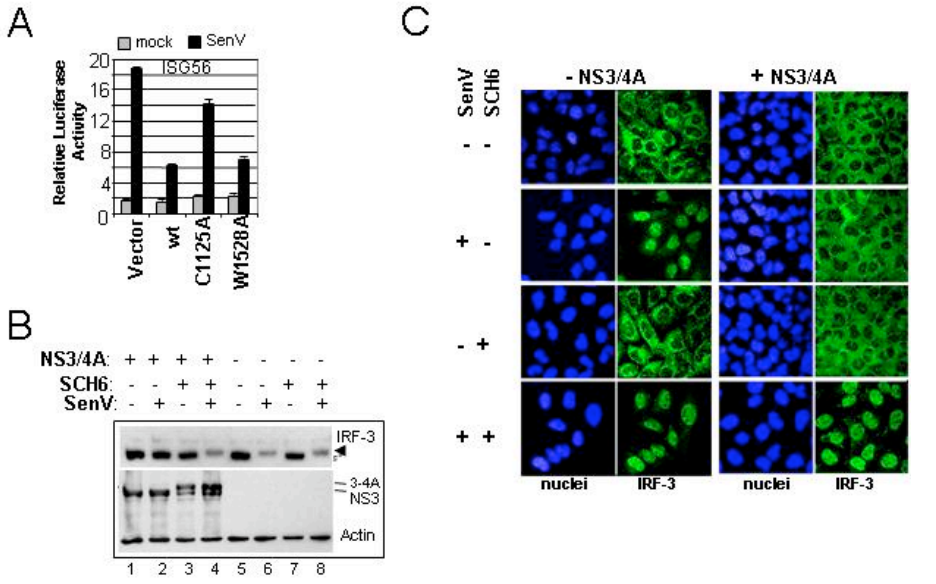


Figure 3-3: The IRF-3 blockade is dependent on HCV NS3/4A protease activity.

A. ISG56 promoter activity was determined in Huh7 cells that were co-transfected with the promoter-luciferase construct and expression plasmids encoding (from left to right) the vector only, the wild-type (wt) NS3/4A, NS3/4A C1125A or NS3/4A W1528A. After transfection cells were either infected with SenV (black bars) or mock-infected (gray bars). **B**. Osteosarcoma cells were cultured to induce (NS3/4A+) or repress (NS3/4A-) NS3/4A expression alone or in the presence of 20 μM of SCH6. Cells were then infected with SenV or were mock infected. Immunoblots of cell extracts were probed with antibodies to IRF-3 (top panel), or NS3 and actin (lower panel). The positions of the hyperphosphylated (activated) and basally phosphorylated (inactive) IRF-3 isoforms are marked by the arrowhead and hash marks, respectively. **C**. The subcellular localization of IRF-3 under these conditions was determined by immunostaining: in each panel set the left panel is stained with DAPI to visualize nuclei, while the right panel shows FITC staining for IRF-3.

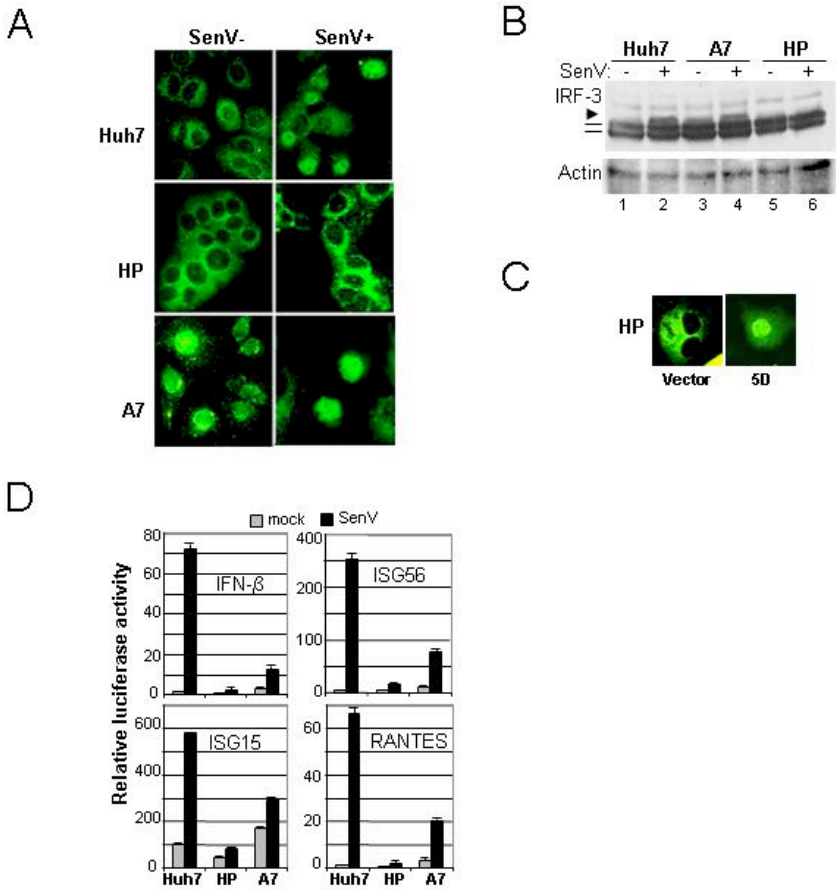


Figure 3-4: Regulation of IRF-3 function directly correlates with HCV fitness.

IRF-3 localization, expression, and HCV RNA replication were assessed in Huh7 cells or Huh7 cells harboring the HP or A7 HCV subgenomic replicons. **A**. IRF-3 subcellular localization (upper panel set) and **B.** protein expression levels (middle panel set) were evaluated within mock-infected or SenV-infected cells. The arrowhead and hash marks next to the IRF-3 blot denote the positions of the hyperphosphylated and basally phosphorylated IRF-3 isoforms, respectively. **C.** The lower panel set shows IRF-3 localization within HP replicon cells transfected with a control plasmid (vector) or a plasmid expressing the constitutively activated IRF-3 5D mutant (5D)(158). **D**. Luciferase activities in extracts of cells that were transfected with promoter-luciferase constructs containing the IFN-β, ISG15, ISG56, or RANTES promoters, and then infected (black bars) or not infected (gray bars) with SenV. Luciferase values are an average from three experiments.

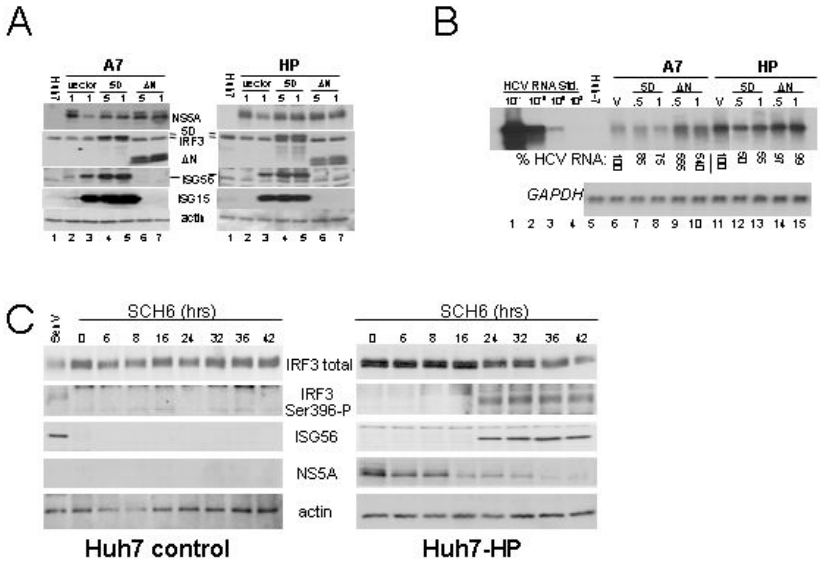


Figure 3-5: Induction of IRF-3 effector programs control HCV RNA replication.

A. Immunoblots for HCV NS5A, IRF-3, ISG56, ISG15, and actin prepared from parallel cultures of Huh7 cells (lane 1) or A7 (left panel set) and HP HCV replicon cells (right panel set). The positions of the endogenous IRF-3, and the IRF-3 5D and ΔN mutant proteins are indicated. Replicon cells were transfected with 1 μg of vector control, or 0.5 or 1 μg of IRF-3 5D or IRF-3 ΔN expression plasmids, as indicated. Lane 3 in each panel set shows protein levels in control cultures treated for 24 hr with 10 U/ml of IFN-α2a. **B.** Northern blots of HCV (upper panel) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) RNAs (lower panel) present in total RNA extracted from Huh7 cells (lane 5), A7 HCV replicon cells (lanes 6-10) or HP HCV replicon cells (lanes 11-15) transfected with 1 μg of the empty expression plasmid alone (vector), or 0.5 or 1 μg of an expression plasmid encoding the IRF-3 5D or IRF-3 ΔN mutants, as indicated above each lane. Values below each lane show the levels of viral RNA present relative to the respective vector control. **C.** Huh7 cells (left panel) or Huh7-HP cells harboring the HP HCV replicon (right panel) were cultured in the presence of 10μM SCH6 for the time indicated. Cells were harvested and extracts subjected to immunoblot analysis for determination of total IRF-3, the phosphoserine 396 isoform of IRF-3 (Ser396-P), ISG56, NS5A and actin levels. A parallel culture of Huh7 cells was infected with SenV and harvested 20 hr later for use as an internal control (far left lane).

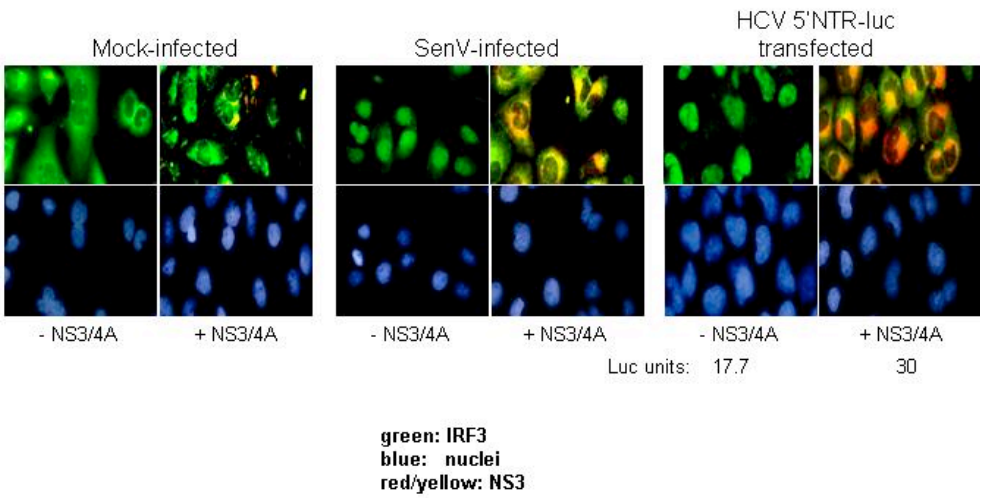


Figure 3-6: NS3/4A controls virus or HCV RNA PAMP signaling to IRF-3.

UNS3/4A cells, cultured to suppress (-NS3/4A) or induce NS3/4A (+NS3/4A) expression, were mock-infected, infected with SenV or transfected with purified in vitro transcribed RNA encoding the HCV 5' NTR appended to the firefly luciferase gene. After 20 h (mock-infected or SenV-infected) or 8 h (HCV RNA transfected) the cells were processed for dual immunostaining using anti-serum to IRF-3 followed by an Alexa 488-conjugated secondary antibody. NS3 was stained using a monoclonal antibody followed by a rhodamine-conjugated secondary antibody. The upper panels in each set show the merged image of IRF-3 (green) and NS3 (red/yellow) obtained from each analysis. The lower panels show DAPI-stained nuclei. Luciferase activity in cells transfected with the HCV 5' NTR-luc RNA was monitored by luciferase assay of extracts derived from 10% of the total transfected cell population and is shown as luciferase (Luc) units beneath the corresponding image set.

CHAPTER 4: Control of antiviral defenses through HCV NS3/4A mediated disruption of dsRNA PAMP receptor signaling pathways

Introduction

Until recently, dsRNA recognition and signaling to IRF-3 was thought to be mediated solely through the TLR3/TRIF pathway. At the time these studies were initiated, the functions of TLR3 as a dsRNA PRR had been recently described(4). The innate antiviral field breathed a collective sigh and it was generally accepted that TLR3 was ***the*** viral dsRNA receptor in cells serving to activate IRF-3 effector function. However, the ligand recognition domain of TLR3 is localized exclusively to the extracellular membrane and within endocytic vesicles making it difficult to reconcile its function as a PAMP receptor for viruses that replicate exclusively in the cytoplasm of cells(163). One explanation to reconcile this could be that TLR3 serves as an early sentinel to recognize viral dsRNA PAMPs before a virus even enters a cell. This could occur through release of viral particles and replication products from neighboring cells that can then be detected in the extracellular milieu by surrounding cells through surface TLR3 expression. These cells can then rapidly respond by initiating antiviral response programs to prevent their own

direct infection. Additionally, the presence of TLR3 within endocytic vesicles positions it to immediately recognize incoming virions, some of which uncoat within these vesicles to possibly expose dsRNA PAMPs. Compounding uncertainty in the exact function of TLR3 for directly sensing viral infection within infected cells is its restricted expression pattern. TLR3 is expressed basally in cells of myeloid origin, primarily in macrophage and dendritic cells. However, it is inducible in nearly all other cell types in response to virus infection or IFN treatment making it more likely to play a secondary or amplification role in antiviral responses rather than a primary role. Indeed, studies demonstrating an important role for TLR3 in antiviral defenses have been reported signifying it as an important mediator in antiviral defenses(4). As such, we wished to evaluate its function in the context of HCV infection and as a potential target for NS3/4A mediated inhibition of IRF-3 function. Additionally, a novel TIR domain adaptor protein TRIF has been identified for TLR3 and TLR4 that specifically directs the activation of IRF-3 and IFN-β gene induction in a MyD88 independent manner suggesting that it may also be a target for NS3/4A regulation(73,214,278). We wished to evaluate the role that the TLR3/TRIF pathway plays in activating the intracellular antiviral response pathway and to assess if either component served as a target for NS3/4A proteolysis.

RIG-I is a cytosolic DExD/H box RNA helicase (Intro Fig. 1-3A) that was recently shown to be a double-stranded (ds) RNA binding protein that functions independently of Toll-like receptor (TLR) 3 and TRIF to signal IFN-β production in response to a variety of RNA viruses, including hepatitis C virus(237,283). RIG-I is basally expressed in most tissues and its expression is further induced by type 1 interferons (IFNs) (287). Our group has recently demonstrated that the RIG-I carboxyl-terminal helicase, or regulatory, domain specifically binds to a pathogen-associated molecular pattern (PAMP) embedded within the structured 5' or 3' nontranslated region of the HCV genome (237) (data from Chapter 3). PAMP binding initiates signaling by the RIG-I amino-terminal caspase recruitment domain (CARD) homologues that direct downstream phosphorylation and activation of IRF-3 and the parallel activation of NF-κB(237,283). Recent studies from our group aimed at characterizing an Huh7 cell line (Huh7.5) defined as highly permissive for HCV replication have shown that the intact processes of RIG-I signaling and the downstream actions of IRF-3 are critical for limiting HCV RNA replication(28,237). The identification of RIG-I as a critical regulator of HCV replication suggests that viral control of this important pathway must be achieved for productive infection and thus is likely targeted by the HCV NS3/4A protease to inhibit IRF-3 activity.

Like RIG-1, MDA5 is a member of the recently described CARD containing, DExH/D box helicase family of intracellular dsRNA receptors (Fig. 1-3B). It similarly promotes the activation of both IRF-3 and NF-κB as demonstrated by ectopic expression of the constitutively active CARD containing amino terminus(282) (E. Foy, unpublished results). However, the relative role of MDA5 in initiating the host response to viral infection remains to be elucidated. RNA silencing studies and data from RIG-I deficient mice reveal the critical requirement for RIG-I in initiating these responses (data above and T. Fujita personal communication). Generation of MDA5 deficient mice and RIG-1/MDA5 doubly deficient mice will be useful in determining the relative contribution of each of these dsRNA PRRs in initiating the host response and in the control of viral infection. At present the role of MDA5 in directly controlling HCV replication has not been evaluated. However, sequence analysis of the MDA5 coding sequence from Huh7 cells has revealed the presence of two point mutations that appear to alter its function (RS and YML unpublished results). Therefore, it is tempting to speculate that MDA5 may represent another cytosolic PRR capable of responding to HCV and its potentially defective signaling in Huh7 cells may additionally explain their relative permissiveness for HCV RNA replication. Indeed, ectopic expression of MDA5 in Huh7.5 cells permits recognition of transfected HCV 5' NTR RNA

to stimulate activation of an IFN-β responsive luciferase promoter. This demonstrates that MDA5 has the capacity to recognize PAMP structures within the HCV genome suggesting that the virus must additionally overcome its function in establishing persistent infection. Similarly, as RIG-I and MDA5 are members of the same family of dsRNA PRRs and signal through tandem CARD motifs the activation of both IRF-3 and NF-κB, it is likely that they share common signaling intermediates that might include the NS3/4A substrate. The ability to target a common signaling factor for two separate host response pathways would represent a clever and efficient mechanism to disarm dsRNA mediated host-signaling pathways.

While the relative importance of each of these dsRNA PRRs in responding to individual viruses and controlling infection remains to be determined, they all appear to play an important role in antiviral defense(4,22,237,283). Interestingly, although the complete signaling pathways have not been fully elucidated for these receptors, they all converge to activate the critical transcription factors IRF-3 and NF-κB. This suggests that activation of both IRF-3 and NF-κB gene induction programs is essential for initiating the host response as multiple viral sensing mechanisms direct their activation. The goal of these studies was to identify which of these pathways is targeted for regulation by the HCV

NS3/4A protease resulting in a blockade to virus induced IRF-3 activity. Additionally, as all of these pathways result in the activation of NF-κB, we wished to address whether virus-induced NF-κB activity stimulated through these receptors was similarly affected by NS3/4A.

Results:

Identification of the TLR3 adaptor protein TRIF as an NS3/4A proteolytic substrate

A genome wide data base search using a consensus protease cleavage sequence initially identified TRIF as a potential NS3/4A substrate. In fact TRIF contains three such sites although one lacks an acidic residue in the P6 position, which is thought to contribute to protease targeting (Fig 4-1A). We utilized a cell based, *in vivo* protease assay to assess the steady state level of transfected TRIF protein in cells conditionally expressing NS3/4A. We observed a consistent decrease in the relative abundance of TRIF protein in cells coexpressing NS3/4A (Fig 4-1B). The identification of TRIF as a bona fide protease substrate was confirmed in *in vitro* studies, which were also used to identify the exact NS3/4A cleavage site(70,152). Surprisingly, the cleavage site occurred at C372S, the site lacking the acidic P6 site. We utilized a mutant, C372R that was shown to be resistant to cleavage *in vitro* but which still

maintained wild type signaling capacity(152) to confirm that the protease could no longer cleave the mutant substrate. Indeed, we observed no difference in the relative levels of C372R TRIF in the presence of NS3/4A confirming the identity of the cleavage site in a cell-based assay (Fig 4-1B). Finally, we utilized metabolic labeling studies to assess the stability of ectopically expressed TRIF from cells conditionally expressing NS3/4A. The TRIF protein is relatively unstable with a half-life of 30min-1hr (Fig 4-1C). However, NS3/4A resulted in a decreased stability of TRIF with a half-life of less than 30 minutes (Fig 4-1C). These data, from both *in vivo* and *in vitro* studies, identify TRIF as an authentic NS3/4A protease substrate.

