5-2018

# Disruption of RNA Metabolism by Zika Virus

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# Disruption of RNA Metabolism by Zika Virus

by

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A Thesis
Submitted to the Honors College of
The University of Southern Mississippi
in Partial Fulfillment
of the Requirement for the Degree of
Bachelor of Science
in the Department of Biological Sciences

May 2018

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## Abstract

Flaviviruses are positive, single-stranded, enveloped RNA viruses that are a part of the family, Flaviviridae. West Nile virus, Dengue, Zika virus and more are a part of this family. Mosquitoes are the vectors for these viruses. In order for the virus to infect mosquitoes, it must evade the RNA interference (RNAi), which is the major antiviral immune mechanism of insects. One study found the 3' untranslated region (UTR) of the West Nile virus that inhibited the RNAi (GP et al. 2016). The goal of this study is to investigate if the 3' and 5' UTR region of the Zika virus (ZIKV) inhibits the RNAi as well. Computational approaches were used to look at changes in short interfering (siRNA) expression in animals followed by infection with Zika, which revealed inhibition by the virus. UTR sequences from Zika virus were cloned and tested for suppression of RNAi in Drosophila S2 cells. Suppression of siRNA production was not observed through northern blotting experiments. This research concluded that the 3' and 5' UTR of Zika virus do not play a part in inhibiting the RNAi. The computational data though, supported that Zika virus does inhibit RNAi, suggesting that a mechanism is a work that is divergent from other flaviviruses.



**Keywords**: flavivirus, zika, RNAi, UTR, siRNA, *Drosophila*

## Acknowledgements

I would like to thank my thesis advisor, Dr. Alex Flynt, for teaching me and guiding me through this research process. You have taught me much and exhibited unending patience. You never failed to answer my many questions, no matter how many times or different ways I found to ask them. I could not imagine working with another adviser on this project.

I would like to thank my graduate student, Matthew de Cruz, for assisting with all of my experiments. You never failed to guide me in each step of the experiments. I gained many lab skills from you. I am thankful that you put up with me, taught me, and made sure we had fun along the way.

Kody Mansfield also deserves a huge thank you for his time and effort put in towards the computational data. This thesis could not have been done without you. You are going to accomplish much at NYU- congrats!

Lastly, I want to thank all of my friends who walked this journey with me. Most of you are not biology majors, so I greatly appreciate your willingness to listen to my ramblings about research even though you had no idea what I was talking about.

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# List of Abbreviations

| | |
|---|---|
| DENV | Dengue virus |
| dsRNA | double-stranded RNA |
| hpRNA | hairpin RNA |
| mRNA | messenger RNA |
| PCR | polymerase chain reaction |
| RNAi | RNA interference |
| sfRNA | subgenomic flavivirus RNA |
| siRNA | small interfering RNA |
| UTR | untranslated region |
| WNV | West Nile virus |
| ZIKV | Zika virus |

## Chapter 1: Introduction

Zika virus (ZIKV) has been recognized recently for its outbreaks that are now reaching into the Americas. It was originally isolated in Uganda from the blood of a particular species of monkeys in 1947. The next year the virus was also found in mosquitoes from the same area (Lanciotti et al. 2008). One year ago, ZIKV spread to the Americas. Specifically, it infected Campina Grande, Brazil. Using phylogenetic and molecular clock analyses, it was found to have possibly been introduced into the Americas between May and December of 2013 (Fario et al. 2016). This is over a year before it was realized. There are not always consequences to this arbovirus but sometimes its side effects can have long term effects. One of the consequences most are worried about is that the virus can cause birth defects in the child of those who are pregnant when bitten. Still to this day, the people of Campino Grande are dealing with the consequences of its introduction. It is approximated that over 1,000 babies have been born with birth defects caused by Zika Virus (Faria et al. 2016). One of the main side effects of this is microcephaly. Microcephaly manifests as an abnormally small head resulting from a brain that did not complete development. There are now public health alerts concerning traveling to places where the virus has spread. One of the more recent places it has been found to have spread to is parts of Florida. The concern of the public has led to ZIKV being a topic of interest in research labs. Research has found mosquitoes, specifically *Aedes africanus*, *Aedes luteocephalus*, and *Aedes aegypti*, to be the vector of the virus (Duffy et al. 2009). Our research group has found inhibition of RNAi in mosquitoes, which are vectors for the virus. When RNAi is inhibited, the mosquitoes are then infected

with the virus. It would be beneficial to determine exactly which gene actually inhibits the RNAi.

