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DEFINING THE IMPORTANCE OF THE HNRNP I INTERACTION TO THE SINDBIS VIRUS SUBGENOMIC VIRAL RNA USING AN INNOVATIVE TETHERING APPROACH

By

Claire Westcott
B.S. University of Missouri, 2016
M.S. University of Louisville, 2019

A Dissertation
Submitted to the Faculty of the
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in Partial Fulfillment of the Requirements
for the Degree of

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Department of Microbiology and Immunology
University of Louisville
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A Dissertation Approved on

July 28th, 2022

By the following Dissertation Committee:

____

Dr. Kevin Sokoloski

____

Dr. Carolyn Casella

____

Dr. Donghoon Chung

____

Dr. Carolyn Klinge

____

Dr. Igor Lukashevich

DEDICATION

This dissertation is dedicated to my parents Mark and Nancy who have always believed in me and encouraged me to achieve my dreams. It is also dedicated to my husband Alex who has been a constant source of support and reassurance. Finally, I dedicate this dissertation to my younger self who did not know this was possible.

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I’m extremely grateful to my mentor, Dr. Kevin Sokoloski, for taking a chance on me to help begin his academic lab at UofL as a technician and then again for allowing me to join the lab as a graduate student. Thank you for always being my advocate and helping me realize that the scientific process can be fun and not always stressful. I am a better scientist, communicator, and leader because of you.

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I am incredibly thankful and appreciative to my family for their love and encouragement. You all have cheered me on, lifted me up, pushed me to finish this undertaking and believed in me, even when I doubted myself. I could not have achieved this without you.

I would be remiss without thanking my fur babies Alex and Finn. Thank you for all of the snuggles and companionship you have given me. All of my problems seemed miniscule as soon as I walked through the door to see your wagging tails.

ABSTRACT

DEFINING THE IMPORTANCE OF THE HNRNP I INTERACTION TO THE SINDBIS VIRUS SUBGENOMIC VIRAL RNA USING AN INNOVATIVE TETHERING APPROACH

Claire Westcott

July 28th, 2022

Old World alphaviruses cause significant outbreaks of illness and debilitating multi-joint arthritis for prolonged periods. Currently, there are no FDA approved vaccines or antiviral therapies; and thus, there is a critical need to identify and characterize the molecular biology of alphaviruses. Alphaviruses rely on the host cell machinery to complete the viral lifecycle and are dependent on interactions with host RNA binding proteins. Accordingly, several host heterogenous nuclear ribonucleoprotein proteins (hnRNPs) have been found to bind to the Sindbis virus (SINV) RNAs. Disrupting the interaction sites in the viral RNAs of these RNA:Protein interactions results in decreased viral titers in tissue culture models of infection. Nonetheless, whether the observed phenotypes were due to loss of hnRNP binding, or the incorporation of polymorphisms into the primary nucleotide sequence of SINV remained unknown.

To determine if the loss of hnRNP binding was the primary cause of attenuation, or if the disruption of the RNA sequence itself was responsible for the observed phenotypes, we utilized an innovative protein tethering approach to

restore the binding of a candidate hnRNP protein in the absence of the native interaction site. Specifically, we reconstituted the hnRNP I interaction with the viral RNA by replacing the native interaction site with the 20nt Bovine Immunodeficiency virus Transactivation RNA Response element (BIV-TAR). Importantly, the BIV-TAR element will bind with high specificity to proteins tagged with a TAT peptide. Reestablishment of the hnRNP I:vRNA interaction via the BIV-TAR / TAT tethering approach restored the phenotype to wild-type like levels. As the reconstitution of the hnRNP I interaction in the absence of the native interaction site repaired the mutant phenotype we can conclude that hnRNP I binding, and not primary sequence, is responsible for the observed mutant phenotype following the loss of the native interaction site. Further examinations of the mutant phenotype revealed that the increased structural protein expression observed following the loss of hnRNP I binding led to an apparent overwhelming of the host glycosylation machinery which in turn caused poor viral particle function as manifested by decreased specific infectivity.

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CHAPTER 1

INTRODUCTION

Alphaviral Infections and Pathogenesis

Alphaviruses are single-stranded positive sense RNA viruses that are transmitted to vertebrate hosts primarily by mosquitoes, and thus are referred to as arthropod borne viruses or arboviruses (1, 2). Typically, alphaviruses infect small mammals and birds, however, notable outbreaks of disease in large mammals such as equines and humans have happened. Large scale outbreaks of alphaviruses infecting thousands or more were first reported in the 1920's, and have happened every decade since then, becoming increasingly larger and more frequent. This is, at least in part, due to the expansion of mosquito populations to new areas as a result of climate change and human interventions (3-5). Belonging to the family *Togaviridae*, the alphaviruses are classified into two groups, the Old World and the New World, based on their geographic location and distributions. In addition, the Old World and New World viruses differ in the disease manifestations they cause. Along with the initial febrile disease, the Old World (OW) alphaviruses, such as Chikungunya virus (CHIKV), Semliki Forest Virus (SFV), and Ross River Virus (RRV), can cause debilitating arthritis in multiple joints which persists months to years after the resolution of acute infection (6-8). Infections of the encephalitic New World (NW) viruses Venezuelan Equine Encephalitis Virus (VEEV), Eastern

Equine Encephalitis Virus (EEEV), and Western Equine Encephalitis Virus (WEEV) exhibit higher mortality and can cause severe neurological sequelae in those that survive initial encephalitic infection (9). Sindbis Virus (SINV) is the infectious agent for diseases like Pogosta, Ocklebo, and Karelian fever, all which are hallmarked by severe arthralgia-like disease; however phylogenetic analyses indicate that it is genetically similar to the NW alphaviruses (10-12). Despite being an arthritogenic alphavirus, SINV is used as a model system for encephalitic infections in mice due to its apparent neurovirulence *in vivo*.

While there are promising candidates for therapeutics and vaccines against alphavirus infections, unfortunately none are currently FDA approved and alphaviral vaccines in the past have exhibited low efficacy and high reactogenicity. Furthermore, not all broad-spectrum immunosuppressant treatments are appropriate for alphaviral infection, as treatment with tumor necrosis factor (TNF) inhibitors makes inflammation worse by reducing the overall control of infection (13-17). Currently, patients infected with alphaviruses take medicine to treat the symptoms of the underlying disease, like antipyretics, steroids, or non-steroidal anti-inflammatory drugs (NSAIDs), as well of antirheumatic drugs when NSAIDs are not sufficient (18-21).

Nevertheless, due to elucidation of alphaviral infections and new technologies, there are many anti-viral strategies in clinical trials. Due to the ability of alphaviruses to infect new mosquito species, and the low level of population immunity, outbreaks are becoming more prevalent .Thus, alphaviruses have become a prime candidate for the development of new anti-viral strategies. Many

of these new compounds target genome replication by targeting the viral replicase components nsP1, nsP2, nsP3 and nsP4, as well as viral entry by targeting the glycoproteins (22-25). Some possible antiviral therapeutics target the host side by either inducing the innate immune response or prohibiting host proteases needed to complete the viral lifecycle (26-29). However, many of these therapeutic compounds are impractical due to cost to manufacture and distribute, low efficacy, and off target effects to the host.

As well as these novel small compound therapeutics, there are many candidate vaccines for alphaviruses. These vaccines are at various stages in development and represent a wide range of strategies; there are inactivated whole virus vaccines, several live-attenuated candidates, measles-vectored vaccines, viral-like particle vaccines, and an emerging mRNA vaccine. The majority of countries / regions afflicted by the alphaviruses are middle- to low-income, and the perfect candidate should be easy and cost-effective to produce and store, as well as distribute and administer (30-36)As well as the logistical problems, many therapeutics and vaccine candidates will fail clinical trials as some candidates will have adverse effects, reactogenicity, and vaccine-induced disease. Furthermore, the extent to which immunity is maintained in the host and whether antibody dependent enhancement will contribute significantly to alphaviral disease remains unknown. This is due to the lack of answers to crucial questions as to how the immune system plays a role in arthritic or encephalitic disease. Thus, there is still a strong need to molecularly characterize alphaviruses and alphaviral infections.

Molecular Lifecycle Overview

Alphavirus infection begins as the viral glycoproteins E1 and E2, which are prominently displayed on the outside of the virion as a trimeric spike of heterodimers, bind to the receptor that is on the host cell membrane via the ectodomain of E2, as shown in Figure 1.1. The specific cell host receptor(s) depends on the alphaviral species and host cell, and there can be multiple receptors for one alphavirus species (2, 37). Interaction with the receptor results in viral uptake into the cell by receptor mediated endocytosis (38, 39). As the virus-containing endosome matures, an ATP-dependent proton pump causes it to acidify, which is critical for a major conformational change of the glycoproteins, causing the viral particle to undergo fusion with the endosomal membrane via the fusion peptide of the E1 glycoprotein. The result is the release of the nucleocapsid core into the host cytoplasm, which then disassembles to release the viral RNA (vRNA) for translation (40-43). Disassembly of the core and consequent protein translation is not well understood; however, host protein engagement and relocalization to the ribosomes are known to be critical for a successful lifecycle (44-46). First, after the release of the viral RNA, the alphaviral nonstructural polyprotein, P1234, is translated from the newly bare vRNA as well as a P123 polyprotein due to slippage at the Opal stop codon. After synthesis the nsP2 component of the polyprotein (and individually in isolation as a monomer) proteolytically cleaves off nsP4 (47). The polyprotein P123 and the RNA-dependent RNA Polymerase nsP4 form to make the initial replicase complex that synthesizes the minus strand RNA, which serves as the template for replication

and transcription of the positive-sense vRNAs (48). After the synthesis of as little as one minus strand RNA, the P123 component of the replicase complex is further processed as nsP1 is cleaved off to generate the short-lived nsP1-P23-nsP4 complex (47, 49). After P23 is processed in trans by another nsP2 protein or nsP2-containing polyprotein, the four nsP proteins together form the fully mature replicase complex that synthesizes both the genomic and subgenomic strand (47, 49-53). This viral RNA synthesis is located in invaginated spherules of the plasma membrane, however the specific site of the spherules is dependent on the particular alphaviral species (54, 55). The structural polyprotein is translated from the subgenomic RNA (sgRNA) strand in the order of Capsid-pE2-6K/TransFrame-E1. Like the nonstructural polyprotein, the structural polyprotein is proteolytically processed into monomeric proteins during infection. After the capsid (CP) protein is translated, it autoproteolytically cleaves itself off from the other proteins, inactivating the protease activity in the process, where it is then free to interact with the genomic RNA (gRNA) to form the nascent nucleocapsid core (51, 56). Consequentially, the leftover structural polyprotein contains a signal sequence that targets the remaining translation to the ER for processing where it is then cleaved by host proteases like signalase and furin proteases, and post-translational modifications are added to the glycoproteins and TransFrame (TF) protein (57-65). After being glycosylated, palmitoylated, and the disulfide bonds rearranged, the glycoproteins are then trafficked and displayed on the host cell surface. Capsid and E2 then interact which is thought to be a major driver of the budding of the newly formed virions from the infected host cell (66-68).

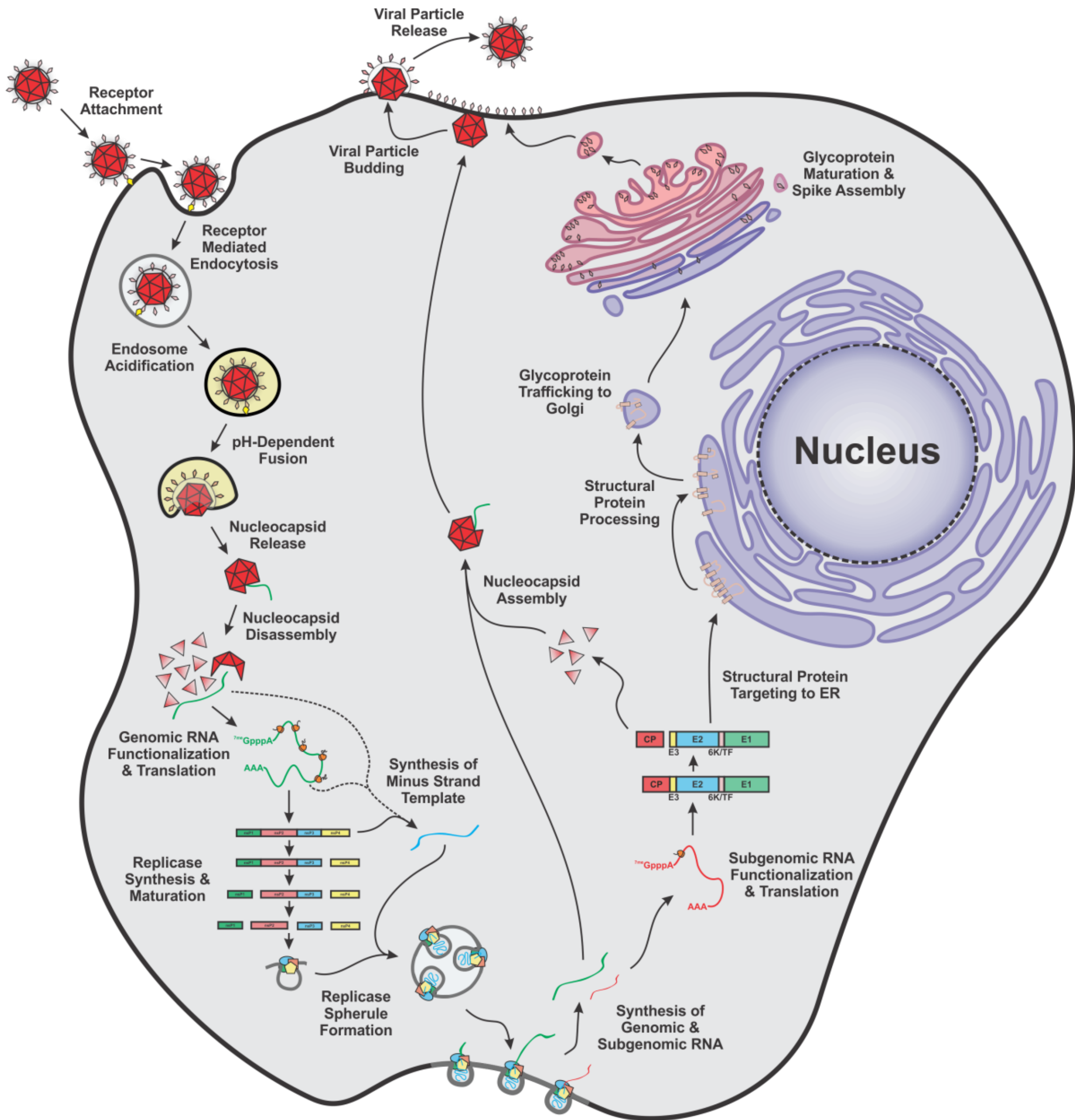


Figure 1.1 Alphaviral Lifecycle A schematic diagram of the major lifecycle events as noted in the text.

Genetic Organization of Alphaviruses

Alphaviruses produce three RNA species during infection that are replicated or translated: the genomic strand, the minus strand, and the subgenomic strand as demonstrated by Figure 1.2. In total, the viral genome is about 11.5kb long which often begins with a type0 7^{me}GpppA cap and ends with a 3' poly(A) tail (51, 69-71). The Alphavirus genomic RNA has two coding regions, of which only the first acts as an open-reading frame (ORF), which encodes the nonstructural proteins that create the viral replicase complex. The second ORF translates from the sgRNA and contains the coding information for the structural proteins that will make up the virion. The Minus Strand RNA is made from the full-length gRNA by the P123-nsP4 polyprotein, and as this RNA is a negative-sense copy of the genomic RNA it acts as the replication template for the synthesis of the other viral RNA species (51).

Alphaviruses have several sequences and RNA structures in coding and non-coding regions that are essential for a productive lifecycle. The 5' terminus of the vRNA is implicated in replication, translation, and virulence. First, the type-0 cap on the 5' end allows for the vRNA to appear and function as a host mRNA, allowing for immediate translation by the host machinery (51). Both of the 5' untranslated regions (UTRs) of the gRNA and sgRNA are highly structured, which is believed to allow for the recruitment of host proteins required for efficient replication and translation (72, 73). The 5' UTR and the complementing negative strand 3' UTR contain core promoter elements that are critical for plus and minus-strand RNA synthesis. Near the beginning of the coding region of nsP1, the 5' end

also contains a conserved sequence element (CSE) that is important for replication in mosquitoes but not the mammalian host (74).

The 3' UTR of the alphaviruses is in general much longer than the 5' UTR proper, and it has several key components that are important to lifecycle and infection. Most alphaviruses have repeated sequence elements (RSE) and a CSE, with the amount of RSEs varying from 2-5 (75). While there is not a complete picture of the RSE function, they are thought to be beneficial to replication in mosquito cells. Deletion of the RSEs reduces replication in mosquito cell lines, and CHIKV has kept the RSEs through multiple adaptations and evolutions, thus they are important for the viral lifecycle within mosquito cell lines. Similar RSEs are noted in closely related viruses, indicating the viruses came from a common ancestor (76, 77). The CSE is 19 nucleotides long and comes immediately before the poly (A) tail. It serves as a promoter for negative strand synthesis, and it is highly conserved in all alphaviruses (75, 78, 79). The 3' tail end is also important in host protein interactions, which is discussed in detail later in Chapter 2.

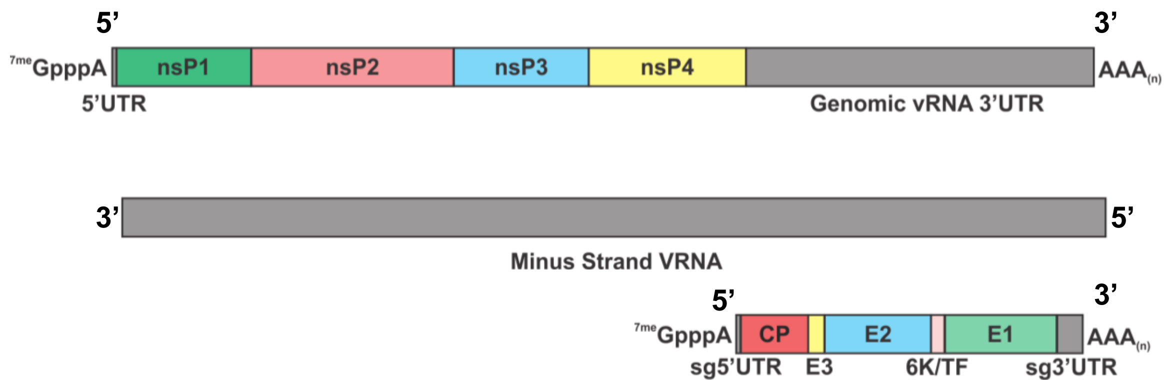


Figure 1.2 Alphavirus Genetic Organization Schematic diagrams of the coding organization of the SINV Genomic (top), Minus (middle), and Subgenomic (bottom) RNAs. The individual components of the nonstructural and structural polyproteins are indicated as are the untranslated regions (UTRs) and major features of the RNAs. Individual components are drawn to scale, relative to one another.

Nonstructural Proteins

The nonstructural polyproteins P123 and P1234, the latter of which is made by a read-through of the Opal stop codon UGA after P123, are essential to viral replication as they constitute the viral replicase complex (50). As described earlier, individually or within the polyprotein, the nsP2 protein has the ability to proteolytically cleave off nsP4 from the polyprotein. The early complex that synthesizes the minus strand RNA is formed by P123 and nsP4, as nsP4 is the viral RNA-dependent RNA polymerase (RdRp) (47). This early complex almost strictly synthesizes the minus strand but is also capable of synthesizing full-length viral genomic RNAs (48, 80). Shortly after replication complex formation, nsP1 is cleaved from P123, which marks the termination of minus strand synthesis. The incredibly short-lived complex nsP1-P23-nsP4 then forms, which can synthesize both the genomic and subgenomic RNA. Finally, P23 is processed into nsP2 and nsP3, and it irreversibly forms the late complex consisting of the monomeric forms of the viral nsPs. Again, the fully mature replicase is responsible for the production of positive-sense genomic and excessive subgenomic vRNAs (47, 49).

The nsP1 Protein

The viral nsP1 protein primarily functions in three ways: as a methyltransferase and guanylyltransferase for viral RNA capping, as an anchor for the replicase complex to the host cell membrane, and the formation of the pore to the replication spherules. The N-terminal domain contains a Rossmann-like

methyltransferase fold that transfers the methyl group to form the m^7 Gppp moiety, and then the guanylyltransferase activity of nsP1 transfers that functional group to the vRNA, forming the cap structure (81, 82). This enzymatic capping activity is independent of nsP1's binding to the cell membrane (83). Initially, the nsP1 protein weakly binds to the host membrane by way of an amphipathic helix, later in which cysteine residues within the helix are palmitoylated to strongly anchor the nsP1 protein to the membrane, presumably via the action of one or more palmitoyl transferases found at the membrane interface, although the mechanism by which is still unknown. The palmitoylation allows nsP1 to look like a host membrane protein and integrate into the host membrane in a more permanent fashion (84-89). Finally, nsP1's ability to bind to the cellular membrane allows for the formation of the replication spherule centers. Twelve copies of nsP1 form a crown-like ring complex with an inner pore that allows for the transfer of RNA and small proteins but restricts access of immune sensors from the dsRNA intermediate during infection (90, 91) The ring formation of nsP1 has a hook-like structure that interacts with one copy each of nsP2 and nsP4 to form the functional replicase machinery (92).

The nsP2 Protein

As stated above, nsP2 is integrally involved in the processing of the nonstructural polyprotein. As well as being a well-defined protease, the nsP2 protein also has helicase and triphosphatase activities during infection. The protease function of the C-terminal domain is primarily responsible for the

processing and cleavage of the nonstructural polyprotein (93, 94). Mutant strains either lacking the proteolytic activity or with differential timing of cleavage have decreased replication or ablated infection (47, 95, 96). Via the nsP2 proteins' helicase activity, the nsP2 protein is heavily involved in RNA replication and translation as it unwinds the vRNA in coordination with the nsP4 protein (97-100). In addition to the other enzymatic functions above, the nsP2 protein also has a triphosphatase motif that is responsible for the removal of the phosphate from the 5' end to become a diphosphate, which prepares the RNA as a substrate for capping by nsP1 (96).

An additional critical function of the nsP2 proteins of OW alphaviruses is their capacity to restrict host transcription. Replicons strictly encoding the nsPs exhibit the same transcriptional shutoff ability as wild-type (WT) virus infection, and several studies with nsP mutants have shown that nsP2 is responsible for the shutoff in OW viruses but not NW viruses (101). This has been specifically shown using a SINV mutant that has a single point mutation in the nsP2 protein, P726G, which is deficient in regard to host cell transcriptional shutoff (102). This key feature is important to infection, as this mutated virus induces decreased cell death and exhibits diminished viral growth in cell culture. The OW nsP2 protein can translocate to the nucleus and degrade RPB1, a subunit of the DNA-dependent RNA polymerase II (103). This degradation shuts down host transcription and inhibits the host's innate anti-viral response. The role of transcriptional shutoff is transferred to the capsid protein in New World alphaviruses and is discussed later in this chapter (104).