Variable alteration in TRIF/TLR3 mediated signaling

Ectopic expression stimulates IFN-β expression in the absence of a stimulus. We took advantage of this to evaluate the effect of NS3/4A on TRIF mediated signaling. Ectopic expression of TRIF in Huh7 results in a robust induction of an IFN-β promoter driven luciferase construct (Fig 4-2A). However, coexpression of NS3/4A attenuates this response suggesting that NS3/4A mediated proteolysis of TRIF affects its ability to signal the downstream induction of antiviral target genes (Fig 4-2A).

Treatment of cells with pIC added directly to the culture media stimulates signaling through TLR3(222). UNS3/4A osteosarcoma cells that conditionally express NS3/4A express TLR3 basally (BF unpublished results). We utilized these cells to assess TLR3/TRIF mediated signaling in response to exogenously added pIC. In the absence of NS3/4A, cells responded to pIC stimulation with a robust induction of ISG56, an IRF-3 target gene, by four hours post treatment as detected by immunoblot analysis (Fig 4-1A left). This response was greatly decreased, but not absent, in cells expressing NS3/4A demonstrating that NS3/4A can modulate signaling events initiated through the TLR3/TRIF pathway that result in IRF-3 target gene expression (Fig 4-1A right).

TLR3 mediated signal transduction through TRIF also results in the activation of the transcription factor NF-κB(73,117,280). We examined the activation of NF-κB through gel-shift analysis of cells treated with exogenous pIC in the presence or absence of NS3/4A to determine if NS3/4A mediated proteolysis of the TRIF adaptor protein similarly resulted in regulation of NF-κB activity. Overall, there was a dramatic decrease in NF-κB binding to the cognate PRDII DNA element in the presence of NS3/4A (Fig 4-1C). Interestingly, at 2 hrs post treatment there were similar levels of NF-κB activation regardless of the presence of NS3/4A,

however at 1 hr and at the later time points, there was a clear blockade to NF-κB activation (Fig 4-1C). This may either reflect a delayed kinetics of NS3/4A activity or instead be due to NF-κB activation mediated through TRIF-independent pathways, which are not affected by NS3/4A. Importantly, this demonstrates that NS3/4A can also regulate the activity of NF-κB in addition to IRF-3 in response to TLR3/TRIF signal transduction.

We utilized stable 293 cell lines expressing TLR3 to assess activation of an IFN-β promoter luciferase construct in response to viral infection and exogenous pIC stimulation. 293 cells do not express TLR3 basally and thus serve as a negative control for TLR3 mediated signaling events. Our results from studies in these cells are surprising for two reasons. First, NS3/4A had no affect on the induction of an IFN-β promoter reporter construct in response to pIC treatment (Fig 4-3A). As expected, parental 293 cells were unresponsive to the addition of pIC to culture media however, the cells stably expressing TLR3 responded nicely with a 5-7 fold stimulation of an IFN-β reporter construct regardless of the presence of NS3/4A (Fig 4-3A). This data conflicts with the data described above which suggests that NS3/4A can regulate TLR3 signaling through proteolysis of the adaptor protein TRIF. Our second surprise

came from virus infection studies in these cells that revealed that 293 cells lacking TLR3 are quite competent to respond to Sendai virus infection and can induce robust expression of an IFN-β promoter luciferase reporter construct (Fig 4-3B). Furthermore, expression of NS3/4A could ablate this response in both 293 and 293TLR3 cells suggesting that regulation is occurring through a TLR3 independent mechanism (Fig 4-3B). Interestingly, the presence of TLR3 in 293 cells consistently enhanced viral induction of IFN-β (Fig 4-3B). These results indicate that TLR3 is not absolutely required for the induction of intracellular antiviral signaling programs however, its presence enhances this response leading to increased induction of IFN-β expression. This supports our earlier hypothesis that TLR3 may not be required for the initial antiviral response but may in fact play a role in the secondary/amplification response. Additionally, our results indicate that NS3/4A can regulate TLR3 signaling in a cell type specific manner.

TRIF is not required for viral induction of IFN-β

In order to directly assess the role of TRIF in the viral induction of IFN-β we utilized TRIF deficient mouse embryonic fibroblasts (TRIF -/- MEFs). Both wild type and TRIF deficient MEFs infected with Sendai virus

efficiently activated the expression of an IFN-β promoter luciferase reporter construct (Fig 4-3C). These data reveal that TRIF is not required for viral induction of IFN-β expression. Importantly, NS3/4A was able to ablate this response in both cell lines demonstrating that the protease is antagonizing a TRIF-independent virus responsive pathway (Fig 4-3C).

We conducted follow-up RNA silencing studies to define the nature of the virus responsive pathways that signal to IRF-3 and IFN-β in human hepatoma (Huh7) cells which we described in the previous chapter support HCV RNA replication(159). Huh7 cells do not basally express TLR3, and therefore do not signal through the TLR3 pathway(146), allowing us to evaluate virus signaling and regulation by HCV independently of TLR3. Transfection of short interfering RNA (siRNA) directed against the TLR adaptor protein, TRIF(280), efficiently ablated its expression but did not affect signaling to the IFN-β promoter induced by infection with Sendai virus (SenV), a potent activator of IRF-3, supporting our observations from the TRIF deficient MEFs described above (Fig. 4-3D)(158). However, siRNA directed against RIG-I abolished virus signaling to the IFN-β promoter in Huh7 cells and prevented the expression of ISG56, an IRF-3 target gene (Fig. 4-3D)(88). These results indicate that NS3/4A disrupts an intracellular virus-responsive pathway

that signals the IFN-β promoter independent of TLR3 or TRIF but involves RIG-I.

HCV NS3/4A disrupts RIG-I signaling

We first evaluated the affect of NS3/4A on signaling initiated by wild type or mutant RIG-I proteins. Ectopic expression of RIG-I conferred super-stimulation of virus signaling to the IFN-β promoter, but this was suppressed in Huh7 cells upon coexpression of increasing amounts of NS3/4A (Fig. 4-4A). NS3/4A also blocked constitutive activation of the IFN-β promoter directed by ectopic expression of an N-RIG mutant representing the amino-terminal CARD homology domains, but had no effect on the carboxyl-terminal helicase domain of RIG-I (C-RIG) which confers a dominant-negative block to virus-induced signaling(283) (Fig. 4-4A). When expressed in an Huh7 variant (Huh7.5 cells) in which RIG-I is defective(237), ectopic RIG-I or N-RIG restored induction of IFN-stimulated gene (ISG) expression (Fig. 4-4B lanes 5 and 9). Complementation of Huh7.5 cells with RIG-I restores viral induction of ISG15 and ISG56 however this response was potently suppressed by NS3/4A (Fig. 4-4B). Additionally, ectopic expression of N RIG results in the constitutive induction of ISG15 and ISG56, which is similarly suppressed by NS3/4A (Fig. 4-4B). Taken together, these results indicate

that NS3/4A antagonizes signaling events that trigger a host response directed by RIG-I.

The RIG-I pathway can modulate HCV replication and is regulated by NS3/4A

Disruption of RIG-I signaling may allow HCV to block IRF-3 activation and IFN defenses that could limit viral replication. In support of this, over-expression of the constitutively active IRF-3-5D or N-RIG proteins(158,237) induced a host response that suppressed the replication of an HCV RNA replicon (A7), described previously, that is poorly adapted to Huh7 cells (Fig. 4-5A and 4-5B). Expression of N RIG or IRF-3 5D induced the expression of ISGs, shown by both Northern and Western blot analysis, which resulted in a concomitant decrease in HCV RNA and protein levels (Fig 4-5A and 4-5B). These data clearly demonstrate that activation of the RIG-I pathway results in the induction of ISGs and an antiviral state capable of suppressing HCV replication. In contrast however, only expression of IRF-3-5D induced a host response restricting replication of a highly adapted replicon (HP), described in Chapter 3, in agreement with our previous findings that this replicon is able to efficiently ablate viral stimulation of IRF-3 and IFN-β expression (Figs. 4-5A and 4-5B). The different responses in these replicons could reflect the reduced

abundance of NS3/4A expressed by A7, or differences in the cell-culture adaptive mutations present within the NS3/4A coding regions of these isogenic replicons that specifically affect downstream IRF-3 regulation (75). Nonetheless, these results define RIG-I as an essential transducer of a virus-responsive cellular pathway that has the capacity to control HCV RNA replication for which HCV has cleverly designed a mechanism to avoid.

NS3/4A inhibits IRF-3 activation through RIG-I

We wished to further characterize the blockade of RIG-I function imposed by the protease and to address if the inhibition of virus induced IRF-3 function that we described previously was in fact due to a disruption of RIG-I signaling. We therefore examined the impact of NS3/4A regulation of RIG-I signaling on IRF-3 activation. As demonstrated previously, NS3/4A prevented the nuclear accumulation of IRF-3 in response to SenV infection and, additionally in cells transfected with HCV specific PAMP, the 5' NTR RNA which we have demonstrated is a stimulus for RIG-I activity (Fig. 3-6). In order to more specifically isolate IRF-3 activation through RIG-I, we utilized ectopic expression of N RIG. In UNS3/4A osteosarcoma cells conditionally expressing NS3/4A, ectopic N-RIG induced the cytosol to nuclear redistribution of IRF-3, but this was

blocked upon NS3/4A expression (Fig. 4-6A). This data confirms that NS3/4A can indeed regulate the activation of IRF-3 function signaled through RIG-I. Additionally, IRF-3 nuclear retention is dependent upon phosphorylation at serine 386 (S386) and stable formation of IRF-3 dimers (179). In control experiments, infection of cells with SenV (shown previously) or New Castle disease virus (NDV), a related paramyxovirus, induced S386 phosphorylation and dimerization of IRF-3 but both were blocked by NS3/4A (Fig 4-6B left panel). Ectopic expression of N-RIG similarly conferred IRF-3 S386 phosphorylation and dimerization but these events were also blocked by NS3/4A (Fig 4-6B right panel). Thus, RIG-I signals IRF-3 activation by directing the S386 phosphorylation event however HCV avoids this consequence by preventing this critical modification through the actions of the NS3/4A holoenzyme complex.

NS3/4A controls RIG-I mediated NF-κB activation

Previous work has demonstrated that the RIG-I pathway bifurcates to signal both IRF-3 and NF-κB activation(283). We further evaluated the role of RIG-I in NF-κB activation and its potential regulation by NS3/4A. In the absence of NS3/4A, SenV infection of osteosarcoma cells induced a rapid biphasic decay in the abundance of the NF-κB inhibitor, IκB-α, accompanied by accumulation of the high-mass, phosphoserine 396

isoforms of IRF-3, thereby confirming the critical carboxyl-terminal phosphorylation and activation of IRF-3(223) (Fig. 4-7A, left panel). This was followed by induction of ISG56 expression, which is positively regulated by the transcription factors IRF-3, NF-κB and ISGF3. As expected NS3/4A prevented virus-mediated IRF-3 phosphorylation however surprisingly, NS3/4A similarly blocked virus induced IκB-α decay (Fig 4-7, right panel). Importantly, NS3/4A did not prevent IκB-α degradation induced by treatment of cells with interleukin (IL)-1β (Fig. 4-7A, right panel). We further wished to evaluate NF-κB functionally and utilized an electrophoretic mobility shift assay to assess NF-κB occupancy on the PRDII DNA element in response to a variety of stimuli. NS3/4A disrupted virus-induced NF-κB binding to the cognate PRDII DNA element but had no affect on IL-1β or TNFα induced activation and binding (Fig. 4-7B). These results demonstrate that NS3/4A regulation of NF-κB activity is not mediated by a global disruption of NF-κB activity as IL-1β and TNFα mediated signaling is intact, but is instead restricted to viral induction of NF-κB. To evaluate the affect of NS3/4A specifically on RIG-I mediated activation of NF-κB, we examined the induction of an NF-κB -dependent PRDII promoter-luciferase construct signaled by ectopically expressed N-RIG in Huh7 cells. Coexpression of NS3/4A abrogated NF-κB promoter

induction in a dose-dependent manner demonstrating that NS3/4A can indeed regulate RIG-I mediated NF-κB activation (Fig 4-7C).

To assess the impact of NS3/4A regulation of NF-κB activity in the context of HCV replication, we examined NF-κB signaling in HCV genomic replicons. Huh7 2-3, cells containing replicating genome-length HCV RNA,(107) similarly exhibited a block in NF-κB activation of the PRDII promoter element luciferase construct both basally in response to endogenous HCV replication and further upon stimulation with SenV (Fig. 4-8A). However, virus-responsive promoter activity was restored in their cured Huh7 2-3c counterparts lacking HCV RNA demonstrating that the block is imposed by HCV protein expression. Protein blot and microarray analyses (Figs. 4-8B and 4-8C, respectively) demonstrated that HCV replication was associated with a blockade in virus-induced accumulation of secreted IL-6, an NF-κB-target gene[15]. This associated with a general attenuation of NF-κB-dependent chemokine and cytokine expression(206) in Huh7 2-3 cells but virus-responsiveness of each was restored in the cured Huh7 2-3c cells.

These studies thus reveal that NS3/4A ablates virus induced, RIG-I dependent signaling to NF-κB, likely by controlling events that signal upstream of IκB degradation. Thus regulation of RIG-I signaling by

NS3/4A attenuates both IRF-3(75) and NF-κB -target gene expression during HCV RNA replication.

NS3/4A protease activity imparts regulation of RIG-I signaling

We next determined whether regulation of RIG-I signaling is also dependent on the protease activity of NS3/4A. In the context of ectopic RIG-I expression, SenV infection of Huh7 cells resulted in IFN-β promoter super-stimulation that was blocked by NS3/4A (Fig. 4-9A lanes 2 and 3 respectively). This blockade was relieved in cells treated with SCH6, a peptidomimetic active site inhibitor of the NS3/4A protease(75) (Fig. 4-9A lane 4). Mutation of the active site Ser to Ala at HCV codon 1165 ablates NS3/4A protease activity(52), and also abrogated NS3/4A control of RIG-I signaling to the IFN-β promoter (Fig. 4-9A lane 5). Expression of the dominant-negative IRF-3-ΔN mutant(158) similarly ablated RIG-I signaling, confirming that IRF-3 is a downstream effector of the RIG-I pathway in human hepatoma cells (Fig. 4-9A lane 6). Additionally, coexpression of the constitutively active N RIG expression construct with NS3/4A resulted in a dose dependent decrease in IFN-β promoter activity (as shown above and Fig. 4-9B). However, similar coexpression with an the active site mutant of NS3/4A (S1165A) does not affect N RIG driven IFN-β promoter

activity further confirming the requirement for intact NS3/4A protease activity in the regulation of RIG-I signaling (Fig. 4-9B).

We also assessed the requirement for intact protease function during NS3/4A regulation of the NF-κB activity. To assess this, we evaluated the activity of an NF-κB responsive PRDII promoter element in response to a viral or N RIG stimulus. The NS3/4A-mediated blockade to SenV-induced NF-κB -dependent promoter activity was relieved in osteosarcoma cells treated with the peptidomimetic ketoamide protease inhibitor SCH6 (Fig. 4-9C). Additionally, coexpression of N RIG with wildtype but not a protease deficient mutant of NS3/4A inhibited the activity of the NF-κB-responsive PRDII promoter element (Fig. 4-9D). Thus, NS3/4A disrupts the host response by mediating protease-dependent control of virus-induced RIG-I signaling to the activation of both IRF-3 and NF-κB. Furthermore, inhibition of protease activity with a therapeutic NS3/4A protease inhibitor relieves the blockade and restores activity of both IRF-3 and NF-κB.

RIG-I is not a direct substrate of the NS3/4A protease

These results prompted us to evaluate the affect of NS3/4A on the abundance and stability of RIG-I. Sequence analysis of RIG-I revealed

the presence of a putative NS3/4A cleavage site within the CARD homology domain. Potential cleavage at this site could explain the ability of NS3/4A to regulate the activity of both FL and N RIG to signal to downstream effectors. We utilized an *in vivo*, cell based assay to evaluate the relative levels of ectopically expressed FL RIG, N RIG and C RIG in both the presence and absence of NS3/4A. There was no detectable decrease in the level of any of the RIG-I proteins upon NS3/4A coexpression (Fig. 4-10A). We also performed metabolic radiolabeling studies to assess the relative stability of N RIG in both the presence and absence of NS3/4A. We utilized this construct because we have already demonstrated that its activity is regulated by the protease and it contains the putative cleavage site. We found no difference in the relative stability or half-life of N-RIG attributable to NS3/4A (Fig. 4-10B). We therefore conclude that the inhibition of signaling by ectopically expressed FL or N RIG through NS3/4A is not due to direct proteolysis of RIG-I.

RIG-I directs an IFN amplification loop that is blocked by NS3/4A

We also wished to evaluate the levels of both basal and induced endogenous RIG-I in cells expressing NS3/4A. RIG-I is itself an interferon stimulated gene (ISG)(283) and, in the absence of NS3/4A, SenV infection or IFN treatment of UNS3/4A cells induced a marked increase in the

abundance of RIG-I protein levels and other ISGs, including ISG56 and PKR. Expression of NS3/4A prevented the virus-induced accumulation of RIG-I and ISG protein expression but failed to prevent their increase after treatment of cultures with exogenous IFN (Fig. 4-11A). Consistent with this, NS3/4A expression blocked virus-induced RIG-I mRNA accumulation but did not prevent the increase in RIG-I mRNA abundance conferred by IFN treatment (Fig. 4-11B).

Similar regulation of RIG-I expression occurred in the context of HCV RNA replication. In these experiments, control Huh7 cells responded to both IFN and SenV infection with induction of RIG-I and ISG protein expression, but cells harboring genetically distinct HCV replicon variants all exhibited a blockade to SenV-induced RIG-I and ISG accumulation while responding normally to exogenous IFN (Fig. 4-11C). These results further indicate that RIG-I is not a substrate for the NS3/4A protease, but that NS3/4A disrupts the activation of an IFN amplification loop that is first signaled by RIG-I and then supported subsequently through an NS3/4A-resistant, IFN-mediated increase in RIG-I mRNA and protein accumulation.

HCV NS3/4A disrupts MDA5 signaling

In order to examine and isolate signaling initiated solely through MDA5, we utilized a mutant devoid of the regulatory carboxyl terminus. N MDA5 is analogous to N RIG, containing the tandem CARD homology domains and constitutively signaling the activation of IRF-3 and NF-κB in the absence of a dsRNA or viral stimulus. Ectopic expression of N MDA5 in Huh7 cells results in the induction of IFN-β luciferase promoter activity. Similar to N RIG, activation of the IFN-β promoter by N MDA5 can be titrated by coexpression with increasing amounts of NS3/4A (Fig. 4-12A). The inhibitory effect was lost when a protease active site mutant was used instead, demonstrating a similar requirement for intact NS3/4A protease activity in mediating the blockade of MDA5 stimulated IFN-β induction (Fig. 4-12A). These results suggest that the HCV NS3/4A protease inhibits signaling through both of the cytosolic dsRNA PRRs known to specifically recognize HCV RNA PAMP structures. This results in a lack of IFN-β and other ISG expression, thus preventing the establishment of the antiviral state within HCV infected cells.

NS3/4A inhibits the activation of IRF-3 by MDA5

We wished to specifically assess the activation of IRF-3 by MDA5 and its regulation by NS3/4A as a mechanism for inhibition of MDA5 signaling to the IFN-β promoter. We examined the intracellular localization of IRF-3 in cells ectopically expressing constitutively active N MDA5. We observed an association of nuclear IRF-3 in cells expressing N MDA5 however at a lower frequency than that observed for N RIG (Fig. 4-12B). Whether this is a reflection of a decreased efficiency of IRF-3 activation by N MDA5 or an artifact of the construct itself remains to be determined. Regardless, expression of N MDA5 in cells coexpressing NS3/4A resulted in a decreased incidence of nuclear localized IRF-3 (Fig. 4-12B). These data correlate with the findings reported above for N RIG and confirm that the protease disrupts signaling events that direct the activation and nuclear localization of IRF-3 in response to viral PRR sensing mechanisms.