A mosquito does not fight a virus the same way a human does. RNA Interference (RNAi) is the mechanism to essentially silence detrimental or unnecessary genes. A mosquito utilizes RNAi to protect itself from the invasion of a virus. It is a process, also known as posttranscriptional gene silencing, that silences gene expression in response to double-stranded RNA (dsRNA). These dsRNA initiate the degradation of mRNA (Gitlin and Andino 2003). The dsRNA is cut into small pieces, called small interfering RNAs (siRNA), by an enzyme referred to as "Dicer". The siRNA then binds to Argonaute proteins, allowing one strand of the dsRNA to be removed. The remaining strand is now able to bind to messenger RNA (mRNA) target sequences. Once bound, the Argonaute protein can either cleave the mRNA, which would destroy it, or recruit accessory factors to regulate the target sequence (Filipowicz et al. 2005).

Zika is not well-studied compared to other flaviviruses such as West Nile virus (WNV) and Dengue (DENV-2) are in this same family and have been studied sufficiently. These particular viruses have been found to inhibit the RNAi of their mosquito vectors. With this knowledge, it is assumed ZIKV would use the same mechanism. The exact mechanism ZKV uses is what is being researched. Information from what has been learned of the other flaviviruses is being used to assist in making deductions.

WNV is a flavivirus with a mosquito as its vector, specifically *Culex pipiens* (Campbell et al. 2002). West Nile fever in its simplest form is associated with fever, headache, myalgia, and oftentimes gastrointestinal symptoms. In an acute form, it will

generally last less than a week. Other, more dangerous cases are characterized by neurological damage, encephalitis, or meningoencephalitis (Campbell et al. 2002). The transmission of WNV to mammals is generally dependent on the infection of the mosquito. In order for the WNV to infect the mosquito, there must be a gene in its sequence that inhibits the RNAi of the mosquito. Research is striving to determine which gene of flaviviruses is the inhibitor. One study of West Nile virus identified the 3' and 5' UTR as viral suppressors of RNAi (GP et al. 2016). Those same regions are found in the Zika virus.

As ZIKV is studied, more is found about its genome. It is composed of three structural proteins and seven nonstructural proteins. The structural proteins consist of a capsid (c), a protein that protects the envelope during assembly (prM), and an envelope glycoprotein which is a target for neutralizing antibodies (E). The nonstructural proteins are NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. They have a role in replication and also invoke cell-mediated immune response. On either side of the genome, there is also a 3' and 5' untranslated region (UTR). In some of the other flaviviruses, untranslated regions have been known to be significant in viral replication and immune variation. Each of the described genes and regions can be seen in sequence in Figure 1. Other than that small description, there is a poor amount of information on the function of these nonstructural proteins in the Zika genome.


**Figure 1.** Genome of Zika virus

The goal of this research is to take both of the UTR, express each of them, and determine if it obstructs the RNAi in S2 cells. Okamura et al. found hairpin RNA (hpRNA) pathways in Drosophila that produce endogenous siRNAs. These hpRNAs, specifically hp-CG4068B, when in S2 cells, were found to be dependent on Dicer-2 and Argonaute-2 (2005). This is beneficial because when we can blot for hp-CG4068B and look for a repression in Dicer-2 and Argonaute-2 to signify an inhibition of the RNAi pathway. Our research is significant because understanding the mechanism could ultimately prevent the spread of flaviviruses such as West Nile virus and Zika virus by targeting the host vector interactions. For this particular study, if the inhibiting gene is determined in the genome of ZIKV, it could be mutated out of the virus, thereby exposing the virus to the insect immune system.

## Chapter 2: Methodology

This research will use two different strategies to determine how Zika virus perturbs the RNA metabolism. The first strategy is a genome wide computational analysis. The second strategy utilizes a cell system.