The nsP3 Protein

While the other alphaviral nsPs have been thoroughly characterized, the function of nsP3 has for many years been evasive and perplexing to the field. The protein is comprised of three domains: the N-terminal macro domain, an Alphavirus Unique Domain (AUD), and the C-terminal Hyper Variable Domain (HVD). The conserved alphaviral macrodomain has both the ability to bind to nucleic acids and exhibits phosphatase capabilities, which could be involved in host protein interactions (105). As well as these functions, the nsP3 macrodomain has a d-ADP-ribosylation activity that counteracts the anti-viral ADP-ribosylation response (106-108). As such, targeting the ribosylase activity has been proposed as a therapeutic strategy. Next in the nsP3, the AUD is strongly conserved among the alphaviruses, and mutations in this area cause inhibition of RNA synthesis, polyprotein processing, as well as neurovirulence (109-111). Finally, the HVD is exactly that: highly varied across the 30 different alphaviral species (112). While there are some conserved elements in the HVD, like being highly phosphorylated, the comparison of the lack of a consensus allows researchers to determine the function of the HVD by identifying cryptic conserved motifs within the HVD (87, 113, 114). Interestingly, the phosphorylation state of the VEEV HVD is important for replication in mosquito cells, but not in vertebrate cells; and mainly, the HVD seems to be a hub for host protein interactions, which are discussed in thorough detail in Chapter 2 (115).

The nsP4 Protein

The final nonstructural protein is the viral RNA dependent RNA polymerase that is responsible for RNA synthesis, as well as formation of the poly(A) tail of the genomic and subgenomic RNA. Due to its molecular nature and function, the nsP4 protein is the most conserved nsP, which strongly resembles all other known RNA-dependent RNA polymerases (116, 117). The N-terminal domain is unique to alphaviruses, and the C-terminal end has the polymerase activity that is similar to other viruses. In addition, nsp4 has adenylyltransferase activity that is responsible for the polyadenylation of the 3' tail end of the virus (80, 118, 119). The high mutation rate of alphaviruses and formation of a quasispecies is due to the lack of any discernable proof-reading capability of the RdRp. Stoichiometrically, there is less nsP4 protein made relative to the other nsPs, as there is a leaky Opal stop codon at the end of nsP3 although not all alphaviruses, like SFV, have the stop codon (50, 51, 109). This will occasionally stop translation before the machinery travels to the nsP4 region for. Studies mutating this codon and forcing readthrough increases the ratio of nsP4 to the polyprotein P123, which increases RNA synthesis (120). However, this has an overall negative effect on infection and causes attenuated disease in mouse models, thus the ratio of nsP4 to P123 plays an important role in severe infection.

Architecturally, nsP4 is located in the center of the membrane pore of the viral replicase complex, as the nsP1 ring tightly holds the protein there by flexible loops. This orients nsP4 to where the RNA entry and exit pockets are facing the

spherule side and the NTP entry is located on the cytoplasmic side. The interaction between nsP1 and nsP4 forms a disk that nsP2 then docks onto and hooks onto the flexible loops of nsP1, which also increases stability for the active conformation of nsP4 (92).

Structural Proteins

The translation of structural proteins is very similar to the nonstructural proteins, although the structural proteins come from the ORF of the 26S subgenomic strand. In SINV, there are approximately three times the amount of subgenomic RNA strands as there are full genomic RNA strands, which is partly responsible for the immense production of the viral structural proteins relative to the viral replication machinery. The result of translating the subgenomic RNA strand is a polyprotein precursor of the structural proteins including capsid, the pE2 glycoprotein predecessor (which consists of E3 and E2), the 6K / transframe (TF) proteins, and the E1 glycoprotein (121). All together and in concert with one another, these structural proteins make up the mature infectious virion. In totality, the alphaviral virion is made up of one genomic vRNA molecule surrounded by a nucleocapsid core composed of the capsid protein(122, 123). This nucleocapsid core is safely packaged in an envelope consisting of the host lipid bilayer in which 240 copies of the E1 and E2 heterodimer are inserted (124-126). The glycoproteins form an icosahedral lattice with a T=4 symmetry in alignment with the underlying capsid proteins of the nucleocapsid core (127). This co-symmetry is oddly unique to alphaviruses, and not observed in any other known enveloped virus.

Capsid

Capsid is the first protein translated in the structural polyprotein, and it autoproteolytically cleaves itself off the polyprotein immediately after synthesis (128, 129). Capsid has two domains: the N-terminal domain which has little structure or conservation besides being highly positively charged and proline-rich, and the C-terminal chymotrypsin-like serine protease. Despite having little overall conservation, the N-terminal domain of the capsid protein has several distinct subdomains / features. The first distinct region of the N-terminal domain is an alpha helix that is involved in the dimerization of the capsid protein (130). The second region is smaller and is involved with the recognition of the RNA packaging signal and the disassembly of the nucleocapsid core during entry (131, 132). The protease function of the C-terminal end only exists for the cleavage of capsid from the structural polyprotein as, to date, no other targets have been identified (133, 134).

Interestingly and importantly, capsid is able to selectively determine and package the genomic vRNA into the nucleocapsid core over sgRNA and cellular RNAs, and it has been shown that deletions or mutations of the protein can cause it to package other RNAs indicating it has high specificity for gRNA (135-137). Unfortunately, the mechanism behind nucleocapsid assembly and gRNA packaging is not fully understood.

In the New World Alphaviruses, capsid is involved in host translational shutoff rather than nsP2 as is known for the OW alphaviruses. In a study conducted by Atasheva et al, VEEV capsid regulates nucleocytoplasmic trafficking by

blocking nuclear import pathways effectively stopping host transcription and translation (138). The loss of the nuclear localization signal and the nuclear export signal in capsid allowed nuclear trafficking. Some OW alphaviruses such as CHIKV also have a nuclear export signal and interact with the nuclear export protein CRM-1, however abolishing this interaction did not have as detrimental as an effect as the New World viruses (139).

TF/6K

The 6K and the alternatively produced TransFrame (TF) proteins are less understood than the other proteins of the structural polyprotein. They are translated from the same coding frame, and 6K is the predominant product of the polyprotein, however, in a minority of times, there is a frameshift upstream of the E1 coding sequence and a stem loop structure that causes TF to be made (58). Although the exact frequency is unknown, and certainly varies amongst the individual alphavirus species, frameshifting is estimated to occur about 30% of the time. As the synthesis of TF precludes the synthesis of the E1 glycoprotein the stoichiometric ratio of E1 and E2 is not the same, however, the precise consequences of differential amounts of the glycoproteins being produced is not well understood (57, 58, 62, 140). It is no accident that TF is made, as the production of TF is conserved across alphaviruses and TF, but not 6K, seems to be mostly packaged in the released virion (57, 62, 141). 6K/TF are required during animal infections but seem to be dispensable in cell culture models (142, 143). The roles of these proteins have not yet been extensively defined, but 6K has been

proposed to act as a viroporin; whereas TF is a virulence factor involved in inhibiting interferon (IFN) synthesis, and the palmitoylation of TF is important for particle assembly (144-146). TF palmitoylation is necessary for localization to the plasma membrane which could 'hide' the virus away from vRNA sensors, or it has been proposed that it can interact with host proteins that sense vRNA.

Glycoproteins

The glycoprotein spikes are made up of 240 copies of E1 and E2, in which E2 is the attachment protein that binds to the host cell receptor, and E1 is a class II fusion protein that mediates membrane fusion (147). Both glycoproteins are critically important for virulence, replication, and dissemination.

After capsid is translated and cleaved, the remaining polyprotein consisting of pE2(the fused form of the E3 and E2 glycoproteins), 6K/TF, and E1 is then translated and relocated to the ER membranes due to an internal signal sequence located in the E3 protein (148, 149). Alphaviruses utilize host proteases to cleave the structural polyprotein into E3, E2, 6K (or TF), and E1. As the proteins are trafficked through the secretory pathway, pE2 and E1 form a heterodimer, and because E3 has not yet been cleaved off, it acts as a chaperone to promote proper folding of the spike complex (150-152). During this time, the glycoproteins undergo several post-translational modifications which are discussed in detail later (60, 153-156). For E1 and E2 to form the fully mature heterodimer, pE2 must be cleaved by a host furin protease, and E1 then goes through disulfide bond

rearrangements to form the quasi-stable prefusion structure (153, 154, 157). To finish the alphaviral lifecycle and for virions to bud from the cell, capsid and the E2 glycoproteins must interact. This interaction is mediated by the endodomain of E2 extends into capsid's hydrophobic cleft within its protease domain, and then loops back to form a hairpin-like turn with the C-terminal end palmitoylated and anchored to the cell plasma membrane (158, 159). This interaction starts the budding and final step of the alphaviral lifecycle, although the exact mechanism behind this is yet to be elucidated.

Structural Protein Processing

After the self-cleavage of capsid from the structural polyprotein and the targeting to the ER membrane, the structural proteins then must be processed before they are fully functional. These processes are carried out by the host and are critical to trafficking through the ER pathway as well as arraying the glycoproteins correctly to form the final virion. The first polyprotein and final glycoprotein forms are illustrated by Figure 1.3.

Disulfide Bond Formation

There are several cysteines in the E1 glycoprotein that go through disulfide bond arrangement to allow for the proper folding of the glycoproteins (154, 160). This is a critical step during the glycoprotein spike formation. E1 can have three different disulfide bond configurations, with the final configuration being the most

compact and most stable. The disulfide bond between E1-E1 interactions is what forms the rigid protein lattice of the virion. Several host molecular chaperones promote disulfide bond formation and proper folding of the glycoproteins, including BiP which binds to E1, and the Cnx/Crt pathway for pE2 (160).

Glycosylation of the Glycoproteins

The alphaviral glycoproteins are N-linked glycosylated, and the sites of these in E1 and E2 are conserved across the alphaviruses. Glycosylation of these proteins is important to function in various ways, including proper folding and increasing structural diversity and therefore altering recognition by the host immune system (161, 162). Many glycosylation mutants have impaired viral replication and infectivity, however interestingly enough a mutation in the SINV E2 region decreasing glycosylation caused increased replication and virulence in mice (162, 163). This was due to the lack of glycosylation causing increased binding efficiency to heparin sulfate, which allowed for better entry into cells. N-linked glycosylation of E1 and E2 are important for conformation of the glycoprotein spike, as mutating the glycosylation sites effect the ability to form functional spikes that can fuse to the host cell (164). Since the E2 glycoprotein is prominently displayed on the glycoprotein spike, it is mainly involved in the attachment of the host receptor. Studies inserting or deleting glycosylation sites on the glycoprotein show that glycosylation can affect viral attachment, viral assembly, and antibody reactivity, all which have an effect on neurovirulence in the host.

Palmitoylation of the Glycoproteins

There are several structural proteins that are palmitoylated, including E1, E2, and TF but not 6K. SINV cysteine residues are palmitoylated at position 430 in E1 and positions 388 and 390 in E2 (59-61). The mutation of the palmitoylation sites caused a decrease in viral growth, and the mutant viral particles were more susceptible to detergent treatment. It was also observed that palmitoylation is dispensable for the viral fusion to the plasma membrane, however no further characterization of the function of palmitoylated glycoproteins has been done. The first evidence of TF being palmitoylated was originally thought to be 6K with two different modification statuses, as the study used an antibody directed to the shared sequence of TF and 6K (165, 166). Follow-up studies found that TF, but not 6K, was always palmitoylated during infection. TF palmitoylation is critical for trafficking, however the exact mechanism is not known (62).

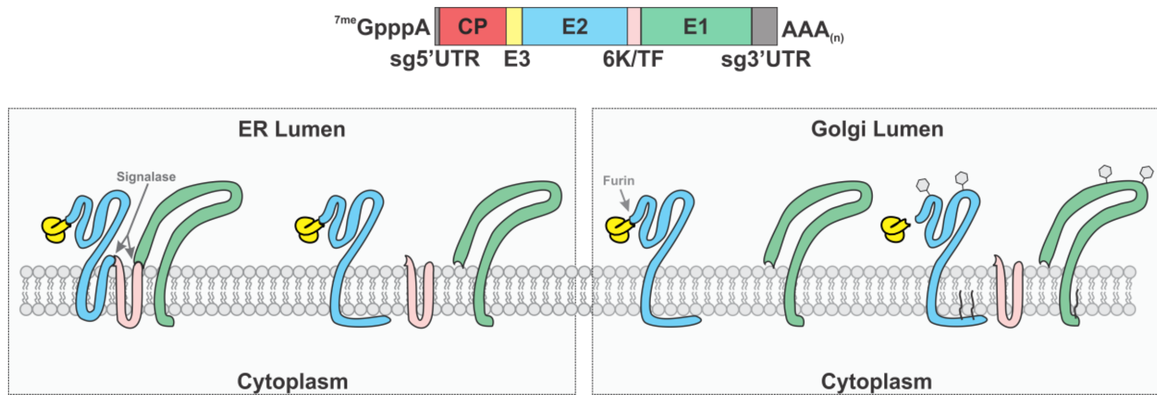


Figure 1.3 Structural Processing of the Viral Glycoproteins A schematic cartoon of structural polyprotein processing during SINV infection in the ER and the Golgi after cleavage of the Capsid protein. Indicated above are the sites of host protease cleavage as needed for glycoprotein maturation. Also shown are sites of glycosylation and palmitoylation, as indicated by hexagons and squiggles, respectively.

Necessity of Host Factors in Alphaviral Infection

Alphaviruses are positive sense, single-stranded RNA viruses that are incredibly dependent on the host to complete the full alphaviral lifecycle successfully. There are host factors interactions with the nonstructural proteins that help promote viral replication and translation, as well as interactions that are critical to shut down the host system to make cells into viral factories (167-169). Alphaviruses also rely on host processes and proteases to process the structural proteins to form an infective virion (62, 64, 65, 153, 157, 161). Studies that characterize host protein interactions also help elucidate viral protein functions, as seen with studies of the nsP3 protein (169, 170). Overall, host protein interactions with alphaviruses are critical for infection.

How host proteins were discovered to be involved in alphaviral infection and their role during infection will be discussed in detail in chapter 2.

CHAPTER 2

A REVIEW OF HOST RNA BINDING PROTEINS AND ALPHAVIRAL INTERACTIONS

Introduction

Like most RNA viruses, alphaviruses rely on host proteins and machinery to translate vRNA and package the virion to complete the viral life cycle. For over 30 years, there have been many studies conducted with the goal of determining what host proteins interact with alphaviruses during infection. Though these studies all look at host protein interactions through different methods, host RNA binding proteins (RBPs) have consistently emerged as an interactant. As such, it is important to comprehensively examine the discovery efforts of host RNA binding proteins interacting with alphaviruses to illuminate the importance of RBPs to alphaviral infections.

Discovery Efforts

Protein: Protein Interactions

Since studies from the Strauss lab first identified host proteins that bound to the vRNA to influence viral RNA biology during infection, efforts expanded to understand the full repertoire of host factors required for alphaviral infection (171).

In the early days of molecular host / pathogen screens for alphaviruses, comprehensive discovery efforts focused mainly on Protein:Protein interactions due to the available technology at the time. Over a series of likewise efforts reported by the Frolova / Frolov group and the MacDonald group, the authors collectively probed nsP3 interactions to identify interacting host factors (169, 170). Both groups employed an approach involving SINV strains containing a green fluorescent protein (GFP) in the nsP3 ORF to enable the purification of Protein:Protein complexes. In Frolova et al. 2006, the authors infected BHK-21 cells using recombinant nsP3-GFP strains of SINV, and 8 hours post infection cell lysates were fractionated and immunoprecipitated with anti-nsP3 or anti-GFP antibodies. Electrophoretic analysis via SDS-PAGE was then used to resolve the interactants of nsP3, which led to the excision and identification of about 30 proteins by matrix-assisted laser desorption / ionization-time of flight (MALDI-TOF) mass spectrometry (MS) (169). Later work by Cristea et al. 2006, similarly utilized nsP3-GFP fusion proteins to evaluate the host / pathogen protein interactions during infections with respect to time. However, this approach differed in that the authors added an additional SINV virus strain, with a freely expressed GFP behind a secondary subgenomic promoter. This allowed the authors to answer the question as to whether a host protein interaction with nsP3 was a true interaction with the viral protein, or whether proteomic changes due to viral infection and the addition of GFP were responsible for the interaction. Briefly, 293HEK or RAT2 cells were infected with a SINV reporter virus containing GFP fused to the nsP3 protein. At several times post infection, following rapid freezing and cryogenic lysate

preparation to preserve biologically relevant complexes the nsP3-GFP complexes were isolated via immunoprecipitation. The resulting materials were assessed using standard SDS-PAGE analyses and MALDI mass spectrometry. The efforts reported in Cristea et al. 2006 led to the identification of around 35 host factors of which 10 of those were previously found by the Frolova group (170).

Both of these studies elucidated several host RNA binding proteins that interact with nsP3 during infection; including G3BP1&2, hnRNP A3, hnRNP A1, and 14-3-3 epsilon, zeta, and eta. In Frolova et al., the authors go on to characterize the biological significance of interacting proteins hsc70 and vimentin by visualizing colocalization. Hsc70 relocated from the nucleus to colocalize with nsP3 in the cytoplasm, however no specific biological significance was determined for vimentin (169). This could be due to vimentin being an artifact of a nonspecific interaction during viral infection as this protein was also found in the control group of the study done by Cristea et al. 2006. As stated above, Cristea et al. 2006 found that host G3PB proteins were consistently involved with the viral nsP3 throughout infection, while co-localizations of the other RBPs during infection were temporally dependent, with the 14-3-3 adapter proteins interacting with nsP3 protein late during infection. Importantly, the authors characterized the nsP3 interaction with the G3BP proteins and determined that the NTF2-domain of G3BP1 interacts with the viral nsP3 and reduces the interaction between G3BP and nuclear pore proteins. While G3BP is normally involved in stress granule formation, this subversion of the protein during infection provides another role, one that which G3BP promotes nonstructural polyprotein processing and viral replication (170).

While alphaviral nsP3 Protein:Protein (P:P) interactions have been extensively described, there have been relatively fewer studies designed to directly assess the P:P interactions of the nsP2 protein. Work by Svetlana Atasheva in the Frolova group identified host / pathogen interactions using an nsP2-GFP fusion protein during SINV infection and SINV replicon system transfections of BHK-21 cells (172). As before with nsP3, nsP2-GFP complexes were immunoprecipitated and several host factors were isolated using SDS-PAGE for identification by MALDI-TOF analysis. Quite a few of these host proteins were identified in the previous publication identifying nsP3 interactants, suggesting that these identified host proteins could be interacting with the whole replicase complex. This has been confirmed by Varjak et al, as they isolated functional replicase complexes and found some of the same host proteins, as discussed later in this section (173). Moreover, several host RNA binding proteins were specifically associated with nsP2, namely hnRNP C and PABP.

Host / pathogen P:P interactions of the RNA-dependent RNA polymerase nsP4 have been furthered characterized by the MacDonald group, as described by Cristea et al. 2010. At first, the group continued parallel efforts to their previous publication by using a GFP-nsP4 fusion protein; however, attempts using this approach were unsuccessful as that virus was nonviable (174). To overcome this challenge, they generated a FLAG-tagged nsP4 fusion virus as it was a smaller epitope for antibody capture. Isolation and immunoprecipitation of the complexes led to the identification of 29 distinct host protein interactions in infections of Rat2 orBHK-21 cells. Importantly, they found several common interactants found in the

nsP2 studies done by the lab, including G3BP1&2 and 14-3-3 adaptor proteins. To further the understanding of functional roles G3BPs may have, Cristea et al silenced G3BP1 and G3BP2 and found that it slightly enhanced SINV polyprotein expression and viral production (174).

The majority of host protein / pathogen interactions have been determined in cellular systems using the model alphavirus, SINV, however it was not known at the time if these interactions were transferable to other similar old-world alphaviruses. In a study done by Bourai et al, it was found that there were mostly conserved, with some distinct, interactions with CHIKV nsP2 (175). This group used a high throughput yeast two-hybrid assay and found 22 host protein interactions with the viral nsP2. Additionally, the authors confirmed CHIKV nsP2 contributed to host transcriptional shut-off, similar to other old-world alphaviruses. To characterize specific host protein interactions during CHIKV infection, Bourai et al. used siRNA to knockdown host proteins, specifically hnRNP K and UBQLN4, and then determined CHIKV replication by luciferase activity. Knocking down these proteins caused decreased viral replication and reduced viral titer, and infection of HeLa cells produced colocalization with CHIKV nsP2 (175).

RNA: Protein Interactions

It is a common premise of old-world alphaviruses to subvert host proteins during infection, specifically RNA binding proteins, however the majority of the beginning studies only looked at P:P interactions. Furthermore, as Varjak et al

pointed out, many interactions with host proteins could have been missed by P:P capture methods since interactions could be transient, or interact with the vRNA and not the viral replicase machinery (173). While the structure of the replicase complex has been recently discovered, it is still unknown where host proteins fit into this structure as they were not resolved in the Cryo-EM reconstructions. During this time there was a larger shift of focus away from P:P interactions to the vRNAs themselves. To this end, Varkjak et al isolated functional replicase complexes via magnetic fractionization and found many hnRNPs that interacted with the SFV RNA and the replicase complex, some of which had been found to interact with the separate proteins of the replicase complex in previous studies. Specifically, they further discussed the colocalization of PCBP1, hnRNP M, hnRNP C, and hnRNP K with SFV, and the effect of silencing on multiple different alphaviruses infection. Interestingly, silencing these proteins caused different effects on infection; however, whether these effects are attributable to silencing host RNA binding proteins with known roles in normal cellular function, or a specific loss of the host factor on alphaviral infection is not distinguishable with this approach (173).

While host / alphaviral interactions have been extensively studied, until recently there have been relatively few findings, and fewer comprehensive discovery studies examining the direct interactions of host proteins and the alphaviral RNAs. Even so, the field at large is focusing on direct RNA interactions due to the newly developed methods. As such, LaPointe et al studied the direct RNA interactions of host proteins by using a cross-link-assisted mRNA purification (CLAMP) assay to determine what host factors were interacting with the alphaviral RNA and

confirmed three hnRNP (K, I, and M) interactions via UV cross-linking and immunoprecipitation sequencing (CLIP-seq) (176). To characterize these interactions, the authors implemented a reverse genetics approach and made silent mutations in the mapped interaction sites. This disrupted hnRNP:vRNA binding, reduced viral titer, and intriguingly increased structural gene expression. This work was also later expanded by Gebhart et al, as the authors studied common interactants of the vRNA of SINV, CHIKV, and VEEV (177). They found 108 common host proteins that interact with the vRNA of the three different alphaviruses. After coimmunoprecipitating the vRNA from three different protein interactions, hnRNP K, hnRNP A1, and ANP32A, they found that even though these viruses share the protein interactions, they might do so at different affinities.