MDA5 is not a direct NS3/4A protease target

The requirement for intact protease function on the inhibition of MDA5 signaling function prompted us to examine its potential as a protease substrate. Like RIG-I, MDA5 encodes a putative cleavage site within its CARD homology signaling domain. We utilized an *in vivo*, cell-

based protease assay to examine steady state levels of FL MDA5, N MDA5 and C MDA5 in the presence or absence of NS3/4A. There was no appreciable difference in the relative levels of any of the MDA5 constructs tested (Fig. 4-13A). Similarly, there was no difference observed in transfected MDA5 protein levels when a viral stimulus was applied to cells in order to effect protein conformational changes that might affect substrate recognition. These data, and that from the RIG-I studies described above, have led us to conclude that NS3/4A does not in fact target these cytoplasmic dsRNA PRRs but rather some shared signaling intermediate that effects both IRF-3 and NF-κB activation.

Signaling through MDA5 has the capacity to control HCV RNA replication

As mentioned above MDA5 contains the ability to recognize and respond to PAMP structures embedded within the 5' NTR of the HCV genome. As MDA5 appears capable of activating similar antiviral gene induction programs as RIG-I, we hypothesized that intrinsic signaling through MDA5 should have the capacity to control HCV RNA replication. To address this, and to specifically isolate the affect of signaling initiated through MDA5 solely, we ectopically expressed N MDA5 in HCV subgenomic replicons and examined its affect on gene induction and HCV

RNA levels through Northern blot analysis. Signaling initiated through MDA5 CARD motifs resulted in the induction of ISGs and a concomitant decrease in HCV RNA replication (Fig. 4-14A). We therefore conclude that induction of antiviral gene programs through both RIG-I and MDA5 have the inherent capacity to control HCV RNA replication however, the virus encodes a mechanism to subvert these responses through the actions of the NS3/4A protease.

NS3/4A mediated disruption of the IFN amplification loop also affects MDA5 induction

We performed immunoblot assays to assess the expression of basal and induced endogenous MDA5 in cells expressing NS3/4A. We were unable to detect basal MDA5 protein in UNS3/4A osteosarcoma cells regardless of the presence of NS3/4A (Fig. 4-15A). However, Sendai virus infection resulted in the induction of MDA5 with similar kinetics as other virally induced genes (RIG-I, P56, ISG15) only in the absence of NS3/4A (Fig. 4-15A). It is possible that the lack of MDA5 observed basally might be due to a low sensitivity of the antibody in this assay. Regardless, we have determined that MDA5 is virus inducible and have further demonstrated that the protease can effectively inhibit its expression in response to virus infection, possibly avoiding its function altogether.

Therefore, as with induction of RIG-I, NS3/4A inhibition of virus response pathways prevents the production of IFN and the subsequent IFN amplification loop which upregulates many of the antiviral response genes.

Discussion

Our results define the intracellular dsRNA PRRs RIG-I and MDA5 as critical components of innate intracellular defense against HCV and show that these pathways are targeted by NS3/4A, thereby allowing HCV to avoid triggering the host antiviral response. Importantly, we have also provided evidence that these are relevant targets for HCV regulation as we have demonstrated that both RIG-I and MDA5 have the capacity to recognize HCV dsRNA structures embedded within the 5' and 3' NTRs and initiate host response programs(237) . We provide further evidence that these pathways branch to signal both IRF-3 and NF-κB activation during virus infection and that NS3/4A can inhibit activation of both transcription factors. The ability of NS3/4A to regulate signaling through both RIG-I and MDA5 to achieve the same effect implies that the protease is targeting a shared component in the signaling pathways emanating from these receptors. Furthermore, NS3/4A control of dsRNA PRR signaling is dependent on its protease activity, implying that NS3/4A may target and

cleave a shared component(s) of these pathways with actions essential for IRF-3 phosphorylation and IκB degradation.

Protease regulation of NF-κB is important mechanistically because it reveals that the protease is impacting the signaling pathway at a point in the pathway before it bifurcates to signal either IRF-3 or NF-κB activation. Importantly, it adds another layer of immune evasion from HCV infection as NF-κB is a critical factor for the induction of important inflammatory cytokines and chemokines that are required for the recruitment and modulation of innate and adaptive immune effector cells. Additionally, the production of IFNα/β at a local site of infection has been shown to be important for efficient modulation of adaptive immune effector cell function(245). Taken together, the absence of these soluble immunomodulatory factors in the milieu of an infected liver through the actions of the NS3/4A protease likely prevents the productive priming and modulation of an anti-HCV adaptive immune response, a phenomenon that has been well documented(11,97,119,124,182,256). An inefficient, or dysfunctional, adaptive immune response also likely contributes to successful HCV immune evasion and subsequently, persistent infection.

These studies examining the relevance of TLR3 signaling and protease mediated TRIF regulation are slightly more complex. Our data

reveals that TLR3/TRIF signaling is not in fact required for the early induction of IFN-β in response to viral infection. Indeed, to date there has been no evidence of a TLR3 dependent mechanism in the detection and early induction of the intracellular antiviral response. Supporting this is the fact that TLR3 and TRIF are not expressed basally in most cell types. However, both proteins are induced in response to viral infection or IFN signaling suggesting that they might play a role in the secondary or antiviral amplification response. Indeed, we consistently observe an increase in IFN-β reporter activity in 293 TLR3 expressing stable cell lines suggesting that the presence of TLR3 and its capacity to signal in response to initial virus infection results in a more pronounced antiviral response, as would be expected for a secondary/amplification response pathway mechanism. The topology of TLR3 with its PAMP recognition domain within endocytic vesicles might be important for recognizing incoming viral particles that are in the process of uncoating within vesicles and exposing viral dsRNA PAMPs. Similarly, intracellular vesicle localization of TLR3 may be responsible for detection of improperly complexed viral dsRNA genomes during viral egress through host cellular membranes. Therefore, while not strictly required for the early response and induction of host antiviral response pathways, TLR3/TRIF meditated signaling has been demonstrated to be important in the innate antiviral

response and host clearance(4,242). Accordingly, TLR3/TRIF mediated signaling may reflect an additional obstacle for HCV in triggering the antiviral response through alternate pathways however, the virus has evolved a unique mechanism to subvert this response through cleavage of the critical TLR3 adaptor protein TRIF.

Yet another consequence of TRIF cleavage and disruption of TLR3 signaling might explain impaired immune effector cell function. It has recently been reported that intact signaling through TLR3 is critical for maturation of dendritic cells and their ability to effectively cross-prime CTL cell responses against cell-associated antigens(105,218). HCV infection of dendritic cells and proteolysis of TRIF could ablate this response. This hypothesis is controversial as many in the field question whether nonhepatocytes are even infected by HCV. However there are numerous reports in the literature citing infection of immune effector cells in HCV infected individuals(97,119,124,182). Additionally, it is well documented that dendritic cells isolated from HCV infected individuals are impaired functionally(6,11,97,256). Therefore it is tempting to speculate that in addition to inhibiting TLR3/TRIF signaling that might be upregulated in HCV infected hepatocytes, NS3/4A mediated proteolysis of TRIF in infected dendritic cells might provide an additional level of immune evasion for this very clever virus.

Three important themes/levels of regulation emerge from our studies. First, HCV is incredibly effective at inhibiting intracellular dsRNA response pathways dedicated to recognizing viral infection and initiating programs to eliminate the invading virus. Our data demonstrates that HCV has the capacity to regulate signaling initiated through the two intracellular dsRNA PRRs that we have previously described are capable of specifically binding to and recognizing HCV dsRNA PAMPs. Second, HCV is efficient in attenuating the antiviral actions of exogenously applied IFN. This is in part due to the fact that RIG-I, MDA5, TLR3 and TRIF are all IFN inducible genes however, their induction during IFN therapy is useless as NS3/4A can block their antiviral effector function. This results in a severely diminished activity of the essential secondary/amplification response in intracellular host defense. Additionally, this might also partially explain the ineffectiveness of IFN therapy observed in the treatment of HCV patients. Third, the end result of NS3/4A regulation of the dsRNA response pathways is the block in the induction of immune response genes that have important antiviral functions. Importantly, this includes the induction and secretion of immunomodulatory cytokines and chemokines with important roles in directing the induction of the adaptive immune response. Thus inhibition of antiviral innate signaling pathways leads to inefficient adaptive immune responses all likely contributing to

HCV persistence. NS3/4A protease inhibitors thus offer a particularly attractive approach to therapeutic intervention, in that their use may lead to restoration of innate and adaptive immune defenses that control HCV replication while also providing a direct antiviral effect.

Immunostaining of cells harboring the HP HCV replicon demonstrated colocalization of endogenous RIG-I and NS3 (Fig. 4-16). The HP replicon is the most efficient replicon at inhibiting the induction of host antiviral response pathways. The codistribution of these two proteins suggests that NS3/4A may regulate virus signaling through proteolytic inactivation of a factor recruited to RIG-I. Evaluation of such factors is difficult as none of the signaling intermediates that mediate the activation of IRF-3 or NF-κB in response to RIG-I signaling are currently known. Our next aim was to delineate factors involved in signal transduction from RIG-I and MDA5 resulting in the activation of IRF-3 and NF-κB in order to elucidate the target of NS3/4A regulation.

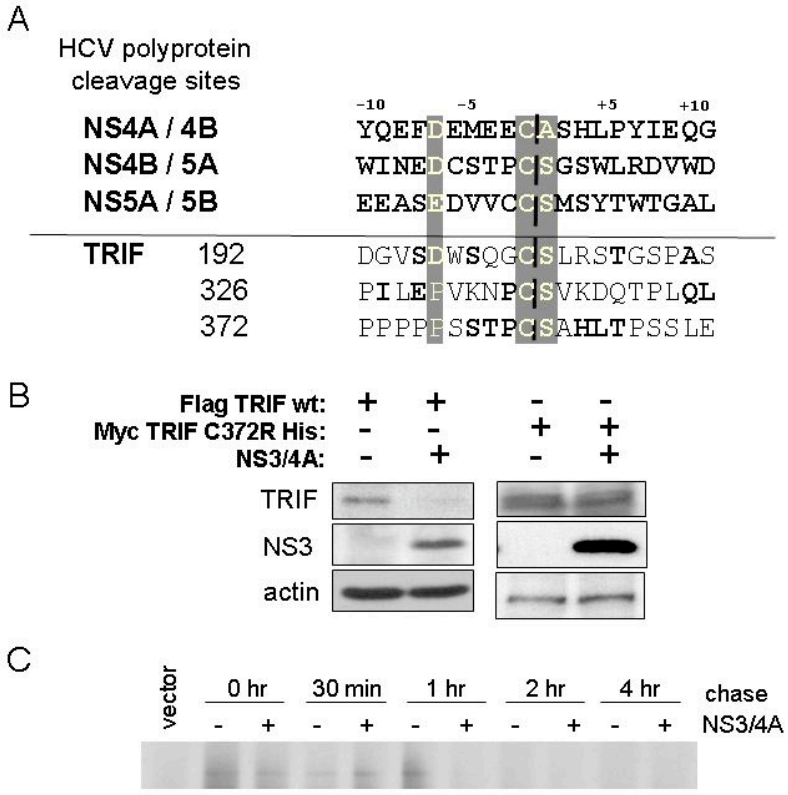


Figure 4-1: Identification of the TLR3 adaptor protein TRIF as an NS3/4A proteolytic substrate.

A. Majority rule consensus sequences for NS3/4A trans-cleavage sites within the genotype 1b HCV polyprotein compared with candidate cleavage sites in TRIF. Conserved Cys/(Ser-Ala) and P6 acidic residues are highlighted. Homologous residues aligning with those in the consensus viral substrates are shown in bold face. **B.** UNS3/4A cells were cultured to suppress or induce NS3/4A expression as indicated. Cells were transfected with expression plasmids encoding an flag TRIF wt and the cleavage mutant Myc TRIF C372R His. 24 hr later the cells were harvested and subjected to immunoblot analysis with specific antibodies to detect the flag, Myc, NS3 and actin. **C.** Cells were metabolically labeled with ^{35}S-methionine for a 30 m pulse and were harvested following a period of culture in the presence of excess unlabeled methionine (chase). 200 μg of protein were immunoprecipitated with anti-flag conjugated agarose beads (Sigma) and products were analyzed by denaturing gel electrophoresis, dried and subjected to autoradiography.

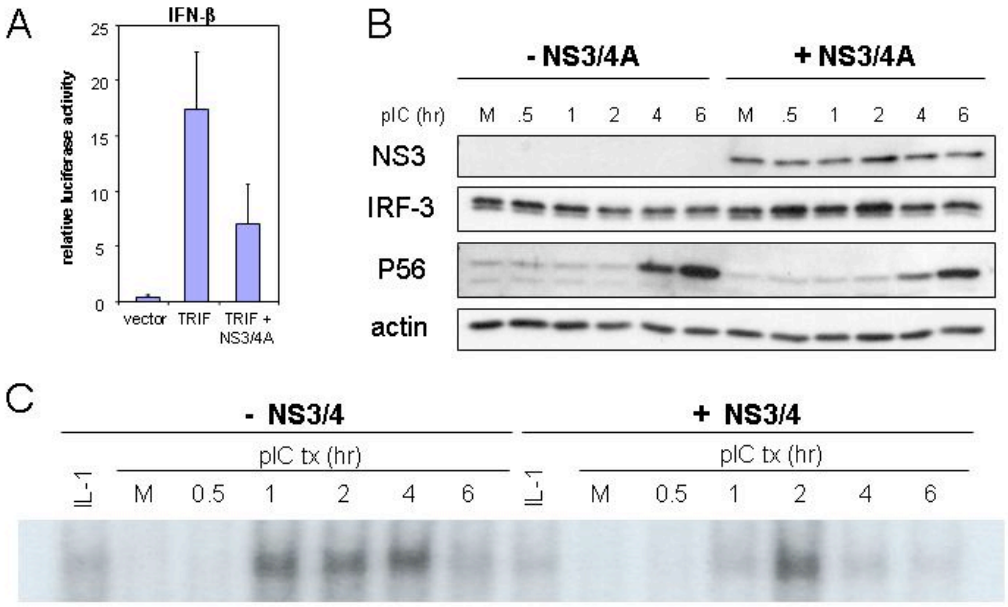


Figure 4-2: Variable alteration in TRIF/TLR3 mediated signaling.
A. Huh7 cells were cotransfected with plasmids encoding the IFN-β luciferase reporter construct, *Renilla* luciferase and empty vector, TRIF and empty vector or TRIF and NS3/4A. B. UNS3/4A cells, cultured to suppress (-NS3/4A) or induce NS3/4A expression (+NS3/4A) were cultured in DMEM or DMEM containing 100μg/ml pIC for the indicated times. Cells were harvested and extracts were subjected to immunoblot analysis. C. Cells were harvest 30 m post-IL-1 treatment or for the indicated times post pIC treatment. Nuclear extracts were prepared and subjected to electrophoretic mobility shift assay using a DNA probe encoding the PRDII element of the IFN-β promoter to detect NF-κB DNA-binding activity.

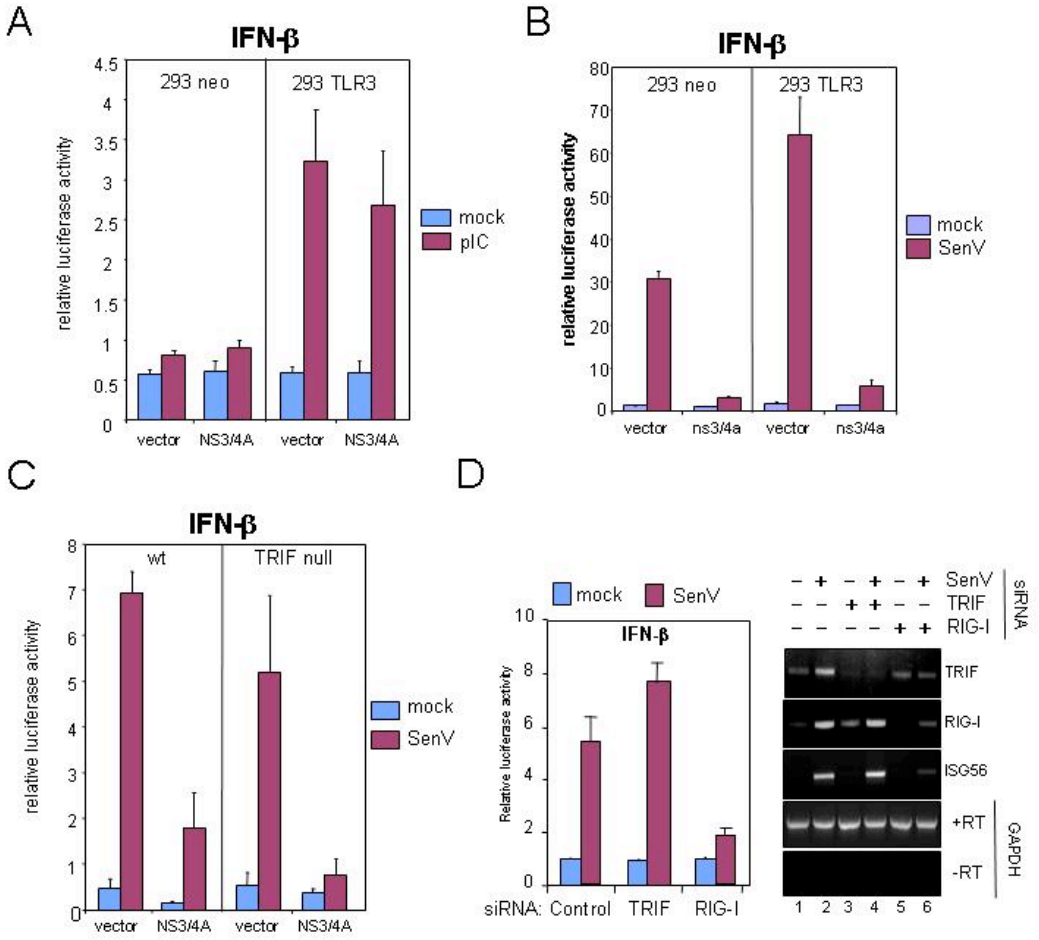


Figure 4-3: TRIF is not required for viral induction of IFN-β.
A. 293 cells stably transfected with TLR3 or empty vector were cotransfected with plasmids encoding the IFN-β luciferase reporter construct, *Renilla* luciferase and 100 ng of NS3/4A or empty vector. Cells were cultured in DMEM or DMEM containing 100μg/ml pIC for 6 h and harvested for luciferase assay. **B.** 293 cells stably transfected with TLR3 or empty vector were cotransfected with plasmids encoding the IFN-β luciferase reporter construct, *Renilla* luciferase and 100 ng of NS3/4A or empty vector. Cells were mock-infected or infected with SenV for 20 h and harvested for luciferase assay. **C.** Wildtype or TRIF deficient MEFs were cotransfected with plasmids encoding the IFN-β luciferase reporter construct, *Renilla* luciferase and 100 ng of NS3/4A or empty vector. Cells were mock-infected or infected with SenV for 20 h and harvested for luciferase assay. **D.** Huh7 cells transfected with control siRNA or siRNA directed against TRIF or RIG-I were subsequently transfected with

plasmids encoding the IFN-β luciferase reporter construct and a constitutively expressed β-galactosidase control construct, mock-infected or infected with SenV and harvested 20 h later for determination of relative luciferase values and RT-PCR analysis of mRNA expression. Bars show average and S.D. relative luciferase activity from three experiments. Panels at right show agarose gel analysis of the indicated RT-PCR product from cells treated as shown above each lane. + and – RT designate control RT-PCR reactions to amplify glyceraldehyde dehydrogenase (GAPDH) mRNA in the presence or absence of reverse transcriptase.

