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The Effect of the Zika Virus on RNA Stress Granule Components

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The Effect of the Zika Virus on RNA Stress Granule Components

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Abstract:

In recent years, Zika virus (ZIKV) has taken over mainstream media. It captivated the world with the images of microcephaly babies born to infected mothers and the appearance of Guillain-Barré syndrome emerging from infected adults. ZIKV is transmitted by the *Aedes* mosquito. This virus is composed of a single-stranded positive-sense RNA genome that belongs to the *Flaviviridae* family. Our long-term goal is to understand the mechanisms in which the virus subverts the host organism's translation machinery by modulation of RNA stress granules (SGs). Stress granules are RNA-protein complexes found in the cytoplasm that form when the cell is exposed to a stressor such as an infection from a virus. Our initial research showed that ZIKV suppresses the formation of these granules. Thus the goal of this research was to investigate the role of various stress granule proteins on ZIKV infection. In particular, we focused on TIA-1, G3BP1, HuR and TIAR. Knockdown of the SG proteins with target-specific siRNAs and subsequent infection with ZIKV showed that depletion of G3BP1 decreased viral protein, RNA and viral titers suggesting this SG protein acts in a pro-viral manner, whereas depletion of HuR increased viral protein, RNA and viral titers present suggesting HuR was acting anti-viral. Luciferase assays following knockdown and overexpression of G3BP1 and HuR showed that these proteins affected ZIKV replication. These studies advance our understanding the role and interactions of RNA stress granules with ZIKV.

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First and foremost, I would like to thank Dr. Cara Pager for giving me the opportunity to work in this wonderful lab. I endlessly appreciate your guidance and kindness throughout these last 3 years. Thank you for always listening and providing constant support in this project and in life. I will truly miss all of our lab parties and Jonah's excitement over his favorite cookies.

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Being a part of undergraduate research has made a huge impact on me and provided me with so much knowledge and skills that I can hold on for life and it is all thanks to my fantastic lab. Thank you to everyone who has assisted me in any way on this journey.

Disclaimer:

The data presented in this honors thesis are part of a manuscript submitted for publication. The manuscript is available on BioRxivs:

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Introduction:

Zika virus (ZIKV) is a single-stranded positive-sense RNA virus and is a member of the *Flavivirdae* family. Similar to other viruses in the *Flaviviridae* family such as West Nile virus and Dengue virus, ZIKV is also an arbovirus. ZIKV may be transmitted following the bite of an infected *Aedes aegypti* or *Aedes albopictus* mosquito. The transmission of ZIKV by *Aedes* mosquitoes predominantly occurs in tropical and subtropical areas, the preferred environment of these mosquitoes, but it has been documented in cases as far north as New York from travelers that were outside of the country [4]. In contrast to other flaviviruses, ZIKV may also be sexually transmitted as well as passed from mother-to-child *in utero* [5]. ZIKV infection is usually asymptomatic although some individuals may experience mild flu-like symptoms such as fever, headache, malaise, or rash which can last for several days and up to a week [4]. However, more serious symptoms such as the development of Guillain-Barré syndrome in adults are also associated with this virus. In Guillain-Barré syndrome, the body's immune system attacks peripheral nerves causing weakness, numbness and in extreme cases paralysis [13]. Other devastating effects of ZIKV include microcephaly that develops in babies born to infected mothers. Microcephaly is a congenital defect that occurs during the first trimester of the pregnancy where babies are born with partially collapsed skulls, decreased brain tissue and damage to the back of the eyes [5]. Because ZIKV may be vertically transmitted to the fetus from the mother and is associated with congenital defect, this re-emerging flavivirus was recently classified as a TORCH agent (toxoplasmosis, other agent, rubella, cytomegalovirus, herpes simplex) [11]. These severe complications together with the lack of vaccines or licensed anti-viral treatments sparked a need to study the virus.

ZIKV was first discovered in 1947 in the Ugandan Zika Forest, from which the virus

takes its name. It was discovered in Rhesus monkey, and by 1952 the first cases of human contraction of the virus were documented [15]. By 2007, ZIKV was found throughout south-east Asia where it made its way across the Pacific and by 2015 ZIKV was documented in South America. The 2015 strain of the virus was found to have the severe symptoms of Guillain-Barré and microcephaly [15]. This research focused on the 2007 Cambodian strain that was associated with the neuronal defects. Throughout the study, we focused on the way in which Cambodian strain of ZIKV produces a successful infection and at points of the Flavivirus life cycle. When a ZIKV viral particle comes into contact with receptors of the host cell it will be allowed to enter the cell through endocytosis. At a certain point the capsid protein, a structural protein that envelopes the virus, will break down and the virus genome is released into the cytoplasm. This genome consists of 3 structural proteins and 7 non-structural proteins [3]. The genome is then translated into a large polyprotein until there is enough polyprotein translated to begin replicating the genome. Immature viral particles then undergo viral assemble to make mature infectious particles that leave the cell to infect surrounding cells [10]. The focus in this project is to better understand how the Zika virus interacts with SGs, and if there is interaction, what proteins and markers are being affected.

The formation and function of cytosolic RNA stress granules (SGs) are essential to the regulation of RNA under poor conditions within cells. Stress granules are made up of stalled initiation complexes containing mRNA, initiation factors and the small ribosomal subunit [14]. SG scaffolding proteins G3BP1, TIA1, and TIAR recognize and bind to stalled initiation complexes and aggregate into large cytosolic RNA-protein complexes. In addition to the proteins listed above, upwards of 260 different proteins are known to localize in SGs [9]. The formation of RNA SGs begins when cellular stress is present activating one of several types of

kinases, these kinases then phosphorylated eIF2α, once phosphorylated eIF2α will take an actively translating polysome and create a stalled translation initiation complex that will bind with other scaffolding and binding proteins to make SGs [12]. These formed SGs can be imaged using fluorescent staining and confocal microscopy and are seen as distinct foci when cellular stress is induced [2].

