CHARACTERIZATION OF V5 TAGGED RIFT VALLEY FEVER VIRUS (RVFV) AND IDENTIFICATION OF HOST PROTEINS THAT INTERACT WITH RVFV

by

Sahar Saleem

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Characterization of V5 Tagged Rift Valley Fever Virus (RVFV) and Identification of Host Proteins That Interact with RVFV

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Bachelor of Science
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DEDICATION

This is dedicated to my wonderful family specially Athar mamun for I wouldn't have made it this far without their unconditional love and unwavering support.

ACKNOWLEDGEMENTS

I would like to thank my thesis director, Dr. Kylene Kehn-Hall, for her mentorship and guidance in the past two years. I am very grateful to be given the opportunity to learn from an amazing mentor like her. I appreciate her taking me under her wing and encouraging me the way she did.

I would also like to thank Dr. Cynthia DeLafuente for holding my hand when I first started in lab and lending invaluable advice and guidance. I would like to thank Brian Carey, Chelsea Pinkham, Lindsay Lundberg and Shi Chao Lin for always being there and helping out in every way possible. I am grateful to have had the opportunity of working with such a wonderful lab group! It was a pleasure spending time with you all in lab and providing me with the numerous chances to learn from your expertise and knowledge.

I want to express my gratitude to my committee members, Dr. Aarthi Narayanan and Dr. Anne Scherer. Thank you for your advice and support along the way.

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LIST OF ABBREVIATIONS

Dulbecco's Modified Eagle Medium.....DMEM
Dimethyl sulfoxide.....DMSO
Fetal Bovine Serum..... FBS
Multiplicity of Infection.....MOI
Phosphate Buffered Saline..... PBS
Rift Valley Fever Virus.....RVFV
Ribonucleic Acid ..... RNA
Human small airway epithelial cells.....HSAECs
Ethylenediaminetetraacetic acid .....EDTA
Bovine serum albumin .....BSA
4',6-diamidino-2-phenylindole..... DAPI
Immunoprecipitation ..... IP
Liquid Chromatography/MassSpectometry..... LC-MS/MS

ABSTRACT

CHARACTERIZATION OF V5 TAGGED RIFT VALLEY FEVER VIRUS (RVFV) AND IDENTIFICATION OF HOST PROTEINS THAT INTERACT WITH RVFV

Sahar Saleem, M.S.

George Mason University, 2019

Thesis Director: Dr. Kylene Kehn-Hall

Rift Valley Fever Virus (RVFV) belongs to the genera *Phlebovirus* in the family *Phenuviridae*. It is an arbovirus, which can be transmitted by vector mosquitoes. RVFV infects livestock and humans. Infection of pregnant ruminants, including goats, sheep and cattle, results in high abortion rates. Rift Valley fever (RVF) ranges from subclinical to fatal and may cause fetal malformation. In adult sheep, the mortality rate is about 20% while lambs have a higher mortality rate. In humans, the incubation period for the virus is about 6-7 days and RVFV presents with a self-limiting febrile illness which includes malaise, headaches and nausea. In some cases the symptoms may progress to neurological disorders, hemorrhagic fever and/ or ocular disease. RVFV has been classified as a category A pathogen by the National Institute of Allergy and Infectious Diseases (NIAID) as it has the potential for an outbreak and poses the highest threat to national security. It is considered an overlap select agent by the Centers of Disease

Control (CDC) and United States Department of Agriculture (USDA). There are currently no FDA approved therapies or vaccines available to the public for RVFV infection. It therefore is important to look at the interaction of viral proteins with host proteins so they can be targeted for therapeutics against RVFV. Gn is one of the glycoproteins of RVFV which plays an important role in the assembly of the virion. The host protein partners of RVFV Gn are largely unknown. Therefore, we aim to identify Gn protein partners using a proteomic-based approach (immunoprecipitation followed by mass spectrometry). To facilitate this analysis and to provide a tool for future research, two V5 tagged MP12 viruses were made namely V5Gn^{105} and V5Gn^{229}. Our data indicates that the recombinant viruses were functionally similar to the wild type MP12. Our research identified E3 Ubiquitin-protein ligase UBR4 (UBR4) as one of the host protein partners that interacts with RVFV Gn. Knockdown of UBR4 from cells decreases the viral titers from infected cells. Based on our findings that UBR4 knockdown cells have an increased retention of virions, we hypothesize that UBR4 plays a role in the egress of virions from the cells.

INTRODUCTION

Background

Rift Valley fever virus (RVFV) belongs to the genera *Phlebovirus* in the family *Phenuviridae*. It is the agent that causes Rift Valley Fever (RVF). It is an arbovirus, which can be transmitted by mosquitoes. RVFV infects livestock and humans (1). Infection of pregnant ruminants, including goats, sheep and cattle, results in high abortion rates (2). RVF ranges from subclinical to fatal and may cause fetal malformation. In adult sheep, the mortality rate is about 20% while lambs have a higher mortality rate (1). In pregnant sheep and cattle the abortion rate is almost 100%, this is also referred to as an "abortion storm" (10).

In humans, the incubation period for the virus is about 6-7 days and RVFV presents with a self-limiting febrile illness which includes malaise, headaches and nausea. In some cases the symptoms may progress to neurological disorders, hemorrhagic fever and/ or ocular disease (1). Patients with milder symptoms recover completely, while those with more debilitating symptoms persist (4). Humans can get infected not only by mosquito bites but also while handling carcasses of dead animals or aborted fetuses (14).

RVFV was first recognized in Rift Valley in Kenya in the 1930s. It has since become endemic to Africa and has spread to the Arabian Peninsula. RVF outbreaks occur

following periods of heavy rainfall when water accumulates and serves as breeding grounds for mosquitoes (13). The spread of RVFV to the Arabian Peninsula across a large body of water shows that the virus is able to cross geographical barriers.

Outbreaks of RVFV pose a huge risk to the livestock and human population as it has the potential to wreak havoc on economies. It is classified as a Category A pathogen by the NIAID and a select agent by the CDC and USDA (4). With the increase in international travel, there has been an increase in the risk of spreading RVF to the United States as mosquitoes that can spread the disease are present along with animals that are capable of mounting high viral loads (10). Currently there are no licensed vaccines for humans so there is a pressing need for the development of vaccines that will help prevent and control the spread of RVFV as the spread of RVFV seems to be on the rise (4). Therefore, it is important to develop vaccines and look for antiviral therapies to deal with this possible threat.