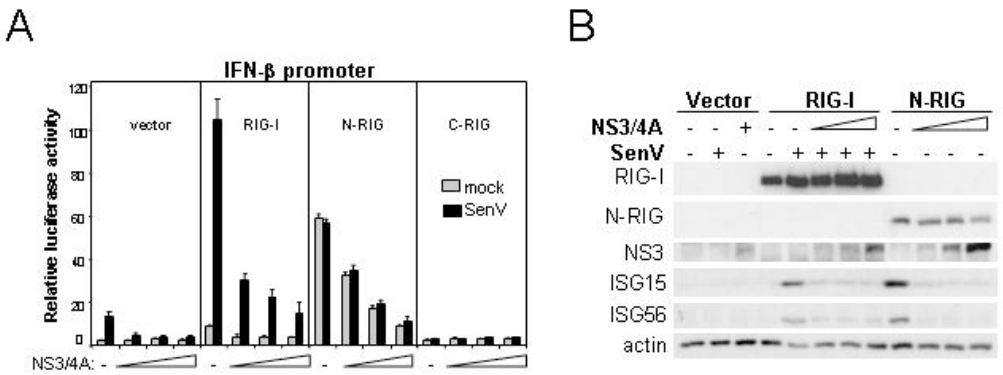


Figure 4-4: NS3/4A disrupts RIG-I signaling.A. Huh7 cells were co-transfected with plasmids encoding the IFN-β luciferase reporter construct, *Renilla* luciferase and 50 ng of the indicated vector or RIG-I expression construct with increasing amounts (0, 50, 100, 200 ng) of plasmid DNA encoding NS3/4A. Cells were mock-infected or infected with SenV and harvested for luciferase assay. Bars show the average relative luciferase activity and S.D. from 3 experiments. **B.** Huh7.5 cells transfected with plasmid constructs encoding vector alone, RIG-I or N-RIG in the presence or absence of an NS3/4A expression construct were infected with SenV as indicated and then were harvest for immunoblot analysis.

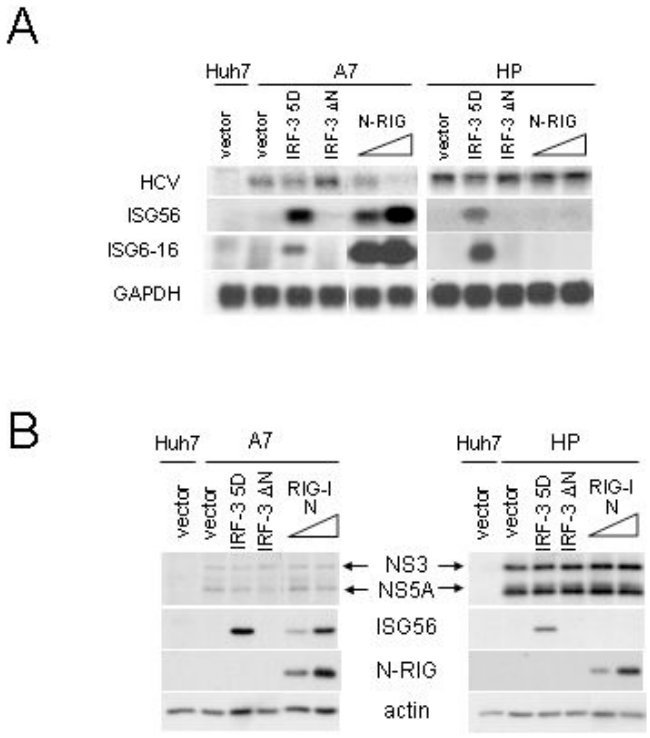


Figure 4-5: The RIG-I pathway can modulate HCV replication.
Control Huh7 cells were transfected with vector only. Huh7-A7 cells harboring the A7 HCV replicon (A7) or Huh7-HP cells harboring the HP HCV replicon (HP) were transfected with 1 μg of plasmid DNA encoding vector alone, IRF-3-5D or IRF-3-ΔN or 1 μg or 2 μg of N-RIG expression plasmid. Cells were harvested 48 h-post transfection, and extracts subjected to Northern blot analysis (**A**) and immunoblot analysis (**B**) using specific DNA or antibody probes, respectively.

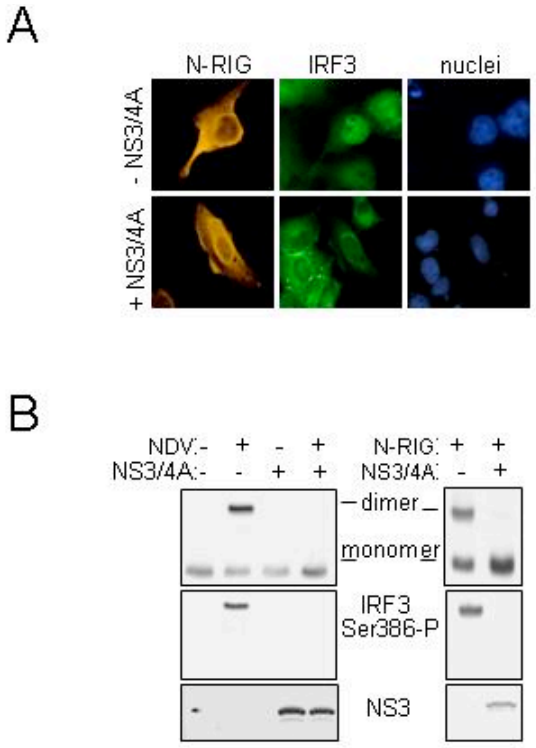


Figure 4-6: Inhibition of RIG-I mediated IRF-3 activity by NS3/4A.
A. UNS3/4A cells, cultured to suppress (-NS3/4A) or induce NS3/4A expression (+NS3/4A), were transfected with the N-RIG expression construct and 24 h later were subjected to dual immunostaining for ectopic N-RIG and endogenous IRF-3. Panels show N-RIG, IRF-3 and DAPI-stained nuclei. **B.** UNS3/4A cells cultured to suppress or induce NS3/4A expression were infected with NDV as shown (left panel set) or were transfected with N-RIG expression plasmid (right panel set). After 24 h cells were harvested and protein extracts separated on nondenaturing gels and subjected to immunoblot analysis to define the dimer, monomer and phosphoserine 386 (Ser386-P) isoforms of IRF-3. The lower panels show NS3 levels derived by standard denaturing gel immunoblot analysis.

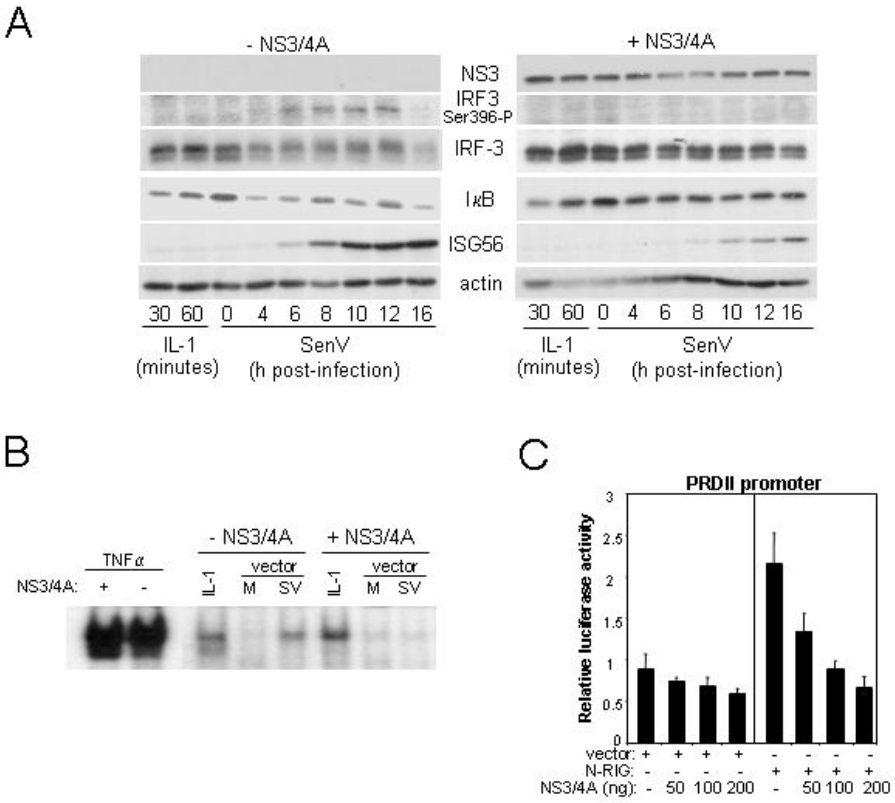


Figure 4-7: NS3/4A controls RIG-I mediated NF-κB activation.A and **B,** UNS3/4A cells, cultured to suppress (-NS3/4A) or induce NS3/4A expression (+ NS3/4A), were treated with TNFα, IL-1, mock-infected or infected with SenV. **A.** Cells were harvested at the times indicated and extracts were subjected to immunoblot analysis to detect NS3, the phosphoserine 396 isoform of IRF-3 (IRF-3 Ser396-P), total IRF-3, IκB-α, ISG56 and actin. **B.** Cells were harvest 30 m post-TNFα or-IL-1 treatment or 16 h post-infection. Nuclear extracts were prepared and subjected to electrophoretic mobility shift assay using a DNA probe encoding the PRDII element of the IFN-β promoter to detect NF-κB DNA-binding activity. **C.** Huh7 cells were co-transfected with plasmids encoding the PRDII-luciferase promoter construct and *Renilla* luciferase along with 50 ng vector only or N-RIG expression plasmid and the indicated amount of NS3/4A expression plasmid. Cells were mock-infected or infected with SenV and processed for luciferase assay 16 h post-infection. Bars show the average relative luciferase and S.D. values from three experiments.

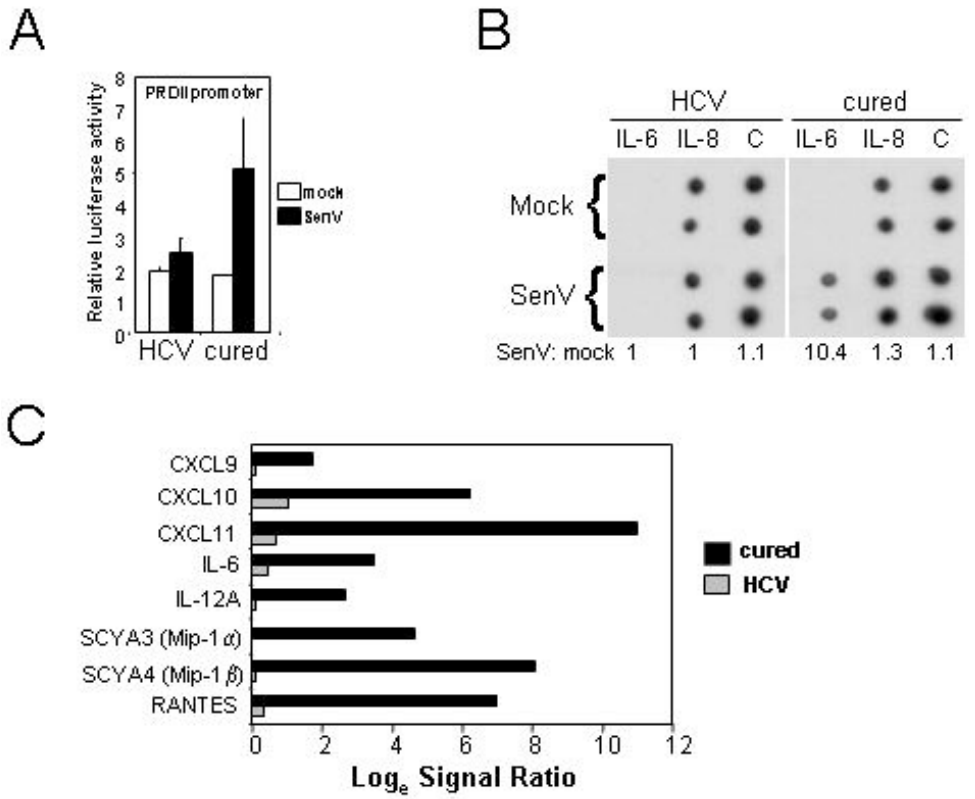


Figure 4-8: HCV replication associates with a blockade to NF-kB function.

A. Huh7 2-3 cells harboring replicating genome-length RNA (HCV) or their IFN-cured Huh7 2-3c counterparts were co-transfected with plasmids encoding the PRDII-luciferase promoter construct and *Renilla* luciferase. Cells were mock-infected or infected with SenV and processed for luciferase assay. Bars show the average relative luciferase and S.D. values from three experiments. **B.** Medium from cultures of mock or SenV-infected Huh7 2-3 (HCV) and Huh 2-3c cells (cured) was subjected to cytokine blot analysis using the Cytokine Array III kit and the manufacturers protocol (Ray Biotech). Numbers show the ratio of mock to SenV signal derived from averaged denisometric values of each spot. C denotes the reference control. **C.** Huh7 2-3 (HCV) and Huh7 2-3c cells (cured) were mock-infected or infected with SenV for 16 h. Total RNA was extracted and subjected to microarray analysis using Affymetrix U133A genechips. Bars show the quantified signal ratio of average hybridization levels for the indicated cytokine and chemokine mRNAs.

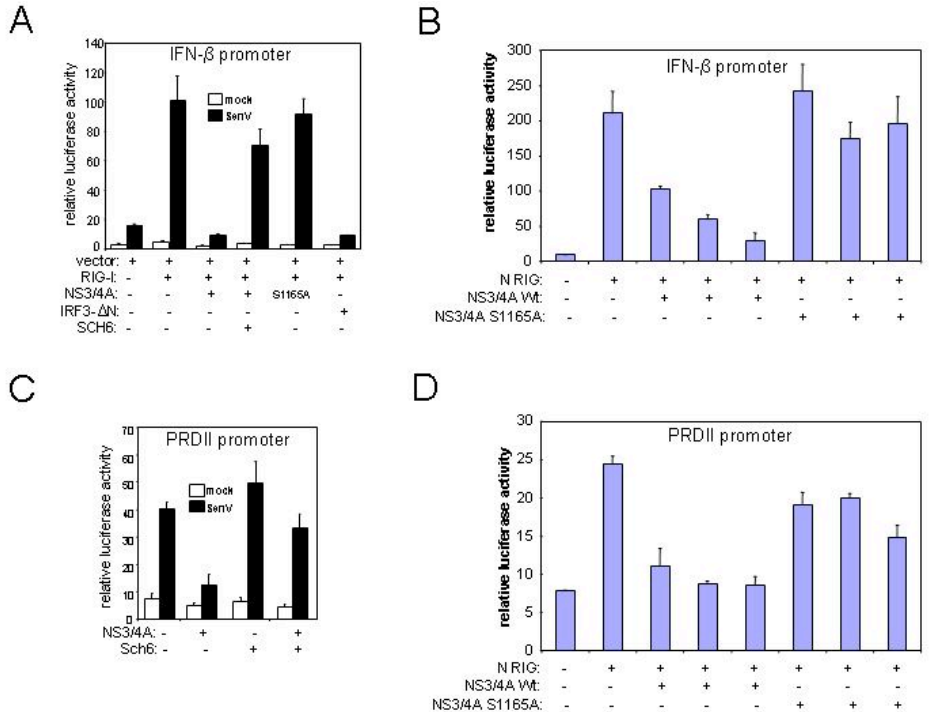


Figure 4-9: NS3/4A protease activity imparts regulation of RIG-I signaling.

A. and **C.** Transfected cells were cultured alone or in the presence of 10 μM SCH6 for 24 h prior to mock-infection or infection with SenV and luciferase assay. Bars show the average relative luciferase and S.D. values from three experiments. **A.** Huh7 cells were co-transfected with plasmids encoding the IFN-β-luciferase promoter construct and *Renilla* luciferase along with 50 ng of plasmid DNA expressing vector only or RIG-I and 200 ng of plasmid DNA encoding wild-type (wt) NS3/4A, S1165A mutant NS3/4A or IRF-3-ΔN as indicated. **B.** Huh7 cells were co-transfected with plasmids encoding the IFN-β-luciferase promoter construct and *Renilla* luciferase along with 50 ng of plasmid DNA expressing vector only or N RIG and 0, 50, 100 or 200 ng of plasmid DNA encoding wild-type (wt) NS3/4A or S1165A mutant NS3/4A or as indicated. **C.** UNS3/4A cells, cultured to suppress or induce NS3/4A expression as indicated, were co-transfected with plasmids encoding the PRDII-luciferase promoter construct and *Renilla* luciferase. **D.** Huh7 cells were co-transfected with plasmids encoding the PRDII-luciferase promoter construct and *Renilla* luciferase along with 50 ng of plasmid DNA expressing vector only or N RIG and 0, 50, 100 or 200 ng of plasmid DNA

encoding wild-type (wt) NS3/4A or S1165A mutant NS3/4A or as indicated.

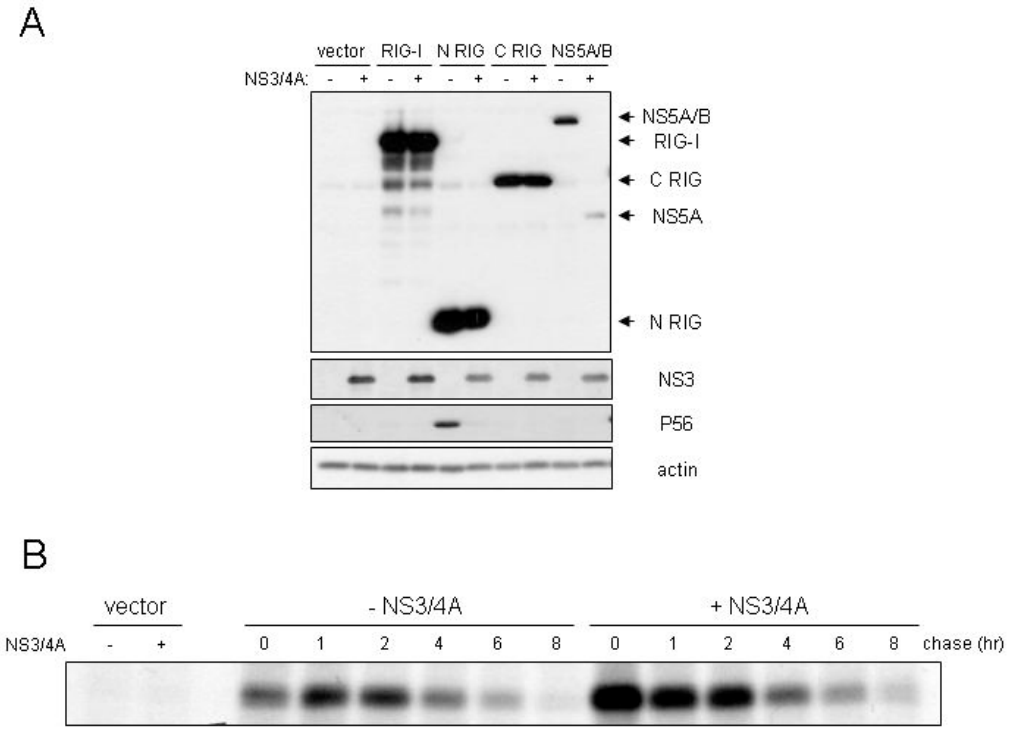


Figure 4-10: RIG-I is not a direct substrate of the NS3/4A protease. UNS3/4A cells were cultured to suppress or induce NS3/4A expression as indicated. **A.** Cells were transfected with expression plasmids encoding an NS5AB polyprotein (control), RIG-I, N-RIG or C-RIG. 24 hr later the cells were harvested and subjected to immunoblot analysis with specific antibodies to detect the NS5AB polyprotein precursor and its NS3-derived NS5A proteolytic cleavage product, NS3, actin and RIG-I, N-RIG and C-RIG (RIG constructs were detected with anti-FLAG antibody). **B.** Cells were metabolically labeled with ^{35}S-methionine for a 30 m pulse and were harvested following a period of culture in the presence of excess unlabeled methionine (chase). 200 μg of protein were immunoprecipitated with anti-flag conjugated agarose beads (Sigma) and products were analyzed by denaturing gel electrophoresis, dried and subjected to autoradiography.