When there is stress on a cell such as an infection, temperature elevation, oxidative stress or the addition of drugs, cellular translation is inhibited translation, and the stalled translation initiation complexes, mRNAs and other RNA binding proteins are sequestered in stress granules. SG formation is a reversible process in that following removal of the stress and optimal conditions are returned to the cell, SGs will disassemble and mRNA translation proceeds. Previous studies conducted on West Nile virus and Dengue virus found that the viruses had evolved machinery in order to inhibit the production of SGs, which prevents host translation complexes from being stalled [6]. It is also known from previous research that several Flaviviruses will manipulate host machinery to translate and replicate their genome. In the case of Zika virus (ZIKV) we wanted to investigate if SGs were contributing to the prevention of ZIKV infection or if they were being sequestered by the virus to have successful infection [7]. This research will advance our understanding of ZIKV mechanisms and lay the groundwork for possible antiviral treatments.

Methods:

Cell Culture:

In this study we used Vero cells (ATCC) derived from the kidney of the *Cercopithecus aethiops*, otherwise known as African green monkeys for viral titering [1]. For siRNA depletion

experiments we used Huh7 cells, which are human hepatoma cell line isolated from a Japanese man in 1982 [8]. Cells were grown in a 37°C incubator with 5% CO_2 levels in 10 cm culture plates that contained 8 mL media of DMEM, 10%FBS, 1x Non-essential amino acids (NEAA) and 1% glutamine (Life Technologies). When the cells were approximately 80% confluent, the cells were split in a sterile biosafety hood. All reagents were sterilized with 70% ethanol before placing them into the biosafety hood. To begin, the old growth media was aspirated from the plate. After this, 1.5 mL of PBS (Life Technologies) was added to each plate being split and rocked to evenly cover the surface of the plate. This step was repeated once more and then aspirated. Hereafter, 1 mL of 0.05% Trypsin (Life Technologies) with EDTA was added, the plate(s) were rocked again, and placed in the incubator for 5 minutes. Trypsin was used to release the adherent cells from the culture plate. After 5 minutes, the plate was removed and gently knocked to dislodge any remaining adherent cells. For a 1:6 split of the cells, 5 mL of media was added, and while holding the plate at a slight angle, the cells were further dislodged from the plate by aspirating the media and expunging onto the highest point of the plate. This step was repeated until all of the cells were collected at the bottom point of the plate. Next, while keeping the plate at an angle, the entire cell suspension was aspirated and the tip of the pipette directly placed onto the surface of the plate. The cell suspension was carefully expunged to break up any cell clumps that were present. These steps were repeated until all cell clumps were dispersed. Next, 7 mL of new media was added to a new 10 cm culture plate, and 1 mL of the cell suspension from the original plate was transferred into the new plate. The cell suspension was aspirated up and down to ensure that all of the cells were distributed throughout the plate. The plate was then placed back into the incubator when completed. The old plate was disposed of in a hazardous waste container and the biosafety hood sterilized.

siRNA and Plasmid DNA Transfections:

To transfect siRNAs into Huh7 cells we first prepared the vehicle master mix reaction containing 980 μL of OptiMEM and 10 μL of Lipofectamine 3000 per siRNA reaction (Life Technologies). The siRNA reaction contained 980 μL of OptiMEM and 20 μL of the particular siRNA (100 nM final concentration). The vehicle master mix was added to each siRNA reaction and incubated at room temperature for approximately 10 minutes. Next, the media from each culture plate was aspirated and the cells washed with 2 mL of PBS. The transfection mix from each reaction was added to the appropriate plate and the plates are then returned to the incubator. The plates were then rocked every 30 minutes for a 2-hour period. After this period is complete, add 6 mL of complete media to each plate.

To transfect Huh7 cells with plasmid DNA, we similarly first prepared the vehicle mastermix was prepared first. We used plasmids CTP 562 and CTP 563 that were used for the overexpression of HuR and G3BP1 respectively. Spectifically, 5.5 μL of Lipofectamine 3000 (Life Technologies)was added to 150 μL OptiMEM media (Life Technologies) and then incubated for 5 minutes at room temperature. The plasmid mastermix was prepared next, where 100 ng of plasmid DNA was added to 150 μL of OptiMEM, followed by 5.5 μL P3000 reagent (Life Technologies). Hereafter, 150 μL of the vehicle mastermix was added to each tube containing the plasmid, and then further incubated at room temperature for 10-15 minutes. After the 10-15 minutes, 300 μL of the transfection mix was added to a 6 cm plate, label and return plate to 37°C incubator. Repeat for the remaining tubes.

Cell Harvest:

To harvest cells, all media from the plate was aspirated and the cells washed with 2 mL of cold PBS. This was repeated one more time and the PBS was aspirated. PBS (1 mL) was

added to each plate and a cell lifter used to carefully scrape the cells from the plate. The scraped cells were collected into a 1.5 mL tube. Hereafter, the cells were briefly centrifuged to pellet the cells. The PBS from the tube was aspirated, while taking special care to not disrupt the cell pellet. The cell pellet was resuspended with 50 μL of protease inhibitor (1x protease inhibitor mix in RIPA buffer with 150mM NaCl; the amount of RIPA added varied depending on the pellet size)(ThermoScientific Pierce). The tubes were placed on ice for 20 minutes, and then centrifuged at 4°C at 14,000 rpm for 20 minutes. When the spin was completed, the supernatant was transferred to a new 1.5 mL tube. The protein concentration was quantified using a BCA assay. Alternatively, the lysates were stored following a quick freeze in Methanol/dry ice bath and then placed in a -20°C freezer.