RVFV has a trisegmented, single stranded RNA genome with a negative or ambisense polarity depending on the segment. L and M segments have a negative polarity while the S segment has ambisense polarity. L codes for the viral RNA-dependent RNA polymerase (RdRp) while the M segment codes for the glycoproteins Gn and Gc. It also codes for a nonstructural protein NSm1 and if merged with Gn, it is called Nsm2. Gn, Gc, NSm and NSm-Gn fusion proteins are processed from the M segment and are produced as a polyprotein which is then cleaved to form individual proteins (18). The S segment encodes for nucleoprotein (N) on the genomic RNA and it codes for NSs on the antigenomic RNA (Figure 1).

RVFV replicates in the cytoplasm and the particles assemble in the cisternae of Golgi apparatus. Gn and Gc, the envelope glycoproteins are expressed as a polyprotein, with the ER localization signal found at the C-terminal end of Gc. In contrast, Gn contains a Golgi localization sequence and Gc localizes to the Golgi via physical interaction with Gn signal (8). The Golgi localization signal on Gn is about 48 amino acids out of which 20 are present in the transmembrane portion of Gn and the remaining 28 are present in the cytoplasmic tail (6). Multiple translation initiation sites are present on the M segment. These result in the production of a nested set of polyproteins via a leaky scanning mechanism (22). The polyprotein is cleaved into Gn and Gc using signal peptidases (22). The Gn and Gc are further modified in the ER and Golgi by N-glycosylation. They glycosylation site on the pre-Gn region prevents the cleavage of NSm and Gn which makes the NSm/Gn fusion protein and Gc (23).

N interacts directly with the cytosolic tails of Gn and Gc because of the absence of a matrix protein. Gn is required for the packing of RdRp and N into the virus particles. The first 30 amino acids on the cytosolic tail of the Gn are required for it to bind to N while the last 40 are required for its binding to RdRp (9). The encapsidated genome contains a packaging signal which is recognized by Gn. The presence of complementary termini on the genome leads to the formation of double stranded RNA structures, referred to as panhandles. These carry sequences necessary for packaging into virions in addition to having the promotors needed for replication and translation (9).

The host protein partners that RVFV Gn interacts with are largely unknown. Therefore we aim to identify Gn protein partners using a proteomic-based approach (immunoprecipitation followed by mass spectrometry). To better our understanding of the role played by Gn in the assembly of the virion, we wanted to identify the host proteins that interact with Gn. This was accomplished using a V5 epitope tag that was inserted into Gn. The V5 tagged viruses were then compared to the parental MP12 virus. It was experimentally determines that the functionality of Gn was not disrupted.

MATERIALS AND METHODS

Cell Culture and Primary Cell Culture:

Vero (African Green Monkey Kidney Cells) and U87MG (human astrocytes) cells were cultured and grown in complete growth medium, Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% fetal bovine solution (FBS). Human Small Airway Epithelial Cells (HSAECs) were cultured and grown in basal Ham's F12 modified media supplemented with 10% FBS, 1% non-essential amino acids (NEAA), 1% L-glutamine, 1% sodium pyruvate, 0.1% β-mercaptoethanol. Huh7 cells were cultured and grown in DMEM supplemented with 10% FBS, 1% L-glutamine, 1% sodium pyruvate and 1% NEAA. C6/36 cells were cultured and grown in Eagle's Minimum Essential Medium (EMEM) media containing 10% FBS. Cell lines were maintained at 37°C in a humidified environment supplemented with 5% CO_2 with the exception of C6/36 cells that were maintained at 28°C in a humidified environment supplemented with 5% CO_2.

Immunofluorescent confocal microscopy:

Huh 7 cells were seeded on glass cover slips in 6 well plates at 5 x 10^5 cells/well. Three wells were infected with MP12 at an MOI of 1. After 24 hours, cells were washed with PBS and fixed with 4% paraformaldehyde for 10 minutes at room temperature. Cells were then permeabilized with 0.1% Triton-X in PBS for 10 minutes and blocked with 1%

BSA and 0.05% Tween-20 for 1 hour at room temperatue. α-Gn (BEI Resources, Catalog #: NR-43190), α-UBR4 (Abcam, Catalog #: ab86738), and α-TGN46 (BioRad, Catalog #: AHP500GT) antibodies were added for two hours at room temperature. 1:500 of secondary antibodies α-mouse, α-rabbit, and α-sheep respectively, (Thermo Fisher Scientific, Catalog #: A21204, A21206, and A21099) were added to the coverslips for one hour in dark. Cells were stained with DAPI (1:1000) and incubated in dark for 10 minutes to visualize nuclei. Flouromount G was used to mount coverslips on slides and a Nikon Eclipse TE 2000-U microscope with a 60x immersion was used to image them. Three images were taken per sample, one of which was shown as a representative image.

siRNA Transfection:

Knockdown of gene expression was achieved by transfection of small interfering RNAs (siRNAs). Negative control siRNA (Dharmacon, Catalog #: D-001810-01-05) as well as siRNA against UBR4 (Dharmacon, Catalog #: L-014021-01-0005) was used. 50nM of the negative control RNA was used and 50nM of siRNA against siUBR4 was used for knockdown. Transfection reagent was replaced with fresh media 24 hours after it was applied. Cells were infected with MP12 (vaccine strain of RVFV) at an MOI of 0.1, 24, 48 or 72 hours after transfection. Protein lysates and viral supernatants were collected at 8 and 24 hours post infection. Plaque assays were used to determine viral titers in supernatants while western blotting was used to analyze protein lysates.