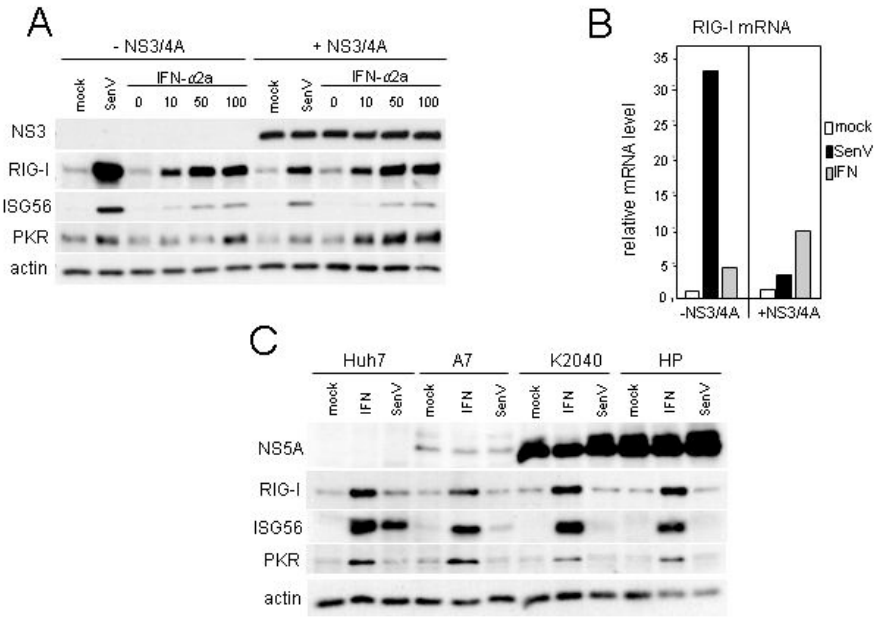


Figure 4-11: RIG-I directs an IFN amplification loop that is blocked by NS3/4A.

A. UNS3/4A cells, cultured to suppress or induce NS3/4A expression as indicated, were mock-infected, infected with SenV or treated with 0, 10, 50 or 100 units/ml of IFN-α2a as shown above each lane. 20 h later cells were harvested for immunoblot analysis of endogenous protein levels. **B.** UNS3/4A cells, cultured to suppress or induce NS3/4A expression, were mock-infected, infected with SenV or treated with 50 units/ml IFN-α2a. 20 h later cells were harvested. Total RNA was isolated and reverse transcribed using an oligo dT primer. Equal amounts of cDNA from each sample were subjected to quantitative real-time PCR analysis of RIG-I and GAPDH mRNA. Bars show the RIG-I mRNA level relative to the GAPDH internal control. **C.** Huh7 control or Huh7-A7, Huh7-K2040 and Huh-HP cells harboring the respective HCV replicon were mock-infected, infected with SenV or treated with 50 units/ml IFN-α2a. After 20 h the cells were harvested and extracts were subjected to immunoblot analysis.

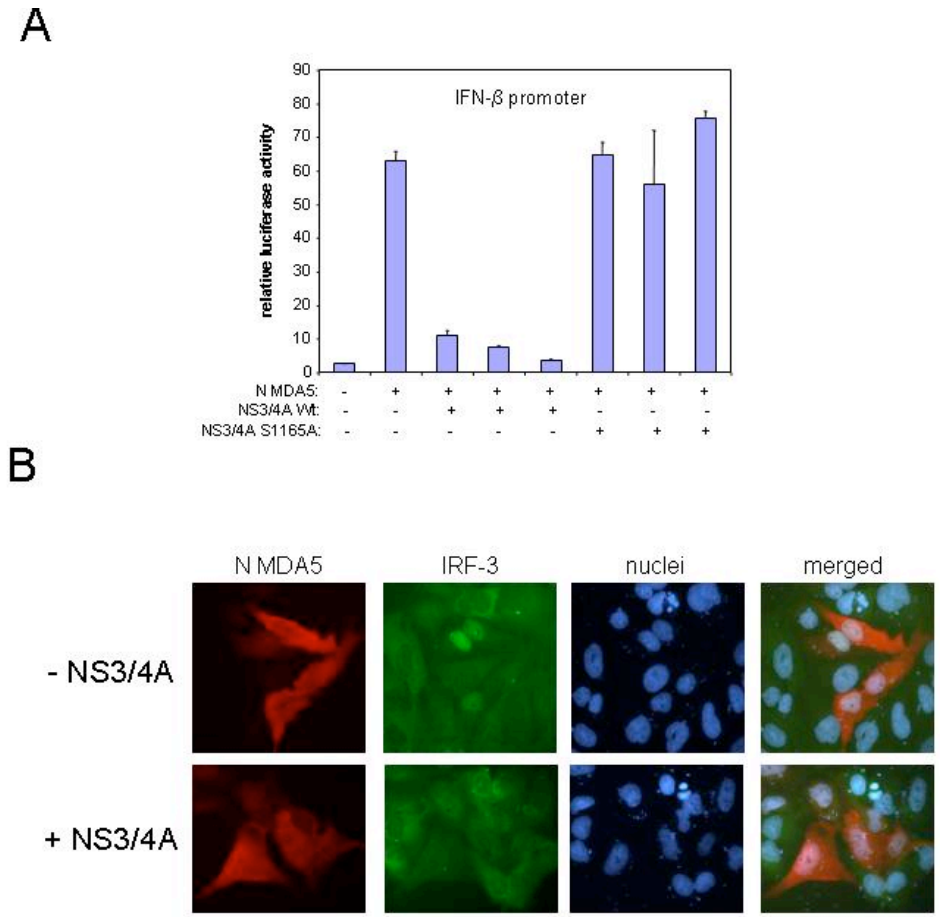


Figure 4-12: NS3/4A disrupts MDA5 signaling.
A. Huh7 cells were co-transfected with plasmids encoding the IFN-β-luciferase promoter construct and *Renilla* luciferase along with 50 ng of plasmid DNA expressing vector only or N MDA5 and 0, 50, 100 or 200 ng of plasmid DNA encoding wild-type (wt) NS3/4A or S1165A mutant NS3/4A or as indicated. **B.** UNS3/4A cells, cultured to suppress (-NS3/4A) or induce NS3/4A expression (+NS3/4A), were transfected with the N-RIG expression construct and 24 h later were subjected to dual immunostaining for ectopic N-MDA5 and endogenous IRF-3. Panels show N-MDA5, IRF-3, DAPI-stained nuclei and a merged image.

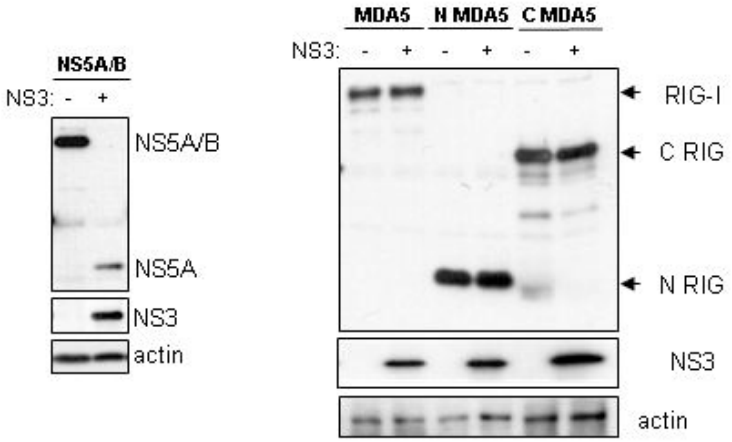


Figure 4-13: MDA5 is not a direct NS3/4A protease target.
UNS3/4A cells were cultured to suppress or induce NS3/4A expression as indicated. Cells were transfected with expression plasmids encoding an NS5AB polyprotein (control), MDA5, N-MDA5 or C-MDA5. 48 hr later the cells were harvested and subjected to immunoblot analysis with specific antibodies to detect the NS5AB polyprotein precursor and its NS3-derived NS5A proteolytic cleavage product (left panel), NS3, actin and MDA5, N-MDA5 or C-MDA5 (right panel; MDA5 constructs were detected with anti-FLAG antibody).

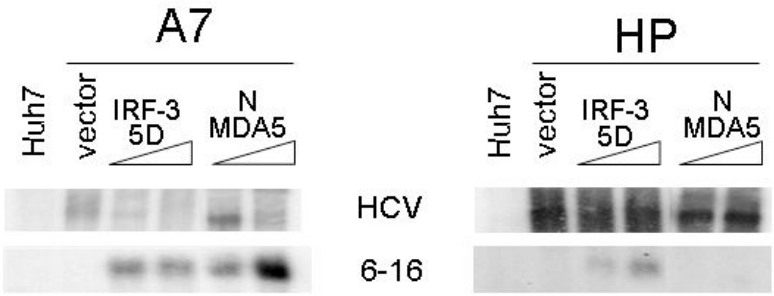


Figure 4-14: Signaling through MDA5 has the capacity to control HCV RNA replication.
Control Huh7 cells were transfected with vector only. Huh7-A7 cells harboring the A7 HCV replicon (A7) or Huh7-HP cells harboring the HP HCV replicon (HP) were transfected with 1 μg of plasmid DNA encoding vector alone, IRF-3-5D or IRF-3-ΔN or 1 μg or 2 μg of N-MDA5 expression plasmid. Cells were harvested 48 h-post transfection, and extracts subjected to Northern blot analysis (**A**) and immunoblot analysis (**B**) using specific DNA or antibody probes, respectively.

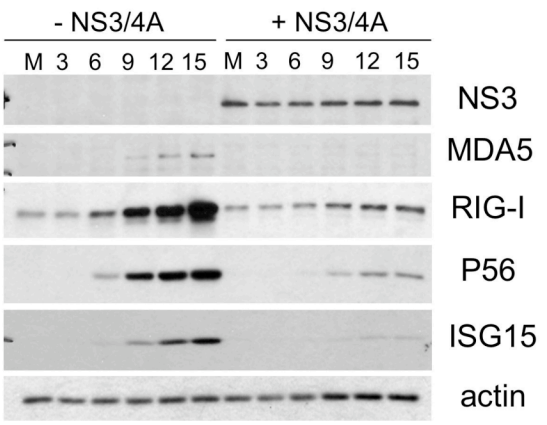


Figure 4-15: NS3/4A mediated disruption of the IFN amplification loop also affects MDA5 induction.
A. UNS3/4A cells, cultured to suppress or induce NS3/4A expression as indicated, were mock-infected or infected with SenV for the indicated times as shown above each lane. Cells were harvested for immunoblot analysis of endogenous protein levels.

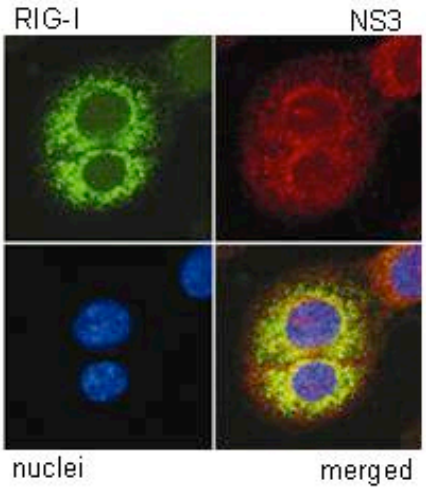


Figure 4-16: Codistribution of RIG-I and NS3/4A in HCV replicon cells.
Huh7-HP cells harboring the HP HCV replicon were cultured on microscope slides and processed for dual immunostaining using an antibody against NS3 followed by a rhodamine-conjugated secondary antibody. RIG-I was stained using a specific rabbit antiserum followed by an Alexa 488-conjugated secondary antibody. Nuclei were stained with DAPI. Digital Images were collected using a Ziess Pascal LSM scanning confocal microscope and Ziess LSM software. The image shown is from a confocal 0.3 micron optical section.

CHAPTER 5: Delineation of the IRF-3 Pathway

Introduction

In the work described previously, we demonstrated that the cellular dsRNA sensors, RIG-I and MDA5 serve as PRRs for structures present in the HCV genome that can signal the activation of the transcription factors IRF-3 and NF-κB. Signaling through these pathways results in the expression of IFN-β which promotes the establishment of an antiviral state within the infected cell and in cells of the surrounding tissue. When the results of these studies were published, two groups simultaneously published papers identifying the non-canonical IKK family members TBK1 and IKKε as IRF-3/IRF-7 kinases(72,225). The activity of these kinases was demonstrated to be critical for virally and dsRNA/TLR3 induced IRF-3 activation and IFN-β induction(72,225). While these kinases were initially identified as IKK homologs, they have not been demonstrated to play a direct role in NF-κB activation and are not required for the activation of NF-κB in response to viral infection(99,167,194). At present, it is not known how these kinases are activated in response to viral infection. Additionally, it has not been demonstrated that these kinases are involved in RIG-I/MDA5 mediated activation of IRF-3 nor are any of the other signaling factors that act downstream of the helicases currently known.

The goals of these studies are to address the role of TBK1 and IKKε as potential targets of NS3/4A regulation and to delineate other factors that might be involved in the signaling cascade that stem from RIG-I and MDA5 to activate IRF-3 and NF-κB.

Results

TBK1 is required for the induction of IFN-β through RIG-I

We utilized TBK1 deficient mouse embryonic fibroblasts (TBK1 -/- MEFs) to assess the role of TBK1 in IFN-β promoter induction signaled through RIG-I. We first confirmed the previously reported requirement of TBK1 for viral induction of IFN-β. As expected, TBK1 -/- MEFs are unable to activate the expression of an IFN-β promoter dependent luciferase construct in response to Sendai virus infection as compared to wild type control MEFs (Fig. 5-1A). However, in contrast to the findings of Yoneyama et al, we were also able to demonstrate a requirement for TBK1 in RIG-I mediated signaling(283). Wild type MEFs, but not TBK1 deficient MEFs are able to induce activation of an IFN-β promoter luciferase construct when transfected with N RIG (Fig. 5-1B). These results indicate a requirement for TBK1 in signaling the induction of IFN-β initiated through the dsRNA PRR RIG-I. Thus, TBK1 likely plays a critical

role in transducing the activation of IRF-3 in response to virus infection through dsRNA sensing programs mediated through RIG-I and likely MDA5.

The noncanonical IKKs TBK1 and IKKε are not proteolytic substrates of NS3/4A

Sequence analysis revealed that both TBK1 and IKKε contain putative NS3/4A protease substrate recognition sequences. We utilized cell based, *in vivo* protease assays to evaluate the steady state levels of the two kinases in the presence or absence of NS3/4A. Immunoblot analyses from cells ectopically expressing epitope-tagged TBK1 and IKKε in the presence of NS3/4A revealed a somewhat surprising result. While the steady state levels of IKKε remain unchanged in the presence of the protease, we consistently saw a slight decrease in the level of TBK1 protein in the presence of NS3/4A (Fig. 5-2A). However, endogenous levels of both TBK1 and IKKε were unaffected by the tetracycline regulated expression of NS3/4A (Fig. 5-2B). Similarly, endogenous levels of TBK1 in a variety of cell lines harboring the HCV subgenomic replicons demonstrated equivalent levels as the parental Huh7 cells (Fig. 5-2C).

Metabolic labeling studies were performed to evaluate the stability of ectopically expressed TBK1 in a tetracycline regulated cell line conditionally expressing NS3/4A. We found no appreciable difference in the relative stability of TBK1 attributable to the presence of NS3/4A (Fig. 5-2D). Follow up in vitro studies similarly revealed that neither TBK1, nor IKKε, are direct NS3/4A substrates whereas a control, native substrate NS5A/B was cleaved efficiently by NS3/4A under the same conditions(152) (Fig. 5-2E). From these studies, it can be concluded that the IRF-3 kinases, TBK1 and IKKε, are not direct NS3/4A protease targets however, we further wished to directly examine their signaling capacity in the presence of NS3/4A.

Induction of IFN-β by TBK1 is regulated by NS3/4A

Ectopic expression of either TBK1 or IKKε results in the induction of IFN-β expression. In order to evaluate the function of the IRF-3 kinases, TBK1 and IKKε, to activate IFN-β expression, we coexpressed TBK1 or IKKε with increasing amounts of NS3/4A in Huh7 cells. Induction of an IFN-β driven luciferase construct by TBK1 was inhibited in a dose-dependent manner by NS3/4A (Fig. 5-3A). However, IKKε mediated induction of an IFN-β promoter luciferase construct was unaffected by

coexpression of NS3/4A (Fig 5-3B). These results were somewhat surprising as we had already ruled out these IRF-3 kinases as direct NS3/4A protease targets (see above). However, the ability of NS3/4A to regulate TBK1 activity, taken together with the slight decrease in steady state levels, suggests that although NS3/4A might not directly target TBK1 proteolytically, it might somehow target a factor that regulates TBK1 activity and/or stability. In support of this, the metabolic radiolabeling studies described above to assess TBK1 stability, revealed differences in the profile of proteins that coimmunoprecipitated with TBK1. We therefore set out to determine what factors might directly interact with TBK1 to affect either its activity or stability with the hope of delineating factors that are also involved in this signaling pathway and potentially identify the NS3/4A protease substrate.

Identification of TANK as an adaptor protein in virus induced signaling pathways

TBK1 had been previously identified and characterized as a TANK binding kinase. TANK, or Traf associated factor of NF-κB activation, has been characterized as an adaptor protein that links upstream signaling factors emanating from the TNFR to NF-κB activation. It has been shown to interact with TRAF2 and NEMO as well as TBK1 and IKKε(44,187,198).

Based on these data, it provides an attractive candidate as a bridging protein for signaling pathways that lead to both IRF-3 and NF-κB activation by directly interacting with kinases responsible for their activation.

In order to delineate the role that TANK might play in the viral induction of IFN-β, we utilized a previously characterized dominant negative mutant of TANK consisting of the truncated carboxyl terminus (C TANK)(46). Overexpression of C TANK prevented the induction of an IFN-β promoter driven luciferase construct in response to SenV infection suggesting that TANK might indeed serve a role as an adaptor protein integrating virally induced signaling events to direct the activation of TBK1 (Fig. 5-4A). As a control, cotransfection with a dominant negative mutant of IRF-3 similarly ablated viral induction of the IFN- promoter (Fig. 5-4A). Similar studies utilizing the constitutively active N RIG and N MDA5 coexpressed with increasing amounts of C TANK also revealed a dose dependent inhibition of IFN-β promoter induction signaled specifically through these receptors (Fig. 5-4B). Surprisingly, when we overexpressed full length TANK (FL TANK) as a control under the same conditions, we similarly saw inhibition of IFN-β promoter induction (Fig. 5-4B). However, this is perhaps not so surprising as overexpression of an adaptor protein

would effectively titrate the necessary interactions between associated proteins required for proper modification and signal integration.