BCA Assay:

The BCA assay kit frm Bio-Rad was used to quantitate protein in harvested cell lysates. To begin, BCA standards were prepared based on the ng/μL desired. All tubes contained 2 μL RIPA and varying amounts of PBS depending on the concentration of the standard. After the standard was prepared, the sample was diluted 1:10 by using 2 μL of the sample and 18 μL of PBS, and then 5 μL transferred to 3 wells. Reagent A (25 μL) was added per well. To prepare this, a 1:50 dilution of S-Reagent was added to A-Reagent. Reagent B (200 μL) was then added to each well and incubated for 20 minutes before measuring the absorbance. The absorbance was measured using a BioTek plate reader at 630 nm, and a standard curve for the samples was generated.

SDS-PAGE and Western Blot Analysis:

A ProtoGel SDS-PAGE gel was prepared with a 10% resolving gel and a 5% stacking gel. The resolving gel was created using ddH_2O, 1.5M Tris with a pH of 8.8 (Fisher Scientific),

ProtoGel (National Diagnostics), SDS at a 20% concentration, 10% APS and TEMED. Typically, 10% gels were used during the course of these experiments. The stacking gel was then made using ddH_2O, 1M Tris with a pH of 6.8 (Fisher Scientific), ProtoGel (National Diagnostics), 20% SDS, 10% APS and TEMED. Samples were separated by SDS-PAGE at 100 V until the 6x loading dye reached the bottom of the gel. The gels were prepared for Western transfer. Each transfer cassette was prepared were on the black side, a sponge was placed, then a filter paper, then the gel, then the PVDF membrane (MilliPore), another filter paper and another sponge. The black side of the cassette faced the black electrode side. An ice pack and a magnetic stir bar are then added. The transfer apparatus was placed in the cold room and run for one hour at 100 V, stirring at medium strength using 1x Transfer buffer (192 mM Glycine, 0.025 M Tris-HCl, 20% methanol). After transfer was completed, the PVDF membrane was placed in methanol and allowed to air dry. Once dried, the membrane was rehydrated in methanol and then transferred to H_2O, before being stained with a Ponceau S stain (Sigma). The membrane was removed from the stain and washed with 1x PBST (0.1% Tween in PBS) 3 times for 5 minute-intervals. The membrane was blocked with 5% skim milk in PBST and for 30 minutes at room temperature. After rocking, the primary antibody diluted in 5% milk in PBST was added and incubated on a rocker overnight in cold room. The blot was washed 3x in PBST for 5 minute-intervals. The secondary antibody was then added and rocked at room temperature for 1 hour. After incubation in the secondary antibody, the blot was washed in 1x PBST 3x for 5 minutes. The blot was developed and exposed to the Clarity ECL reagent (Bio-Rad) and visualized using the ChemiDoc (BioRad). Re-Blot Plus Mild (Millipore) was used if the blot needed to be stripped for further testing. Specifically, 9 mL of PBS was added to 1 mL of Re-Blot plus, and this mixture added to the blot. The blot was incubated on a rocker for 30 minutes to fully strip off

the previous antibodies used. The antibodies used in this study are in Table 1.

Table 1:

Protein	Supplied (Product ID)	Species	Western Dilution
G3BP1	Bethyl (A302-034A)	Rabbit	1:5,000
TIAR	Bethyl (A303-614A)	Rabbit	1:1,000
YB1	Cell Signaling (D299)	Rabbit	N/A
Ataxin-2	ProteinTech (21776-1-AP)	Rabbit	1:5,000
HuR	ProteinTech (11910-1-AP)	Rabbit	1:6,000
TIA-1 (C-20)	Santa Cruz (Sc-1751)	Goat	1:3,000
GAPDH	Millipore Sigma	Mouse	1:10,000
ZIKV Capsid	GeneTex (GTX133317)	Rabbit	1:5,000
Envelope	BioFront (1176-76)	Mouse	N/A
Anti-Rabbit Peroxidase	Santa Cruz (SC-2313)	Donkey	1:10,000
Anti-Goat Peroxidase	Santa Cruz (SC-2354)	Mouse	1:5,000
Anti-Mouse Peroxidase	Santa Cruz (SC-2314)	Donkey	1:10,000

Expression of MR766 GLuc Reporter Genomes in Huh7 Cells:

To investigate the effect of particular SG proteins on ZIKV translation or replication, Huh7 cells were first transfected with either the siRNA or with plasmid DNA. On the second day, the cells were trypsinized and the number of cells in each plate was counted using a hemacytometer. Specifically, 10 μL of the cell suspension was added to the hemacytometer and cells in each of the four quadrants was counted. The number of cells counted was multiplued by 2,500 to calulate cells/mL. Hereafter we seeded 7x10^4 cells/mL in one well of a 24-well plate. Remaining cells were cultured in the original 6 cm plate with 3 mL of media, and used to verify siRNA knockdown or protein overexpression.

The MR766 GLuc reporter genome plasmids (pCDNA6.2 MR766 clGLuc Intron3127 HDVr; wild-type replication competent and Pol(-) replication incompetent; gift from Dr. Matthew J. Evans, Mount Sinai School of Medicine) were transfected 24 hours post-seeding of

cells into 24-well plates. As before, the vehicle mastermix contained 25 μL of OptiMEM and 0.5 μL Lipofectamine 3000 and was incubated at room temperature for 5 minutes. The mastermix containing the plasmid, 200 ng of plasmid was added to 25 μL of OptiMEM followed by 0.5 μL P3000 reagent per reaction. The two master mixes where mixed 1:1 and incubated at room temperature for 10-15 minutes. Hereafter, 50 μL of each reaction was added to the appropriate well of each 24-well plate. The media from the weels was collected at 6, 24, 48 and 96 hours, and stored at -20°C freezer until the luciferase activity was measure.