To date, RBPs have proved essential to alphavirus infection. Most studies have focused on host / pathogen interactions from the viral side as in what host proteins are interacting with alphaviruses. Work done in the Castello lab have tried to identify system wide changes in RBPs due to SINV infection (178). To this end, the study done by Garcia-Moreno, Noerenberg, Ni et al. used a method called RNA-interactome capture to determine what RBPs were important during SINV infection compared to non-infected cells. This method uses a mixture of isotype labeling during infection and then crosslinks the interactions by UV. After cell lysis and oligo(dT) capture, the samples were quantified by proteomic analysis. Using UV crosslinking identifies more specific interactions that happen in large quantities, however it misses interactions that are transient or have small amounts of cellular proteins. Despite this, the authors found that there are around 250 total RBPs that

have ‘differential’ binding during SINV infection. While the authors did find novel RBP interactions through this approach, they did not identify several previously found RBPs that have emerged during numerous studies (178). For instance, this approach failed to detect hnRNPA1 in viral replication areas, which has been very well established by the Li group, however the authors attribute this to the EGFP tag effecting protein localization (179). Garcia-Moreno et. al, go on to describe how XRN1 is important for SINV to replicate, and GEMIN5 regulates capsid protein expression (178).

Even as new approaches are developed and the field has moved away from Protein:Protein interactions, the characterization of RNA:Protein interactions is relatively new for the field. As such, there has been some limited descriptions and attributions of functions of RBPs during alphaviral infections, of which the known and unknowns are extensively discussed in the next section.

Host Factor		Viral Component					
Protein Name	UnitProt ID	nsP1	nsP2	nsP3	nsP4	RepC	vRNA
AQR	O60306						X
BCLAF1	Q9NYF8						X
CHTOP	Q9Y3Y2						X
CSTF2	P33240						X
CSTF2T	Q9H0L4						X
DDX1	Q92499						X
DDX10	Q13206						X
DDX17	Q92841			X			
DDX18	Q9NVP1						X
DDX24	Q9GZR7						X
DDX31	Q9H8H2						X
DDX3X	O00571					X	
DDX5	P17844		X	X			
DDX50	Q9BQ39						X
DDX52	Q9Y2R4						X
DDX54	Q8TDD1						X
DDX56	Q9NY93						X
DHX9	Q08211					X	
DKC1	O60832						X
ELAV1	Q15717						X
EWSR1	Q01844						
FBL	P22087				X		X
FBLL1	A6NHQ2						X
FXR1	P51114			X			
G3BP1	Q13283		X	X	X	X	
G3BP2	Q9UN86		X	X	X	X	
GAR1	Q9NY12						X
GEMIN5	Q8TEQ6						X
HABP4	Q5JVS0						X
hnRNP A0	Q13151			X		X	
hnRNP A1	P09651			X		X	X
hnRNP A2B1	P22626			X		X	X
hnRNP A3	P51991		X	X			
hnRNP C	P07910		X			X	X
hnRNP G	P38159			X			
hnRNP H1	P31943					X	X
hnRNP K	P61978					X	X
hnRNP M	P52272					X	X
hnRNP R	O43390					X	
hnRNP U	Q00839		X	X		X	X
HNRNP AB	Q99729						X

Host Factor (Cont')		Viral Component (Cont')					
Protein Name	UnitProt ID	nsP1	nsP2	nsP3	nsP4	RepC	vRNA
HNRNP D	Q14103						X
HNRNP DL	O14979						X
HNRNP LL	Q8WVV9						X
HNRNP UL1	Q9BUJ2						X
IMPDH2	P12268						X
KHDRBS1	Q07666						X
KHSRP	Q92945						X
KRR1	Q13601						X
LARP4B	Q92615						X
LARP7	Q4G0J3						X
LRPPRC	P42704					X	
LSM5	Q9Y4Y9						X
LSM8	O95777						X
MATR3	P43243						X
MBNL1	Q9NR56						X
MYEF2	Q9P2K5						X
NCL	P19338			X			X
NONO	Q15233						X
NOP2	P46087						X
NOP9	Q86U38						X
NPM1	P06748			X			
NUDT5	Q9UKK9						X
NUFIP2	Q7Z417						X
PA2G4	Q9UQ80						X
PABPC1	P11940		X	X		X	X
PABPC4	Q13310						X
PABPN1	Q86U42						X
PATL1	Q86TB9						X
PCBP1	Q15365					X	
PCBP2	Q15366					X	
PPP1R10	Q96QC0						X
PRPF8	Q6P2Q9						X
PSPC1	Q8WXF1						X
HNRNP I	P26599					X	X
PUF60	Q9UHX1						X
PUM1	Q14671						X
PUM3	Q15397						X
RALY	Q9UKM9						X
RAVER1	Q8IY67						X
RBM10	P98175						X
RBM12B	Q8IXT5		X				

Host Factor (Cont')		Viral Component					
Protein Name	UnitProt ID	nsP1	nsP2	nsP3	nsP4	RepC	vRNA
RBM14	Q96PK6						X
RBM15	Q96T37						X
RBM19	Q9Y4C8						X
RBM28	Q9NW13						X
RBM3	P98179						X
RBM34	P42696						X
RBM39	Q14498						X
RBM8A	Q9Y5S9						X
RNPS1	Q15287						X
RPL12	P30050						X
RPL5	P46777		X	X			
RPL8	P62917						X
RPS3	P23396		X	X	X		X
RPS9	P46781		X	X	X		
RRP7A	Q9Y3A4						X
SARNP	P82979						X
STF1	Q13285						X
SF1	Q15637						X
SF3B4	Q15427						X
SFPQ	P23246			X		X	X
SFRS10	P62995				X		
SNRPB	P14678						X
SNRPD3	P62318						X
SNRPE	P62304						X
SNRPF	P62306						X
SRP54	P61011						X
SRSF2	Q01130						X
SRSF4	Q08170						X
SSB	P05455						X
STAU1	O95793						X
SYNCRIP	O60506					X	
TARS	P26639						X
TIA1	P31483					X	
TIAL1	Q01085						X
TRMT1L	Q7Z2T5						X
U2AF2	P26368						X
UPF1	Q92900						X
YBX1	P67809		X	X			
YTHDC2	Q9H6S0						X
YTHDF2	Q9Y5A9						X
ZC3HAV1	Q7Z2W4						X

Table 2.1 RBP Interactions with Alphavirus Table of mammalian RBPs and indicated interactions with alphaviral nonstructural proteins, replicase complex (RepC), or vRNA.

Specific Roles of RBPs During Alphavirus Infection

While discovery efforts to determine the host proteins involved in alphaviral infection are important (Table 2.1), it is also vital to determine the function of these host protein interactions to viral infection. Thus, the second goal of this chapter is to explain in depth the studies performed to characterize these interactions.

1. *La- A Possible Replication Regulator of 3' Minus Strand:*

   In the early 1990's, efforts from the Strauss lab identified which cellular proteins interacted with SINV RNAs, and found that several host factors bound to the 3' end of the Minus Strand RNA of SINV. As reported in later work by Pardigon et al., one of these host proteins were determined to be the cellular La protein. La was hypothesized to be important for SINV RNA replication as it normally binds to the 3' end of host transcripts and regulates transcription; however, unfortunately further studies could not be concluded due to high intracellular concentrations preventing complete silencing / knockdown and the La protein's essential role during embryogenesis. Thus, despite being the first recorded instance of a host RBP affecting viral RNA function, much is still unknown regarding the importance of La to infection (168, 171).

2. *HuR / ELAV1–- Stabilizing the Alphaviral RNAs from 3' RNA Decay:*

   The HuR / ELAV1 proteins, and its closely related homologs with tissue specific expression patterns, are RNA recognition motif (RRM) containing proteins that have been identified as potent regulators of RNA

stability and gene expression (180, 181). RNA stability conferred by HuR is imparted by direct interactions of the HuR protein with poly(U)-rich (URE) and AU-rich elements (AREs) found in the 3'UTRs of cellular transcripts (181-184).

Using a series of tissue culture model systems and biochemical approaches it was found that the host protein HuR not only binds to 3' UTR and AU-rich regions of cellular mRNAs and stabilizes them, but also binds to the 3' UTR of alphaviruses to stabilize them for successful infection (185). The HuR protein is conserved in both mosquitoes and humans, and both homologs have been found to associate with similar elements in the alphaviral 3'UTRs (186). This conservation implies that HuR binding could be important for infections across multiple host systems. During infection, HuR will bind to either the RSE, the URE and/or the CSE depending on the alphavirus and prevent deadenylation to keep alphaviral mRNAs from being degraded (187). While HuR is located in the cytoplasm in mosquito cells, it is located in the nucleus in mammalian cells and relocates to the cytoplasm after dephosphorylation during SINV infection (188). Relocalization of the HuR protein is not due to cellular stress, but is a response specifically found in alphaviral infections as infection with Measles or Dengue viruses did not cause relocalization (188). Knockdown of HuR or the deletion of the binding site in the SINV 3'UTR caused reduced viral titer in both mammalian and mosquito tissue culture models. While HuR binds to the URE region of

SINV, the CSE region is needed for binding of RRV and CHIKV as well as the third RSE for CHIKV.

3. *G3BP1/2- Regulation of Alphaviral Replicase and Minus Strand Synthesis:*

Ras GTPase-activating protein-binding protein 1 and 2 (G3BP1/2) are critical for the formation and activation of stress granules due to multiple different environmental stressors, including viral infection (189-191). These proteins form homo- and hetero-multimers with each other to induce stress granule formation (192).

G3BP1 and G3BP2 are homologous and have some redundancy in alphaviral infection so in most studies they are collectively referred to and studied as G3BP. Studies involving the role of the G3BP protein during alphaviral infection have been a prolific area of study, and as such the G3BPs have been found to interact with the nsP3 proteins of around 17 different alphaviruses, and also replicase proteins nsP2 and nsP4 (170, 172, 174). Studies performed by Cristea et al in the MacDonald lab found that the knockdown of G3BP caused viral nonstructural protein expression and titer to be significantly increased starting early in infection and kept the trend through late infection(174). However, knockdown only slightly effected RNA levels, but not enough to attribute the change in titer. The mosquito homologue of G3BP is Rasputin (Rin), and knockdown of Rin also did not significantly affect CHIKV RNA levels during infection (193). To determine at what point G3BP is affecting the viral lifecycle, Scholte et al determined

that G3BP was not linked to CHIKV entry or nonstructural protein translation but was involved the switch to negative strand synthesis (194). The McInerney lab comprehensively studied the effect of G3BP knockdown on several alphaviral nsP3s interactions and found that for most old-world alphaviruses replication and transcription were reduced (195). Further characterizing this interaction, the authors found a link between nonstructural polyprotein processing and G3BP dependence. Overall, they concluded that G3BP is proviral at several points through the alphaviral lifecycle, as it most likely is involved in activation of the replicase and negative strand synthesis, but not the switch from RNA translation to replication (195, 196). Despite G3BP's role in binding to the alphaviral replicase complex being a subject of extensive investigation, the role of G3BP specifically as an RBP is largely overlooked. While G3BP may be involved in RNA replication and minus strand synthesis, it is intimately linked to the replicase as a complex. At this point with current technology, it would be nearly impossible to distinguish the impact of G3BP on RNA binding without disrupting the viral replicase.

4. *TIA1/R- Promotes Infection by Forming Stress Granule Decoy:*

T-cell-restricted intracellular antigen 1 (TIA1) and TIA1-Related protein (TIAR) proteins regulate protein translation, RNA splicing, and stress granule formation (197-199). These proteins are included into initiation complexes, which then collect and sequester these initiation

components away in SGs in response to environmental or intracellular stress stimuli. During alphaviral infection, SGs are disassembled in the vicinity of viral replication complexes despite viral infection inducing a clear state of cellular stress (200). McInerney et al explored the importance of these proteins during SFV infection and found that TIA1/R diffused away from SGs throughout the cell when viral replication was active. In TIA1 knockdown MEF cell lines host cell translational shutoff was delayed, and early SG formation was decreased. This led the authors to conclude that TIA1/R forming SGs at the beginning of viral infection was fulfilling a pro-viral role, as it removes cellular mRNAs so the subgenomic vRNAs do not have to compete for translation with the active polysomes (200). Although TIA1/R have RNA binding abilities and are involved in RNA splicing, RNA binding roles on alphaviral RNAs have not been evaluated.

5. *GEMIN5- Regulation of Protein Expression Through 5' UTR Interaction:*

Gem-associated protein 5 (GEMIN5) is an RBP that catalyzes the formation of the spliceosome, can bind to the 7-methylguanosine cap of RNA strands, and may can control protein synthesis by interacting with the ribosome (201-203). Garcia-Moreno et al found that GEMIN5 was significantly stimulated by SINV infection and over-expressing a GEMIN5-EGFP fused protein caused a delay in viral subgenomic gene expression and inhibited capsid expression (178). While GEMIN5 binds to the 3' UTR of some host mRNAs, this RBP was found to interact with the 5' ends of

both of the SINV RNA species. GEMIN5 effects viral protein expression through this interaction at the 5' end, however GEMIN5's role as an RBP has not been characterized in depth.

6. *FXR1- Interaction with the nsP3 HVD Promotes Infection:*

The Fragile X-Related Protein 1 (FXR1) is involved in post-transcriptional mRNA regulation (204). After discovering FXR1 that interacts with SINV nsP3, the Frolova/Frolov group continued to functionally characterize this interaction (169). The Protein:Protein interaction of FXR1 mapped specifically to the VEEV nsP3 HVD, and FXR1 moves from the nucleus to the viral replicase complexes during infection (205). Knockout of the FXR1 family only reduced VEEV viral growth titers while unaffecting the Old-World alphaviruses SINV and CHIKV. Addition of these proteins reinstated viral titer almost back to WT like levels, which indicates that the FXR1 family is important for VEEV infection. Removal of the interaction site of FXR1 in the HVD region of nsP3 causes diminished pathogenesis of VEEV and EEEV (206, 207). Further elucidating this interaction, the authors found that FXR1 is important RNA synthesis and replicase complex formation. This interaction also holds true for EEEV, albeit the interaction is a separate distinct site. Direct impact of FXR1 as an RBP has not been well characterized in alphaviral infections.

7. *DEAD-box helicases- Promotion of Spreading Infection to Neighboring Cells:*

The family of dExD/H-box RNA helicases are involved in many RNA processing roles like splicing, transcription, RNA export, translation, stress granule formation, and innate immune sensing (208-210). As such, many of these helicases have been isolated during alphavirus interaction studies as shown in Table 2.1. In studies performed by Amaya et al, they found that DDX1 and DDX3 interacted with VEEV nsP3 (211). The removal of DDX3 and DDX1 by siRNA knockdown reduced viral titer, intracellular and extracellular viral RNA. It is interesting that the decrease of intracellular viral RNA seemed to be more significant at earlier time points in the infection whereas extracellular viral RNA had a more considerable decrease at 24hpi. This observation was not as evident in the double DDX1/DDX3 knockdown however the same trend of decreased viral titer and RNA levels late during infection was evident. From this data, the authors conclude that the role of these helicases are important during multiple rounds of infection, due to the low amount of virus inoculated and the decreasing trend becoming more significant at later times during infection (211).

RNA helicase DHX9 is known to unwind DNA and RNA structures and has been implicated during many other viral infections including picornaviruses, orthomyxoviruses, pestiviruses, flaviviruses, and retroviruses (212-215). Studies completed by Varjak et al and Matkovic et al both show that DHX9 interacts with the SFV and CHIKV replicase

complex, respectively (173, 216). Matkovic et al go on to show that DHX9 relocates to viral replicase complexes during infection, and knockdown of this helicase promotes infection while overexpression of DHX9 inhibits it (216). DHX9 also negatively regulates both positive and negative RNA strand synthesis. Since DHX9 can sense dsRNA, it was hypothesized that DHX9 is involved in innate immune sensing and establishment of an anti-viral state. However, IFNβ levels were not affected during knockdown of DHX9 by CRISPR/Cas9 editing. Interestingly, knockdown reduced genome translation while overexpression enhanced translation of the viral nsPs (216). Enhancement of translation but overall reduction in infection with the lack of DHX9 points to this protein having a role during alphaviral infection, however an RBP role has not been characterized.

Heterogenous Nuclear RibonucleoProteins (hnRNPs)

Many individual proteins make up this family of RBPs, and they are all heavily involved in RNA regulation. Individually and altogether, they have roles that include splicing, mRNA stability, translational and transcriptional regulation, and mRNA decay (217). The majority of the hnRNPs are located in the nucleus due to the presence of nuclear localization signals; however, they can shuttle to the cytoplasm for various reasons during their normal life as cellular RBPs. Some hnRNPs can also be post-translationally modified which in turn changes their binding preferences, functions or locations. There are many hnRNPs that are implicated not only in alphaviral infection

but many other viral infections as well. The remainder of this chapter will discuss the hnRNPs involved in alphaviral infection in detail.

hnRNP A1- Promotion of RNA Replication and Synthesis:

This protein is in the hnRNP A/B subfamily, and not only did hnRNP A1 show up in the majority of the alphaviral interaction discovery efforts, but it also has been shown to promote infection of viruses such as mouse hepatitis virus, hepatitis C virus (HCV), dengue virus, human papilloma virus 16 (HPV), and vesicular stomatitis virus (218-222). Studies performed by Lin et al have concluded that hnRNP A1 relocates from the nucleus to the cytoplasm during infection where it interacts specifically with the 5' UTR of the SINV genomic RNA (179). The knockdown of hnRNP A1 by siRNA reduced viral gene expression, vRNA synthesis, and viral titer to a significant extent. Continued work out of Mei-Ling Li's lab determined that hnRNP A1 interacts with both the genomic and subgenomic promoters of SINV positive strand (223). Gui et al went on to suggest that the removal of this protein from host cells reduces genomic and subgenomic RNA synthesis, and the addition of hnRNP A1 in knockout cells can rescue the vRNA synthesis.

hnRNP C- Negative Regulator of Alphaviral Infection:

Regulatory molecular switches like m^6A help hnRNP C bind to the mRNA and regulate splicing events and mRNA stability during normal host biology (224). Efforts undertaken by Varjak et al characterized the impact of hnRNP C on alphaviral infection (173). During hnRNP C knockdown, viral genomic and subgenomic protein expression was increased as compared to control cells infected with SFV, CHIKV, and SINV. In addition to hnRNP C's effects on translation, viral titer and both vRNA synthesis were increased as well. Altogether, it is interesting that removal of hnRNP C allowed for better alphaviral infection, when during normal biology it is involved in cellular mRNA stability. Thus, for alphaviruses at least, hnRNP C expression negatively influences viral infection (173). Along a similar vein, hnRNP C is also implicated in other viral infections, where it seems to have mixed uses, as like during alphaviral infection, the knockdown of hnRNP C increased adenoviral protein expression and viral titer; however during Dengue virus infection, removal of hnRNP C was detrimental to viral replication and protein expression (225, 226).

hnRNP K- Phosphorylated Form Regulates RNA Synthesis and Gene Translation:

The hnRNP K protein has three KH domains, which allows this protein to not only be involved with RNA regulation and processing, but also bind to DNA to regulate transcription as well (227). The hnRNP K protein

also interacts with many different host proteins and has a wide array of functions in the nucleus and cytoplasm. Since the finding that hnRNP K interacts with the replicase complex in SFV infection by Burnham et al, hnRNP K has been found to interact with alphaviruses in every discovery effort since (228). Differentially phosphorylated states of hnRNP K were both found to have been increased in the cytosolic fraction after SINV infection, though mostly the phosphorylated form interacts with the viral replicase machinery containing the subgenomic RNA strand. Silencing of hnRNP K also decreases the number of SINV infected cells at 6hpi, indicating an important role for hnRNP K in the early stages of viral infection. In studies performed by Bourai et al, the authors knocked down CHIKV nsP2 interactants, which included hnRNP K (175). In this study, knockdown caused decreased viral gene expression over time, and lower viral titer of CHIKV. Varjak et al also found that hnRNP K knockdown inhibited CHIKV and SINV infection by decreasing protein expression (173). Interestingly, Varjak et al reported that the knockdown of hnRNP K did not affect SFV viral titer and RNA synthesis over time and did not significantly affect SFV viral protein expression until 8hpi, which correlated with the localization of hnRNP K to areas of replication. Localization has not been characterized previous to 6hpi, so the precise timing as to when hnRNP K, and really other hnRNPs proteins, moves early in infection is unknown.

Unlike many of the other proteins reviewed here, the hnRNP K protein has been evaluated in regard to its capacity to bind to and influence

viral RNAs during infection. Specifically, as reported in LaPointe et al, the hnRNP K protein interacts with a distinct interaction site on the subgenomic vRNA (176). Disrupting this site with the incorporation of silent mutations through the primary binding site caused lower viral titer in mammalian cells and intriguingly increased structural protein expression. Both of these studies validate the previous findings that hnRNP K is beneficial for old world alphaviral infection. Overall, hnRNP K regulation most likely depends on the phosphorylation status of the protein, and which alphavirus it interacts with.

hnRNP M- Regulation of RNA Synthesis and Translation:

The hnRNP M protein has been indicated to be involved in the splicing of immune gene transcripts, and it dampens the innate immune response during some RNA viral infections (229, 230). During SFV infection, hnRNP M was identified as a component of the replicase complexes and was shown to colocalize with the viral replicase machinery(173). Silencing hnRNP M increased viral gene expression during SFV, CHIKV, and SINV infection, although the effect was minimal with the Old-World alphaviruses. Viral titer was also increased as compared to WT SFV infection. Nonetheless, hnRNP M knockdown only effected RNA synthesis slightly by increasing at 8hpi.

As with hnRNP K, the specific binding affinity of the hnRNP M protein have been directly evaluated. Indeed, reports from LaPointe et al. have

found that hnRNP M directly interacts with the vRNA, primarily with the subgenomic strand (176). As before with hnRNP K, disrupting the interaction site by mutating the vRNA caused a decrease in hnRNP M binding, inhibition of viral growth kinetics, and an increase in structural protein expression.

LaPointe et al. reported some opposing results from the previously discussed results from Varjak et al (176). After disruption of the interaction site, the loss of interaction caused a reduction in viral titer in both mammalian and mosquito tissue culture models, although no effect on vRNA synthesis was observed. Viral protein expression, specifically the structural proteins, was significantly increased. These opposing results could be due to that the LaPointe studies used an interaction disruption approach, whereas the Varjak approach of hnRNP M knockdown could have impacted cell biology beyond viral infection, resulting in off-target or indirect consequences on viral infection. Accordingly, we argue that disrupting the interaction while leaving host biology unperturbed allows for a more direct conclusions to be made regarding the importance of RBPs to viral infection.