While these data suggest a role for TANK as an adaptor protein for signal integration between viral dsRNA PRRs and the activation of TBK1, it does not exclude the possibility that the inhibitory effects of the TANK constructs might in fact be due to simply artificially sequestering factors (such as TRAFs) that are directly required for signal transduction from properly interacting with downstream mediators. Indeed, sequestration of TRAFs by TANK has been demonstrated to be a modulatory mechanism to regulate signaling through the TNFR(198). Nonetheless, the results presented above and the fact that TBK1 has been shown to directly interact with TANK provides a plausible role for TANK in this signaling pathway.

TANK is not a proteolytic substrate of NS3/4A

As the results presented above seemed to indicate a role for TANK in signal transduction to regulate induction of the IFN-β promoter, we examined its potential as a direct target of protease regulation. Protein sequence analysis revealed a putative NS3/4A cleavage site in the TRAF binding domain of TANK providing a very tantalizing target for regulation.

However, cell based protease assays revealed no significant differences in the protein levels of ectopically expressed TANK when NS3/4A was present (Fig. 5-5A). We further examined the steady state levels of endogenous TANK in cell lines conditionally expressing NS3/4A under conditions of both mock and viral infection. We observed no difference in the levels of TANK protein regardless of the presence of NS3/4A (Fig 5-5B). Finally, we assessed the levels of endogenous TANK protein in cells harboring a variety of HCV subgenomic replicons. We found no difference in the levels of TANK in the replicon cells as compared to the parental Huh7 control cells (Fig. 5-5C). Interestingly, when we examined endogenous TANK protein in Huh7 cells infected with SenV or treated with IFNα2a, we noticed an increase in steady state levels/mobility shift upon IFN treatment (Fig. 5-5C).

The interaction between TANK and TBK1 is unaffected by NS3/4A

A possible explanation for the regulation of TBK1 activity described above could potentially occur through disruption of important regulatory interactions with TANK and other associated proteins. Therefore, we wished to assess the functional interaction between TBK1 and TANK. We used commercially available anti-flag antibody coated agarose beads (Sigma) to immunoprecipitate TANK from cells coexpressing T7 epitope

tagged TBK1 and flag epitope tagged TANK and conditionally expressing NS3/4A. Immunoblot analysis of the bead fractions against T7 revealed that TBK1 coimmunoprecipitated with TANK regardless of the presence of NS3/4A (Fig. 5-6A). Additionally, when the membrane was reprobed for TBK1, we found that endogenous TBK1 associated with transfected TANK protein and was efficiently coimmunoprecipitated in both the presence and absence of NS3/4A (Fig. 5-6A). These data reveal that there is a basal association between TANK and TBK1 and further suggests that these proteins might remain in a quiescent state in the cytoplasm awaiting further interactions to modify their activity.

We therefore wished to assess their association in response to a viral stimulus in both the presence and absence of NS3/4A. We ectopically expressed flag TANK in cells conditionally expressing NS3/4A and performed a Sendai virus time course. TANK was immunoprecipitated from cell extracts using anti-flag beads and the pulldown fractions were immunoblotted and probed for endogenous TBK1. We observed no differences in the association of TBK1 with TANK either in response to viral stimulus or in the presence of NS3/4A (Fig. 5-6B). These data instead suggest that the TANK/TBK1 interaction occurs in the cell basally and in a “ready state” for integration of signaling events directing activation of TBK1. It is thus likely that upon stimulation, a factor

is recruited to TANK that modulates TBK1 activity. It is interesting that disassociation of TBK1 does not appear to be required for this function, at least as assessed through this assay, suggesting the existence of a macromolecular signaling complex in cells that directs the activation of IRF-3. It is noteworthy however that when these samples were probed for IRF-3, it was not detected in association with the TANK/TBK1 complex. This is likely due to a transient interaction between TBK1 and IRF-3 as phosphorylated IRF-3 becomes activated and translocates to the nucleus to induce target gene expression. In support of this, we have not been able to demonstrate a stable interaction between IRF-3 and TBK1 in either mock or virally infected cells (data not shown). Regardless, these studies reveal that modulation of TBK1 activity through NS3/4A does not appear to be regulated through disruption of TBK1/TANK interaction. Instead, it is likely that the protease regulates the function of a factor recruited to this complex that in turn directly modulates TBK1 function.

NS3/4A does not affect the association between TANK and NEMO

We wished to further characterize the role that TANK might serve as a bridging protein for signal integration events resulting in viral activation of both TBK1/IRF-3 and IKK/NF-κB. TANK has been previously shown to serve as an adaptor molecule in bridging signaling factors induced by

TNFα stimulation to the activation of the IKK complex through a direct interaction with the IKK regulatory subunit NEMO (IKKγ)(40). We therefore sought to elucidate its potential role in the virally induced activation of IKK signaling

We examined the association between TANK and NEMO in cells conditionally expressing NS3/4A under mock or viral stimulation. We again utilized a flag epitope tagged TANK expression construct for immunoprecipitation from cell extracts using anti-flag conjugated agarose beads. TANK immunoprecipitated fractions were subjected to immunoblot analysis and probed for associated endogenous NEMO protein. We observed a basal association between TANK and NEMO in unstimulated cells that was not affected by the presence of NS3/4A (Fig. 5-7). Furthermore, viral stimulation of cells altered the levels of associated NEMO in a similar manner regardless of the presence of NS3/4A (Fig. 5-7). These findings are similar to what we observed for endogenous TBK1 protein in that there is a basal association of these proteins, likely in a preformed complex, which is further maintained in response to viral infection to achieve activation of the important transcription factors IRF-3 and NF-κB. Indeed, these interactions occur under unstimulated conditions, in which no detectable activation of IRF-3 and NF-κB is observed, suggesting they are subject to further regulatory mechanisms.

However, the existence of a basal complex provides for rapid integration of signaling events and the timely induction of antiviral programs. These data also support the hypothesis that viral stimulation through RIG-I and MDA5 results in the activation of factors and their recruitment to this complex to in turn induce the activation of TBK1 and IKK to direct the activity of IRF-3 and NF-κB.

Discussion

The studies described above establish TBK1 as an essential mediator in viral induction of IFN-β and are in agreement with previously published reports(99,167,194) (and Fig 5-2). As we, and others, have demonstrated that RIG-I is an essential viral dsRNA PRR, is perhaps not surprising that we were able to extend these observations and describe TBK1 as a critical factor for IFN-β induction through these PAMP receptors. However, this result is in disagreement with a published report stating that TBK1 is not required for N RIG, the constitutively active mutant of RIG-I, mediated activation of IRF-3 and induction of IFN-β(283). We have found that upon repeated passage, the viral responsive properties of TBK1 deficient MEFs change. We hypothesize that this may be due to compensatory mechanisms that result in the induction of IKKε expression

over time. Indeed, a similar phenomenon has been reported for IRF-3 knockout MEFs which have been shown to upregulate the expression of IRF-7 after extended culture(213). We have shared our findings with Fujita's group and they now agree that TBK1 is involved in RIG-I mediated signaling to IRF-3. We therefore conclude that the activity of TBK1 as an IRF-3 kinase is essential for the induction of IFN-β in response to viral infection, triggered through either RIG-I or MDA5 mediated recognition of viral dsRNA stimuli.

Additionally, we have described a potential role for the adaptor protein TANK in dsRNA PRR signal transduction. We have demonstrated that TANK associates with both TBK1 and NEMO and through these interactions likely directs the activation of the transcription factors IRF-3 and NF-κB. Our studies have revealed that TANK, TBK1 and NEMO exist in a complex in the cytoplasm of unstimulated cells, in a "ready state" to facilitate rapid integration of signal transduction pathways and early initiation of host response programs. Activation of TBK1 and the IKK complex likely requires the recruitment of factor(s) that modify them to direct their activation of the downstream transcription factors. We believe that the protease must target such a factor that is required for both the activation of TBK1 and the IKK complex, thus effectively shutting down the function of two important transcription factors that initiate innate response

programs. Our studies demonstrating NS3/4A regulation of TBK1 function support this model. Overexpression of TBK1 results in its activation, likely through forced interactions with factors that regulate its function. NS3/4A alteration of such a factor would result in decreased TBK1 function as assessed by induction of gene expression in luciferase assays and immunoblot analysis demonstrated above. Similarly, this could also explain why we observe lower steady state levels of ectopically expressed TBK1 but not endogenous protein. By uncoupling an important modulatory mechanism, the protease would inhibit downstream signal transduction and prevent the accumulation of ectopic TBK1 protein.

It is interesting that we do not observe the same protease mediated regulation of IKKε function. Indeed, IKKε has also been demonstrated to interact with TANK and it is thought that this also likely directs its activity. However, we and others have observed enhanced activity of the IKKε kinase as compared to TBK1 as detected through a variety of biochemical assays(31) (E. Foy unpublished observations, S. McWhirter personal communication). It is possible that the activity of IKKε is regulated more directly through transcriptional induction with a lesser requirement for stimulus dependent post-translational modification. IKKε is an ISG and thus not likely to be expressed in most cell types in the absence of a viral threat. Thus, it should only be expressed under conditions where it is

likely to be activated and as such may not be as strictly dependent on such post-translational modifications. Indeed, TBK1 and IKKε only share 64% sequence homology and it is possible that these differences encode for differential regulatory control mechanisms. Unfortunately, without fully understanding the mechanisms that regulate TBK1 activation, we cannot directly test this hypothesis but it will be interesting to do so once the pathway is more fully elucidated. Indeed our data suggests the elucidation of the factors that modulate TBK1 activity will likely reveal the identity of the NS3/4A target.

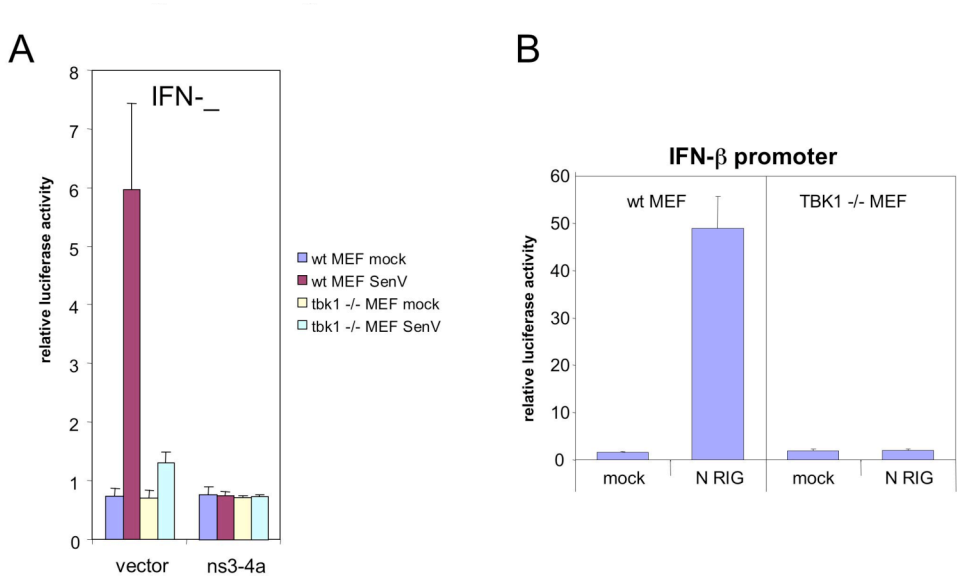
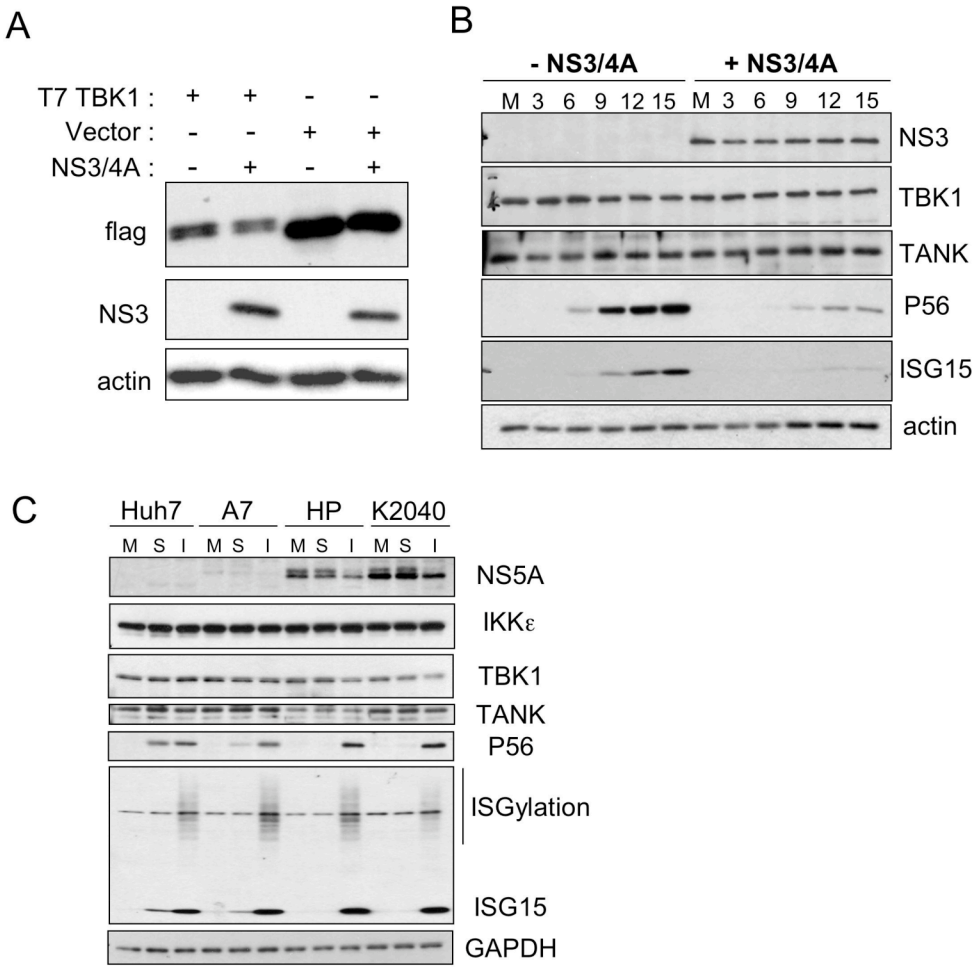


Figure 5-1: TBK1 is required for the induction of IFN-β in response to virus infection or signaled through RIG-I

A. Wildtype or TBK1 deficient MEFs were cotransfected with plasmids encoding the IFN-β luciferase reporter construct, *Renilla* luciferase and 100 ng of NS3/4A or empty vector. Cells were mock-infected or infected with SenV for 20 h and harvested for luciferase assay. B. Wildtype or TBK1 deficient MEFs were cotransfected with plasmids encoding the IFN-β luciferase reporter construct, *Renilla* luciferase and 100 ng of N RIG or empty vector. After 24 h, cells were harvested for luciferase assay.


A
T7 TBK1 : + + - -
Vector : - - + +
NS3/4A : - + - +
flag
NS3
actin
B
- NS3/4A
+ NS3/4A
M 3 6 9 12 15 M 3 6 9 12 15
NS3
TBK1
TANK
P56
ISG15
actin
C
Huh7 A7 HP K2040
M S I M S I M S I M S I
NS5A
IKKε
TBK1
TANK
P56
ISGylation
ISG15
GAPDH

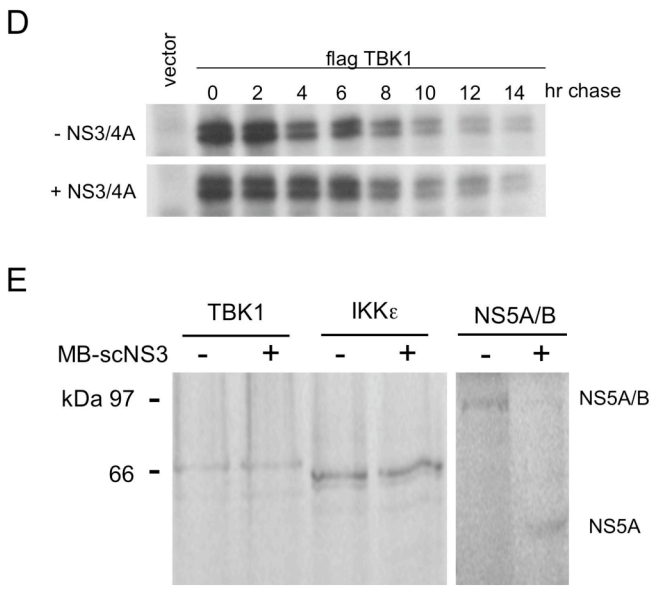


Figure 5-2: The noncannonical IKKs TBK1 and IKKε are not proteolytic substrates of NS3/4A

A. UNS3/4A cells were cultured to suppress (-NS3/4A) or induce NS3/4A expression (+NS3/4A) and transfected with expression plasmids encoding flag-TBK1 or flag IKKε. 24 hr later the cells were harvested and subjected to immunoblot analysis with specific antibodies to detect the flag, NS3 and actin. **B.** UNS3/4A cells, cultured to suppress (-NS3/4A) or induce NS3/4A expression (+ NS3/4A), and either mock-infected or infected with SenV. Cells were harvested at the times indicated and extracts were subjected to immunoblot analysis to detect NS3, TBK1, TANK, P56, ISG15 and actin. **C.** Huh7 control or Huh7-A7, Huh7-K2040 and Huh-HP cells harboring the respective HCV replicon were mock-infected, infected with SenV or treated with 50 units/ml IFN-α2a. After 20 h the cells were harvested and extracts were subjected to immunoblot analysis. **D.** Cells were metabolically labeled with ^{35}S-methionine for a 1 h pulse and were harvested following a period of culture in the presence of excess unlabeled methionine (chase). 200 μg of protein were immunoprecipitated with normal rabbit serum (NRS; control) or anti-TBK1 serum (from T. Maniatis) and products were analyzed by denaturing gel electrophoresis and autoradiography. **E.** TBK1, IKKε or control HCV NS5A/B were synthesized in reticulocyte lysate and incubated with MB-scNS3. The reactions were then separated by SDS PAGE.

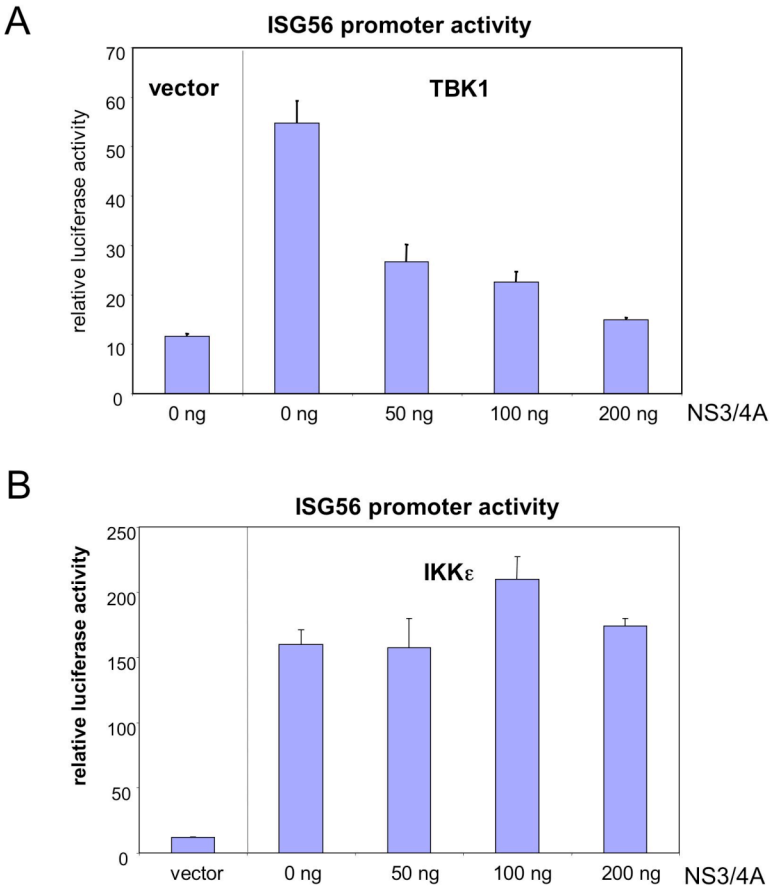


Figure 5-3: Induction of IFN-β by TBK1 is regulated by NS3/4A
Huh7 cells were co-transfected with plasmids encoding the IFN-β-luciferase promoter construct and *Renilla* luciferase along with 100 ng of plasmid DNA expressing vector only, (**A**) TBK1, or (**B**) IKKε and 0, 50, 100 or 200 ng of plasmid DNA encoding wild-type NS3/4A as indicated.