Immunoprecipitation and Western Immunoblotting:

10^7 Huh 7 cells were seeded in two T225 flasks. One flask was infected with $V5Gn^{229}$ at an MOI of 1 and one was kept with media as a control. Cells were lysed 24 hours post infection with a buffer containing 50mM Tris-HCl, 120mM NaCl, 5mM EDTA, 0.5% NP-40, 50mM NaF, 0.2mM Na3VO4, 1 Protease Cocktail tablet. 10mg of the α-V5 antibody (BioRad, MCA1360) was added to 2 mg of whole cell lysates and incubated for 2 hours. 50μL of protein G conjugated dynabeads (Thermo Fisher Scientific, Catalog #: 1004D) were then added to the lysates and the mixture was incubated for 45 minutes. Antibody bound complexes were washed 3 times with TNE150 (Tris-HCl, 150 mM NaCl and EDTA) and 0.1% NP40 and 1 time with TNE50 (Tris-HCl, 50 mM NaCl and EDTA) and 0.1% NP40. 30μL of blue lysis buffer was then added to the samples and they were boiled for 10 minutes. Blue lysis buffer was made using 25ml 2x Novex Tris-Glycine Sample Loading Buffer SDS (Invitrogen, Catalog #: LC2676), 20ml T-PER Tissure Protein Extraction Reagent (Thermo Scientific, Catalog #: 78510), 200ul 0.5M EDTA pH 8.0, 2 complete Protease Cocktail tablets, 80ul 0.1M Na3VO4, 400ul 0.1M NaF, 1.3ml 1M DTT. 30μL from each sample was run on a 3-12% tris glycine gel and transferred onto a Polyvinylidene difluoride (PVDF) membrane. After the membranes were blocked with PBS + 0.1% Tween-20 containing 5% non-fat dried milk (NFDM) at room temperature for 30 minutes, they were incubated with 1:20,000 α-Gn primary antibodies [BEI Resources, Catalog #: NR-43190] in PBS-T 5% NFDM overnight at 4°C. Then, the blots were incubated with 1:5000 secondary HRP-coupled antibody cocktail in PBS-T 5% NFDM for 2 hours at room temperature. The western blots were visualized by

chemiluminescence using SuperSignal West Femto Maximum Sensitivity Substrate kit (ThermoScientific) and a Bio Rad Molecular Imager ChemiDoc XRS system.

Plaque Assays:

$1.5*10^5$ Vero cells were seeded in 12 well plates 24 hours before infection. Serial dilutions of viral supernatants collected were made from 10^{-1} to 10^{-6}. 200 μL of each dilution was added to a well. The plates were placed in the 37°C incubator for one hour with occasional rocking. One hour post infection, the wells were overlaid with a 1:1 mixture of 0.1% agarose and 2x EMEM supplemented with 5% FBS, 1% L-glutamine, 1% penicillin/streptomycin, 1% non-essential amino acids, and 1% sodium pyruvate. Plaques were fixed 72 hpi with 10% formaldehyde for two hours at room temperature. The agarose plugs were removed and the plaques were stained with a solution of 20% methanol and 1% crystal violet to be visualized. The viral titer in pfu/mL was calculated by multiplying the counted number of plaques to the dilution factor (5) to the dilution.

MP12 and V5 tagged MP12:

The V5 tagged viruses were made using the reverse genetics system. The BSR T7/5 cells were transfected with plasmids including 2ug pProT7-M(+), 2ug pProT7-L(+), 2ug pProT7-S(+), 2ug pT7-IRES-vN, 1ug pT7-IRES-vL, and 1ug pCAGGS-vG to make the MP12 virus. For the $V5Gn^{105}$ and the $V5Gn^{229}$, the pProT7-M(+)_$V5Gn^{105}$ or pProT7-M(+)_$V5Gn^{229}$ were used. BSR-T7/5 cells were seeded at a density of $1x10^6$ cells in a T25 flask one day before transfection. 30 uL of TransIT-LT Mirus was in 385 uL of

MEM media was used to make the transfection mix. This was added to the flask with BSR-T7/5 cells and incubated at 37°C and 5% CO_2. The media was replaced 24 hours post transfection and supernatants were harvested at 4 days post infection. This was then used to make the passage 1 viral stocks.

Cell Viability Assay:

Huh7 cells were plated in a 96-well plate and incubated at 37°C and 5% CO_2. After 24 hours, the cells were transfected with siNTC or siUB4 and the transfection media was left on for 48 hours after which it was replaced with complete media. At 72 hours post transfection, the cells were analyzed using CellTiter-Glo Luminescent Viability Assay (Promega, Catalog #: G7570) according to the protocol provided by the manufacturer. The reagents and cells were brought to room temperature and the supernatant in wells was replaced with a 1:1 ratio of complete media and CellTiter-Glo reagent. Cells were lysed on the plate shaker for two minutes and then read using the Beckham Coulter after 10 minutes to let the luminescence signal stabilize. Data was recorded and analyzed.

Intracellular Infectivity Assay:

Huh7 cells were plated in a 6-well plate and incubated at 37°C and 5% CO_2. After 24 hours, the cells were transfected with siNTC or siUBR4 and the transfection media was left on for 48 hours after which it was replaced with complete media. At 72 hours post transfection, cells were infected with MP12 at an MOI of 0.1. Intracellular virus was collected at 24 hpi along with extracellular viral supernatants. To collect intracellular

virus, cells were trypsinized and complete media was used to neutralize the trypsin. The cells were pelleted and washed three times with complete media. 500 uL of media was added after the last wash. Cells were then processed through four freeze thaw cycles. They were frozen in an ethanol and dry ice bath and thawed in the water bath at 36°C to release the intracellular virions. The debris was pelleted and the supernatant was used for a plaque assay. % intracellular infectivity was calculated using the absolute pfu.

RESULTS

MP12 viruses with V5-tagged Gn (V5Gn^{105} and V5Gn^{229}) have similar viral replication kinetics as MP12:

MP12, the vaccine strain of RVFV (non-FDA approved for public), was chosen for this study as it can be handled at biosafety level 2 (BSL-2). Gn is present on the surface of the virion and has three known antigenic epitope regions present on the surface (labeled as regions I, II, and III in Figure 1). The V5 tag is an epitope tag derived from the p and v proteins of the paramyxovirus of Simian virus 5. It is 16 amino acids in length. The V5 tag was inserted in the 105th or 229th positions of Gn (Figure 1) as a means to effectively isolate Gn as commercially available Gn antibodies are limited. These viruses were named V5Gn^{105} and V5Gn^{229}. The positions for the V5 tag insertion were selected so the V5 tag would end up exposed on the surface of the virion for easy detection by the V5 antibody.