Luciferase Assay:

Once all time points had been harvested, the media was thawed at room temperature. To measure Gaussia luciferase activity, the BioLux buffer and Stabilizer reagents (NEB) were thawed at room temperature. Each sample was measured in triplicate. Therefore the volume of the reagent needed was multiplied by the number of 3 times the number of samples. Then the total number of reactions and was multiplied by 50 μL to give the reagent volume needed. After determining the the total volume, we added 1.25 mL required to prime the plate reader. After this is calculated, the amount of stabilizer must be calculated. For each reaction 8 μL of stabilizer is needed. We then take the total volume of the reagent and divide by 50, then multiply that number by 8 to get the amount of stabilizer needed for the reaction. Then we determined the amount of substrate needed. The substrate is at a 100x concentration; we must have 0.5 μL per reaction. Following this, we determined the amount of buffer required. To calculate this, we added the volume of the stabilizer and substrate together and subtract from the total reagent. As the substrate is light sensitive, wrap a new 15mL conical tube in aluminum foil. Add the amount of buffer to the tube, followed then by the amount of stabilizer and substrate. Tightly cap the solution and allow to sit at room temperature for 20 minutes. Once all of the samples have

thawed, transfer 30 μL of each into 3 wells of a white 96-well plate. For sample controls, transfer only the 30 μL of sample into three wells, but for the reagent controls just leave 3 wells blank.

Bacterial Transformation of Plasmids:

Following a protocol provided by New England BioLabs, we thawed NEB 5- α competent *E. coli* cells on ice for 10 minutes. Following this, 100 ng of plasmid DNA (pG3BP1-Flag and pHuR-Flag; Genscript) was added to the bacteria, and the bacteria were placed on ice for 30 minutes. After 30 minutes, the bacteria were heat shocked for exactly 30 seconds and placed back on ice for 5 additional minutes. Then, 950 μL of room temperature SOC media was added and the bacteria were allowed to recover at 37ºC and 250 rpm for 1 hour. Following this, the bacteria were plated on LB/carbenicillin plates and incubated overnight at 37ºC.

DNA Plasmid Miniprep:

To isolate the plasmid DNA from bacteria, we conducted minipreps following the Omega Biotek E.Z.N.A. kit protocol. A single bacterial colony was picked from the transformed bacteria and grown overnight in 5 mL LB/ampicillin at 37ºC and 250 rpm. Hereafter, the bacteria were pelleted at 10,000xg for 1 minute at room temperature. The culture media was aspirated, but pellet was untouched. We then added 250 μL of Solution I mixed with RNase A, and thoroughly resuspended the bacterial pellet by vortexing. The suspension was transferred to a new 1.5 mL microcentrifuge tube, and 250 μL of Solution II was added and the suspension egently inverted 3-5 times until a clear lysate was formed. Once there was a clear lysate, 350 μL of Solution III was added, and the tube inverted several times until a fluffy white precipitate formed. The solution was then microfuged at maximum speed, approximately ≥13,000xg, for 10 minutes. While the tube was being centrifuged, a HiBind DNA Mini Column was inserted in a 2 mL

collection tube. Once lysate was clarified, the clear supernatant was directly added onto the HiBind DNA Mini Column and centrifuge at max speed for 1 minute. The flow through was discarded and 500 μL HBC Buffer diluted with isopropanol was added to the column, centrifuged again at maximum speed for 1 minute and the flow through discarded. Following this, 700 μL of DNA Wash Buffer diluted with ethanol was added to the column, centrifuged at max speed for 30 seconds and the flow through poured off. This step was repeated for another wash and then the column with no additive was centrifuged at max speed for 2 minutes to dry. The column was transferred to a new 1.5 mL microcentrifuge tube and the plasmid DNA eluted using 30-100 μL nanopure water. A NanoDrop Microvolume Spectrophotometer was used to determine DNA concentration. The plasmid DNA was stored at -20ºC.

Results:

siRNA mediated knockdown of SGs and effects on ZIKV capsid protein levels

To investigate whether the amount of Zika proteins would be affected by knockdown of specific SG components we transfected cells with siRNAs targeting Gaussian Luciferase 2 (GL2; control), Ataxin2, G3BP1, HuR, TIA-1, TIAR, and YB-1, and then infected the cells with ZIKV at a multiplicity of infection (MOI) of 5. Cells were harvested two days post-infection and protein analyzed via Western blot (Figure 1). Using antibodies to each of the SG proteins we observed protein depletion with the respective siRNA. In the blot examining the levels of Ataxin-2 we noted an increase in Ataxin-2 when TIAR was depleted, but a decrease when YB1 was knocked-down. Notably levels of each of the SG proteins was not affected by knockdown of either G3BP1 or HuR. GAPDH, the loading control for the western blot (Figure 1A), showed the bands had the same overall intensity, indicating the lanes were evening loaded. The data shown

in Figure 1 are representative of the experiment undertaken in triplicate.