PCBP1/hnRNP E1- Unknown Promotion of Alphaviral Infection:

Along with hnRNP K, hnRNP E1 and E2 are the only hnRNPs that have an RNA binding KH domain (231). The hnRNP E1 has been shown to be involved in translational control of viruses like poliovirus, HPV and HCV

(232-234). In 2013, Varjak et al found that PCBP1 does not colocalize with SFV nsP3 proteins, however it is still important for infection (173). During PCBP1 knockdown, viral gene expression was negatively impacted. Viral growth kinetics were also decreased compared to WT SFV infection. Silencing of hnRNP E1 caused a slight reduction in RNA synthesis early at 4hpi, however this effect seemed to disappear later in infection. Infections with CHIKV and SINV also continued the trend of lower luciferase expression with the lack of hnRNP E1 present. This data led the authors to conclude that hnRNP E1 supports alphaviral infection (173). However, there are no known experiments studying the effect of overexpression of this protein, and there is not data on the role of hnRNP E1 as an RBP.

PTBP1/hnRNP I- Interaction with vRNA Regulations Translation:

PTBP1 (or hnRNP I as it is mainly called in this thesis) plays a role in alternative splicing and can regulate RNA stability, replication, and translation (235). HnRNP I preferentially binds to polypyrimidine tracts by the four RNA binding domains and each domain has a high affinity for sequences containing 15 to 25 pyrimidines (236). The third and fourth binding domain of hnRNP I can even bind the same RNA and remodel the structure to form a loop (237). As well as alternatively splicing host mRNA, the majority of the functions of hnRNP I are enhancing RNA stability and translation by binding and preventing RNA degradation (238). Not only does hnRNP I promote host mRNA translation, but it has been shown to bind to

the picornavirus IRES site and stimulate translation (239, 240). As well as picornaviruses, hnRNP I is implicated in several viral infections including being upregulated during ZIKV infection, interacting with several coronaviruses, regulating hepatitis B virus, and interacting with the influenza viral protein NS1 (241-245).

Studies out of our lab have characterized hnRNP I interactions with alphaviruses as RBPs (176). Similar to the other characterized hnRNP interactions with SINV, the hnRNP I protein interacts with SINV subgenomic vRNA however its primary binding site is in the 3' UTR region. The deletion of this interaction from the vRNA causes diminished protein binding and decreased viral growth in mammalian and mosquito cells lines. Despite having lower viral titer and somewhat of a downward trend of vRNA synthesis late during infection, viral structural gene expression significantly increased (176). The characterization of the hnRNP I:vRNA interaction site, and the impact of hnRNP I on SINV infection is a major focus of this dissertation as described in detail in Chapter 3 (246).

Rationale

Alphaviruses cannot complete the lifecycle without RBP interactions, whether they interact with the viral proteins or RNA. Many studies have sought out to determine what RBPs are important to infection and characterize their role. In addition, previous studies have used knockdown methods to disrupt RBP function

which could harm cellular function to the point where it is not known if the effects on alphavirus infection are due to the true ablation of this interaction or disrupting the host.

Previously, we have found a distinct hnRNP I interaction site on the subgenomic vRNA via CLIP-seq, as seen in Figure 2.1, and mutating the vRNA at this site demonstrates that it is important to infection (176). Reduction of the hnRNP I:vRNA interaction caused increased viral titer and decreased structural protein expression. These observations led to question whether the hnRNP I interaction was dependent on the primary sequence or the structure of the vRNA, and how increased structural protein expression cause an overall decrease in viral growth. Focusing on the hnRNP I interaction site for reasons described below, we utilized a protein tethering method to characterize the hnRNP I interaction site which allowed us to define the role of hnRNP I during infection without disturbing host biology and effecting alphaviral infection by perturbing the host. Defining the role of hnRNP I during infection is important as illustrating the molecular interactions of alphaviruses provides more information about how the virus can cause severe disease and can possibly lead us to innovative therapeutics and vaccine candidates.

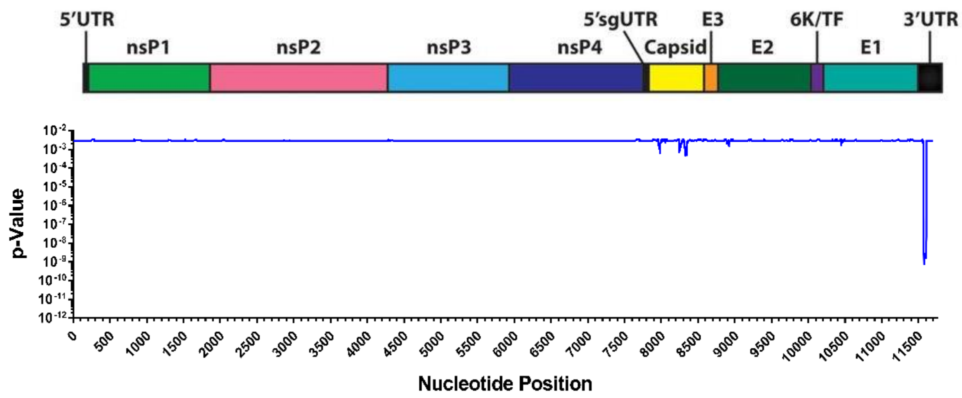


Figure 2.1 Identification of the hnRNP:vRNA Binding Sites via CLIP-seq A map of the SINV genomic RNA with relative site of the hnRNP I interaction indicated in blue. The *y*-axis represents the statistical significance of the fold enrichment at the nucleotide position, represented by the *x*-axis. Adapted from LaPointe et al. 2018.

To Bind With Specificity Without a Specific Binding Site- Developing the Model System

While disrupting the vRNA hnRNP interaction site gives the ability to determine the role of the specific hnRNP during infection without disrupting the host, it still does not answer the question of exactly how the hnRNPs are affecting infection. We implemented a protein tethering approach to determine if the impact of hnRNP I bound to the vRNA was enough to abrogate the phenotypes discovered, or if there was more to the sequence or structure of the vRNA. While the interaction sites for hnRNP K and hnRNP M are in the subgenomic ORF, fortunately the hnRNP I interaction site is in the 3' UTR, and in a region that is not imperative to infection. Thus, to determine the role of hnRNP interactions during alphaviral infection by protein tethering, the hnRNP I site was chosen for its sequence malleability. The hnRNP I interaction site is between the second and third RSE, and this site could easily be replaced without interrupting too much of the original viral sequence.

CHAPTER 3

BINDING OF HNRNP I–VRNA REGULATES SINDBIS VIRUS STRUCTURAL PROTEIN EXPRESSION TO PROMOTE PARTICLE INFECTIVITY [1]

Summary

Alphaviruses cause significant outbreaks of febrile illness and debilitating multi-joint arthritis for prolonged periods after initial infection. We have previously reported that several host hnRNP proteins bind to the subgenomic Sindbis virus (SINV) RNAs and disrupting the sites of these RNA–protein interactions results in decreased viral titers in tissue culture models of infection. Intriguingly, the primary molecular defect associated with the disruption of the hnRNP interactions is enhanced viral structural protein expression; however, the precise underlying mechanisms spurring the enhanced gene expression remain unknown. Moreover, our previous efforts were unable to functionally dissect whether the observed phenotypes were due to the loss of hnRNP binding or the incorporation of polymorphisms into the primary nucleotide sequence of SINV. To determine if the loss of hnRNP binding was the primary cause of attenuation or if the disruption of the RNA sequence itself was responsible for the observed phenotypes, we utilized

[1] This chapter is adapted from work previously published in Viruses, 2022, Volume 14, no. 7 with the same title under a Creative Commons Attribution (CC BY). Westcott, C.E. et al., *Binding of hnRNP I–vRNA Regulates Sindbis Virus Structural Protein Expression to Promote Particle Infectivity.* Viruses, 2022. 14(7).

an innovative protein tethering approach to restore the binding of the hnRNP proteins in the absence of the native interaction site. Specifically, we reconstituted the hnRNP I interaction by incorporating the 20nt bovine immunodeficiency virus transactivation RNA response (BIV-TAR) at the site of the native hnRNP I interaction sequence, which will bind with high specificity to proteins tagged with a TAT peptide. The reestablishment of the hnRNP I–vRNA interaction via the BIV-TAR/TAT tethering approach restored the phenotype back to wild-type levels. This included an apparent decrease in structural protein expression back to the baseline observed during wild type infection in the absence of the native primary nucleotide sequences corresponding to the hnRNP I interaction site. Collectively, the characterization of the hnRNP I interaction site elucidated the role of hnRNPs during viral infection.

Introduction

Alphaviruses are positive-sense, single-stranded RNA viruses that have, and will likely continue to, cause significant outbreaks of clinically severe disease (5, 10, 247). A primary reason for the sustained emergence of mosquito-borne viruses may largely be due to the wide geographical distribution of competent mosquito vectors aggravated by climatological change and global trade, which have led to the dissemination of vector mosquitos (4, 248, 249). Based on clinical presentation, there are two subgroups of the alphaviruses, namely the encephalitic and arthritogenic subgroups. Sindbis virus (SINV), Chikungunya virus (CHIKV), Ross River virus (RRV), Semliki Forest virus (SFV), and Mayaro virus (MAYV) are

all considered to be members of the arthritogenic group, and infection can result in moderate to severe febrile illness often followed by long-term multi-joint arthritis, which may persist for several years past the resolution of acute infection (11, 12, 250). While the arthritogenic alphaviruses are not typically as deadly as those of the encephalitic subgroup, the arthritogenic alphaviruses still cause significant burdens to community health systems and reduced quality of life to infected individuals (7, 8). As stated earlier, due to the widespread distribution of vector-competent mosquitos, the majority of the world's population is at risk for at least one alphaviral infection. Despite there being significant clinical disease, there are no FDA-approved treatments or safe, effective vaccines to limit the public health burden of the alphaviruses.

The identification and study of host protein interactions with viral RNAs or viral proteins is not a novel concept, and many studies have identified host factors with known RNA-binding properties (52, 102, 169, 170, 172-175, 177, 179, 196, 223, 228, 251-254). While these prior efforts have established the importance of these factors to alphaviral biology, many have overlooked the potential impact of the host RNA-binding proteins engaging with the viral RNAs (vRNAs) on viral biology and have instead utilized RNAi or gene knockout studies to evaluate the importance of specific host factors to infection. In addition to not directly defining the importance of the protein–vRNA interaction, this approach has the disadvantage of potentially disrupting the host system if the target protein is deeply involved in the regulation of host RNA biology (255-258). Thus, it remains possible

that the knockdown or knockout of host factors essential to cellular homeostasis may cause artefacts to viral replication.

Previously, we published a study that determined that there are several host hnRNP proteins that directly bind to the SINV subgenomic vRNAs in a site-specific manner (176). It was found that disrupting the hnRNP binding sites in the alphaviral RNAs led to decreased growth kinetics, and surprisingly this phenotype correlated largely with increased structural protein expression. Nonetheless, whether the phenotypes observed following the mutation of the hnRNP interaction sites was specifically due to the loss of hnRNP–vRNA binding or due to the mutations in the primary nucleotide sequences or RNA secondary structures remained unknown. The primary goal of this study was to determine whether the observed phenotypes were genuinely ascribable to the loss of hnRNP protein binding through the reconstitution of the protein–RNA interaction in the absence of the native interaction site. To this end, we employed a modified protein tethering approach to develop a mutant SINV, where a native hnRNP interaction site was replaced with the bovine immunodeficiency transactivating response RNA element (TAR) (259-261). As the inclusion of the BIV-TAR element would alter the primary amino acid sequence of the target, we prioritized the hnRNP I interaction for evaluation, as the hnRNP I interaction site is located in the SINV 3′UTR (176). Importantly, the inclusion of the BIV-TAR element enabled the direct assessment of the importance of the hnRNP–vRNA binding to SINV infection. Altogether, our data indicate that the loss of hnRNP I protein binding to the vRNA is directly responsible for the phenotype observed following the mutation of the native interaction site.

Furthermore, the data from these efforts further define the biological and molecular importance of the hnRNP proteins to alphaviral infection.

Materials and Methods

Tissue Culture Cells

BHK-21 (ATCC CCL-10) and HEK293 (ATCC CRL-1573) tissue culture cells were cultured in minimal essential medium (MEM; Cellgro Mediatech, Inc, Manassas, VA, USA) supplemented with 10% fetal bovine serum (FBS; Corning, Corning, NY, USA), 1× penicillin–streptomycin (Pen/Strep; Corning, Corning, NY, USA), 1× nonessential amino acids (NEAA; Corning, Corning, NY, USA), and l-glutamine (Corning, Corning, NY, USA). HEK293T cells were cultured in Dulbecco's modified Eagle's medium (Corning, Corning, NY, USA) supplemented with 10% fetal bovine serum, 1× penicillin–streptomycin, 1× nonessential amino acids, and 5 mM L-glutamine. All cells were maintained at 37 °C in a humidified incubator at 5% CO2.

Where specifically noted, tissue culture dishes receiving HEK293 cells were pre-treated with poly-l Lysine (Advanced Biomatrix, Carlsbad, CA, USA) to aid cell adherence and prevent premature detachment during handling. Briefly, tissue culture dishes were pre-treated with 0.1 mg/mL poly-l Lysine for 30 min at 4 °C. After poly-l lysine treatment, the stock solution was removed and the wells were briefly rinsed twice with 1× PBS and allowed to dry under sterile conditions prior to seeding the dishes with HEK293 cells for use the next day.

Sindbis Virus Mutant Construction and Preparation

To generate a SINV mutant with the native hnRNP I interaction site replaced with the 21nt BIV-TAR element, we utilized a two-step mutational approach. First, using site-directed mutagenesis, the primary nucleotide sequence of the native hnRNP I interaction site of SINV.TE12-nanoluciferase, consisting of nucleotides 11,557 to 11,586, was replaced with a NotI restriction digestion site to generate the hnRNP I interaction-deficient SINV.hnRNP I^{Δ} (262). After sequencing to confirm the veracity of the clone, a restriction enzyme/DNA ligase strategy was utilized to insert the BIV-TAR element into the NotI (New England Biolabs, Ipswich, MA, USA) site of the hnRNP I interaction-deficient subclone. The specific sequence of the BIV-TAR element, including the NotI restriction enzyme sequences and flanking sequences, was 5′-gcggccgcaacactGGCTCGTGTAGCTCATTAGCTCCGAGCCtatcctgcggccgc-3′, with the BIV-TAR-specific sequences capitalized for reference. The resulting virus, SINV.hnRNP I^{TAR}, was sequenced to confirm the presence of the BIV-TAR element and to verify that the orientation of the element was correct.

All viruses utilized in this study were generated via the electroporation of in-vitro-transcribed RNAs derived from cDNA infectious clones, as previously described (263). Briefly, approximately 10 ug of in-vitro-transcribed RNA was electroporated into BHK-21 cells by a single pulse from a Gene Pulser Xcell electroporation system set to deliver a single square-wave discharge of 125 V for a period of 12.50 ms. After the development of significant cytopathic effects, the

tissue culture supernatants were harvested and clarified of cell debris via centrifugation prior to aliquoting and storage at −80 °C for later use.

Control and hnRNP I^{TAT} Transfection of HEK293 Cells

To reconstitute the hnRNP I interaction via the BIV-TAR/TAT system and test the importance of the hnRNP I interaction to SINV infection, HEK293 cells were transfected with an expression plasmid encoding the full-length hnRNP I protein with a c-terminal TAT peptide tag (pEXPR.hnRNPI-TAT) using Lipofectamine 3000 (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. Transfections were conducted in a 12-well format at 80% confluence. Transfection efficiency was confirmed via initial co-transfections of EGFP reporter plasmids (with efficiency estimated at >90%), and hnRNP I-TAT expression was confirmed by western blot. Each well was transfected with DNA–lipid complexes generated by mixing 0.5 ug DNA supplemented with 2 µL of P3000 reagent and 1.5 µL of Lipofectamine 3000 reagent in separate volumes of 50 µL of Optimem (Thermo Fisher Scientific, Waltham, MA, USA). Control transfections lacking the hnRNP I expression clone were conducted in parallel. The cells were transfected in a minimal volume of 1 ml of whole growth medium and allowed to incubate overnight prior to replacing the media with fresh growth medium before continuing with further experimentation.

Quantitative Immunoprecipitation of hnRNP I–vRNA Complexes

Transfected HEK293 cells were infected with either wild-type SINV or SINV.hnRNP I^{TAR} at a multiplicity of infection (MOI) of 5 plaque-forming units (PFU) per cell in a 12-well format. At 16 h post-infection (hpi), the tissue culture monolayers were harvested via gentle scraping and centrifugation at 300× g for five minutes. The media were aspirated and the cells were washed with 1× phosphate-buffered saline (PBS; Corning, Corning, NY USA) to remove contaminating media. The washed cell pellets were gently resuspended in 1×PBS supplemented with 1.0% formaldehyde and incubated under gentle agitation for 7 min. The cross-linked cell pellets were then recollected via centrifugation at 1000× g for 3 min, and the supernatant was promptly removed and replaced with 1×PBS supplemented with 0.25 M glycine to quench any excess formaldehyde. After a 5 min incubation, the cells were again collected via centrifugation as above and resuspended in 400 µL of RIPA buffer (50 mM Tris pH 7.6, 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) and lysed via brief sonication, as previously described (176, 264).

The resulting lysates were clarified via high-speed centrifugation (5 min at 16,000× g) to remove insoluble debris, and subsequently immunoprecipitated with 10 µL of either anti-hnRNP I (anti-PTBP1; rabbit polyclonal; PA5-95949; Thermo Fisher Scientific, Waltham, MA, USA) or anti-NALP1 (rabbit polyclonal; PA5-20005; Thermo Fisher Scientific, Waltham, MA, USA) as a nonspecific control. Antibody complexes were then precipitated from the lysate via the addition of paramagnetic protein G agarose beads. The beads were washed a minimum of

five times prior to the elution of the immunoprecipitated materials via incubation at 70 °C for 30 min. The total RNA was extracted from the eluate using TRIzol reagent (Promega, Madison, WI, USA), as indicated by the manufacturer's instructions.

The purified RNAs were used as the inputs for the synthesis of cDNA for analysis by qRT-PCR, as previously described. The relative quantitative immunoprecipitation was determined by comparing the amount of viral RNAs detected across the indicated experimental conditions, after normalization to the sample specific inputs and nonspecific control immunoprecipitations as determined by qRT-PCR.

Analysis of Viral Growth Kinetics

The viral replication kinetics were assayed using one-step growth kinetics assays in HEK293 cells bound to poly-l lysine plates. After transfection the cell, monolayers were infected with either wild-type SINV or SINV.hnRNP I^{TAR} at an MOI of 10 PFU per cell. After a one-hour adsorption period, the cells were carefully washed twice with 1×PBS prior to the addition of whole medium supplemented with 25 mM HEPES to enable the use of an automated liquid handling system lacking a CO2 atmosphere. At the indicated times post-infection, the cell supernatant was collected and stored at 4 °C, and fresh replacement media was added. Viral titers were determined via plaque assay using BHK-21 cells overlaid with a 2% Avicel (FMC, Philadelphia, PA, USA) suspension (in whole media). After

a 30 h incubation period, the samples were fixed with 3.7% formaldehyde (in 1×PBS) and visualized by crystal violet staining.

Quantitative Analysis of SINV Structural Protein Expression

The assessment of structural protein expression was performed as previously described, with several specific modifications (176). Briefly, HEK293 cells were cultured on poly-l-lysine-treated plates and transfected as described above. The tissue culture monolayers were then infected with either wild-type SINV or SINV.hnRNP I^{TAR} at a MOI of 10 PFU per cell. After removal of the unbound virus particles, fresh tissue culture medium was added and the cells were incubated under normal conditions. At the indicated times post-infection, the supernatant was removed and discarded and the cell monolayers were washed with 1×PBS. Whole-cell lysates were then harvested by scraping in 1×PBS supplemented with 0.15% Triton X-100 (Avantor; Radnor Township, PA, USA). The lysates were collected in microcentrifuge tubes and frozen at −80 °C. After the completion of the time course, the cell lysates were thawed, vortexed, and clarified via centrifugation at 17,000× g for 3 min to remove insoluble materials. Equivalent amounts of cell lysate, as confirmed by Bradford assay (Avantor; Radnor Township, PA, USA), were then assessed using the Nano-Glo nanoluciferase assay system (Promega, Madison, WI, USA) according to the manufacturer's instructions. The nanoluciferase activity was detected in a BioTek Synergy H1 microplate reader.

Quantification of Viral RNA Synthesis or Accumulation and Particle Numbers

The lysates generated for the quantitative assessment of structural protein expression, as described above, were treated with TRIzol reagent and extracted using a Direct-zol-96 MagBead RNA kit (Zymol Research; R2102; Irvine, CA, USA) via a Kingfisher Duo Prime automated nucleic acid extractor system. The quantitative detection of the individual RNA species was accomplished using strand-specific reverse transcription and standard curve qRT-PCR, as previously described (176). The RNA levels were normalized to the 18S rRNA levels.

The particle numbers, as determined by genome equivalents per ml, were quantitatively assessed similarly to those described previously, and as generally described above, with two major differences. First, the input materials consisted of tissue culture supernatants that had been boiled prior to the synthesis of genome-specific cDNAs. Second, the samples were not normalized to an endogenous control transcript and were instead normalized through the use of equal volumes.

Purification of SINV Particles, Morphological Assessments via Transmission Electron Microscopy, and SDS-PAGE

The concentration and purification of SINV particles were adapted from the low-speed, low-temperature centrifugation protocol (265). Briefly, HEK293 cells were cultured in 100 mm dishes infected (2 dishes per virus, per prep) to 95% confluence. The monolayers were then infected with either wild-type or

SINV.hnRNP I^{TAR} at a MOI of 5 PFU units per cell. After the adsorption period, the inoculum was removed and replaced with Virus Production Serum-Free Media (VP-SFM; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 1× penicillin–streptomycin, 1× nonessential amino acids, and 5 mM L-glutamine. After a 20 h incubation period, the supernatants were harvested and clarified via centrifuge to remove cell debris. The clarified supernatants were then transferred to Oakridge tubes and the virus particles were gently pelleted via centrifugation at 5300× g for 18 h at 4 °C. After centrifugation, the tubes were promptly removed, the supernatant was carefully decanted, and the residual moisture was gently blotted with a Kimwipe wrapped around a pipette tip. The pellets were resuspended in HEPES-NaCl-EDTA resuspension buffer (HNE; pH = 7.5; 20 nM HEPES, 150 mM NaCl, 0.1 mM EDTA).

For the transmission electron microscopy (TEM) analysis, the SINV particles were applies to Formvar- and carbon-coated 400-mesh copper grids and stained with 1% uranyl acetate. The prepared grids were imaged using a JEOL 1010 transmission electron microscope operating at 80 kV. The images were recorded via a Gatan Ultrascan 4000 CCD camera. The image processing and the measurement of the particle diameter were performed in ImageJ.