In order to determine if there were any differences in viral kinetics of the MP12 virus compared to the V5 tagged viruses, three cell lines were infected with either MP12, V5Gn^{105} or V5Gn^{229}. We analyzed Huh7 (hepatocyte derived carcinoma cell line, which provide a good model for replication of RVFV in the liver), HSAECs (Human Small Airway Epithelial cells, which provide a model for the infection of RVFV through aerosol exposure), and C6/36 (cells from the Aedes albopictus mosquito to show

replication kinetics in mosquito cells as RVFV can replicate efficiently in mosquitoes). The viral titers of the V5 tagged viruses were similar to the MP12 tagged viruses. The viral titer increased from 3hpi (hours post infection) to 48hpi (Figure 2). There was a rapid increase in the viral titer for all three viruses between 3 and 24hpi and the viral titers reached a plateau between 24hpi and 48hpi.

Confirmation of V5 presence in V5Gn^{105} and V5Gn^{229}:

In order to make sure that the recombinant viruses expressed the V5 tag and to assess its impact on Gn expression, HSAECs, Huh7s, and C6/36 cells were infected with V5Gn^{105} and V5Gn^{229}. Cell lysates were collected and western blot analysis performed to determine the presence of the V5 tag. The blots show that levels of Gn 24hpi are similar to the show Gn as a control. The expression of Gn and V5 tagged Gn is similar between different viruses across the three cell lines (Figure 3).

Gn and Gc produced from V5Gn^{105} and V5Gn^{229} are similar in localization to Gn and Gc produced from parental MP12:

To determine if addition of the V5 tag to Gn alters the spread of Gn or Gc in the cells, confocal microscopy was utilized. It is important to look at the localization of Gc in addition to Gn because Gn has the Golgi localization signal which guides both the glycoproteins to the Golgi. HSAECs were infected with MP12, V5Gn^{105} or V5Gn^{229} at an MOI of 1. Cells were fixed 24 hours post infection and analyzed using immunofluorescence microscopy. Staining was performed for calnexin and TGN46, as

markers of the endoplasmic reticulum (ER) and Golgi, respectively, in addition to Gn and Gc staining. This was done because viral glycoproteins are synthesized in the ER before they localize to the Golgi; therefore it is expected that Gn and Gc colocalization with ER and Golgi will be observed. The ER and the glycoproteins were observed to localize in a similar fashion in both the parental and V5 tagged viruses (Figure 4A and 4B). In addition, the glycoproteins Gn and Gc were found localizing with the Golgi in all viruses (Figure 4C and 4D). The results indicate that insertion of the V5 tag did not disrupt the localization of Gn and Gc.

UBR4 interacts with Gn:

The V5 tagged viruses were created to study host proteins that interact with Gn. Even though more Gn can be visualized after immunoprecipitation with the Gn antibody, the Gn antibody shows nonspecific bands when the efficiency of isolation is looked at using immunoprecipitation with the Gn antibody and western blotting with UBR4. The V5 tagged viruses are a better way to isolate proteins that interact with viral Gn and it provides cleaner blots when analyzed with western blotting (Figure 5A).

In order to determine the protein partners that interacted with Gn, Huh7 cells were infected with MP12 and V5Gn^{229} at an MOI of 1. Next, immunoprecipitation with the V5 antibody was performed and samples subjected to mass spectrometry analysis which revealed the proteins that interacted with the V5 tagged Gn. Untagged MP12 virally infected cells were processed in parallel as a control. Proteins identified in this sample

were considered background and subtracted from our analysis. Viral and host proteins were among those that were identified (Table 1). The proteins that were identified included host cytoskeletal proteins like actin and myosin and some cellular trafficking proteins like Clathrin. E3 Ubiquitin-protein ligase UBR4 (UBR4) is one of the host proteins that was seen as interacting with RVFV Gn. It is one of the more novel proteins identified, the role of which has not been investigated in RVFV infection.

The ability of Gn to interact with UBR4 was confirmed using immunoprecipitation and western blot analysis. Once again Huh7 cells that were infected with the V5 tagged MP12 (V5-229) were immunoprecipitated with the V5 antibody and a western blot performed for UBR4. The western blot confirmed the interaction of the V5 tagged MP12 with UBR4 (Figure 5B). MP12 infected Huh7 cells along with uninfected Huh7 cells were used as a negative control for immunoprecipitation with V5. Mock Huh7 cells were used as a negative control for immunoprecipitation with Gn and then western blot for UBR4. MP12 and V5Gn^{229} both can be shown interacting with UBR4.

Optimization of UBR4 knockdown and cell viability post UBR4 knockdown:

In order to see how the presence of UBR4 influences the efficiency of viral replication, UBR4 was knocked down from Huh7 cells using siRNA against UBR4 and siNTC (non-template control) as a control. Huh7 cells were transfected with different concentrations of siRNA against UBR4 including 25, 50 and 75 nanomoles all at 48, 72 and 96 hours post transfection. The cell lysates were collected and western blot analysis

performed for UBR4. The most efficient knockdown was achieved at 72 hours post transfection and with 50 nanomoles of siUBR4. The transfection efficiency turned out to be more than 80% (Figure 6).

To determine if loss of UBR4 affected cell viability, we measure ATP production using the Cell titer Glo assay. Huh7 cells were examined at 72 hours post siRNA transfection. Compared to the cells that had been transfected with siNTC, cells that had been transfected with siUBR4 were more viable (Figure 7). That means the cells were still viable after transfection and UBR4 knockdown did not harm the cells.

Decrease in viral titer after UBR4 knockdown:

To determine if UBR4 knockdown affects the replication of RVFV, cells were transfected with siUBR4. The cells were then infected with MP12 at an MOI of 0.1. Extracellular supernatants were collected at 8 and 24 hours post infection (hpi) and plaque assay was used to determine the viral titers. The viral titer after siUBR4 transfection decreased between four- and five-fold compared to transfection with the siNTC at 24 hpi, with no difference observed at 8 hpi (Figure 9). This shows that removal of UBR4 from cells interferes with the production of RVFV virions.