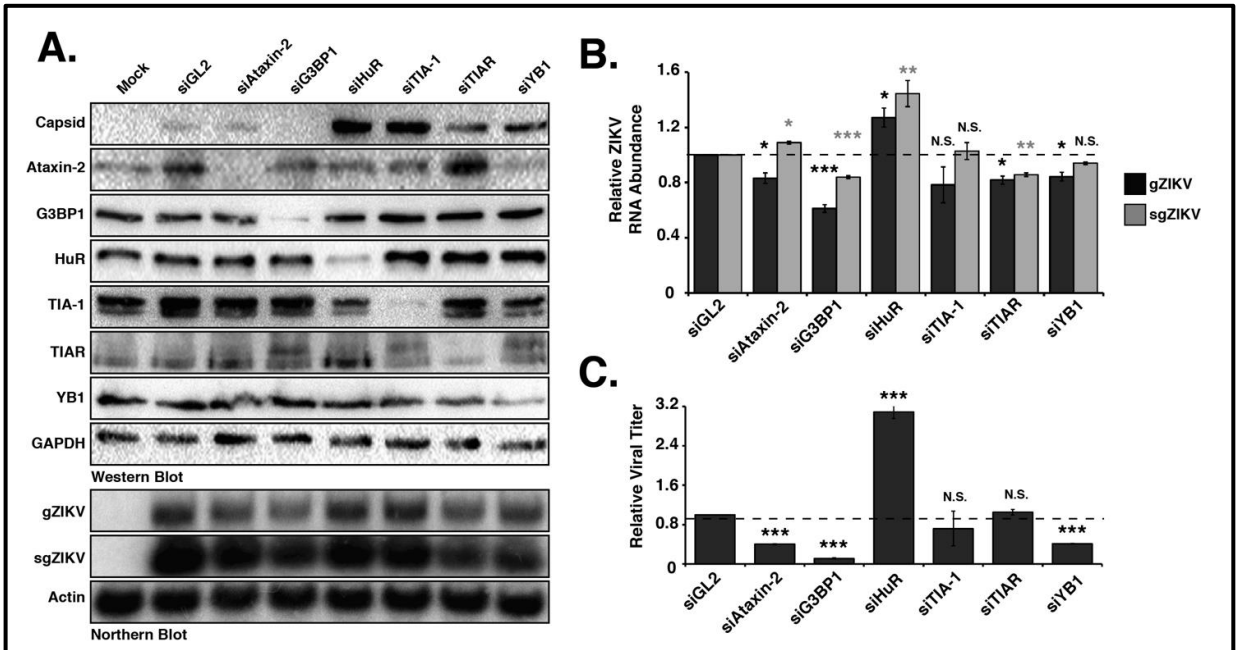

Figure 1: ZIKV protein and RNA abundance changes following depletion of specific stress granule proteins. Cells depleted of specific stress granule proteins were infected with ZIKV. A) Cellular and viral proteins and RNA were analyzed by western and northern blot. The northern blot shown was performed by Gaston Bonenfant. B) Quantification of ZIKV genomic and subgenomic RNA from the northern blot, and represented with respect to the control siGL2. C) Effect of SG protein knockdown on ZIKV titer determined by plaque assay; experiment conducted by Gaston Bonenfant.

To investigate the effect on ZIKV protein expression, we used an antibody to the ZIKV capsid protein. As expected, no capsid protein was detected in the mock-infected lane (Figure 1A). We consistently observed a decrease in ZIKV capsid levels when G3BP1 was knocked-down. In contrast the levels of ZIKV capsid increased when HuR, TIA-1, TIAR and YB-1 were knocked-down. Depletion of Ataxin-2 had no effect on ZIKV capsid levels.

siRNA mediated knockdown of SGs and viral RNA abundance

Figure 1A and 1B shows northern blot and quantification data genomic ZIKV (gZIKV) and sub-genomic ZIKV (sgZIKV) RNA following knock-down of SG proteins. These analyses were performed by Gaston Bonenfant. The gZIKV RNA reflects the entire RNA genome of the virus whereas the sgZIKV RNA shows the 3' UTR that is not degraded by the cellular 5'-to-3'

exonuclease Xrn1, otherwise known as the subflavivirus RNA. In Figure 1B, ZIKV RNA was quantified relative to actin mRNA and normalized to the amount of viral RNA in relation to the siGL2 control that was set to 1.00. We observed a decrease in both gZIKV and sgZIKV RNA when Ataxin2 and G3BP1 were knocked-down. Depletion of G3BP1 and the corresponding decrease in ZIKV RNA levels suggesed this SG protein is pro-viral. The intensity of the gZIKV and sgZIKV increased when HuR was depleted, indicated that HuR restricts ZIKV during infection.

siRNA mediated knockdown of SGs and effects on ZIKV titers

Plaque assays (Figure 1C) were done by Gaston Bonenfant to determine the number of infectious viral particles, or viral titers. The siRNA for GL2 was used as a control in this experiment and was set to 1.00. It was shown that the number of infectious particles was nearly 3 times higher in siHuR in comparison to the control. For G3BP1, Ataxin-2 and YB1, there was a decrease in viral particles that were found.

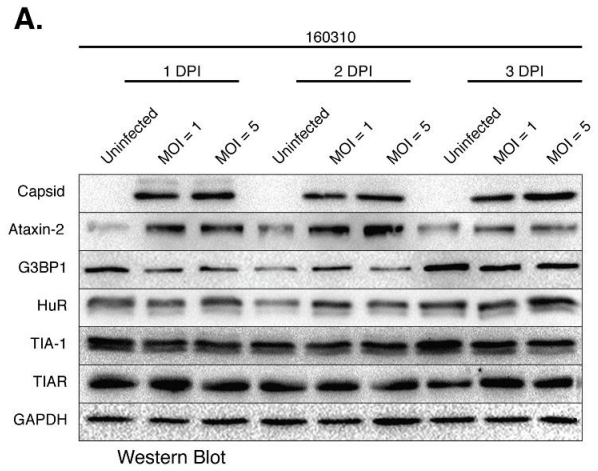

Figure 2: ZIKV infection does not affect the levels of SG proteins. Huh7 cells were infected at 2 different MOIs, and cellular and viral proteins were analyzed by western blots at 1, 2 and 3 days post-infection.

Time-course of ZIKV infection and changes SG protein abundance

Depletion of different SG proteins showed that ZIKV gene expression was modulated by these proteins. To investigate whether the virus affects the levels of SG proteins during infection we examined the expression of SG protein levels by western blot analysis at 1, 2 and 3 days post-infection of Huh7 cells with the Cambodian

strain of ZIKV (Figure 2). We also examined whether infected at two different MOIs would impact SG protein abundance (Figure 2). While ZIKV did not change the amount of most of the SG proteins over a three infection, we did observe an increase in the levels of Ataxin-2 during infection. This is interesting particularly because depletion of Ataxin-2 did not affect ZIKV proteins, but did decrease ZIKV RNA levels and viral titers (Figure 1B and 1C).