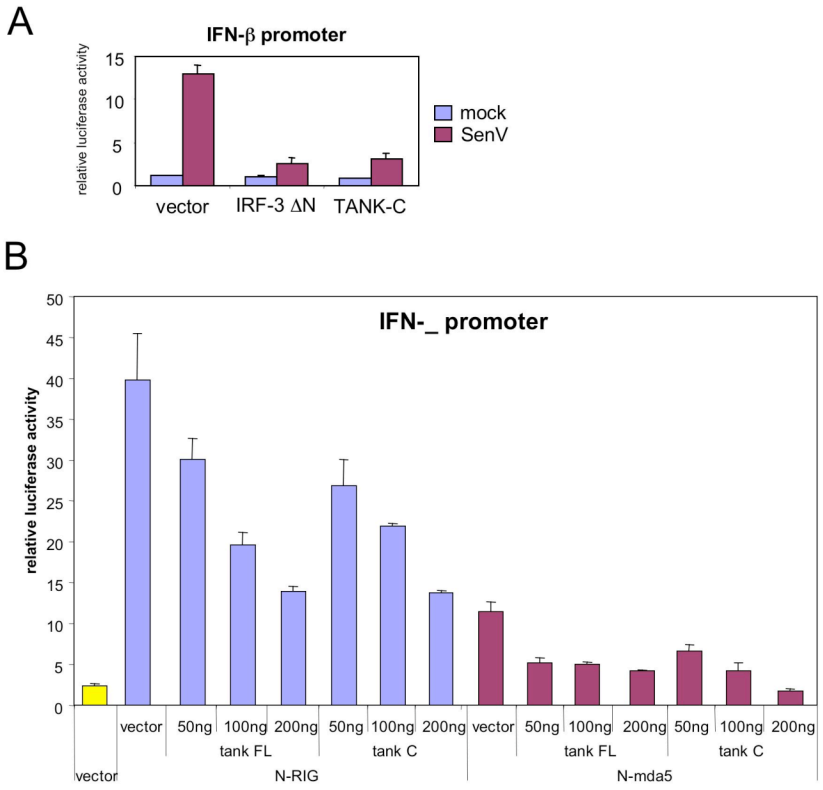


Figure 5-4: Identification of TANK as an adaptor protein in virus induced signaling pathways

A. Huh7 cells were co-transfected with plasmids encoding the IFN-β-luciferase promoter construct and *Renilla* luciferase along with 100 ng of plasmid DNA expressing vector only, IRF-3 ΔN, or TANK C. After 24 h, cells were either mock infected or infected with Sendai virus for 20 h and harvested for luciferase assay. **B.** Huh7 cells were cotransfected with plasmids encoding the IFN-β-luciferase promoter construct and *Renilla* luciferase along with 50 ng of plasmid DNA expressing vector only, N RIG, or N MDA5 and 0, 50, 100 or 200 ng of plasmid DNA encoding TANK FL or TANK C as indicated. Cells were harvested after 24 h for luciferase assay.

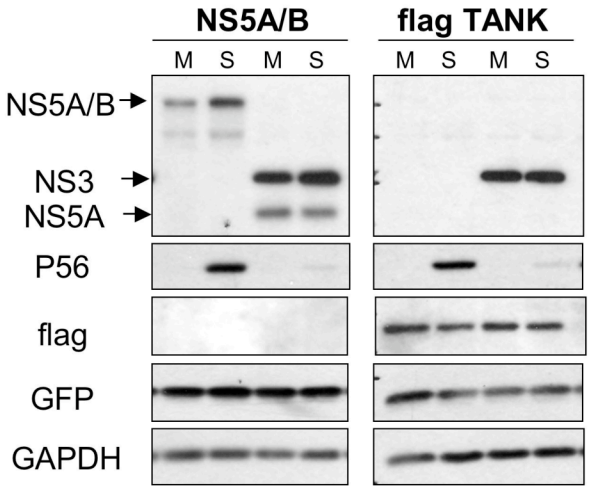


Figure 5-5: TANK is not a proteolytic substrate of NS3/4A
UNS3/4A cells were cultured to suppress (-NS3/4A) or induce NS3/4A expression (+NS3/4A) and transfected with expression plasmids encoding flag-NS5A/B or flag TANK. 24 hr later the cells were harvested and subjected to immunoblot analysis with specific antibodies to detect the flag, P56, GFP, GAPDH and anti-HCV polyclonal patient serum.

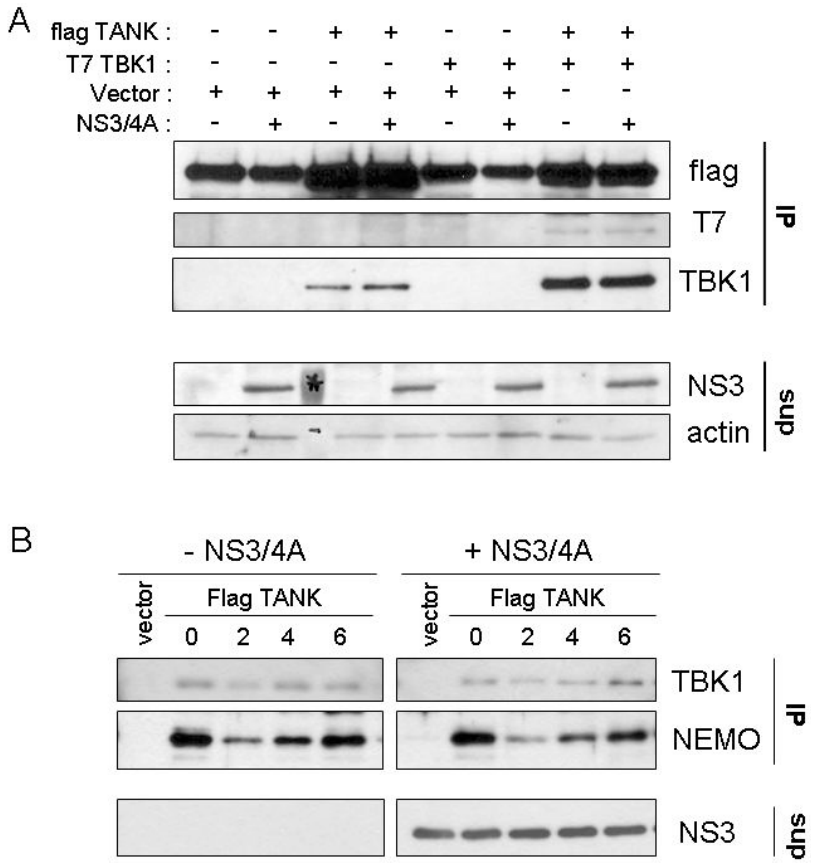


Figure 5-6: Association between TANK and TBK1 or, TANK and NEMO is unaffected by NS3/4A

A. UNS3/4A cells were cultured to suppress (-NS3/4A) or induce NS3/4A expression (+NS3/4A) and transfected with expression plasmids encoding empty vector, flag TANK or T7 TBK1 as indicated. Cells were harvested after 30 h and immunoprecipitated with anti-Flag conjugated agarose beads (Sigma). Bead and supernatent fractions were subjected to immunoblot analysis with specific antibodies to detect Flag, T7, TBK1, NS3 and actin. **B.** UNS3/4A cells were cultured to suppress (-NS3/4A) or induce NS3/4A expression (+NS3/4A) and transfected with an expression plasmid encoding flag TANK. 24 hr later the cells were either mock infected or infected with Sendai virus for the indicated times. Cells were harvested and immunoprecipitated with anti-Flag conjugated agarose beads (Sigma). Bead and supernatent fractions were subjected to immunoblot analysis with specific antibodies to detect TBK1, NEMO and NS3.

CHAPTER 6: Discussion and Future Directions

Discussion

Role of the IRF-3 pathway in controlling HCV infection

The goal of this thesis was to assess IRF-3 function during HCV infection. Chapter 3 details our efforts to characterize IRF-3 present in HCV replicon cells. We found that there is a blockade in the activation of IRF-3 imposed by HCV and mapped this activity to the viral NS3/4A protease. We determined that the NS3/4A protease could prevent the activation of IRF-3 at a point upstream of virus-induced hyperphosphorylation which prevented IRF-3 dimerization, nuclear accumulation and induction of innate effector genes (Fig. 6-1). We further demonstrated the relevance of this regulation by showing that IRF-3 has the capacity to control HCV replication through forced expression of a constitutively active IRF-3 mutant (IRF-3 5D) which resulted in a 50% reduction of HCV genomic RNA in our most fit HP replicon within 48 hours. This suggests that in order to maintain a persistent infection the virus must avoid the actions of the IRF-3 pathway. In support of this, we observed a greater than fivefold increase in viral replication and overall fitness of a poorly adapted replicon (A7) that associated with a basal activation of the IRF-3 pathway when we inhibited endogenous IRF-3 function with a dominant negative IRF-3 mutant. These studies clearly define control of the IRF-3 pathway as a critical determinant to HCV infection outcome.

Additionally, we were able to exploit the requirement for protease activity in the regulation of IRF-3 function by utilizing an experimental, therapeutic NS3/4A protease inhibitor to alleviate the inhibition of IRF-3 imposed by the protease and concomitantly restore the host antiviral

response. This was associated with a decrease in HCV replication demonstrated by the absence of HCV proteins in our replicon model system. We are hopeful that the protease inhibitors will be similarly efficacious in patients by both directly inhibiting viral replication and through restoration of host response signaling pathways. Additionally, patients will have the advantage of a restored innate immune response in priming the adaptive immune response that should result in a more effective clearance of the virus.

Identification of cellular sensors for dsRNA/viral infection and their regulation by NS3/4A

Our goal in Chapter 4 was to determine the antiviral host pathway(s) targeted for regulation by the HCV NS3/4A protease. Our results were somewhat surprising in that each dsRNA responsive pathway that we evaluated was in some manner regulated by the NS3/4A protease. Additionally, this regulation imparts a blockade to both IRF-3 and NF-κB activation in all cases. Perhaps this is not surprising for a virus like HCV that is so efficient in maintaining a persistent infection throughout the lifetime of its host. The virus must be able to antagonize cellular dsRNA sensing mechanisms to avoid induction of antiviral response programs that would otherwise eliminate the virus. The studies outlined in Chapter 4 describe the elucidation of two types of cellular sensing mechanisms for dsRNA/viral infection and coincidently, two mechanisms for protease regulation of these pathways.

Perhaps the most relevant dsRNA sensors for HCV infection are those encoded by the RIG-I and MDA5 CARD containing DEXD/H box helicases. These proteins function as dsRNA PRRs within the cytoplasm

that detect dsRNA and respond by initiating signal transduction cascades resulting in the activation of IRF-3 and NF-κB(237,282,283). Our studies reveal that the NS3/4A protease can specifically regulate signal transduction events through both of these receptors preventing the activation of IRF-3, and additionally NF-κB in response to virus infection (Fig. 6-1). We have also demonstrated that signaling programs initiated through RIG-I and MDA5 have the capacity to modulate HCV replication through the productive induction of antiviral response programs. However, HCV NS3/4A protease regulation occurs downstream of RIG-I and MDA5 signaling and can efficiently prevent the induction of antiviral response programs through these intracellular dsRNA PRRs thereby allowing the virus to persist within the infected cell.

Likewise, we demonstrated NS3/4A regulation of TLR3 signaling through NS3/4A mediated proteolysis of the TRIF adaptor protein. However at present, we do not fully understand the role of this regulatory mechanism in the context of HCV infection, as it is not likely that TLR3/TRIF are expressed appreciably in hepatocytes(151) (our own unpublished observations). One explanation is that the TLR3/TRIF pathway functions as a secondary response pathway that serves to enhance, broaden and amplify the cell's ability to respond to viral invasion and that NS3/4A prevents this important role through proteolysis of TRIF. Another explanation could be that NS3/4A present in infected dendritic cells prevents signaling through TLR3 and effective cross-priming of CTLs against HCV. The important role for intact TRIF function during the immune response to viral infection has been demonstrated in response to other viruses(102,103) and regardless of the exact nature of its role, HCV encodes a mechanism to prevent its function.

Finally, by directly inhibiting IFN-β production signaled through the dsRNA PRRs described above, our data also suggests that NS3/4A prevents the generation of the auto-amplification loop generated in response to direct IFN signaling. Additionally, many of the factors that recognize and signal in response to viral infection (RIG-I, MDA5, TLR3, TRIF, IKKε, IRF-7, etc) are themselves ISGs. Therefore the application of exogenous IFN therapeutically is diminished as the actions of many of the antiviral effector proteins induced are blocked by the HCV NS3/4A protease.

Elucidation of intracellular antiviral dsRNA signaling pathway components

The studies described in Chapter 5 describe the critical role for the IRF-3 kinase TBK1 in cellular antiviral responses. We have demonstrated that the activation of IRF-3 mediated through both RIG-I and MDA5 is dependent on TBK1. Additionally, we have established a potential role for the TBK1 adaptor protein TANK in viral signal integration culminating in the activation of both IRF-3 and NF-κB. This is mediated through the association of TANK with the IRF-3 kinase TBK1 and the IKK regulatory subunit NEMO. As such, TANK likely serves as a scaffold protein for the interaction of TBK1 and IKK with upstream mediators recruited as a result of dsRNA mediated signal transduction. These factors likely modify TBK1 and IKK to direct their activation. Our data demonstrating protease regulation of ectopically expressed TBK1 suggests that NS3/4A targets a factor that directs the activity of TBK1. Therefore, it is likely that such a factor recruited to TANK that modulates both TBK1 and IKK activity or, a factor just upstream of it is targeted for proteolysis by NS3/4A. Our future

studies are therefore aimed at elucidating the regulatory mechanisms that activate TBK1 and IKK in response to dsRNA response pathways and characterizing the signaling intermediates that mediate this response.

Current and Future directions

Role of FADD/RIP1 in pathway

Balachandran et al have recently reported an involvement for the death domain containing adaptor protein FADD and the death domain-containing serine/threonine kinase RIP1 in the induction of innate immune signaling in response to intracellular dsRNA or virus infection(12). Although a role for RIP1 has been reported for activation of NF-κB signaling initiated through TLR3, it's involvement in signaling in response to intracellular dsRNA/viral stimuli had not been previously demonstrated(172). RIP1 is perhaps better characterized for its role in the activation of IKK and p38 MAPK induced by TNFα(56,131,134,149,150). Interestingly, the kinase activity of RIP1 was shown to be dispensible for this function however, a role for TRAF2 mediated ubiquitination of RIP1 is involved(150).

We assessed the activation of both an IFN-β promoter and NF-κB promoter element driven luciferase construct in response to viral infection in the presence of a dominant negative FADD (dn) mutant. We found that neither was affected by coexpression of dn FADD. To more adequately address the role of FADD in these response pathways, we need to utilize FADD deficient MEFs and ask whether or not they can activate IRF-3 and NF-κB dependent gene expression in response to signaling initiated specifically through RIG-I, MDA5 and TBK1. Balachandran et al

demonstrated that these cells were competent to induce the activation of an IFN-β promoter in response to ectopic expression of the TLR3 adaptor TRIF suggesting that FADD is not required for the induction of IFN-β in response to TLR3 signaling(12). Furthermore, close examination of the gene induction profiles of the FADD deficient MEFs reported in their studies reveals only a partial defect in IFN-β gene induction in response to either viral infection or transfection of cells with dsRNA. Additionally, induction of IFN-β at early time points post treatment appears similar in both wildtype and FADD deficient MEFs and it is not until expression is examined at slightly later time points that a defect is observed. Instead, the more drastic defect in antiviral gene induction reported is more indicative of response genes that result from either IFN signaling directly or secondary antiviral response pathways. Alternate explanations from their data could be that 1.) FADD is instead involved a noncanonical IFN signaling pathway and required for proper induction of a subset of ISGs, 2.) FADD is required for signaling initiated through a secondary/amplified response pathway (i.e., IRF-5/7), or 3.) the attenuation reported in IFN-β induction may be attributed to a defect specifically in NF-κB activation suggesting a role for FADD in NF-κB activation but not IRF-3 activation. Results from our lab do in fact suggest that FADD deficient cells are competent to activate IRF-3 in response to viral infection as determined from IRF-3 nuclear accumulation in immunofluorescence studies (B.F. unpublished observations). It is clear that more studies are needed to fully elucidate the role of FADD in these immune response pathways.

An interaction between RIP1 and FADD has been demonstrated to be involved in regulating the initiation of either cell survival signaling or induction of the apoptotic response pathway(104,162,173). We examined

the association between FADD and RIP1 during viral infection, in the presence or absence of NS3/4A. We observed a basal association between FADD and RIP1 that was not affected by either the viral stimulus or NS3/4A. Additionally, an interaction between RIP1 and NEMO has been reported and so we also examined the association of RIP1 and NEMO under the same conditions(55,287). We found a decreased association between RIP1 and NEMO in the presence of NS3/4A. As NEMO is the regulatory subunit of the IKK complex, these data might shed light on the regulation of NF-κB activation by NS3/4A. If RIP1 does indeed play a role in signal transduction mediating the activation of NF-κB, it would appear that the protease is targeting a factor that regulates this process.

Finally, as ubiquitination of RIP1 has been reported to modulate its activity, we assessed whether RIP1 was ubiquitinated in response to viral infection and what affect NS3/4A might have on this(274,287). We immunoprecipitated RIP1 and probed for endogenous ubiquitin. We observed ubiquitinated RIP1 in response to viral infection in the absence of NS3/4A but not in the presence of NS3/4A. Interestingly, overexpression of RIP1 did not result in the activation of an IFN-β promoter driven luciferase construct. However, overexpression of RIP1 did result in the induction of an NF-κB regulated promoter luciferase construct. The activity of this promoter was not affected by the presence of NS3/4A. Taken together, these data suggest that RIP1 may play a role in the intracellular innate response with regards to the activation of NF-κB but likely does not play a role in the activation of IRF-3. Interestingly, while NS3/4A can inhibit viral activation of NF-κB, it does not appear to regulate the activation of NF-κB in response to overexpressed RIP1.

Examination of innate antiviral signaling pathways and the activation of these transcription factors in RIP1 deficient MEFs should shed light on the relative importance of this kinase in these pathways. However, what we can take away from these studies is the fact that the protease appears to affect processes that result in the ubiquitination of RIP1 that occur in response to viral infection. The role of ubiquitination in the regulation of intracellular dsRNA signaling pathways has not been evaluated however, it is an intriguing possibility that it plays a role in the activation of these antiviral effector pathways and this process may serve as a target for protease regulation.

TRAFs as potential protease targets---role in pathway?