Increased intracellular viral titers after UBR4 knockdown from Huh7 cells:

To determine if UBR4 knockdown changes trafficking of RVFV through the cells or affects viral spread or replication in the cells, intracellular infectivity was measured. Huh7 cells transfected with siUBR4 were infected with MP12 at an MOI of 0.1. This

time intracellular virus was collected in addition to extracellular virus and the viral titers in siUBR4 and siNTC cells were compared. Sorafenib treated cells were used as positive control for increased intracellular infectivity as Sorafenib inhibits the egress of virions from the cells (21). There was an increase in the viral levels inside the cells after UBR4 was knocked down (Figure 10). Huh7 cells that had been infected with MP12 (MOI 0.1) and treated with sorafenib acted as a positive control for increased intracellular retention of virus. There was a significant difference between the percent infectivity of the siUBR4 cells compared to siNTC. It is suspected that UBR4 plays a role in helping the virus egress from the cell.

FIGURES

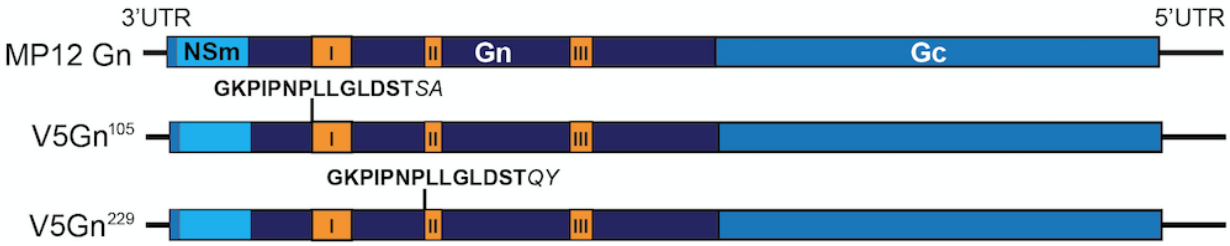

Figure 1: Gn of the V5-229 and V5-105 tagged viruses along with the Gn from MP12 (parental) strain of the virus.
I, II, and III represent the antigenic epitopes.

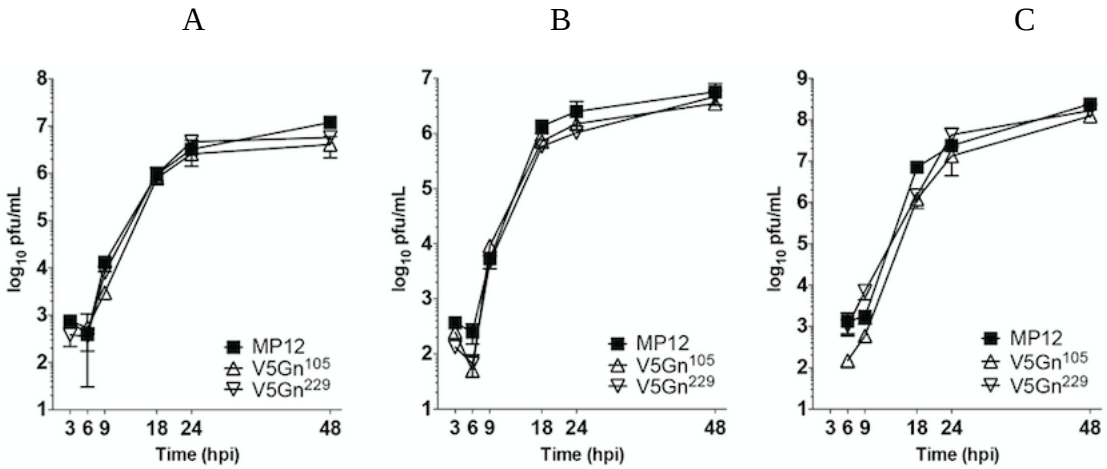

Figure 2: Viral replication kinetics of MP12, V5-105, and V5-229 viruses.
HSAECS (A), Huh7 (B), and C6/36 (C) were seeded in 12 well plates and infected with the wild type or V5 tagged viruses at an MOI of 0.1, supernatents were collected at the indicated timepoints and plaqued in Vero cells to determine the viral titer in pfu/mL. The figure shows an average of the three replicates for each time point with the standard deviation shown by the bars.

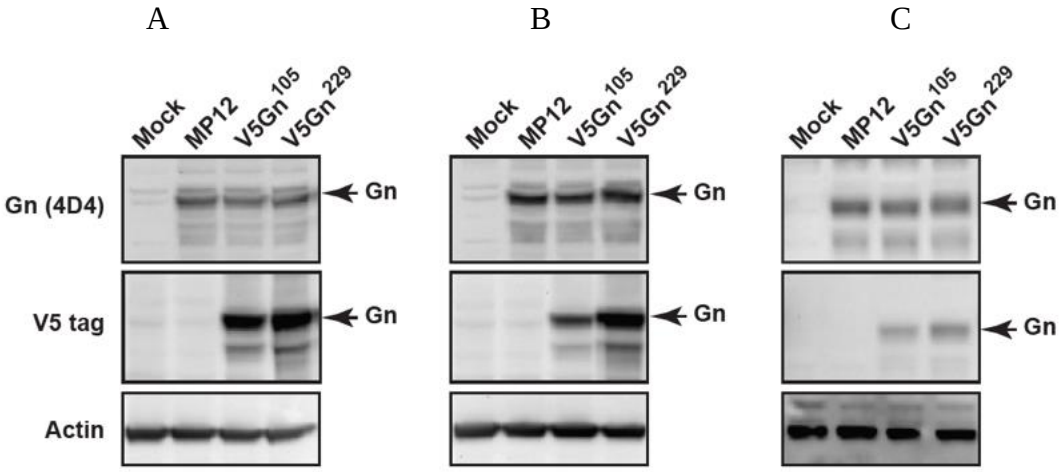

Figure 3: Gn and V5-Gn expression from MP12, V5-105, and V5-229 viruses. To see the expression of V5 in the tagged viruses, A) HSAECS, B) Huh7s and C) C6/36 cells were infected with MP12, V5-105 and V5-229 at an MOI of 1. Lysates were western blotted for V5, Gn or actin as a loading control.