Luciferase reporter shows HuR expression changes throughout translation and replication

To investigate effects on ZIKV translation and replication, we expressed the used the replication-competent (WT) ZIKV reporter plasmid (pCDNA6.2 MR766 clGLuc Intron3127 HDVr) expressing *Gaussia* luciferase (GLuc). The cDNA clone containing the full-length genome of the Uganda MR766 strain under the control of the cytomegalovirus (CMV) promoter was used to create the pCDNA6.2 MR766 clGLuc Intron3127 HDVr constructs. Within this construct, the GLuc gene was cloned as a translational fusion between the NS1 and NS2A genes, and the NS1 and NS2A junction was duplicated on both sides of the reporter gene thus providing the mode by which GLuc was proteolytically processed out of the polyprotein. Following transfection of pCDNA6.2 MR766 clGLuc Intron3127 HDVr, cellular RNA polymerase II via the CMV promoter directs transcription and 5'-end capping of ZIKV genomic RNA. The correct 3'-end of the ZIKV genomic RNA is derived by the hepatitis D virus ribozyme (HDVr) at the end of the 3' UTR. In this assay GLuc expression is used as a proxy for measuring viral RNA function. In particular at 6 hours post-transfection translation of capped ZIKV RNA may be assayed. However following synthesis of the ZIKV polyprotein and activation of the RNA-dependent-RNA polymerase (RdRp) NS5, the reporter genome is replicated where the increase in viral RNA, and GLuc activity, is measured at 48- and 96-hours post-transfection.

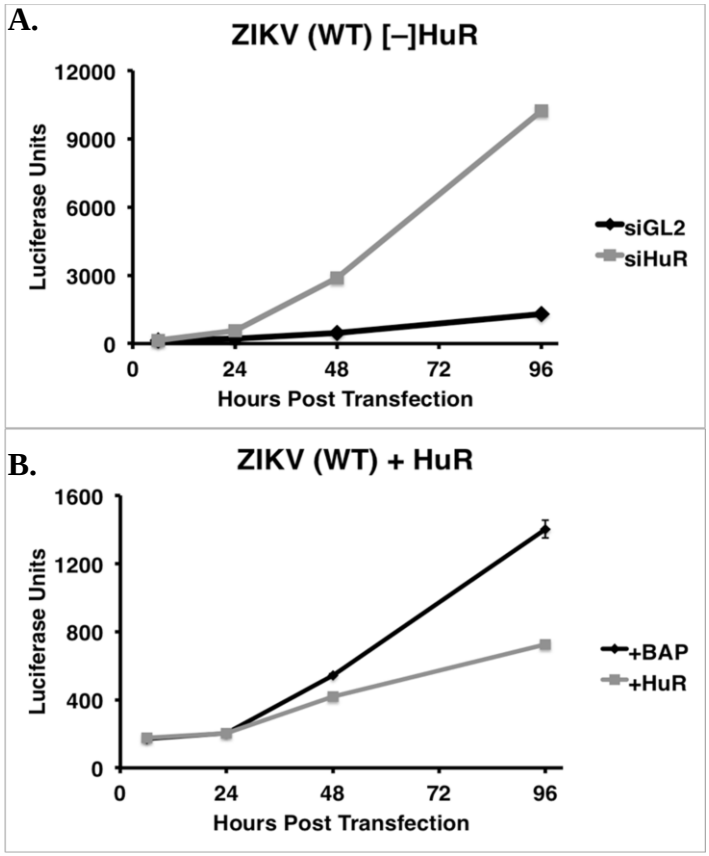

Figure 3: Effects of HuR on ZIKV translation and replication. GLuc activity from the wild-type MR766 GLuc reporter genome was examined following depletion ([-]HuR) or overexpression (+ HuR) of HuR. GLuc activity at 6 and 24 hours measures effects on ZIKV translation while effects on ZIKV replication may be analyzed at 48- and 96-hours post-transfection. siGL2 and p3xFlag-bacterial alkaline phosphatase were used as transfection controls.

To determine whether modulation of HuR levels affects ZIKV gene expression, we either depleted HuR with target-specific siRNAs or overexpressed HuR-Flag (Figure 3). Following expression of either the control siRNA or 3xFlag-bacterial alkaline phosphatase (BAP) we observed a continued increase in luciferase units throughout the 96-hour time points. For knockdown of HuR (Figure 3A), seen in black, we similarly observed an increase throughout the experiment however at slightly higher levels than siGL2. In contrast, when HuR was overexpressed (Figure 3B), GLuc levels were decreased compared to 3xFlag-BAP. Together these data support our earlier data indicating an antiviral role for HuR by affecting ZIKV replication.

G3BP1 modulates ZIKV replication

In Figure 4 we investigated the effect of knockdown and overexpression G3BP1 on ZIKV translation and replication. Following transfection of the GL2 control siRNA, we observed the continuous increase in luciferase units throughout the 96 hours post transfection (Figure 4A). In contrast, following knockdown of G3BP1 GLuc activity decreased beginning at the 24-hour