The compositional assessment of SINV particles was accomplished via standard SDS-PAGE with nonspecific Coomassie staining. Equal particle numbers of either wild-type or SINV.hnRNP I^{TAR} were boiled in 2× Laemmli buffer prior to the resolution of proteins by molecular weight via SDS-PAGE on 10% pre-cast gels (CriterionTM TGXTM; Bio-Rad Laboratories, Hercules, CA, USA). After

electrophoresis, the gels were stained using Coomassie blue and visualized using a flatbed scanner.

Quantitative Assessment of Viral Attachment

The HEK293 cells from untreated plates were scraped, aspirated, and transferred into sterile microfuge tubes. After ensuring that the cells were evenly resuspended, the cell aliquot was evenly divided and inoculated with either wild-type or SINV.hnRNP I^{TAR} at a MOI of 0.1 PFU per cell and incubated with gentle mixing at 4 °C to allow binding but not entry of the viral particles. After the incubation period, one aliquot was immediately treated with TRIzol to generate an input sample. The cells in the second aliquot were gently pelleted via centrifugation at 300× g for 5 min at 4 °C, and extensively washed three times with excess volumes of 1×PBS. Prior to being treated with TRIzol reagent, the cell pellets were resuspended in an equivalent volume (relative to the input control) of whole media. The total RNA from input and bound samples was extracted as described above, and the number of viral particles bound to the host cells was determined via qRT-PCR, as described above.

To determine the relative efficiency with which each viral particle population bound to the host cell, the percent binding was calculated for each specific pair by comparing the input and bound samples. A comparative analysis of binding was performed by normalizing the percent bound to that detected for wild-type particles.

For simplicity, comparisons were restricted to host cell derivation (as per mock or hnRNP I^{TAT}-transfected cells).

Deglycosylation of Viral Particles

Viral particles were deglycosylated via treatment with PNGase F (Recombinant; New England Biolabs, Ipswich, MA, USA) under nondenaturing reaction conditions. Briefly, equal amounts of viral particles were diluted into PNGase nondenaturing reaction buffer, which was pre-prepared as close to 1× as possible to prevent the destruction of the viral particles due to osmotic pressure. The mixtures were then split into two parallel reactions, and 1% of total reaction volume of PNGase F was added to one reaction. Both samples were then incubated for a minimum of 18 h at room temperature prior to the determination of the viral titer via serial dilution assays.

Statistical Analyses

All quantitative data shown are from a minimum of three independent biological replicates, unless more replicates are specifically indicated. Data shown represent the quantitative mean, with the error bars representing the standard deviation of the means. Where appropriate, a statistical analysis of the ratios was performed using variable bootstrapping, as described previously (266). Pairwise statistical analyses were conducted using unpaired Student's t-tests, with a minimum threshold p-value of < 0.05 being considered statistically significant. A

statistical analysis of the viral growth kinetics was accomplished using an area under the curve (AUC) analysis.

Results

Developing a Protein Tethering System to Study the Impact of hnRNP I Binding to SINV RNAs

On the basis of our prior data, we concluded that the disruption of hnRNP–vRNA interaction sites, and ergo the loss of hnRNP–vRNA binding, resulted in decreased viral growth kinetics, potentially as the result of increased structural protein expression during SINV infection. However, from these data, conclusions could not be made as to whether this phenotype was due to the direct loss of hnRNP binding to the viral RNA, or due to some other consequence of mutating the primary nucleotide sequences of the interaction sites themselves. As such, we sought to develop a system by which the protein–RNA interaction of the hnRNP proteins could be functionally and phenotypically restored in the absence of the native interaction site to address whether hnRNP–vRNA binding or a cryptic feature of the nucleotide primary sequence or structure was primarily responsible for the observed defects in growth kinetics following the disruption of the hnRNP–vRNA interaction sites.

In our previous study, we identified the hnRNP–vRNA interaction sites between hnRNP K, hnRNP I, and hnRNP M and the SINV viral RNAs using next-generation sequencing approaches (176). The interaction sites for the hnRNP K

and hnRNP M proteins were found within the structural ORF coding region of the viral subgenomic RNA, whereas the interaction site for hnRNP I was determined to be in the viral 3′UTR. Due to the constraints associated with manipulating the coding regions of the viral RNAs, we elected to continue these studies by focusing on the hnRNP I interaction site because of its location in the 3′UTR, as this region of the genome has a greater degree of sequence plasticity.

Our previous approach to eliminate the hnRNP I interaction relied on the deletion of the entire interaction site as identified by way of CLIP-Seq. Specifically, in the original hnRNP I interaction mutant, nucleotides 11,545 to 11,608 were deleted from the SINV 3′UTR. While the majority of this nucleotide range exists between the repeat sequence elements (RSEs) 2 and 3, the tail end of the original hnRNP I interaction deletion mutant included approximately 12 nt of RSE3. Thus, as detailed above, the phenotype observed with the original hnRNP I interaction site mutant could be due to either the loss of hnRNP I binding, the disruption of sequences or structures important to the alphaviral biology, or a combination of the two possibilities. As the primary goal of this study was to functionally dissect the importance of hnRNP I binding from the viral RNA sequence, we developed a new set of mutants to determine the specific impacts of the hnRNP I–vRNA interaction. These mutants utilized a more focused definition of the hnRNP I interaction site, as depicted in Figure 3.1A, to avoid altering the sequence and putative structures of the RSEs.

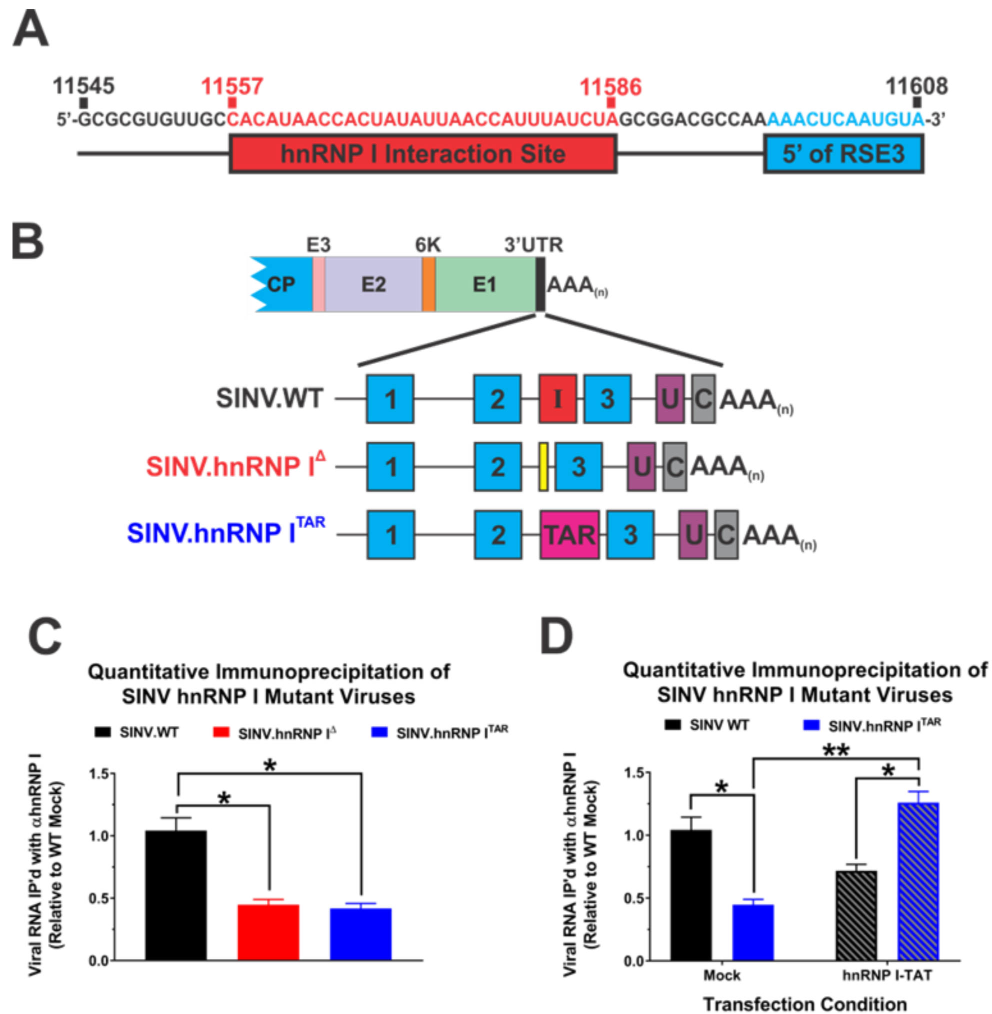
To determine the specific impact of hnRNP I binding on SINV infection, we employed a modified protein tethering approach that binds the hnRNP protein to

the vRNA in a targeted manner in the absence of the native interaction site or sequence. As diagrammed in Figure 3.1B, the native SINV hnRNP I interaction site was replaced with the 20 nucleotide bovine immunodeficiency virus transactivation response element (BIV-TAR) sequence to create SINV.hnRNP I^{TAR}. Importantly, in addition to ablating the native hnRNP I interaction site, the TAR element enables the site-specific tethering of proteins tagged with a bovine immunodeficiency transactivator (TAT) peptide motif by creating an RNA aptamer / structure that the TAT peptide recognizes and binds to with high affinity (259). Thus, by expressing an hnRNP I protein tagged with the TAT peptide motif (hnRNP I^{TAT}) we may reconstitute the hnRNP–vRNA interaction, enabling direct comparisons of infections with the native hnRNP I interaction, no hnRNP I interaction, and a forced hnRNP I interaction to determine the explicit importance of hnRNP–vRNA binding.

First, to confirm that the BIV-TAR/TAT system reestablished the interaction between the viral RNA and hnRNP I, we quantitatively assessed the interaction via immunoprecipitation. To this end, cells were either mock-transfected or transfected with an expression plasmid encoding the hnRNP I^{TAT} fusion protein, and then infected with either wild-type SINV, SINV.hnRNP I^{Δ}, or SINV.hnRNP I^{TAR}. At 16 h post-infection, the cells were crosslinked with formaldehyde and whole-cell lysates were generated via the addition of detergent and gentle sonication (267). RNA–protein complexes were immunoprecipitated via an hnRNP I-specific antibody, and the amount of viral RNA that co-immunoprecipitated with hnRNP I was determined via qRT-PCR. To ensure the specificity, the quantitative detection of the vRNAs

was normalized to parallel control immunoprecipitations using a nonspecific antibody. As shown in Figure 3.1C, the deletion of the previously identified hnRNP I interaction site (as per SINV.hnRNP I^{Δ} and SINV.hnRNP I^{TAR}) negatively impacted the immunoprecipitation of SINV vRNA with anti-hnRNP I antibody by approximately 2-fold in comparison with the wild-type SINV (SINV.WT). In contrast, quantitative immunoprecipitations of hnRNP I protein–RNA complexes in lysates generated from HEK293 cells that were transiently transfected with an expression plasmid encoding the hnRNP I^{TAT} fusion protein indicated that the BIV TAR/TAT system was capable of reconstituting the hnRNP–vRNA interaction in the absence of the native interaction site. Specifically, as shown in Figure 3.1D, the co-immunoprecipitation of the SINV vRNA with hnRNP I antibody was significantly increased for SINV.hnRNP I^{TAR} in the presence of hnRNP I^{TAT} relative to SINV.WT and SINV.hnRNP I^{TAR} in the absence of hnRNP I^{TAT}. Interestingly, the co-immunoprecipitation of SINV.WT vRNAs was modestly decreased in the presence of hnRNP I^{TAT}. The precise underlying the reasons behind this phenomenon are unclear, but the potential causes of this decrease are speculated on in the discussion section.

Altogether, these data confirm that the BIV-TAR/TAT system is a means by which the interaction between the SINV vRNAs and the hnRNP I protein may be restored in the absence of the native interaction site. Nonetheless, while confirming that we may functionally dissect the binding from the vRNA primary sequence, the specific consequences of restoring the hnRNP I–vRNA interaction on the viral biology remain unaddressed.



<u>Figure 3.1. Protein tethering restores hnRNP I protein binding in the absence of the native interaction site.</u> (A) A nucleotide map of the hnRNP I interaction site in the SINV TE12 3′UTR as defined by prior CLIP-Seq efforts. The specific sequences targeted for deletion in this study are highlighted in red, and sequences belonging to RSE3 (which were included in the original deletion mutant) are highlighted in cyan. (B) A schematic diagram of the viruses used in these studies, including wild-type SINV (SINV.WT) and the hnRNP I interaction-deficient mutants SINV.hnRNP I^{Δ} and SINV.hnRNP I^{TAR}, which incorporated a bovine immunodeficiency virus transactivation response element (BIV-TAR) in lieu of the native interaction site. The SINV repeat sequence elements (RSEs) are denoted by cyan boxes with their relative number labeled inside, similarly the hnRNP I interaction site, the SINV U-rich element and 19-nt 3′ conserved sequence element

are indicated with red, purple, and gray boxes labeled with an I, U, or C, respectively. Elements are drawn to scale. (C) Immunoprecipitation of vRNA–hnRNP I complexes derived from mock-transfected HEK293 cells infected with the indicated viruses. (D) Immunoprecipitation of vRNA–hnRNP I complexes derived from hnRNP I^{TAT}-transfected HEK293 cells infected with the indicated viruses. Quantitative detection of vRNA relative to the SINV.WT level was accomplished using qRT-PCR. Quantitative data shown are the means of three independent infections or co-immunoprecipitations, with the error bars representing the standard deviation of the means. Statistical significance, as determined by Student's t-test, is indicated above the specific comparisons (with * ≤ 0.05; ** ≤ 0.01).

Reconstitution of hnRNP I Binding Restores Growth Kinetics in Tissue Culture Models of Infection

As the data above confirmed that the hnRNP I protein–RNA interaction could be reconstituted in the absence of the native interaction site via the BIV-TAR/TAT system, we next sought to examine whether hnRNP–vRNA binding impacted the viral growth kinetics. Briefly, HEK293 cells were either mock-transfected or transfected with an expression plasmid encoding hnRNP I^{TAT}, and then subsequently infected with either SINV.WT or SINV.hnRNP I^{TAR} at a multiplicity of infection of 10 PFU/cell. Over a period of 24 h, the supernatants were collected every six hours and the viral titer was quantitatively determined using plaque assays. As shown in Figure 3.2A, the hnRNP I-binding-deficient mutant SINV.hnRNP I^{TAR} exhibited a statistically significant ~3.5-fold decrease in viral titer relative to the wild-type SINV. In contrast, when the hnRNP I interaction was restored through the BIV-TAR/TAT system in hnRNP I^{TAT}-transfected cells, the viral growth kinetics observed for SINV.WT and SINV.hnRNP I^{TAR} were comparable (Figure 3.2B).

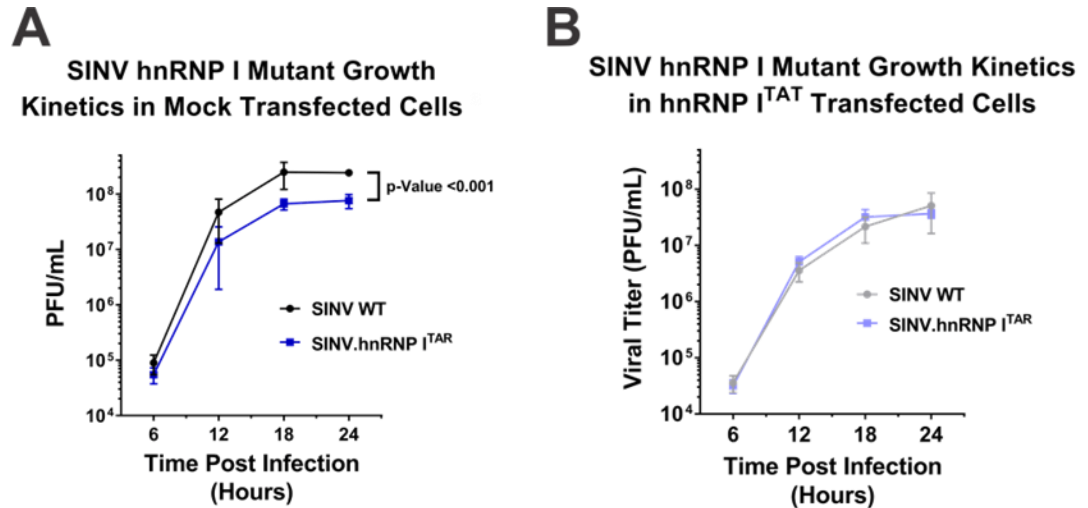


Figure 3.2. Restoration of hnRNP I binding results in wild-type-like growth kinetics. The capacity of the hnRNP–vRNA interaction site mutant viruses to replicate in HEK293 cells was assessed using one-step growth curves in (A) mock-transfected or (B) hnRNP I^{TAT}-transfected cells. The titer was quantified using standard plaque assays. Quantitative data shown are the means of at least three minimum biological replicates, with the error bars representing the standard deviation of the means. Statistical significance, as determined by the area under the curve analysis, is shown above.

Overexpression of hnRNP I Negatively Impacts Overall Growth Kinetics

Interestingly, despite using parallel conditions for both the control transfection and hnRNP I^{TAT}-transfected cells, the overall titers were lower for both viruses in the hnRNP I^{TAT}-expressing cells. The precise underlying cause of this phenomenon is unclear; however, the overexpression of hnRNP I appears to negatively impact cellular homeostasis, as observed via the cell division and morphology.

An unfortunate consequence of the apparent toxicity of hnRNP I^{TAT} overexpression is that critical assessments of the one-step growth kinetics data presented in Figure 3.2 do not enable the direct conclusion that reconstituting the hnRNP I interaction restores the wild-type-like growth kinetics. Indeed, an alternative conclusion could be that hnRNP I^{TAT} overexpression negatively impacted wild-type replication, while the replication of SINV.hnRNP I^{TAR} was unperturbed. To directly test whether SINV.hnRNP I^{TAR} improved to wild-type levels or wild-type deteriorated to meet SINV.hnRNP I^{TAR} levels, we assessed the impact of hnRNP I^{TAT} expression on the parental hnRNP I interaction site mutant SINV.hnRNP I^{Δ}. As shown in Figure 3.3A, hnRNP I^{TAT} expression uniformly negatively impacted viral replication for all SINV mutants utilized in this study. Importantly, while growth differences were readily observed between wild-type SINV and both hnRNP I interaction-deficient viruses under mock-transfected conditions (Figure 3.3B), in the presence of hnRNP I^{TAT}, both wild-type SINV and SINV.hnRNP I^{TAR} replicated to similar extents, while SINV.hnRNP I^{Δ} remained phenotypically distinct and lower than wild-type SINV (Figure 3.3C). Thus, from

these data, we are able to conclude that reconstituting the hnRNP I–vRNA interaction genuinely restored SINV.hnRNP I^{TAR} replication to wild-type levels.

From these data and the previous section, we are able to conclude that replacing the native hnRNP I interaction site with the BIV-TAR element negatively impacts the viral growth kinetics in highly permissive tissue culture models of infection. More importantly, the reconstitution of the hnRNP–vRNA interaction via the BIV-TAR/TAT system in the presence of hnRNP I^{TAT} restored the wild-type-like growth kinetics, ultimately providing strong evidence that the direct loss of the interaction between the viral RNA and the hnRNP I protein is primarily responsible for the previously established phenotype.

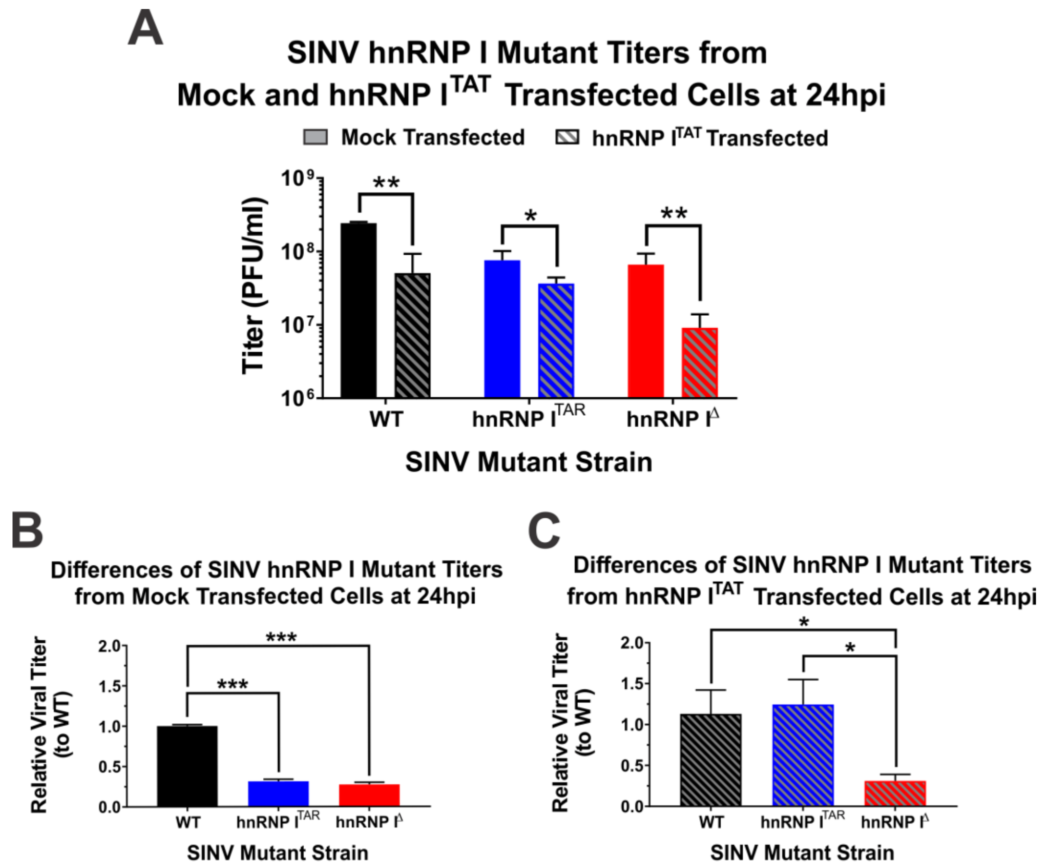


Figure 3.3. Specific reconstitution of hnRNP I–vRNA binding restores wild-type growth kinetics in a mutant lacking the native interaction site. (A) Viral titers of wild-type and hnRNP–vRNA interaction site mutant viruses SINV.hnRNP I^{TAR} and SINV.hnRNP I$^{\Delta}$ at 24 h post-infection of mock and hnRNP I^{TAT}-transfected HEK293 cells infected at an MOI of 10 PFU per cell. (B) Comparative analysis of the viral titer for each of the aforementioned SINVs in mock-transfected HEK293 cells relative to wild-type SINV. (C) Identical to (B), with the exception that the HEK293 cells were transfected with hnRNP I^{TAT}. Quantitative data shown are the means of at least three minimum biological replicates, with the error bars representing the standard deviation of the means. Statistical significance, as determined by Student's t-test, are indicated above the specific comparisons (with * ≤ 0.05; ** ≤ 0.01; *** ≤ 0.001).