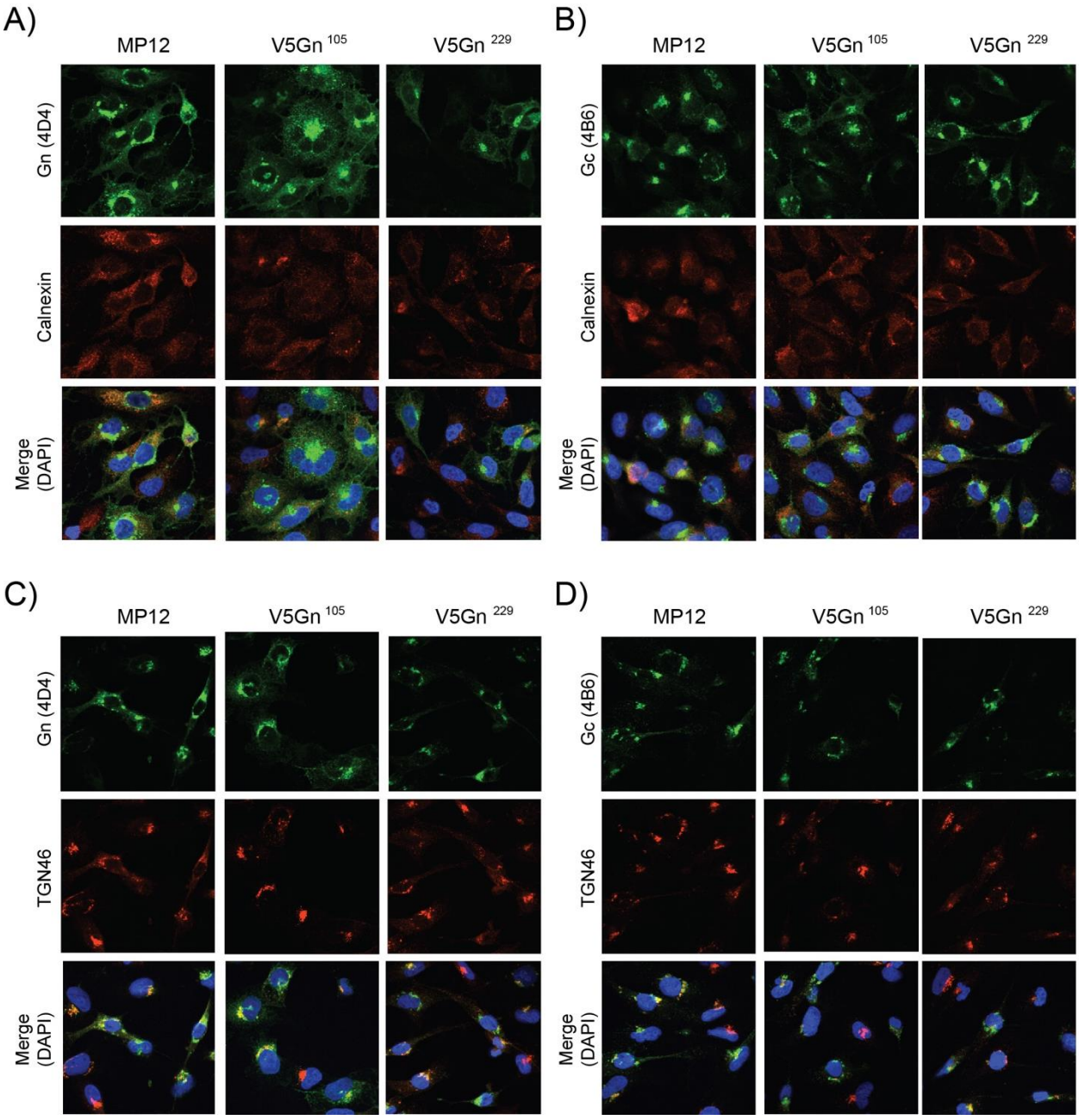

Figure 4: Gn and Gc localization in MP12, V5-105, and V5-229 viruses.
The glycoproteins Gn and Gc colocalize with Endoplasmic Reticulum (Calnexin) in A and B and Golgi (TGN46) in C and D as shown. HSAECs were infected with MP12, V5Gn^{105} and V5Gn^{229} at an MOI of 1. Cells were fixed 24 hpi and analyzed using immunofluorescence.

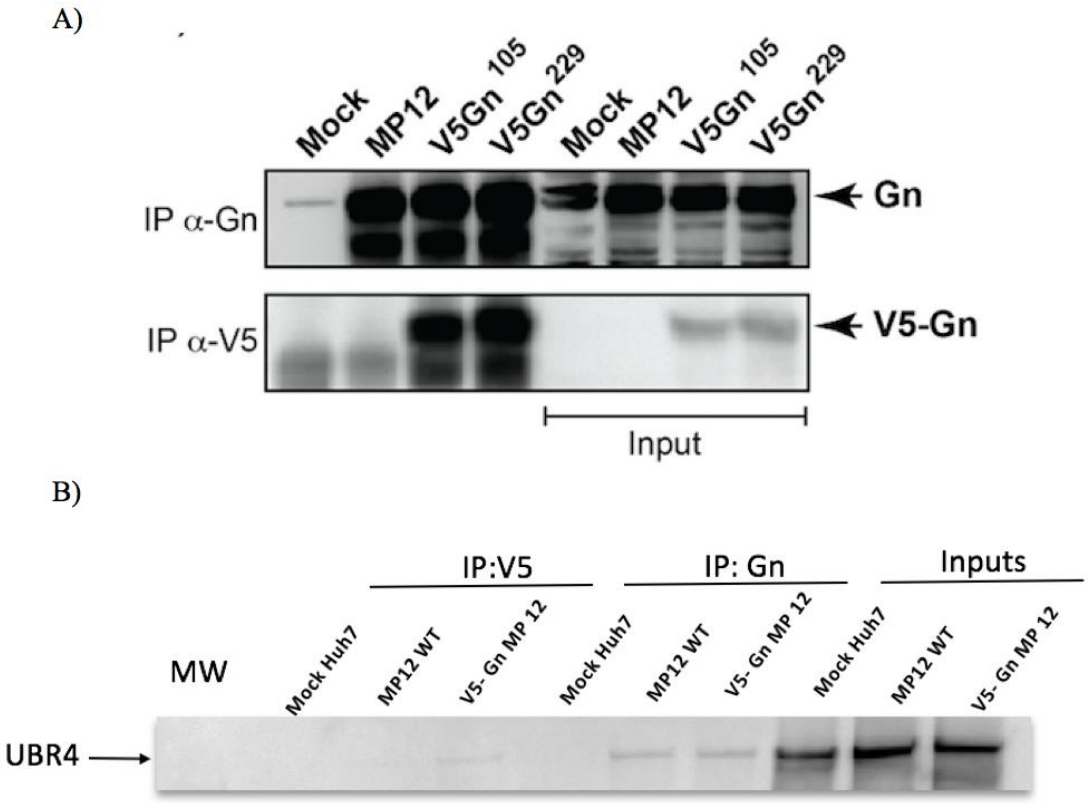

Figure 5: Gn interacts with UBR4.
A) Huh7 cells were infected with MP12, V5Gn^{105} or V5Gn^{229} at an MOI of 1. Lysates were collected at 24 hpi and immunoprecipitated with either the Gn antibody or the V5 antibody. B) UBR4 association with Gn was confirmed using Huh-7 lysates that were immunoprecipitated with V5 antibody or Gn antibody.