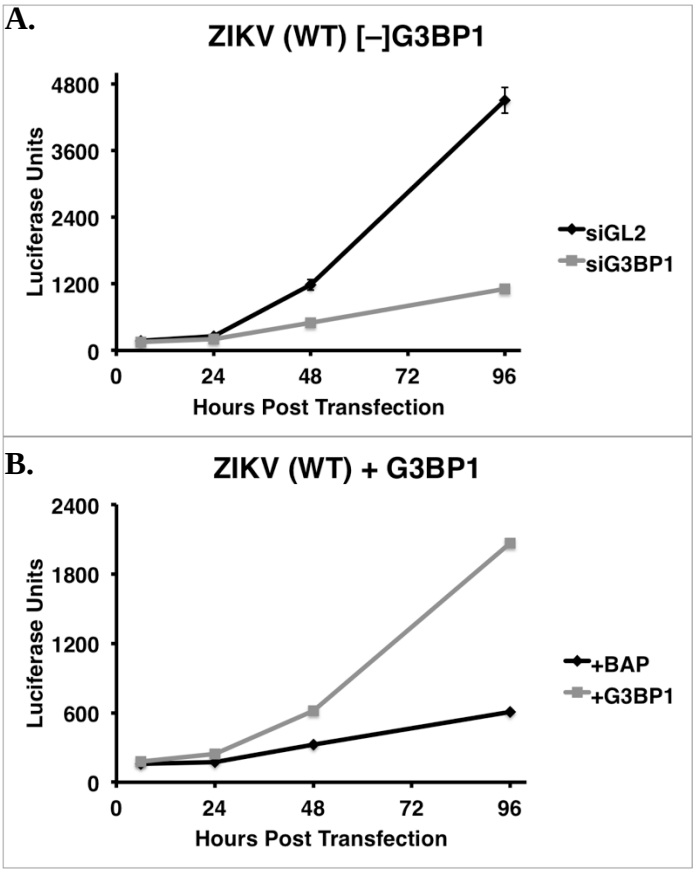

Figure 4: Effects of G3BP1 on ZIKV translation and replication. GLuc activity from the wild-type MR766 GLuc reporter genome was examined following depletion ([-]G3BP1) or overexpression (+ G3BP1) of G3BP1. GLuc activity at 6 and 24 hours measures effects on ZIKV translation while effects on ZIKV replication may be analyzed at 48- and 96-hours post-transfection. siGL2 and p3xFlag-bacterial alkaline phosphatase were used as transfection

time point. Following overexpression of G3BP1, we observed a 4-fold increase in GLuc activity (and thus replication of ZIKV) compared to 3xFlag-BAP control value (Figure 4B). These data indicate that G3BP1 promotes replication and thus modulates ZIKV protein and RNA levels.

Discussion:

The objective of this research was to investigate the role of SG proteins during ZIKV infection. Our data show that two RNA binding proteins, G3BP1 and HuR, function to promote and limit ZIKV replication.

In Figure 2 we examine the consequence of decreasing the levels of six different SG

proteins. To determine if siRNA depletion of the SG proteins affected ZIKV protein we blotted for the capsid protein. Our data showed consistently less capsid when G3BP1 was knockdown and an increase in ZIKV protein in the HuR depleted cells. This result suggests that G3BP1 is a pro-viral cellular factor while HuR is antiviral. We also observed an increase in ZIKV capsid protein TIA-1 was knockdown. TIA-1 depletion had no effect on ZIKV RNA or viral titers (Figure 1). These data contrast that observed for West Nile virus [6], indicating that although ZIKV and West Nile are both within the same virus family and genus, distinct cellular factors affect viral gene expression. We also examined the effect on viral RNA abundance by northern blot. By northern blot we visualized both the genomic and subgenomic ZIKV RNAs. The sub-genomic RNA (sgRNA)is composed of the virus' 3' untranslated region that is approximately 340 base pairs in length, and thus migrates further in the gel compared to the larger full ZIKV RNA genome (gZIKV) which is approximately 11,000 nucleotides in length. By northern blot we observed an increase in the intensity of the ZIKV gRNA and sgRNA when HuR was depleted compred to transfection of the siGL2 contol (Figure 1A). Consistent with the western blot we also observed a decrease in ZIKV RNA following knockdown of G3BP1 compared to siGL2, albeit the effect was not as dramatic as that on capsid levels. The less dramatic effct on the viral RNA may in part be because the viral RNA is protected during viral replication in membrane vesicles [7]. Notably the effects on gRNA and sgRNA are better visualized, and thus easier to dissect what is occurring throughout the cell during infection, by quantifying the viral RNA based on the siGL2 control (Figure 1C).

While the most dramatic effects on ZIKV gene expression were observed following depletion of G3BP1 and HuR, our results showed that knockdown of other SG proteins also affect ZIKV. Depletion of Ataxin2, TIAR and YB-1 decrease ZIKV protein expression

suggesting these proteins likely function to promote translation of the viral polyprotein (Figure 1A). Interestingly however the increase in viral translation did not affect replication. Further studies will need to be undertaken to further discern how these protein modulate viral translation, and whether knockdown of Ataxin2, TIAR and YB-1 similalry change translation of cellular mRNAs during ZIKV infection. Moreover it will be interesting to investigate whether the increase in viral proteins changes the composition or formation of replication complexes to affect stability of the viral RNA or synthesis of new viral genomes.

Gaston Bonenfant conducted plaque assays in order to determine changes in the viral titer when each stress granule protein is depleted. Similar to the other experiments, siGL2 was used as a control. There was a 3-fold increase in the relative PFU/mL for siHuR, while there was a distinct decrease in the viral titer for siAtaxin-2 and siG3BP1. These support that the HuR stress granule may be antiviral in nature, while G3BP1 may have pro-viral roles. Depletion of Ataxin-2 also affected viral titers. This decrease might be the consequence on decreased replication or a direct role in assembly of new viral particles.

In Figure 2, we conducted an infection time course on the Cambodian strain of the ZIKV. We compared the levels of SG proteins between mock-infection and cells infected at a low and high MOI, at 1, 2 and 3 days post-infection. Overall the levels of the SG proteins did not change, indicating that ZIKV infection did not induce the overexpression or degradation of SG proteins. Notably, the abundance of Ataxin-2 increased in ZIKV infected cells, although the biological implication of this increase on cellular and viral function is at this point unclear and warrants additional studies particularly as this protein is associated with neurological disease.