Based on the RIP1 ubiquitination data described above, perhaps the most attractive candidate(s) to date as a target for NS3/4A protease regulation are TNF receptor associated factors, TRAFs. TRAFs have a well characterized role in mediating the activation of NF-κB in response to signals emanating from cell surface receptors including TNFR, CD40, TLR, IL-1, TCR, BCR, etc(35,106,111,208,211). Additionally, TRAFs have also been shown to be involved in signaling the activation of MAPK signaling pathways resulting in the activation of the AP-1 family of transcription factors(18,97,186,286). The mechanism through which TRAFs mediate these effects is rather novel and involves the catalysis of ubiquitin to lysine residues of the target protein. Interestingly, many of the well characterized targets of TRAF mediated ubiquitination have been shown to be positively regulated by the modification(97,197,217,241). Such positive modulation as a result of ubiquitin conjugation has been elegantly shown to be through differential modification. Whereas classical

proteosome mediated ubiquitination results from K48 linked ubiquitin, K63 linked ubiquitin modification has been demonstrated to impart positive regulation to the affected proteins activity(97,196,217,240). A requirement for TRAF mediated ubiquitin modification in the signaling pathways described above suggests that it serves an important role in pathogen sensing, immune modulation and inflammatory responses. Additionally, as RIP1 has previously been demonstrated to be a target for ubiquitination by TRAF proteins in response to TNFα signaling and our results described above suggest a role for RIP1 ubiquitination in response to viral infection, it is tempting to speculate that intracellular dsRNA signaling pathways might also utilize this evolutionarily conserved pathway to modulate signaling.

In support of this idea, there is both direct and indirect evidence that TRAF proteins indeed play a role in such responses. Direct evidence of a role for TRAFs in signal transduction from intracellular dsRNA PRRs come from studies of PKR. As described previously, protein kinase regulated by RNA (PKR) is an intracellular dsRNA PRR that activates the transcription factors NF-κB and IRF-1 in response to viral infection however, the mechanism underlying this was previously unknown. It has recently been demonstrated that PKR contains two TRAF interaction motifs that facilitate interaction with TRAFs 2, 5 and 6(82). The interaction between PKR and TRAF proteins is inducible and dependent on PKR oligomerization. Furthermore, the association between PKR and TRAFs was demonstrated to be required for the activation of NF-κB. Interestingly, overexpression of CYLD, a TRAF deubiquitinating enzyme, was shown to negatively regulate PKR mediated activation of NF-κB. This suggests that it is the ubiquitinating function of TRAF proteins that is

required for PKR mediated NF-κB activation. Additionally, dsRNA transfection of TRAF2/TRAF5 double knockout MEFs showed a complete defect in NF-κB activation. While this undoubtedly reflects impaired PKR activity, it also likely reflects a defect in RIG-I and MDA5 signaling as well as both of these dsRNA PRRs also direct the activation of NF-κB in response to intracellular dsRNA stimuli. These data suggest that TRAF2 and/or TRAF5 mediate an important role in signal transduction initiated in response to intracellular dsRNA detection.

Indeed, TRAF2 has been demonstrated as a component in a complex containing TANK, NEMO and TBK1(40,82,97). Importantly, our studies have described a role for TBK1, TANK and NEMO in viral dsRNA responsive signal transduction pathways. While a direct role for TRAF2 function in the modulation of TBK1 activity has not been demonstrated, it is possible that TRAF mediated signal transduction may in fact also direct TBK1 activity. Preliminary studies have demonstrated that TBK1 is not directly ubiquitinated in response to viral infection however it is possible that a signaling intermediate that directs TBK1 activation is instead ubiquitinated. Indirect evidence of a role for ubiquitination in the modulation of TBK1/IRF-3 activation signaled through RIG-I was recently reported(101). Hiscott's group has demonstrated that signaling to IRF-3 initiated through RIG-I is inhibited by another deubiquitinating enzyme, A20. It is also interesting that Ganes Sen's group has also reported a correlation between IRF-3 activity and A20 levels in cells(65). While certainly not conclusive, these data are suggestive of a role for TRAF protein function in signal transduction in response to signaling initiated through dsRNA PRRs.

In light of these observations, we have begun to evaluate the role of TRAF proteins in intracellular dsRNA response pathways. We have examined endogenous TRAF2 and TRAF6 from cells by immunoblot analysis to determine if they are modified in response to viral infection. We have not observed any obvious mobility shifts associated with either phosphorylation or ubiquitin modifications. Furthermore, we have also immunoprecipiated TRAF2 from virally infected cells and probed for endogenous ubiquitin modification. Thus far, we have no evidence that either TRAF2 or TRAF6 are modified in response to viral infection. Similarly we have not observed any changes in the steady state levels of either endogenous TRAF2/6 or transfected TRAF2 protein in the presence of NS3/4A as assessed through immunoblot analysis.

It is worth noting that TRAF2 possesses a very interesting potential NS3/4A cleavage site in the Zn ion coordination site of the RING domain that encodes the E3 ligase function of TRAF2. Cleavage at this site would disrupt the ubiquitin ligase function of TRAF2 providing an attractive model for NS3/4A protease regulation as well as a potential explanation for our observations with RIP1 described above. Comparative sequence analysis of the other TRAF family members has revealed that only TRAF5 possesses the same putative cleavage site although the more commonly recognized CS is replaced with CA, which has also been shown to be an NS3/4A recognition and cleavage site within the HCV polyprotein. Importantly, both sites contain an acidic glutamic acid residue at position P6 which in vitro studies have revealed correlates with increased proteolysis. The presence of this site exclusively in TRAF2/5 is especially interesting given the report that TRAF2/5 doubly deficient MEFs are unable to activate NF-κB in response to an intracellular dsRNA

stimulus(82). It would be interesting to evaluate the activation of IRF-3 and the induction of IFN-β in these cells to evaluate the role of these signaling adaptor proteins in the dsRNA antiviral response pathways. Given the evidence described above, it is highly likely that these factors are at least involved in dsRNA response signal transduction pathways and potentially also a target of NS3/4A protease regulation and future studies are planned to evaluate these possibilities. Indeed, viral modulation of TRAF function has been previously reported. The best characterized example is the Epstein Barr virus (EBV) LMP1 protein which encodes a TRAF interaction sequence(32,97,210). LMP1 has been shown to interact with TRAF family members to positively modulate NF-κB signaling(54,97,181). It is likely that this contributes to EBV pathogenesis. Additionally, the rotavirus V4 protein and the V8 cleavage product also contain a TRAF interacting sequence. This has been demonstrated to mediate V protein interaction with TRAFs to direct the activation of NF-κB through NIK however, this interaction negatively regulates AP-1 transcriptional induction(145). This provides further direct evidence for viral regulation of these signaling adaptor proteins and provides further proof of their role in modulation of the host response.

TAK1 as potential protease target---role in pathway?

While TRAF2/5 provide attractive targets for protease regulation, there is one additional cellular protein that may serve as an NS3/4A substrate. Transforming growth factor (TGF)-β activated kinase 1 (TAK1) is a serine/threonine kinase that has been demonstrated to function downstream of TRAF signaling(265). TAK1 signal transduction has been shown to direct the activation of both MAPK and IKK signaling pathways

resulting in the induction of AP-1 and NF-κB transcriptional programs(265). Additionally, TAK1 has been demonstrated to be involved in innate immune response signaling pathways. In support of this, Drosophilia deficient in dTAK1 have impaired innate immune responses to bacterial infections(97,191,230,264). TAK1 has a well characterized role in TNFα, IL-1, TLR and IL-18 inflammatory cytokine signaling pathways leading to the activation of NF-κB and AP-1(61,97,118,180,229). TAK1 has also recently been described to play a role in signal transduction emanating from PRRs. Studies have revealed that TAK1 plays a critical role in the activation of NF-κB in response to signaling through membrane associated TLR2, TLR3, TLR4 and the through the intracellular PRR, NOD2(41,42,97,117,118). Additionally, TAK1 has been also been reported to be required for the induction of the chemokine RANTES in response to TLR4 signaling(116). In addition to NF-κB, transcriptional induction of RANTES is also dependent on IRF-3(156). While there is no direct evidence that TAK1 plays a role in the activation of IRF-3, it is tempting to speculate that TAK1 might indeed play a critical role as a signal transducer in the regulation of innate immune responses by acting an integrator of divergent stimuli to signal the activation of AP-1, NF-κB and IRF-3. Indeed, we have preliminary evidence that overexpression of TAK1 has the capacity to activate transcription of an IFN-β luciferase construct, an event that requires the activity of IRF-3. Furthermore, a kinase dead, dominant negative TAK1 construct also prevents the induction of IFN-β in response to Sendai virus infection. We are currently dissecting this response to determine if TAK1 results in the activation of IRF-3, NF-κB and AP-1 in response to viral infection and if these events

are regulated by the protease. This is obviously an area that warrants further study.

Regulation of the AP-1 transcription factor by the NS3/4A protease

As discussed in the introduction, the AP-1 family of transcription factors bind to the PRDIV element of the IFN-β promoter and have been demonstrated to be a key component of the IFN-β enhanceosome. It is well known that virus infection results in MAPK signaling and activation of AP-1 although the processes mediating this are currently unknown. As described above, the two primary candidates for NS3/4A regulation also direct the activation of MAPK signaling pathways that result in the induction of AP-1 transcriptional activity. It would be interesting to characterize the role of these factors (TRAFs and TAK1) in viral induction of AP-1 activity as it seems plausible that viral sensing mechanisms signaled through PRRs (specifically RIG-I and MDA5) might initiate multiple signaling cascades that result in the activation of transcription factors that culminate in the induction of IFN-β expression. As IFN-β induction is the primary response in initiating host defense programs, it would make sense for the cell to coordinate the activation of factors that regulate its expression. As such it would be interesting to assess if RIG-I and MDA5 can initiate MAPK signaling to direct the activation of AP-1.

Additionally, if signaling through RIG-I and MDA5 is found to direct MAPK signaling to activate AP-1 function, it would be worthwhile to assess potential NS3/4A regulation of this activity. If the protease is indeed regulating TRAF2/5 or TAK1 function to inhibit IRF-3 and NF-κB activity, it stands to reason that AP-1 activity might be similarly regulated. Regardless, evaluation of AP-1 function in the presence of the protease

would be helpful in further delineating at what point in host signaling pathways the protease is impacting.

Potential other pathway components

With the above signaling components in mind, a review of the literature provides potential clues to other dsRNA PRR signaling components. Starting from the top with CARD containing dsRNA PRRs, and assuming that TRAF2/5 and TAK1 are involved, several proteins fall into place. With the assumption that RIG-I and MDA5 interact with a CARD containing adaptor protein and given the relative paucity of CARD proteins recognized in the human genome, the CARD containing proteins RIP2, CARMA1 (CARD11) and BCL10 jump out. Given its role in NOD signaling and the obvious similarities with RIG-I and MDA5, RIP2 is perhaps the most obvious candidate(45,97,109,138). However, RIP2 deficient MEFs maintain intact antiviral responses culminating in IRF-3 activation and IFN-β production in response to viral challenge (T. Fujita, personal communication). CARMA1 and BCL10 both contain CARD domains and have been shown to play a critical role in TCR signaling(251). BCL10 recruits the death domain containing protein MALT1 which importantly has been demonstrated to interact with TRAF2 and TRAF6(97,241,251). In the context of TCR signaling, ubiquitinated TRAF6 recruits TAK1 which directs the downstream activation of NF-κB(241). Additionally, it has been reported that TRAF2 mediated ubiquitination of RIP1 results in its interaction with TAK1(122). Therefore a potential model for dsRNA CARD containing PRR signaling that emerges from our data and observations from the literature is as follows: RIG-I/MDA5 multimerize in response to dsRNA substrate interactions.

The CARD domains recruit the adaptor proteins CARMA1 or BCL10 which in turn recruit MALT1. MALT1 binds to TRAF2 which mediates the activation and ubiquitination of RIP1. TAK1, through TAB2/3, associates with the complex and becomes activated. TAK1 then directs the phosphorylation of TBK1, MAPK and IKKβ through interaction with the TANK complex which results in the activation of IRF3, AP-1 and NF-κB. These transcription factors form the IFN-β enhanceosome to direct transcription of IFN-β and induction of the antiviral response. While this model is purely speculative, it is certainly testable and future studies examining the role of these factors should demonstrate whether or not they play a role in these signaling pathways.

Context dependent regulation of protease substrate

The fact that the protease specifically regulates activation of NF-κB in response to viral infection but not in response to TNFα or IL-1 mediated signaling initially prompted us to eliminate as candidates some of the signaling factors discussed above. However, our data has led us to reevaluate this assumption. We currently believe that dsRNA PRR signaling pathways utilize shared signaling components of other immune/inflammatory pathways to direct the activation of both similar (MAPK, IKK) and divergent (IRF-3) transcriptional programs. While different pathways may rely on similar preformed complexes within the cell to achieve similar effects, there are likely obvious differences that account for differential incorporation of certain signaling factors that result in a tailor made and stimulus appropriate response. Therefore, these differences in complex composition may account for differential regulation

imparted by the NS3/4A protease in certain contexts but not others. Similarly, subcellular localization may impart another level of regulation as signaling of membrane associated receptors (TNFR, IL-1R, TLR) occur in vastly different compartments within the cell than viral simulation of cytoplasmic receptors (RIG-I, MDA5) resulting from an ER membrane associated virus. Finally, NS3/4A recognition of its substrate may also be dependent on stimulus dependent modifications or conformational changes that only occur in the context of a specific complex, which represents a much smaller pool of the actual protein that the protease must deal with.

Concluding remarks

This work provides mechanistic insight into how hepatitis C virus can efficiently establish and maintain persistent infection. The virally encoded NS3/4A protease utilizes a novel mechanism to subvert host antiviral signaling pathways that direct the activation of the host antiviral response. As much of this pathway still remains to be characterized the NS3/4A protease therefore provides a powerful tool to probe signal transduction events to ultimately elucidate pathway signaling components. Additionally, we can take advantage of the requirement for an enzymatic activity for this regulation in the development of antiviral therapeutics to target protease function. The fact that the virus also relies on NS3/4A protease activity to support viral replication provides hope that therapeutic protease inhibitor drugs will be successful in treating and ultimately eliminating HCV infection.

It will also be interesting to further characterize the role that NS3/4A may play in regulating adaptive immune responses. Whether immune effector cell dysfunction is simply related to inappropriate cytokine/chemokine costimulation of infiltrating effector cells within infected tissues, or due to direct infection and viral antagonism of TCR/BCR/TLR signaling pathways remains to be determined. Both explanations are certainly plausible and a better understanding of the underlying mechanism could result in the development of additional HCV therapeutics aimed at restoring immune effector cell function.

Finally, identification of the protease target should provide much insight as to variation in initial viral clearance which occurs in roughly 15% of infected individuals. It is tempting to speculate that variation might arise from naturally occurring polymorphisms in the protease recognition and/or cleavage site that affect NS3/4A activity. For example, the TRAF2/5 putative cleavage site includes a strictly conserved cysteine residue that is absolutetly required for Zn ion coordination and is similarly present in all other TRAF family members, except TRAF1 which lacks the RING domain. However, the surrounding sequence exhibits relatively high sequence variability that is obviously tolerated for E3 ligase function. Therefore, it is possible that polymorphisms that affect NS3/4A recognition, interaction or cleavage might occur that otherwise have no affect on the protein's normal function. The inability of NS3/4A to ablate the protein's function would result in the generation of a productive immune response to eventually eliminate the virus. Additionally, such polymorphisms could potentially explain differences observed in the chimp model studies. Obviously, this is just one example and hepatitis C viral clearance is likely mediated by a variety of other host genetic differences.

In summary, the outcome of hepatitis C virus infection is determined by both viral and host factors that regulate innate and adaptive immune responses. The outcome of the virus/host battle determines the outcome of infection. A better understanding of the “tactics” used by each side to regulate these processes should not only aid in the fight against HCV but against many other human viral pathogens as well.

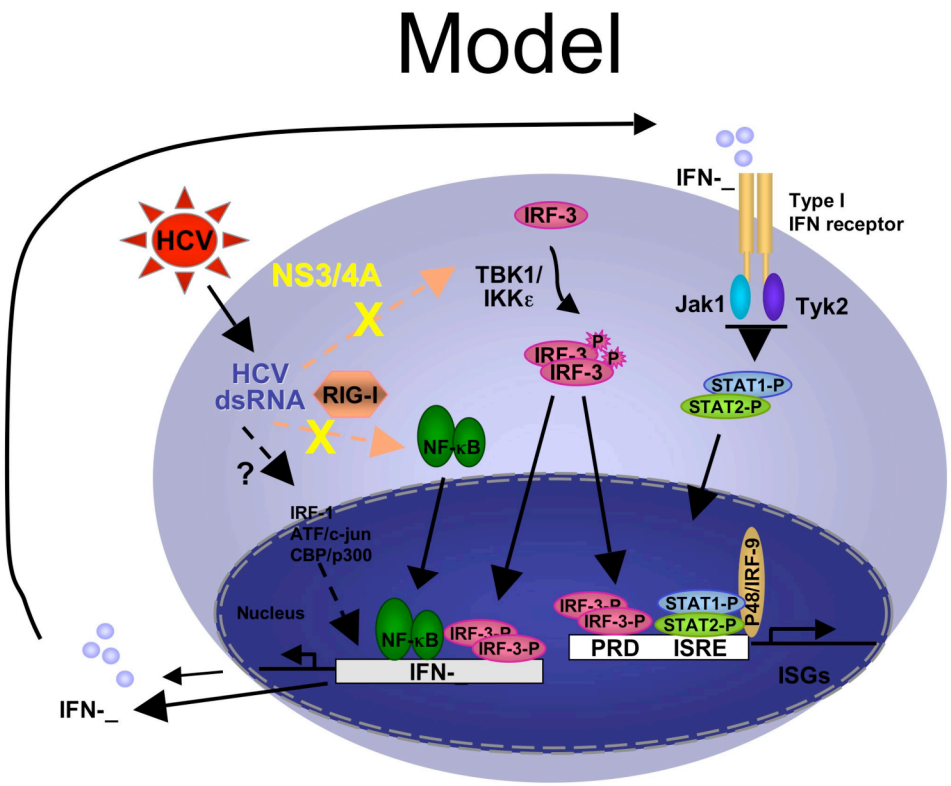


Figure 6-1: Model of the host response to HCV infection.
Our studies have demonstrated that PAMPs embedded within the HCV genome have the capacity to activate innate host antiviral signaling pathways resulting in the activation of IRF-3 and induction of IFN-β expression. We have further shown that the RIG-I pathway is essential in mediating this response and signals the activation of both IRF-3 and NF-κB. HCV evades these actions by disrupting signaling events emanating from RIG-I and other cellular dsRNA PRRs to prevent activation of IRF-3 and NF-κB and induction of antiviral effector programs that would otherwise limit HCV infection.

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VITAE

Eileen Marie Foy was born in East Orange, New Jersey. She grew up in southern California but moved to Woodstown, New Jersey at the start of high school. After a year and a half in New Jersey, her family moved to Scottsdale, Arizona where she attended and graduated from Horizon High School. After graduation, she and her family moved back to southern California where she attended Mount San Antonio College in Walnut, California. From there she transferred to the University of California, Los Angeles where she graduated *summa cum laude* with a B.S. in Microbiology and Molecular Genetics in 1998. During college she participated in campus undergraduate research and was awarded two summer research fellowships at the University of California, San Francisco and City of Hope/Beckman Research Institute. She was also awarded the President's Undergraduate Fellowship at UCLA. She worked as a laboratory technician at the City of Hope/Beckman Research Institute for one year before joining the medical scientist training program at the University of Texas, Southwestern Medical Center where she is currently enrolled. Eileen has two loving parents, John and Chris Foy and two younger sisters, Aimee and Megan who are attending college at California State University, Fullerton. She is currently engaged to Jeff Milush whom she met as a graduate student at UTSW.

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