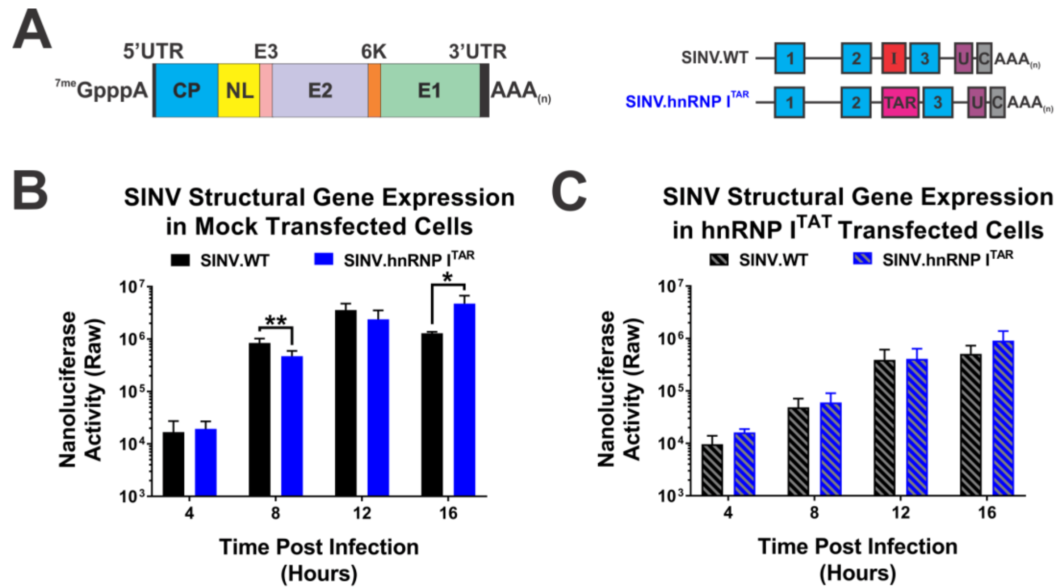
Binding of hnRNP I Correlates with Translational Repression of the SINV Subgenomic RNA

As demonstrated by the data presented in Figures 3.1 and 3.2, the BIV-TAR/TAT system is a means by which the specific impacts of hnRNP I binding to the viral RNAs may be assessed. Previously, we showed that disrupting the hnRNP I–vRNA interaction site resulted in increased structural protein expression; however, it was unknown whether the altered structural protein expression was specifically due to the loss of hnRNP I binding or the mutation of a cryptic regulatory element in the 3′UTR (176). To delineate the impact of the hnRNP I binding on the structural protein expression, we utilized a reporter strain of SINV that expresses nanoluciferase from the subgenomic RNA strand (Figure 3.4A). Similar to what was previously reported, the loss of hnRNP I binding correlated with a biologically and statistically significant enhancement of SINV structural protein expression (Figure 3.4B). Indeed, at 16 hours post-infection (hpi), the subgenomic gene expression during the SINV.hnRNP I^{TAR} infection of HEK293 cells was significantly enhanced by approximately 4-fold relative to the wild-type SINV. However, at early times during the infection, this effect was notably absent, as at 4 hpi there was no difference in structural protein expression between SINV.WT and SINV.hnRNP I^{TAR}. At both 8 and 12 hpi, the wild-type SINV exhibited slightly increased protein expression relative to SINV.hnRNP I^{TAR}, yet only the difference observed at 8 hpi was found to be statistically significant.

The examination of the structural protein expression during SINV infection after the reconstitution of the hnRNP I interaction via the BIV-TAR/TAT system

revealed that the loss of hnRNP I binding was directly responsible for the enhancement of the structural protein expression late during infection. Specifically, in cells expressing hnRNP I^{TAT} there was no significant biological or statistical difference between wild-type or SINV.hnRNP I^{TAR} structural protein expression at any time (Figure 3.4C). Nonetheless, as observed during the analysis of viral growth kinetics above, the expression of hnRNP I^{TAT} reduced structural protein expression for both wild-type SINV and SINV.hnRNP I^{TAR}.

Together these data suggest that hnRNP I-binding is tied to the regulation of viral structural protein expression during infection, and that the enhancement of the structural protein expression due to the loss of hnRNP I binding is time-dependent and specific to the very late stages of infection.



<u>Figure 3.4. Restoration of hnRNP I binding abrogates the enhanced structural protein expression observed late during infection.</u> (A) A graphic schematic of the nanoluciferase-based reporter strain derived from SINV TE12 that expresses nanoluciferase in parallel with the SINV Capsid protein during the translation of the subgenomic strand. (B) Mock-transfected or (C) hnRNP I^{TAT}-transfected HEK293 cells were infected with the designated virus and nanoluciferase activity was quantified at the times indicated post-infection. Quantitative data shown are the means of three independent infections, with the error bars representing the standard deviation of the means. Statistical significance as determined by Student's t-test is indicated above the specific comparisons (with * ≤ 0.05; ** ≤ 0.01).

Binding of hnRNP I Does Not Contribute to the Regulation of Viral RNA Synthesis

An established role for the hnRNP proteins during alphaviral infection centers around viral RNA synthesis; however, it should be noted that these studies relied upon RNAi-mediated knockdown strategies, which as described earlier could lead to substantial off-target impacts on the cellular environment (173, 179, 217). Accordingly, to refine the understanding of the role of the hnRNP I protein in viral transcription and replication, we examined the RNA synthesis profiles of wild-type SINV and the hnRNP I interaction-deficient viruses during infections of HEK293 cells either mock-transfected or transfected with an hnRNP I^{TAT} expression plasmid. The detection of the individual viral RNA species was accomplished using standard qRT-PCR detection using previously reported methods over four-hour intervals from 4 hpi to 16 hpi.

As previously reported, the loss of the hnRNP I interaction did not significantly alter the synthesis or accumulation of the individual viral RNAs, as exhibited by the general RNA profiles observed for SINV.WT and SINV.hnRNP I^{TAR} with respect to time (Figure 3.5A,B) (176). In contrast, as shown in Figure 3.5C,D, both SINV.WT and SINV.hnRNP I^{TAR} exhibited altered accumulation profiles in the presence of hnRNP I^{TAT}. In the presence of hnRNP I^{TAT} expression, the synthesis and accumulation of both the genomic and subgenomic RNA species was negatively impacted, with average reduction rates of approximately 4- and 6-fold, respectively, for SINV.WT and SINV.hnRNP I^{TAR}. Nonetheless, despite the

clear impact of the hnRNP I^{TAT} expression on RNA synthesis, the overall magnitudes of the impact were similar.

To enable a more direct comparison of the viral RNA species during SINV.WT and SINV.hnRNP IT^{TAR} analyses, we assessed the quantitative data for the individual viruses using pairwise statistical analyses (Figure 3.5E,F). These analyses revealed that only a single pairwise sample was statistically different between SINV.WT and SINV.hnRNP I^{TAR}, specifically the quantity of genomic viral RNA at 16 hpi in the mock-transfected condition. All other comparisons, including those for the subgenomic RNAs, were not different to any statistically significant degree (with a minimum $\alpha \leq 0.05$ on a one-tailed analysis).

In summary, these data indicate that the synthesis and accumulation of viral RNA species is not negatively impacted by the loss of hnRNP I binding or the mutation of the native interaction sequence. However, the overexpression of hnRNP I negatively impacted the viral RNA synthesis and accumulation in a generalized manner.

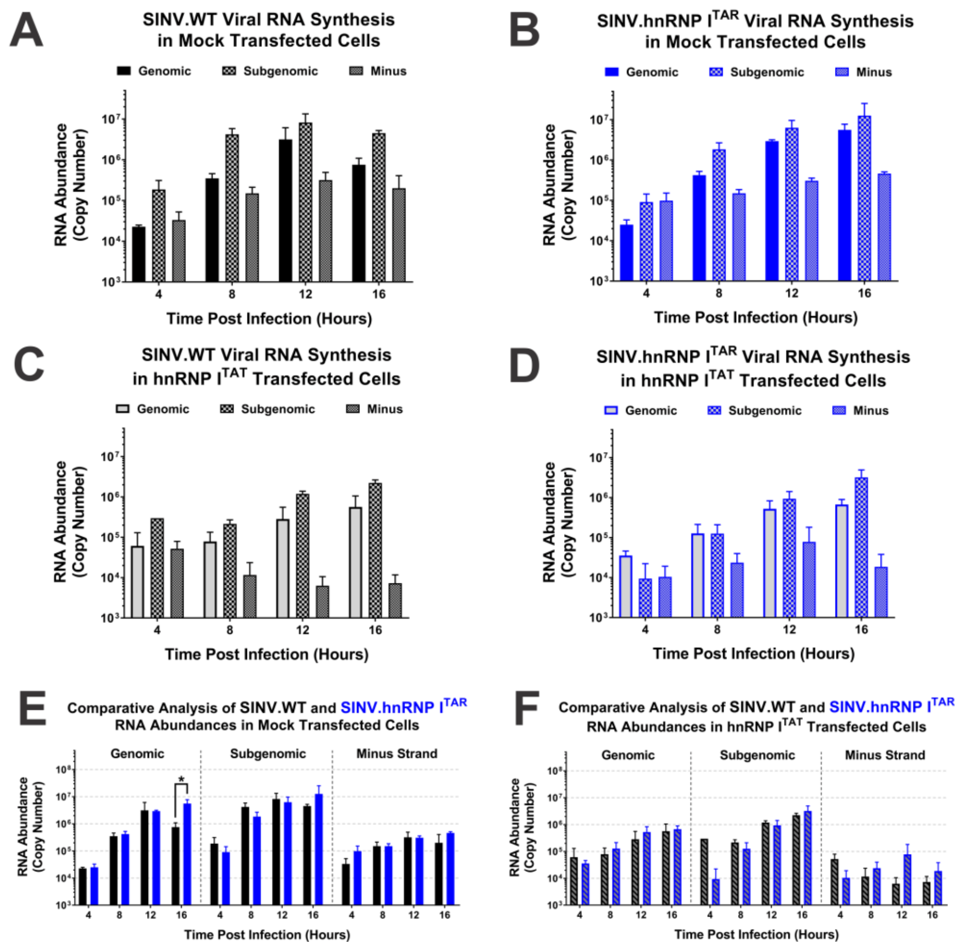


Figure 3.5. Viral RNA synthesis is not impacted by the hnRNP–vRNA interaction. Strand-specific quantitative analysis of the three SINV vRNA species in mock-transfected HEK293 cells infected with (A) SINV.WT or (B) SINV.hnRNP I^{TAR} viruses at an MOI of 10 PFU per cell. (C,D) Identical to the previously described panels, with the primary difference being that hnRNP I^{TAT}-transfected HEK293 cells were used. (E,F) Data from the previous panels reconfigured to allow direct comparisons between the viruses in either cell condition. Quantitative data shown are the means of three independent infections, with the error bars representing the standard deviation of the means. Statistical significance as determined by Student's t-test is indicated above the specific comparisons (with * ≤ 0.05).

Binding of hnRNP I is Important to the Viral Particle Function or Specific Infectivity

Precisely how the loss of the hnRNP I protein–RNA binding negatively impacts the SINV infection despite enhancing the structural protein expression has always been an interesting yet puzzling question. Since structural protein expression is directly linked to viral particle assembly, we sought to determine whether or not the production of viral particles was negatively impacted by the loss of hnRNP I binding (268, 269). To address this research question, we measured the total particle production via the detection of genome equivalents by way of qRT-PCR. Briefly, control-transfected and hnRNP I^{TAT}-expressing cells were infected with either SINV.WT or SINV.hnRNP I^{TAR}, and tissue culture supernatants were collected at 24 hpi. The number of viral genomic RNAs was then measured via standard curve qRT-PCR to determine the number of viral particles. As shown in Figure 3.6A, the loss of hnRNP I binding does not negatively affect the particle production, as there is no difference in particle numbers between SINV.WT and SINV.hnRNP I^{TAR} in either the presence or absence of hnRNP I^{TAT}. Consistent with our above data the expression of hnRNP I reduced the particle production relative to the control-transfected cells, as there was an approximately half-log decrease in particle production for both SINV.WT and SINV.hnRNP I^{TAR}.

While the production of total viral particles was seemingly unaffected by the loss and restoration of hnRNP I binding, we hypothesized that the viral particle function, as defined by the capacity of a viral particle to complete the viral lifecycle, is negatively impacted by the loss of hnRNP I binding and subsequent structural

protein overexpression. To define the functional potential of the viral particles generated in the presence and absence of the hnRNP I interaction, we measured the titer of the viral particles (Figure 3.6B) and determined the specific infectivity of the particles by calculating the ratio of particles-per-PFU for the individual samples. In this instance, a higher specific infectivity value means that it takes more particles to make a single plaque forming unit, meaning the viral particle population has poor infectious potential.

As shown in Figure 3.6C, SINV.WT particles derived from control-transfected cells exhibited an infectivity ratio of approximately 170:1 particles-per-PFU, whereas SINV.hnRNP I^{TAR} was significantly less infectious, with a particle-per-PFU ratio of greater than 600:1. Nonetheless, when the hnRNP I protein–RNA interaction was restored via the BIV-TAR/TAT system, the specific infectivity of SINV.hnRNP I^{TAR} significantly improved to a ratio of 200:1 and exhibited an infectivity ratio highly similar to that of SINV.WT. It is notable that SINV.WT particles exhibited a similar infectivity ratio regardless of whether they were produced in control-transfected or hnRNP I^{TAT}-transfected cells.

Altogether these data indicate that the particle functionality, as measure by the infectious potential of the population, is negatively impacted by the direct loss of hnRNP I binding and not the loss of specific primary nucleotide sequences or secondary structures in the SINV 3′UTR. Moreover, these data infer that while the particle production and viral titer may be generally reduced in systems that express high levels of hnRNP I, the infectious potentials of wild-type viral particles are unperturbed.

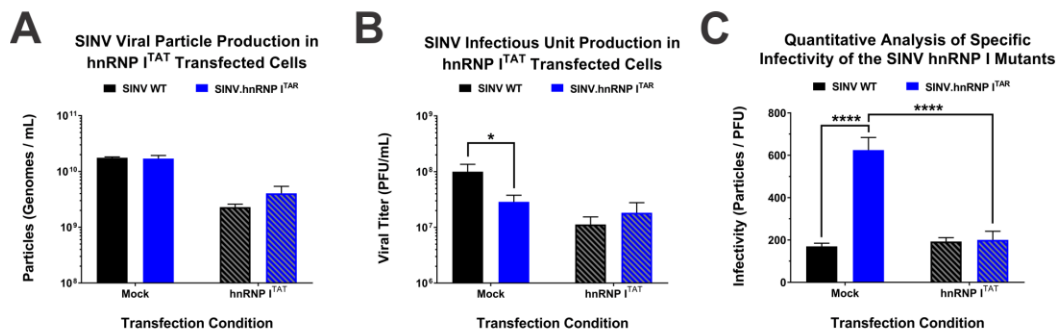


Figure 3.6. Reestablishment of the hnRNP–vRNA interaction restores viral particle infectivity. (A) Virus particles, as defined by the genome equivalents per ml, derived from either mock or hnRNP I^{TAT}-transfected HEK293 cells were quantified via qRT-PCR. (B) Paired viral titer analysis of the samples examined in (A) as measured using standard plaque assays. (C) Quantitative analysis of virus-specific infectivity, as measured by the ratio of particles per infectious unit, for the samples described in the above panels. Quantitative data shown are the means of three independent infections, with the error bars representing the standard deviation of the means. Statistical significance as determined by Student's t-test is indicated above the specific comparisons (with * ≤ 0.05; **** ≤ 0.001).

The Loss of hnRNP I Binding Does Not Negatively Impact Particle Assembly or Structure

As reported above, the loss of hnRNP I binding negatively impacted the specific infectivity of the viral particles. In light of these data, we hypothesized that the overexpression of SINV structural proteins leads to the formation of viral particles with decreased infectious potential, either through the formation of aberrant multicore viral particles, the inclusion or exclusion of host or viral proteins, or the production of irregular viral proteins during infection (as diagrammed in Figure 3.7A). To test this hypothesis, we set about characterizing the viral particles produced by wild-type SINV and SINV.hnRNP I^{TAR} in the presence and absence of hnRNP I^{TAT}.

The production of multicore particles would readily explain our previous observations, in that a single PFU would be composed of multiple genome equivalents, as several nucleocapsid cores would be packed into an envelope, resulting in a poor specific infectivity, as measured by the particle-per-PFU ratio (270). To this end, we examined the morphologies of wild-type and hnRNP I interaction-deficient viral particles via transmission electron microscopy (TEM). As shown in Figure 3.7B, the overall morphologies of viral particles derived from hnRNP I binding and nonbinding SINVs were highly similar, and multicore particles were not observed. Curiously, the quantitative analysis of the particle diameter indicates that viral particles derived in the absence of hnRNP I binding exhibited increased heterogeneity, albeit to a minor extent.

As the formation of multicore particles was not observed in the absence of hnRNP I binding, we next characterized the protein composition of the viral particles. Briefly, low-speed purified viral particles were denatured and analyzed via SDS-PAGE and the total protein content was visualized by Coomassie staining. As shown in Figure 3.7C, the viral particles produced in the presence and absence of hnRNP I binding were highly similar, and no significant unexpected proteins were observed. The quantitative analysis of the ratios of the viral glycoproteins to capsid protein provides further evidence against the formation of multicore particles, as the ratios between the particle populations are highly consistent.

Notwithstanding the products of these efforts being largely negative data in regard to our hypothesis, these data were informative, as they effectively rule out the possibility that gross particle defects were arising due to increased structural protein expression. However, there could be minute differences in post-translational modifications that could not be detected using an SDS-PAGE gel. Nonetheless, from these data we cannot rule out that the malformation or misprocessing of the viral structural proteins during assembly negatively impacts the viral particle function.

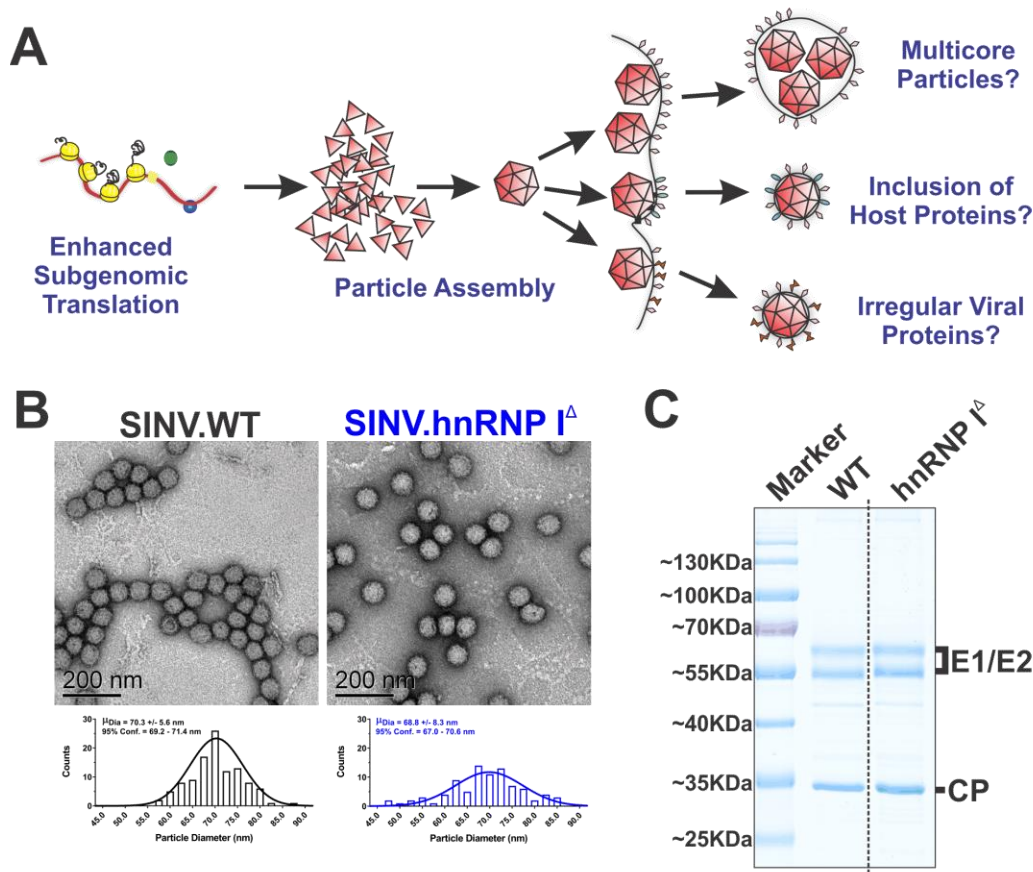


Figure 3.7. Loss of hnRNP I binding does not negatively impact the viral morphology or composition. (A) A graphic model of several working hypotheses as to how increased structural protein expression leads to poor particle infectivity. (B) Representative TEM micrographs of wild-type or SINV.hnRNP I^{TAR} particles purified via low-speed low-temperature centrifugation. Below each micrograph is a histogram of measured particle diameters with the mean and 95% confidence intervals reported inset to each graph. (C) Concentrated SINV.WT and hnRNP I interaction site deletion mutant viral particles were resolved via SDS-PAGE gel and stained with Coomassie blue. Data shown are representative of multiple independent viral preps. (C) The dashed line is indicative of where the gel was cropped and merged to remove intervening lanes for the final presented image.

The Loss of hnRNP I Binding Negatively Impacts the Early Stages of the Viral Lifecycle

Although the viral particles derived from the hnRNP I binding-deficient mutant are less infectious, the mechanism behind why they are poorly infectious is yet to be known. During the viral lifecycle there are several points with high potential to influence the specific infectious potential of a viral particle, and importantly these alphaviral lifecycle events can be parsed apart at certain points to determine where in the lifecycle the particles are functioning poorly. As our data above strongly indicates that the viral replication and gene expression are not explicitly negatively impacted by the loss of hnRNP I binding, it can be reasonably concluded that these events are not the primary defects leading to poor infectivity. As such, we hypothesized that an earlier event in the viral lifecycle was responsible for the observed deficits in specific infectivity.

To test our hypothesis, we quantitatively examined the first step of the viral lifecycle, which is the viral attachment to the cell. To accomplish this, we exposed HEK293 cells to either SINV.WT or SINV.hnRNP I^{TAR} particles derived from control-transfected or hnRNP I^{TAT}-transfected cells at 4 °C for one hour to allow for attachment without entry or internalization of the viral particles. Paired tissue culture monolayers were then processed in parallel to generate input and bound samples, with the bound samples being generated from exposed monolayers that were extensively washed to remove unbound particles prior to RNA extraction. The viral RNAs from the input and bound samples virus were quantitatively assessed by qRT-PCR to determine the relative binding of the viral particles via the retention

of genome equivalents. As shown by Figure 3.8A, the particles derived from infections lacking the hnRNP I interaction bound approximately two-fold less to cells relative to the particles derived from SINV.WT infection. Nonetheless, SINV.hnRNP I^{TAR} particles derived from hnRNP I TAT-transfected cells bound equivalently to SINV.WT (Figure 3.8B).