	% Knockdown		
	siUBR4 concentration		
Hours post transfection	25 nM	50 nM	75 nM
48	31	10	12
72	80	86	51
96	27	38	35

Figure 6: Knockdown efficiencies at different concentrations of siUBR4.
Huh7 cells were treated with 25, 50 or 75nM of siRNA against UBR4 for 48, 72 or 96 hours. The cell lysates were then collected and western blotted for UBR4. The band intensity was used to calculate the percent knockdown.

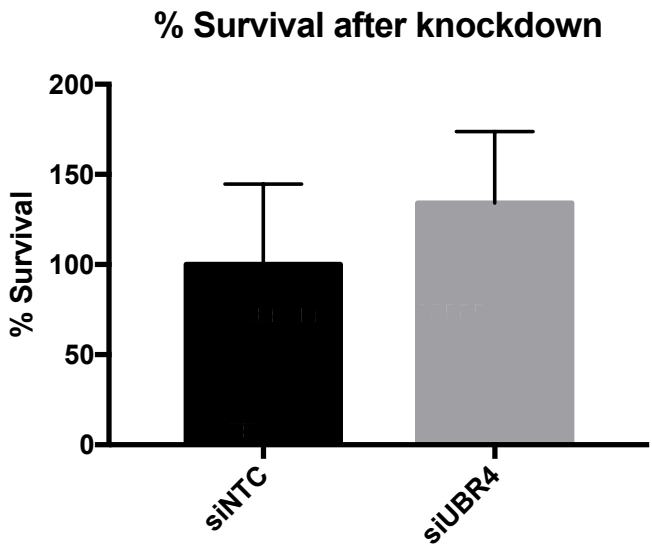

Figure 7: Loss of UBR4 does not affect cell survival.
The percentage survival of cells after knockdown of UBR4 was calculated using the CellTiter-Glo assay. Huh7 cells were treated with siUBR4 for 72 hours post infection. The supernatant was replaced with a 1:1 ratio of complete media and CellTiter-Glo reagent. The luminescence was measured using the Beckham Coulter 10 minutes after. The surviving cells in the control were normalized to 100.

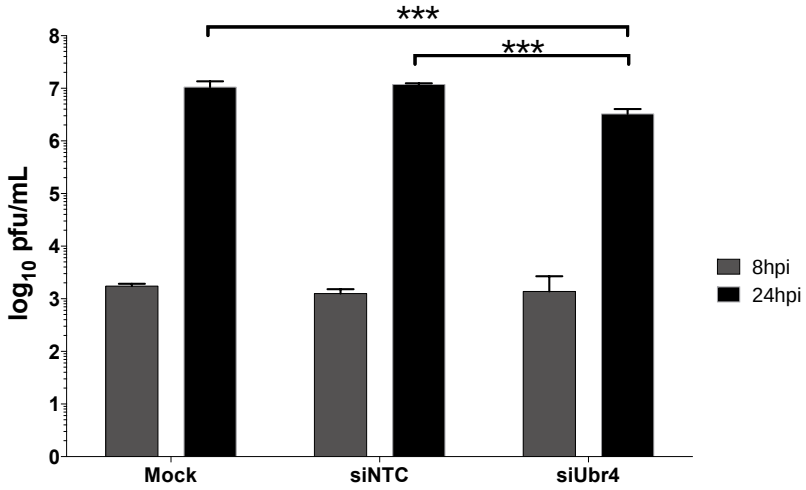

Figure 8: Loss of UBR4 decreases RVFV titers.
Huh-7 cells transfected with 50 nM siUBR4 and siNTC (for control) were infected with MP12 at an MOI of 0.1 72 hours post transfection. Supernatants were collected at 8 and 24hpi and titered in Vero cells. (*** represent significant difference between the mock and UBR4 and siNTC and UBR4, $p < 0.05$)

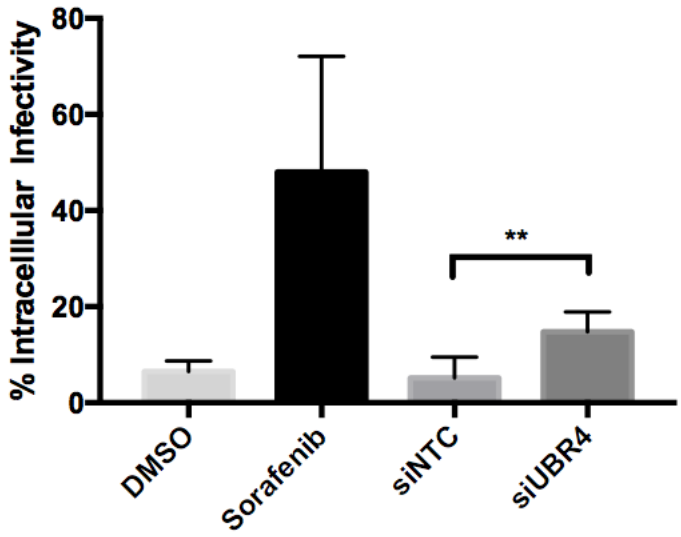

Figure 9: Loss of UBR4 results in increased intracellular infectivity.
Huh-7 cells transfected with 50 nM siUBR4 and SiNTC (for control) were infected with MP12 at an MOI of 0.1 72 hours post transfection. Intra and Extracellular virus was collected at 24 hours post infection and titered on Vero cells and percent infectivity was calculated by dividing the intracellular virus with the total virus. (** represents significant difference between siNTC and siUBR4, $p < 0.01$). DMSO or sorafenib (10 mM) treated samples were included as controls.