Our data in Figure 1 show a pro- and antiviral role for G3BP1 and HuR in ZIKV

infection. To further investigate whether HuR affected ZIKV translation or replication we undertook studies using a GLuc reporter genome (Figure 3). Using this reporter genome, we were able to examine the effects on ZIKV translation at 6 and 24 hours post-transfection of the plasmid, and replication at 48 and 96 hours post-transfection. In this assay, GLuc activity in the media served as a proxy for ZIKV translation and replication. Following transfection of the siGL2 control (Figure 3A), GLuc activity increased continuously through the 96-hour time points. Following knockdown of HuR however, we similarly observed continuous increase in Gluc activity, although at significantly higher levels compared to the siGL2 control (Figure 3A). This suggests that when HuR is knocked-down that ZIKV is able to be translated and replicated with no complications, and that decreased levels of HuR promote the accumulation of viral RNA. To confirm these data we performed a reciprocal experiment (Figure 3B), where HuR overexpression starkly decreased GLuc activity. This suggests that HuR is acting antiviral and effecting the ZIKV infection at the point of replication. While the binding of HuR to 3' UTRs is known to stabilize cellular mRNAs, our data indicate the the presence of HuR decreases or limits the accumulation of ZIKV RNA. It is possible that depletion of HuR directly or indirectly affects ZIKV gene expression, where the absence of HuR stabilizes the viral RNA and thus promotes ZIKV replication. Alternatively, depletion of HuR might destabilize a cellular mRNA that promotes ZIKV gene expression. Regardless, our data are the first to ascribe an antiviral for HuR.

In Figure 4, we similarly wanted to investigate whether G3BP1 functioned in ZIKV translation or replication. Figure 4A shows GLuc activity following siRNA-mediated knockdown of G3BP1 and the siGL2 control. As in Figure 3A, siGL2 control showed an increase in GLuc activity throughout, but when G3BP1 was knockdown decreased GLuc activity

was observed at 48 and 96 hours post-transfection of the reporter genome. Conversely, in Figure 4B, when G3BP1 is overexpressed we observed a four-fold increase in replication. Together these datasupport a proviral role for G3BP1 in ZIKV replication.

The research undertaken in this Honor's thesis increases our understanding of ZIKV-host interactions, and is particularly relevant as to date there is no licensed vaccine or anti-viral treatment. For those living in at risk areas, particularly women that are looking to get pregnant, preventative measures would be extremely comforting and protect both mother and baby from any fetal defects. Through this study, we gained an understanding on how proteins associated with stress granules interact with ZIKV, and that HuR and G3BP1 specifically act on the replication of the virus. Future research will focus on replicating these results in both the Puerto Rican (2015) strain as well as the Ugandan (1947) strain. This study has additionally opened up a new avenue of research investigating the role of neuronal-specific HuR proteins, and whether these proteins similarly show antiviral activities and the role these neuronal HuR proteins might play in neurological defects observed in newborns following verticle transmission.

References:

1. Ammerman, N. C., Beier-Sexton, M., & Azad, A. F. (2008). Growth and maintenance of Vero cell lines.*Current Protocols in Microbiology*, Appendix 4E.
2. Anderson, P., & Kedersha, N. (2009). Stress Granules. *Current Biology* 19(10): R397-398.
3. Boss, S., et al. (2018). The structural proteins of epidemic and historical strains of Zika virus differ in their ability to initiate viral infection in human host cells. *Virology* 516: 265-273.
4. CDC. (2017, April 24). Zika Virus. https://www.cdc.gov/zika/hc-providers/preparing-for-zika/clinicalevaluationdisease.html
5. CDC. (2018, September 27). Zika and Pregnancy. https://www.cdc.gov/pregnancy/zika/testing-follow-up/zika-syndrome-birth-defects.html
6. Emara, M. M., & Brinton, M. A. (2007). Interaction of TIA-1/TIAR with West Nile and dengue virus products in infected cells interferes with stress granule formation and processing body assembly. *Proceedings of the National Academy of Sciences of the United States of America* 104(21): 9041-9046.
7. Fernandez-Garcia, M., Mazzon, M., Jacobs, M., & Amara, A. Pathogenesis of Flavivirus Infections: Using and Abusing the Host Cell. *Cell Host and Microbe* 5(4): 318-328.
8. Krelle, A. C., Okoli, A. S., & Mendz, G. L. (2013). Huh-7 Human Liver Cancer Cells: A Model System to Understand Hepatocellular Carcinoma and Therapy. *Journal of Cancer Therapy* 04(02): 606-631.
9. Markmiller, S., Soltanieh, S., Server, K., & Mak, R. (2018). Context-Dependent and Disease-Specific Diversity in Protein Interactions within Stress Granules. *Cell* 172(3): 590-604.
10. Medigeshi, G. (2011). Mosquito-borne flaviviruses: Overview of viral life-cycle and host–virus interactions. *Future Virology* 6(9)

11. Mehrjardi, M. Z., (2017). Is Zika Virus an Emerging TORCH Agent? *Virology: Research and Treatment* 8: 1178122X17708993.
12. Poltronieri, P., Sun, B., & Mallardo, M. (2015). RNA Viruses: RNA Roles in Pathogenesis, Coreplication and Viral Load. *Current Genomics* 16(5): 327-335.
13. Uncini, A., Shahrizaila, N., Kuwabara, S. (2017). Zika virus infection and Guillain-Barré syndrome: a review focused on clinical and electrophysiological subtypes. *BMJ Neurology Neurosurgery Psychiatry* 88:266-271.
14. Reineke, L. C., & Lloyd, R. E. (2013). Diversion of stress granules and P-bodies during viral infection. *Virology* 436(2): 255-267.
15. World Health Organization. (2016). The history of Zika virus. https://www.who.int/emergencies/zika-virus/timeline/en/