As in order for a viral particle to be infectious it must first be able to attach and enter into a host cell these data confirm our hypothesis that the loss of hnRNP I binding negatively impacts an early event in the viral lifecycle, resulting in poor specific infectivity. This assertion is evidenced by the reestablished particle attachment, which correlates with the above restoration of the infectivity of SINV.hnRNP I^{TAR} in hnRNP I^{TAT}-transfected cells.

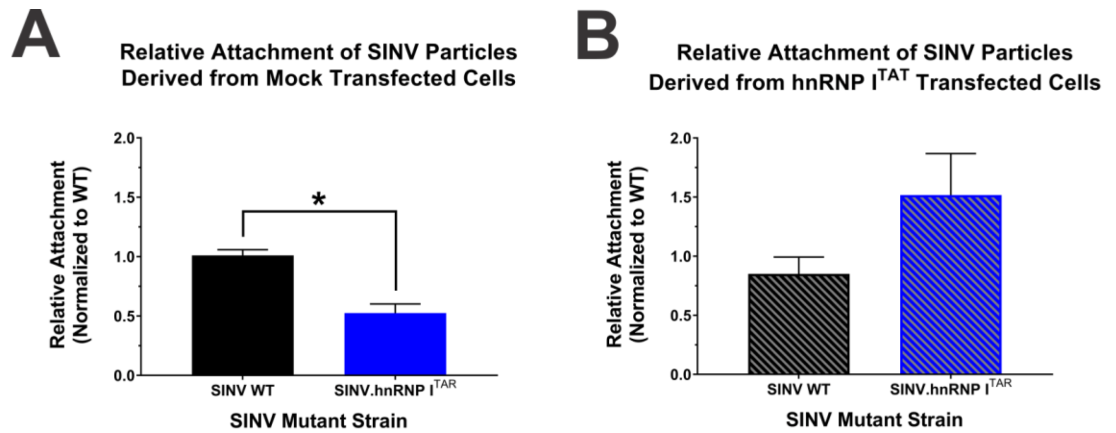


Figure 3.8. Loss of hnRNP I binding negatively impacts viral particle attachment. Quantitative analysis of viral attachment via qRT-PCR of total RNAs extracted from HEK293 cells that were incubated with viral particles derived from either (A) mock-transfected or (B) hnRNP I^{TAT}-transfected HEK293 cells. Quantitative data shown are the means of three independent attachment assays, with the error bars representing the standard deviation of the means. Statistical significance as determined by Student's t-test is indicated above the specific comparisons (with * ≤ 0.05).

Deglycosylation of the hnRNP I Mutant Particles Does Not Impact Their Infectivity

The alphaviral entry pathway is initiated and governed by the viral glycoproteins through their engagement with the host receptor during attachment (270-272). As our data are indicative of a defect at the level of the cell attachment, we hypothesized that the viral glycoproteins may be somehow altered in the absence of hnRNP I binding due to the overexpression of structural proteins during late infection. The viral glycoproteins are known to be post-translationally modified during their maturation process, including being palmitoylated and glycosylated as they traffic to the cell membrane for later envelopment of the nascent nucleocapsid cores (60, 128, 162, 273, 274). As glycosylation has been previously identified as a major contributor to cell attachment, we prioritized efforts to examine the impact of glycosylation on the hnRNP I mutant particle function.

To define the extent to which glycosylation differences were contributing to the observed deficits in particle function, we enzymatically deglycosylated SINV.WT and SINV.hnRNP I^{TAR} viral particles and assessed their infectious potentials. Concisely, aliquots of the viral particles were either mock-treated or treated with PNGase F under native protein conditions overnight, and the viral titer was subsequently assessed. The deglycosylation of SINV.WT particles via PNGase F negatively impacted the viral titer, as evidenced by a decrease of approximately 5-fold (as depicted in Figure 3.9A). In contrast, there was little to no decrease in the apparent viral titer when SINV.hnRNP I^{TAR} viral particles were treated with PNGase F. Indeed, comparing the relative effects of the

deglycosylation on SINV.WT and SINV.hnRNP I^{TAR} titer revealed that the deglycosylation did not appreciably affect the specific infectivity of the SINV.hnRNP I^{TAR} particles (Figure 3.9B).

On the whole, these data strongly suggest that the differences in glycosylation may be responsible for the underlying defects observed following the loss of hnRNP I protein binding. Nonetheless, whether this is due to the absence of glycosylation or the presence of faulty glycosylation is unknown at this time.

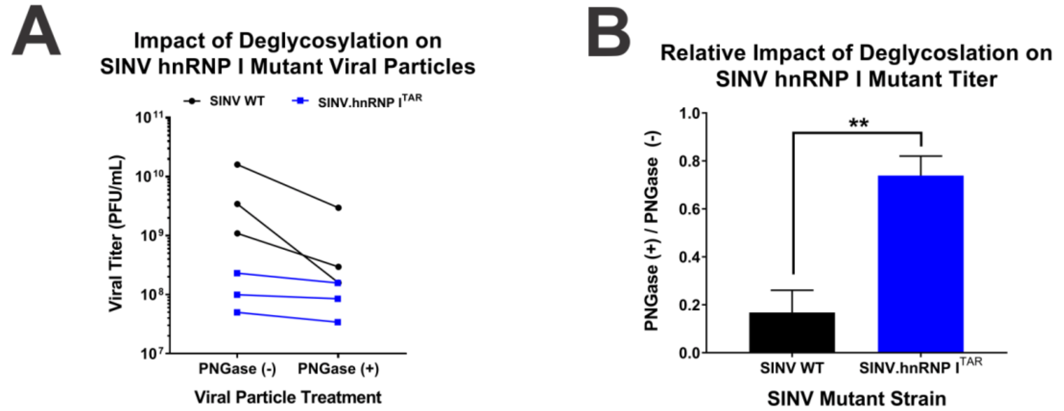


Figure 3.9. The loss of hnRNP I binding negatively impacts the glycosylation of the viral glycoproteins. (A) SINV.WT or SINV.hnRNP I^{TAR} viruses were incubated in the presence or absence of PNGase F overnight at room temperature under nondenaturing conditions. After treatment the viral titer was quantified and the change in viral titer is presented for each pairwise sample. (B) The relative impact of deglycosylation, as determined by the average ratio of treated and untreated samples. Quantitative data shown are the means of three independent PNGase F assays, with the error bars representing the standard deviation of the means. Statistical significance as determined by Student's t-test is indicated above the specific comparisons (with ** ≤ 0.01).

Discussion

As has been previously published, several host hnRNP proteins are known to interact with the SINV vRNAs during infection, with the hnRNP K, I, and M proteins interacting with discrete sites of the SINV subgenomic RNA (176). The ubiquity and specificity of the hnRNP protein interactions was indicative of an important role during the SINV lifecycle. Nonetheless, due to the involvement of the hnRNP proteins in the synthesis and maturation of many cellular transcripts, RNAi- or CRISPR-based approaches would undoubtedly perturb the underlying cell system through the loss of hnRNP function. As shown here and published previously by our lab, an approach that enables the assessment of the contributions of the hnRNP proteins in the absence of an altered host system is to target the hnRNP–vRNA interaction sequence without disrupting the coding capacity of the virus. The application of this approach diminished the hnRNP–vRNA interactions, leading to significantly decreased viral growth kinetics in tissue culture models of infection. Curiously, the primary molecular defect associated with the disruption of the hnRNP interactions was increased structural protein expression, which positively correlated with decreased viral growth; however, the precise underlying mechanisms behind these phenomena were unknown. Altogether these observations raised several key questions, including the following: (1) Are the observed phenotypes due to the loss of hnRNP binding or due to disrupting the native RNA sequences? (2) How does the enhanced structural protein expression in effect result in a decreased viral titer?

To address our research questions, we utilized a modified protein tethering approach to reconstitute the hnRNP interaction in the absence of the native sequence. Since the current protein tethering methodologies are largely incompatible with use in coding regions, we focused our efforts on assessing the hnRNP I–vRNA interaction, as the primary interaction site for hnRNP I is located in the 3′UTR of the subgenomic RNA (176). Specifically, the BIV-TAR element was incorporated into the vRNA at the site of the hnRNP I interaction site, where the BIV-TAR element could act as a highly specific binding site for proteins such as hnRNP I, provided the protein is tagged with a TAT peptide (259). To confirm the capacity of the BIV-TAR/TAT system to reconstitute the hnRNP I–vRNA interaction, we assessed the interaction via quantitative co-immunoprecipitation. In the presence of hnRNP I^{TAT}, the co-immunoprecipitation of the SINV.hnRNP I^{TAR} vRNA was equivalent to that of the wild-type interaction in the absence of hnRNP I^{TAT} and greater than that of the wild-type SINV in the presence of hnRNP I^{TAT}. In other words, more SINV vRNA was pulled down during the forced interaction between SINV.hnRNP I^{TAR} and hnRNP I^{TAT} than that of SINV.WT in the presence of hnRNP I^{TAT}. The underlying cause of the reduced wild-type SINV co-immunoprecipitation is unclear, and potentially due to several mechanisms. First, this could be due to the interaction between the BIV-TAR RNA and TAT fusion peptide being a stronger interaction than the native hnRNP I and vRNA interaction, resulting in greater occupancy and increased co-immunoprecipitation. In addition, the overexpression of hnRNP I could interfere with the immunoprecipitation by

reducing the amount of RNA–protein complex binding relative to the total hnRNP I immunoprecipitation via an effective antibody dilution effect.

Regardless, this system allowed us to directly compare the phenotypes observed between SINV infections with native hnRNP I interactions, those lacking native hnRNP I infections, and those with a forced hnRNP I interaction. As such, it is unsurprising that after confirming the validity of the BIV-TAR/TAT approach, we then tested the effect of the hnRNP I tethering on the viral growth kinetics. As observed before, there was a decrease in SINV.hnRNP I^{TAR} titer compared to SINV.WT in mock-transfected cells. However, this difference in infectious titer between wild-type and SINV.hnRNP I^{TAR} was not observed in hnRNP I^{TAT}-transfected cells, indicating that the tethering of hnRNP I was capable of restoring the wild-type growth kinetics.

Despite alleviating the growth defect resulting from the loss of the native hnRNP I interaction site, the overall titers for both the wild-type and interaction-deficient mutants were decreased in the presence of hnRNP I^{TAT} relative to mock-transfected cells. This was despite an experimental design that included using the same MOIs to infect either condition. Thus, the hnRNP I overexpression appears to be deleterious to SINV infection in a generalized manner. This observation is echoed by our assessments of viral gene expression, vRNA synthesis and accumulation, and viral particle production. As alluded to above, the steady-state levels of the hnRNP proteins, including hnRNP I, are likely important to the homeostasis of the host cell, and altering the levels of hnRNP I upwards or downwards may negatively impact the cytosolic environment. In support of this

notion is the general observation that hnRNP I^{TAT}-transfected cells looked morphologically abnormal and less confluent when compared with cells that had been mock-transfected. Accordingly, our leading hypothesis as to why viral titers were reduced overall is that hnRNP I overexpression negatively impacts host cell processes. In any case, the generalized impact of hnRNP I overexpression may be negated by ensuring that phenotypic comparisons are made with those between the viruses in a single transfection condition and not those between transfection conditions.

Binding of hnRNP I is Specifically Important for the Regulation of Viral Structural Protein Expression

Previously, we reported that disrupting the hnRNP I–vRNA interaction site led to increased structural protein expression; however, this prior effort examined viral gene expression in a limited manner late during infection, meaning the full picture of the potential role of hnRNP I in the regulation of viral gene expression throughout the lifecycle remained unknown. To enhance the understanding of the role of hnRNP I in the regulation of viral translation, we examined the viral gene expression with respect to time in systems with native hnRNP I interactions, those lacking native hnRNP I infections, and those with a forced hnRNP I interaction.

In mock-transfected cells, there were no biologically significant differences in viral structural protein expression at 4, 8, or 12 hpi. However, at 16 hpi the SINV mutant lacking the hnRNP I interaction again exhibited enhanced structural protein

expression. The timing of this effect may be indicative of the unavailability of the hnRNP I protein to the vRNAs, as hnRNP I relocalization to the cytoplasm has not likely occurred at these earlier stages of infection. As observed above for the viral growth kinetics, there was no difference in structural protein expression between SINV.WT and SINV.hnRNP I^{TAR} in hnRNP I^{TAT}-transfected cells, despite a generalized decrease in viral structural protein expression. Hence, we conclude that the hnRNP I protein binding to the viral RNA is important for the regulation of the viral structural protein expression at late stages of viral infection.

Binding of hnRNP I is Dispensable to SINV vRNA Synthesis and Accumulation

As the hnRNPs are RNA binding proteins that are involved in the processing of many cellular RNAs, it was imperative to examine the potential impacts of hnRNP I in viral RNA synthesis (230, 238, 255, 256, 275, 276). Previous studies have shown that knockdown or silencing of hnRNPs will cause decreases in alphaviral RNA synthesis; however, as discussed previously, this could be the result of disrupting the host cell biology through the loss of hnRNP function (173). As with the viral gene expression, our prior efforts examining the role of the hnRNP proteins were limited to a singular time post-infection. Here, we expanded these analyses by examining the impact of hnRNP I on SINV replication and RNA synthesis with respect to time by using our model infection systems. Consistent with our prior examination of hnRNP I interaction-deficient mutants, we observed no explanative differences in RNA synthesis or accumulation for any of the vRNA

species at any time post-infection in any of the conditions assessed. Collectively, these data infer that under conditions of equal infectious units, the viral RNA synthesis is unperturbed by the loss of hnRNP I binding.

Nonetheless, whether specific differences in viral RNA synthesis are present at the very early stages of viral infection remains unknown. As a primary difference between the native particles and those produced in the absence of hnRNP I binding is decreased infectious potential, one could envision a scenario where the viral RNA levels at the earliest stages of infection differ to a significant extent. The inequality of the viral particle function would, a priori, suggest that the viral RNA levels at the earliest instances of infection should differ by 2- to 3-fold, as per the observed differences in attachment and specific infectivity. However, these differences are not reflected by our data. There are several reasons as to why these differences are not propagated to the times post-infection assessed in this study, which were chosen on the basis of them representing times post-infection where all viral RNA species are readily detectable via qRT-PCR. First, it is unclear as to whether the infectious particles would effectively deliver their RNA cargos to the host system, thereby contributing them to the pool of cytoplasmic viral RNAs from which replication may proceed. To control for this possibility, the experimental designs were standardized to utilize equal numbers of infectious units (as PFU) to create a level playing field between the hnRNP I mutant and wild-type SINVs. Secondly, the alphaviral RNA synthesis kinetics are inherently very robust, and as such it remains possible that the RNA synthesis is capable of overcoming any early deficits through the inherent momentum of replication.

Overall, from our data we conclude that the loss of hnRNP I binding does not significantly impact the RNA synthesis over time. However, as with our other data, there is an observable general impact of the hnRNP I overexpression on the vRNA synthesis.

Loss of hnRNP I Binding Results in the Production of Poorly Infectious Virus Particles

All together, we can conclude that the phenotypic differences observed following the mutation of the hnRNP I interaction site were due directly to the loss of hnRNP I binding and not due to a loss of secondary RNA structures or primary sequences. Nonetheless, while our first major research question had been addressed, the question of how precisely an increase in structural proteins negatively effects viral infection remained elusive. To address this ongoing research question, we comparatively examined viral particles produced in the presence and absence of hnRNP I binding via the BIV-TAR/TAT system.

A quantitative analysis of the viral particle production yielded an unexpected result as the increased viral structural protein expression did not correlate with a parallel increase in particle production. This observation was puzzling because despite there being more structural proteins to make more viral particles, there was no difference in particle production. Nonetheless, the differences in viral titer led to the hypothesis that the particles made in the absence of hnRNP I binding were less functional than wild-type viral particles. The virus-specific infectivity, as

defined by the number of viral particles to infectious units, is a ready means by which the functionality of the viral particles in total may be assessed. These data presented above indicate that the viral particles produced in the absence of hnRNP I binding are poorly functional relative to the wild-type particles. In short, when there was no hnRNP I–vRNA interaction, many more particles were needed to make one infectious unit, and when the hnRNP I interaction was restored through the BIV-TAR/TAT system, the number of viral particles per infectious unit was similar to that of the wild-type SINV. Not only do these data reinforce the conclusion that the direct loss of hnRNP I binding is the specific cause of the observed mutant phenotype, these data provide valuable insight towards the elucidation of the underlying mechanism as to why there are decreased viral growth kinetics.

We have established so far that hnRNP I is important to the regulation of the viral structural protein expression, and without that hnRNP I–vRNA interaction, there is an influx of structural proteins at the later stages of infection relative to the wild-type infection. Since alphaviral infections rely heavily on host processes to develop mature virions, these excess structural proteins could overwhelm the host biology and create a bottleneck in virus production. This in turn could create poorly functioning viral particles via several different mechanisms, which we alluded to in detail in the results section. Notably, many of our efforts were designed to identify whether these overt defects were revealed wild-type-like phenotypes for the particles produced in the absence of the hnRNP I interaction. Nonetheless, in this case even the negative data were meaningful data, as they narrowed down the potential causes of the defective particles.

Despite being able to rule out the formation of multicore particles or malformed particles, there was still no clear explanation of why these particles were poorly infectious. The molecular data presented here indicate that an early event of the viral lifecycle is negatively impacted by the loss of hnRNP I binding. By turning to the beginning of the alphaviral lifecycle and examining the viral attachment, we determined that the viral particles produced in the absence of hnRNP I binding were less able to bind to the host cells in the tissue culture models of infection. Importantly, this reduced attachment is 'fixed' when hnRNP I binding is restored via the BIV-TAR/TAT system. The reduced attachment to the host cell strongly suggested that the viral glycoproteins of the mutant particles were stoichiometrically inferior, malfunctioning, or malformed. The examination of the viral particle composition did not reveal altered capsid-to-glycoprotein ratios, indicating that the mutant viral particles were likely not lacking viral glycoproteins on the whole. As the alphaviral glycoproteins mature, they are post-translationally processed prior to their incorporation into the viral particles (60, 162, 273). Of these potential post-translational modifications, the glycosylation of the E1 and E2 glycoproteins has been previously established to directly influence the viral attachment to the host cell, and the alphavirus glycosylation site mutants are poorly infectious owing largely to the altered host cell attachment (156). Importantly, the deglycosylation of SINV viral particles generated in the presence and absence of hnRNP I binding reveals the difference in the glycosylation states to be a primary difference between the two particle populations. As the SINV particles derived from wild-type infection were sensitive to deglycosylation, whereas those generated in

the absence of hnRNP I binding were insensitive, the defective particles formed during enhanced structural protein expression may lack or possess erroneous glycosylation profiles. Further studies are ongoing to determine precisely how an increase in structural protein expression results in this phenotype, whether the phenotype is the result of a bottleneck during processing or an active host response to infection, and whether this phenotypic defect is caused by altered glycosylation or a lack thereof.

It is worth noting that in addition to the defects in particle function related to glycosylation, other defects may also be present and may contribute to the phenotype observed during the loss of hnRNP I binding. These include aspects of infectivity related to viral lifecycle events prior to and after host cell attachment. For instance, our research has previously established that encapsidated host factors and viral RNA features, such as the 5′ cap structure, influence the particle infectivity (277, 278). Whether or not these phenomena are also altered in response to the increased structural protein expression is unknown at this time.

Is hnRNP–vRNA Binding a Host Response to Infection, or the Recruitment of a Pro-Viral Factor?

The sum of our observations raises an interesting question- is the repression of the viral translation via hnRNP I binding beneficial or detrimental to the virus? On face value, the molecular impacts of the hnRNP I binding, in that the viral structural protein expression is reduced, are reminiscent of an anti-viral

response by the host. However, the output of the increased structural protein expression is the formation of poorly functioning viral particles, which infers that the hnRNP I interaction is beneficial to the viral infection through a complex means that maintains the functional integrity. The engagement of other host RNA-binding proteins to the alphaviral RNAs has been established to be largely pro-viral. Thus, we posit that the engagement of the hnRNP proteins to the viral RNAs is pro-viral in nature due to the body of knowledge regarding alphaviral RNA-binding protein interactions, as well as the summative phenotype resulting from the loss of hnRNP I binding presented here. However, further work is needed to fully understand the precise roles of cellular RNA-binding proteins during viral infection, including the likely reality that the consequences of the RNA-binding protein function is redefined during infection through post-translational modifications or the formation of contextually novel ribonucleoprotein complexes on viral RNAs.

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

Research Summary

Since alphaviruses are reliant on host proteins to complete the viral lifecycle, the identification of these host / pathogen interactions and the subsequent characterization of their importance to viral infection has been an intensive area of study for decades. Many of these host protein interaction studies were Protein:Protein interactions, but as more RBPs became apparent as host interactants through these studies, the field shifted to focus on the role of the RBPs via their potential RNA:protein interactions. For a significant period of time the leading technological approaches consisted of knocking out or knocking down the expression of the host factor prior to infection. As such, the majority of these studies utilized RNAi or CRISPR based approaches to test the importance of the RBP to infection; however, doing so likely harmed the normal cellular environment and functions as many RBPs are critically essential to maintaining host gene expression / homeostasis (169, 173, 174). Most hnRNPs have been studied during normal function or during a disease state such as cancer; however, it is not known if they serve these same “normal” functions during alphaviral infection. To maneuver around the problem of disrupting the interaction without disturbing host

biology, we and others pioneered an approach that consisted of incorporating silent mutations, or sequence deletions in non-coding areas, to test the impact of the interaction without modifying the host system (246). Interrupting the interaction of the targeted RBPs caused decreased viral titer, and surprisingly, the primary molecular defect was found to be increased structural protein expression. While these early works identified the target proteins as important to alphaviral biology, the precise mechanism behind this phenotype was unknown. Due to the nature of the approach, several questions arose including whether the phenotypes observed were due to a genuine loss of interaction or to the loss of an RNA sequence and / or structure, and thus indirect to RBP binding. Additionally, the question as to how increased structural protein expression could result in decreased viral titer remained unanswered.

A major goal of this dissertation project was to determine whether the phenotype associated with the mutation of the interaction site was due specifically to the loss of hnRNP I binding. By employing a protein tethering system, we were able to reconstitute the hnRNP I:vRNA interaction in the absence of the original interaction sequence in the vRNA. We found that reconstituting this interaction via the BIV-TAR system restored a wild-type like phenotype despite the absence of the native RNA sequence or potential secondary structures. The results discussed herein have led to the conclusion that hnRNP I directly binds to the vRNA, and the phenotypic impact is directly due to the hnRNP binding and not dependent on the loss of viral RNA structure or sequence.