Table 1: Gn interacting partners identified via LC-MS/MS.

Gene Ontology	UniProt #	Protein Symbol	Full name	Score XC	MW (kDa)	Peptides Identified
Cytoskeleton	P63261	ACTG1	Actin, gamma 1 propeptide	60.31	41.8	9
	P63267	ACTG2	Actin, gamma 2 propeptide	90.24	41.8	14
	P35579	MYH9	Myosin, heavy polypeptide 9, non-muscle	80.33	226.4	11
	P19105	MYL12A	Myosin, light chain 12A, regulatory, non-sarcomeric	60.25	19.8	15
	P60660	MYL6	Myosin, light chain 6, alkali, smooth muscle and non-muscle isoform 1	60.22	16.9	7
	P35580	MYH10	Myosin, heavy polypeptide 10, non-muscle	30.19	228.9	4
	P67936	TPM4	Tropomyosin 4 isoform 2	80.28	28.5	18
	P14649	MYL6B	Myosin alkali light chain 6B	20.14	22.7	2
	P09493	TPM1	Tropomyosin 1 alpha chain isoform 5	20.20	32.8	2
Trafficking	Q00610	CLTC	Clathrin heavy chain 1	20.19	191.5	2
	P55735	SEC13	SEC13 protein isoform 1	20.21	35.5	2
Protein Folding/Stability	Q5T4S7	UBR4	Ubiquitin Protein Ligase E3 Component N-Recognin 4	10.15	573.5	7
	P11021	HSPA5	Heat shock 70kDa protein 5	20.14	72.3	3
Viral	P21401	GP_RVFVZ	ZH548/MP12 strain M polyprotein	60.24	130.7	7

DISCUSSION

The aim of the project was to determine the characteristics of the V5 tagged RVFV and identify the host proteins that interact with Gn of RVFV which could provide us with targets for antivirals in the long run. The insertion of the small, 16 amino acid V5 tag provides multiple advantages including eliminating the need for Gn specific antibodies that are available in limited quantities commercially. V5 is a relatively small tag about 16 amino acids in size compared to other tags including the GFP tag which is 238 amino acids in size. This possibly reduces the disruption in the protein structure. Another advantage of using the V5 tag is that it minimally impacts the protein interaction of Gn with other proteins.

The addition of the V5 tag to Gn also posed some challenges. We had to make sure that the V5 tag did not disrupt the structure of Gn and the virion and it had to be added to a region where it would be exposed on the protein surface for easier identification and recognition by the V5 antibody. We opted not to add the tag to the N- or C-terminal of Gn as that could potentially interfere with the assembly of the virus. The N-terminals of Gn interact with the N protein and is important for the budding of the virus into the Golgi and the C-terminal of Gn interacts with Gc and helps it localize to the ER.

From our results, it can be concluded that the insertion of the V5 tag into Gn did not significantly disrupt the formation of the virion in the two different forms of V5 tagged viruses.

The viral titer of the recombinant viruses was similar to wild type MP12 when compared in different cell lines, the localization of Gn and Gc in the recombinant viruses was also similar to the wild type MP12 and the level of Gn protein was also similar.

An important outcome of our study was the identification of UBR4 as a Gn interacting protein. UBR4 is a relatively uncharacterized protein and not much is known about the different domains of the protein and how they function. From what is known, UBR4 belongs to a group of seven identified proteins that all contain the UBR box named UBR1 to UBR7. The UBR box is a unique motif that is thought to identify N-terminals of proteins that need to be degraded (18).

These proteins have been characterized as E3 ligases that recognize the proteins that need to be degraded and add ubiquitin to them so they can be processed through the 26S proteasome and be degraded.

These N-terminals of proteins that are recognized to be degraded are called N-degrons and the E3 ligases that recognizes the N-terminal that needs to be degraded are called N-recognins. The specificity of amino acids on the N-terminals of N-degrons determines which proteins can be degraded. This is determined by what is known as the N-end rule pathway (17). According to this pathway, proteins with primary destabilizing residues (Arginine, Histidine, and Lysine) are recognized directly and ubiquitylated,

those with secondary destabilizing residues (Aspartate, Glutamate and Cysteine) are converted to type one residues and then recognized and degraded while tertiary destabilizing residues (Glutamine, Asparagine and Cysteine) are converted to secondary residues and then to primary ones to be recognized and ubiquitylated (16).

Proteins that are degraded also have a Lysine residue in close proximity to the N-terminal and this is the residue on which ubiquitin chains can be added to the protein. This process tags the N-degrons for degradation through the 26S proteasome (19).

The knockdown of UBR4 in cells decreased the viral titer of RVFV which suggests that UBR4 is possibly not degrading Gn because if it was, then the viral titer should increase after UBR4 knockdown. Instead it seems that UBR4 is degrading an antiviral host protein which helps the virus form. Influenza A virus takes advantage of UBR4 to replicate, the knockdown of UBR4 decreases the virus released from infected cells. Tripathi et al. hypothesized that UBR4 is targeting a host restrictive factor in the ER which enables the cells to shuttle the virions from the ER to the Golgi and help in their egress from the cells (12).

UBR4 is also known as p600 and is present in the nucleus and the cytoplasm. In the nucleus it acts as a chromatin scaffold along which DNA organizes (15). In the cytoplasm UBR4 is present at the leading edge of cells where it associates with clathrin to form structures which seem to be important for the structural organization of the cell membrane. The association between clathrin and UBR4 is flexible and enables cells to change membrane morphologies (15). It is known that UBR4 also binds calmodulin. The arrangement of UBR4 in cells alongside fibrous actin and microtubules also suggests its

involvement with the cytoskeleton (15). Both of these roles suggest the structural importance of UBR4 in cells and are different from the E3 ligase activity of the protein. Therefore it is also possible that UBR4 has a role in trafficking virus through the cells because of its association with clathrin, actin and microtubules.

We have shown that UBR4 knockdown from cells decreases the viral titer of RVFV by almost half a log. After looking at the decreased intracellular infectivity titers after UBR4 knockdown from cells, it can be hypothesized that UBR4 is playing a role in the egress of virus from the cell. Future studies will focus on elucidating the specific role of UBR4 in RVFV egress. Continued efforts to outline the role of UBR4 could help provide a target for antivirals against RVFV.

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