As stated above, another ongoing area of interest is the specific mechanism as to how the loss of hnRNP I binding negatively impacted infection. As detailed earlier, the primary defect associated with the loss of hnRNP I binding was decreased viral particle specific infectivity. Or in other words, viral particles produced in the absence of the hnRNP I interaction were inferior to wild type viral particles in regards to cell attachment, and presumably entry. Extensive characterizations of wild type, mutant, and reconstituted mutant viral particles revealed that a potential defect in viral glycoprotein processing may be responsible for the observed phenotype. From the data described earlier, it can also be concluded that the increased structural protein expression due to the loss of hnRNP I binding causes abnormal, incomplete, or nonexistent glycosylation, culminating in poor particle function.

What is the Ultimate role of hnRNP I During Alphaviral Infection?

Unfortunately, despite clearly establishing the importance of hnRNP I binding to viral infection, the direct role(s) the hnRNP I protein performs during infection has not yet been completely elucidated, and how exactly hnRNP I binding influences viral structural protein expression remains unknown. Nonetheless, there are several potential mechanisms by which this might occur. For cellular transcripts, the hnRNP I protein has been shown to increase RNA stability by binding to the 3' UTR, however, this mechanism is not likely the case during alphaviral infection, as RNA abundance of the three viral strands is not affected by the loss of hnRNP I binding (238). If the loss of hnRNP I was important to RNA

synthesis and replication, there would be a significant increase or decrease of RNA levels. Even more, the hnRNP I protein is most likely not involved in the translation of the nsPs, as more of the replicase complex would also lead to increased RNA levels for all three species of viral RNAs.

During normal host function, hnRNP I typically enhances translation, however the hnRNP I protein interacting with the viral subgenomic RNAs during alphaviral infection negatively regulated protein expression. The hnRNP I protein has been shown to be involved in the translational regulation of many different viruses, and some of these instances are due to the binding of hnRNP I protein to IRES elements in the 5'UTRs of the individual viruses (242, 279, 280). To date, it is understood that the alphavirus subgenomic RNA is likely capped and no functional 5' IRES elements have been found (279). Nonetheless, as cap-mediated translation has been shut off at the time of subgenomic translation due to the inactivation of phosphorylated eIF2α by PKR, a potential role for the hnRNP I protein during subgenomic RNA translation persists (200). While specifically unknown for the alphaviruses, there are studies indicating that other RNA viruses can use elements in their 3' UTRs to direct translation via an IRES-like mechanism (281). In alignment with this instance, perhaps the hnRNP proteins may act as a complex to regulate translation by binding to the 3' UTR and throughout the subgenomic strand. If the hnRNP proteins function in this manner as a complex it may also explain why the disruption of individual hnRNP interactions creates a battery of mutants with similar phenotypes. The presence of a cryptic translational element in the 3'UTR also creates a plausible explanation as to why the hnRNP

proteins preferentially bind to the subgenome during infection. Nonetheless, the fact that the loss of hnRNP I binding enhances translational activity is difficult to reconcile with this possibility, and thus further examinations are warranted.

To study deeper aspects of the hnRNP I protein being involved in translational regulation, the impact of hnRNP I binding should be examined outside of the context of the viral RNAs during infection. For instance, the BIV/TAR tethering system could be used to tether the hnRNP I protein to a nonspecific reporter RNA, and the translational activity of the reporter quantified in the presence and absence of viral infection. Due to the normal function of hnRNP I in the absence of infection, one could assume that the translation of the reporter strand would be increased with hnRNP I binding. Alternatively, if translation of the reporter was decreased in the absence of infection, you could assume that hnRNP I acts as a general translational repressor when bound in this context. If there was a specific decrease of reporter translation during infection compared to increased translation in the absence of infection, one could include that viral infection perturbs or instills new function to the hnRNP I protein, as hnRNP I is shown to increase translation, not inhibit it. Alternatively, if the hnRNP I tethering failed to have any consequence outside of a viral context, it would suggest that the effects of hnRNP I are specifically involved in the regulation of translation of the virus through a cap-independent non-IRES element mechanism unique to Sindbis virus.

Perspectives and Limits of Understanding

Picking a Dance Partner- Who or What Decides Viral RNA Binding Specificity?

While host protein interaction studies have started to focus on specific RBPs and their interactions directly with the RNA, an interesting question is raised when multiple vRNAs are expressed from a single template- How might colinear RNAs have different binding repertoires despite having identical sequences? For example, hnRNP I selectively interacts with the subgenomic RNA and not the genome, despite them being the same RNA sequence (176). Most likely, RBPs have preferential binding sites due to their function during alphaviral infection, or even the function of the RNA. Studies from several different labs have found that different RBPs like hnRNP K, hnRNP I, and hnRNP M all preferentially bind to the subgenomic vRNA in distinct interactions to promote alphaviral infection (176, 228). The La protein, one of the first host proteins to be found to interact with vRNA, preferentially binds the 3' end of the negative strand to most likely promote viral replication (171). Additionally at the 3' region, HuR binds to the URE and promotes RNA stability (185, 186). At the opposite end, hnRNP A1 is involved in translation by binding to the 5' UTR of genomic vRNA (179, 223). While it is unknown exactly why these interactions are specific to a particular RNA strand, we can hypothesize that RBPs preferentially bind to the vRNA depending on the RBP function or even the RNA function as well. As a large part of the genomic vRNA strand and the minus strand do not translate, however the subgenomic strand does, it stands to reason that they may have very different repertoires of RBPs. Additionally, the

specificity conundrum may also be solved through the existence of dual occupancy interaction sites, such as that posed by the SINV capsid protein and the host hnRNP M protein (176, 264). In this instance binding specificity may be determined by the presence of the competing RNA binding proteins during infection. It should be noted that a secondary interaction partner for the cognate hnRNP I interaction site has not been identified, although the explorations of RBP binding remains woefully incomplete.

Backwards and in High Heels- Differential Host Functions and Requirements

As demonstrated by our data, and by others, RBP interactions are important during mammalian infection, but also there are many RBP homologues in other hosts of alphaviral infection, like mosquitoes. Proteins like HuR, the G3BP homologue Rasputin, hnRNP A1, and PTBP1 all have been characterized during infection of mosquito cell culture models (179, 185, 186, 193, 223, 246). These studies have varied in whether they utilized knockdown or disrupted protein binding by mutating the interaction site sequence, but almost always perturbing the RBP or the RBP interaction negatively affected alphaviral infection. The effects seen during mosquito cell line infection reflect the same effect in mammalian tissue culture, and the conclusions from the vertebrate system are assumed to be true for the invertebrate. However, it is not known if all RBP interactions have the same role in multiple hosts or if there are host dependent RBP interactions therefore, it is important to not apply the same conclusion to each system.

On a similar note, within a single host system there can be considerable variation in regard to RBP expression levels and patterns. This reality creates a patchwork of host systems at the cellular level that may, or may not, support viral replication should a critically necessary RBP be present or absent. A consequence of this may be tissue or cellular restriction, in addition to the possibility that the viral lifecycle is differentially regulated in different host cell systems.

Despite the ubiquity and essentiality of RBPs such as hnRNP I, the host specificities, proclivities, and functions of the RBPs differ to a great extent across and within the individual host. Thus, the importance of RBPs to viral infection needs to be better characterized across all potential host systems.

Taking Center Stage- Location of RBPs

In most cases, the roles of the RBP during infection and the interaction themselves are time dependent. During normal cellular function the majority of the RBPs, especially the hnRNPs, spend the better part of their time in the nucleus, and are shuttled back and forth to the cytoplasm during their normal functions in host RNA biology. During infections with alphaviruses, many of these RBPs shuttle out of the nucleus and stay in the cytoplasm around replication complexes for the remainder of infection. Many also have certain roles either early or late during infection, as experimental time courses have discerned the difference. The relocalization of RBPs during infection could be due to a number of factors including post-translational modifications like phosphorylation.

Proteins like HuR and hnRNP K have been shown to have different roles and localizations depending on the differential phosphorylation states of the proteins. During normal biology, HuR can be phosphorylated and this is associated with the localization of the protein. Studies by Dickson et al 2012 show that during SINV infection, HuR is dephosphorylated, and it is hypothesized that this causes the protein to relocate from the nucleus to the cytoplasm during infection, in which the vRNA sequesters HuR away like a sponge (188). Both forms of unphosphorylated and phosphorylated hnRNP K were found in the cytosol during SINV infection, although mostly the phosphorylated form interacted with the replicase machinery and subgenomic vRNA (228). It has been shown that hnRNP K has multiple phosphorylation sites, and these are all important for the different functions and locations of hnRNP K. While we do know that hnRNP K is phosphorylated during infection, we do not know the specific role the phosphorylated protein plays. In normal host biology, phosphorylation states can determine the role of RBPs, almost like a switch, and effects binding to RNA or shuttling out of the nucleus. These differential states most likely also effect alphaviral infection, although they have not been described or studied in detail.

Don’t Say ‘Break a Leg!’- Perspective Role of RBPs During Infection

One of the biggest questions that arises from studies of host proteins during alphaviral infection is whether these interactions are pro- or anti-viral. Unfortunately, despite these interactions being researched for decades, the answers to this question are extremely nuanced. Depending on the study, the

alphavirus, and the way of inhibiting the RBP, the effect could be interpreted as being either. For instance, HnRNP U has been shown to be antiviral and activate type-I IFNs during infection with DNA and RNA viruses, although this specific interaction has not been shown for alphaviruses (282). In Varjak et al, the knockdown of hnRNP M proved to be beneficial by increasing viral genomic and subgenomic expression and viral titer; however, studies from our lab have shown that disrupting the interaction with vRNA causes decreased viral titer, albeit while increasing structural protein expression (173, 176). Thus, while both studies concur that hnRNP M is important to infection, the precise nature of the interaction to alphaviral infection is nuanced. There may also be roles for the hnRNPs to repress innate immune sensing, as it binds to vRNA sensing proteins and it represses splicing and processing of immune transcripts. In the majority of studies, RBPs act in a pro-viral way since interrupting the interaction causes decreased viral growth along with other phenotypes as described in previous chapters. The dual evolution of host protein and alphaviral interactions have caused an already complex system to become more multifaceted. Alphaviruses have most likely hijacked the host system to benefit infection, although some RBPs still may have innate or intrinsic antiviral properties.

Future Directions

The project described in detail here characterizes the interaction between the host hnRNP I protein and the vRNA; however, as we have found there are many more RBP interactions with SINV vRNA that need to be characterized in

depth. In our previously published research, we found that disrupting the hnRNP:vRNA interactions decreased viral titer and despite hnRNP K, hnRNP I, and hnRNP M all having distinct interaction sites they all manifested a similar phenotype upon the disruption of their binding site. This led to the hypothesis that hnRNPs are possibly acting as a complex to regulate subgenomic translation.

All Together Now- hnRNP Complexes During Infection

HnRNPs, for example hnRNP I, have been previously shown to interact with each other, as the hnRNP K protein has a K-interactive (KI) region that acts as a docking site for other proteins (283). Determining if RBPs act as a complex would be beneficial for other alphaviral infections as well, as the interaction sites do not overlap among the different viral species. As found by Gebhart et al, there are many conserved RBP interactions, however they are not all in the same distinct region (177). An approach to determine this is to isolate the RNA:protein complexes by the RNA, and determine if there are multiple proteins attached, for example as in hnRNP K and hnRNP I together, by immunoprecipitation or mass spectrometry. Another interesting approach would be to replace the TAT peptide tagged hnRNP I with another RBP such as hnRNP K or hnRNP M and determine if the interaction is from a complex or from a singular protein. For this approach, similar experiments like the ones described in Chapter 3 would be executed: viral growth, RNA levels, and structural protein expression to determine if forcing the 'new' hnRNP would restore the phenotype back to wild-type like levels. Although the hnRNP K and hnRNP M interactions were in the coding regions, the new TAT

fused peptides would still have to be in the 3' UTR, as replacing the original hnRNP interaction sites with the BIVTAR sequence would disrupt critical coding regions of the structural proteins. However, because the original binding site would not be in the same location of the forced interaction, the interactions may not be equivalent.

Changing the Tempo of Structural Gene Expression Negatively Impacts Glycosylation

Although the work here establishes that hnRNP I interaction with the vRNA is important to infection, it still is unknown how exactly increased structural protein synthesis causes aberrant glycosylation. One possible mechanism is that an increase of structural proteins could overwhelm host processes, as alphaviruses are reliant on the host to glycosylate the viral glycoproteins prior to assembly of the viral particle (162). This could lead to several outcomes affecting the glycosylation profile including the partial or full lack of glycosylation, or erroneous or immature glycosylation of the viral glycoproteins. As seen in the data described in Chapter 3, increasing structural protein expression by disrupting the hnRNP:vRNA interaction site caused a loss of sensitivity to the deglycosylation of the viral particles by PNGase F treatment implying that the glycosylation status of the viral particle was already disturbed. Concluding from these data, one could imply that there could be no glycosylation residues present in the mutant particles, that the particles could be partially glycosylated resulting in the incorporation of incomplete or nonfunctional glycoprotein spikes, or that the host glycosylation machinery is transferring immature glycans that natively decrease infection and

attachment to cells. Determining exactly how glycosylation is disrupted in the hnRNP I viral mutant would be beneficial to understanding the role of hnRNP I during infection. The isolation and digestion of the viral glycoproteins of the wild type and hnRNP I interaction deficient mutants for mass spectrometry could initially tell us if the viral glycoproteins have aberrant or deficient glycosylation states. If a molecular bottleneck is to blame, then the addition of either the oligosaccharide parts (to enhance metabolic availability) or the overexpression of *N*-glycosyltransferase enzymes during the infection with the hnRNP I interaction deficient virus could rescue viral attachment and growth kinetics. While this may not determine the precise effect of the loss of hnRNP I binding on viral infection, it does elucidate how the interaction may impact glycosylation and its role to infection.

Glycosylation modifications are important for functional viral glycoproteins as they contribute to the correct folding of the glycoproteins to form a fully functional viral particle. In fact, a study from Ren et al 2022 confirms this notion by interrupting the interdimer interaction that is between E2 of one heterodimer and E1 of another heterodimer via mutating six E2 amino acid residues at the interdimer interface (284). Very similar to the results we have found, the interdimer interaction disruption caused nonspherical and fragile viral particles by disrupting the folding of the trimeric spikes of the glycoprotein dimers. Revertant site mutants interfered with the glycosylation sites of the viral glycoproteins, and these revertant mutations allowed for better protein folding and glycoprotein trimerization in the mutants (284). Not only is glycosylation important for the correct folding of the

glycoproteins, but it is important for attachment to the host cells. Attachment and infection of cells is important for virulence, as mouse models infected with RRV mutants lacking glycosylation have reduced disease and increased viral clearance (161, 285). Glycosylation is closely linked to virulence and recognition by the host immune system. Given the impact of the loss of hnRNP I binding on glycosylation, it is important to continue this interaction which may lead to possible vaccine candidates. However, glycosylation states may also contribute to host tropism, as expression of receptors that bind to N-linked glycosylation on nonpermissive cells will allow for alphaviruses to infect them (286).

Concluding Remarks

Host RBPs are critical for a successful alphaviral infection. While the direct consequences of many host RBPs binding to the viral RNAs remains unknown to infection, the work in this dissertation elucidates the role of hnRNP I binding to the subgenomic vRNA and defines how the hnRNP I:vRNA interaction impacts infection. Through the results presented here, we can conclude that hnRNP I regulates structural protein translation, although the exact mechanism(s) underlying this phenomenon are yet to be fully discovered.

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GLOSSARY

ARE	AU-rich element
AUC	area under the curve
AUD	Alphavirus Unique Domain
BIV-TAR	Bovine Immunodeficiency virus Transactivation RNA Response element
CHIKV	Chikungunya virus
CLAMP	Cross-link-assisted mRNA purification
CLIP-seq	Cross-linking and immunoprecipitation sequencing
CP	Capsid
CSE	Conserved sequence element
EEEV	Eastern Equine Encephalitis Virus
GFP	Green fluorescent protein
gRNA	Genomic RNA
HCV	Hepatitis C virus
hnRNP	Heterogenous nuclear ribonucleoproteins
hpi	Hours post infection
HPV	Human papillomavirus
HVD	Hyper Variable Domain
IFN	Interferon

KI	K-interactive
MALDI-TOF	Matrix-assisted laser desorption / ionization-time of flight
MAYV	Mayaro Virus
MOI	multiplicity of infection
MS	Mass spectrometry
NSAIDs	Non-steroidal anti-inflammatory drugs
NW	New World
ORF	Open reading frame
OW	Old World
P:P	Protein:Protein
PFU	plaque forming units
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
RBP	RNA binding proteins
RDRP	RNA-dependent RNA polymerase
RepC	Replicase complex
RRM	RNA recognition motif
RRV	Ross River virus
RSE	Repeated sequence element
SFV	Semliki Forest virus
sgRNA	Subgenomic RNA
SINV	Sindbis virus
TAT	bovine immunodeficiency transactivator
TEM	transmission electron microscopy

TF	TransFrame
TNF	Tumor necrosis factors
URE	U-rich element
UTR	Untranslated region
VEEV	Venezuelan Equine Encephalitis Virus
vRNA	Viral RNA
WEEV	Western Equine Encephalitis Virus

CURRICULUM VITAE

Claire Westcott

Microbiology and Immunology
University of Louisville
Clinical Translational Research Building Rm 642 E
Louisville, KY 40292

Education

PhD, Microbiology and Immunology (July 2022), University of Louisville School of Medicine
BS, Biological Sciences (2016), University of Missouri

Research Experience

Graduate Student (2017-2022), Department of Microbiology and Immunology, University of Louisville

Research Technician (2016-2017), Sokoloski Lab , University of Louisville

Research Assistant (2014-2016), Mawhinney Lab, University of Missouri

Collaboration and Leadership

Louisville Science Pathways Director (January 2020 – August 2021)
Science Policy and Outreach Group, University of Louisville
Admissions Committee Student Representative (July 2020 – June 2021)
Microbiology and Immunology Student Organization, University of Louisville
President (July 2019 – June 2020)
Microbiology and Immunology Student Organization, University of Louisville
Director (July 2018 – June 2020)
Science Policy and Outreach Group, University of Louisville
Organizational Board Member for Science Policy and Outreach Group (December 2019 – May 2020)
Louisville Regional Science and Engineering Fair (LRSEF)
Policy Coordinator (July 2017 – June 2018)
Science Policy and Outreach Group, University of Louisville
Chief Panhellenic Officer (December 2013 – August 2014)
Kappa Alpha Theta, Alpha Mu chapter, University of Missouri

Publications and Presentations

Publications

Westcott CE, Qazi S, Maiocco AM, Mukhopadhyay S, Sokoloski KJ. Binding of hnRNP I–vRNA Regulates Sindbis Virus Structural Protein Expression to Promote Particle Infectivity. *Viruses*. 2022; 14(7):1423. https://doi.org/10.3390/v14071423

LaPointe AT, Landers VD, **Westcott CE**, Sokoloski KJ. Production of Noncapped Genomic RNAs Is Critical to Sindbis Virus Disease and Pathogenicity. *mBio*. 2020 Dec 1;11(6):e02675-20. DOI: 10.1128/mBio.02675-20. PMID: 33262258; PMCID: PMC7733944.

Sokoloski, K., Nease, L. M., **Jones, C**., Gebhart, N. N., and Hardy, R. W. Identification of Cytoplasmic Sindbis Virus Capsid/vRNA Interactions as a Novel Virulence Determinant. *PLoS Pathog* 2017, 13(6):e1006473.

Oral Presentations

Westcott, C., K. Sokoloski (May 2022) Reconstituting Sindbis vRNA:Protein Interactions in Deficient Mutants via Protein Tethering ASV, Flash Talk in Togaviridae Section

Westcott, C., K. Sokoloski (July 2021) Reconstituting Sindbis vRNA:Protein Interactions in Deficient Mutants via Protein Tethering ASV, Flash Talk in Togaviridae Section

Westcott, C., K. Sokoloski (April 2021) It's Not Me, It's You- Breaking up Sindbis Viral RNA and Host Protein Interactions. Regional ASM, Virtual Presentation in Microbes and Molecular Biology Section

Westcott, C., K. Sokoloski (December 2020) Characterizing Sindbis Viral RNA and Host Protein Interactions. Regional ASM, Virtual Presentation in Alphaviruses Section

Westcott, C., K. Sokoloski (June 2020) Disrupting the Status Quo: Breaking Sindbis Viral RNA and Host Protein Interactions. ASV, Virtual Presentation in Togaviridae Section

Jones, C., K. Sokoloski (July 2019) Disruption of Sindbis Virus hnRNP-vRNA Interactions Results in Dysfunctional Particles. ASV Conference, Minneapolis MN, Presentation in Togaviridae Section

Poster Presentation

Westcott, C., K. Sokoloski (May 2022) Elucidating the Role of a Host Protein during Sindbis Infection via Protein Tethering. Colloquium on Inflammation and Pathogenesis, Louisville KY, 1st Place

Westcott, C., K. Sokoloski (October 2021) Characterization of Sindbis vRNA:Protein Interactions in Deficient Mutants via Protein Tethering Research!Louisville, Louisville KY

Jones, C., K. Sokoloski (September 2019) Disruption of Sindbis Virus hnRNP-vRNA Interactions Results in Dysfunctional Particles. Research!Louisville, Louisville KY

Jones, C., K. Sokoloski (November 2018) Disruption of Sindbis Virus hnRNP-vRNA Interactions Results in Misformed Particles. Regional ASM Conference, Murfreesboro TN

Jones, C., K. Sokoloski (October 2018) Disruption of Sindbis Virus hnRNP-vRNA Interactions Results in Misformed Particles. Research! Louisville, Louisville KY

Jones, C., K. Sokoloski (August 2017) Lost in Translation- Analysis of the Sindbis Virus nt10400 Capsid:vRNA Interaction Site. Midwest Microbial Pathogenesis Conference, South Bend IN

Honors

Best Poster Presentation Award, Colloquium on Inflammation and Pathogenesis (2022)
Flash Talk Upgraded to Full Presentation, American Society for Virology, Togavirus Virtual Sessions (2020)
Invited Guest, American Association of University Women, Louisville Chapter (2020)
American Society for Virology Student Travel Grant (2019, 2022)
AAAS/Science Program for Excellence in Science (2019)
University of Louisville Graduate Student Council Travel Award (2019)

Professional Memberships

American Medical Writers Association
American Society for Microbiology
American Society for Virology
American Association for the Advancement of Science
Graduate Women in Science
American Society for Cell Biology

Certifications

AMWA Express Skills Certificate, *In Progress*
American Medical Writers Association
ASCB Biotech Course Certificate, 2021
American Society for Cell Biology, Keck Graduate Institute, UMass Manning School of Business