The first project was a computational analysis of the RNA. Figure 2 displays the process taken to analyze the RNA. A dataset from a published study was utilized in the analysis as annotations. Saldana et al. performed a study on the interactions between ZIKV and *Aedes aegypti* mosquitoes (2017). They found that ZIKV, similar to other flaviviruses, does induct a host defense in mosquitoes (Saldana et al. 2017). The genome of the mock and infected animals was mapped using Bowtie2 (Langmead and Salzberg 2012). When a genome is mapped it is essentially placing specific markers along it.

Annotations were retrieved from the genome browser. These annotations provide precise information concerning the genome, such as where a gene begins or ends. The mapping and annotations were combined using Bedtools (Quinlan and Hall 2010). The "R" module through DESeq (Anders and Huber 2010), which normalizes the data retrieved from Bedtools, was ultimately utilized to determine the presence of differential expression.

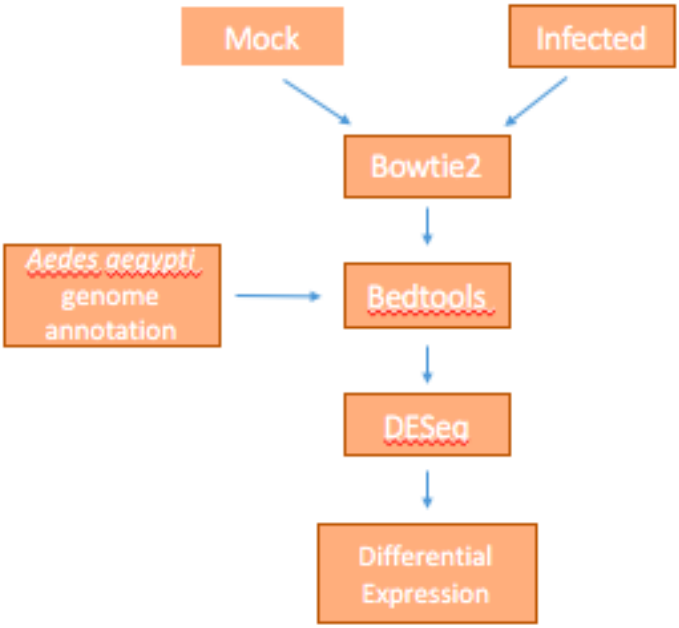


**Figure 2.** Flowchart of computational pipeline.

The second phase of the project consisted of multiple wet lab experiments. Reverse transcription is used to generate a complementary DNA from an RNA template of cells infected with ZIKV. Polymerase chain reaction (PCR) is utilized to amplify the DNA in order to make multiple copies. PCR is made from the combination of multiple agents being added together in a PCR tube. Generally, 10 microliters of 5x buffer, 1 microliter of dNTP, 1 microliter of primer, 1 microliter of fragments, 37 microliters of

RNase free water, and 5 microliters of Phire II enzyme are added together. The primer is a combination of the forward and reverse strand. The amounts of each of these may vary, but it must always total about 55 microliters. It is then put in the PCR machine in order to incubate.

RNA from infected cells was reverse transcribed. This reaction was subjected to PCR to amplify each Zika gene. Sequences from each were used to assist in designing the primers. For these particular primers, a single strand positive virus is being used to make a double strand so it can be put into animals. First, the sequence or ECORI is needed, followed by a start codon and then the beginning sequence of a genome sequence. These are going in the forward direction. Then, the end of the genome sequence is needed, followed by a stop codon and the sequence of NOT1. These are going in the reverse direction so they have to be reverse complimented. Once all of those sequences have been pieced together, a primer has been made. Each primer pair will add homology arms for insertion into PUAST.

Once the DNA is cloned, a SLiCE reaction is inserted. SLiCE is a method of cloning that makes use of bacterial cell extracts in order to fit numerous DNA fragments into molecules of recombinant DNA in one reaction (Zhang et al. 2014). The SLiCE reaction individually cloned a protein or region from the ZIKV genome. Figure 1 displays the ZIKV genome and its composition. Specifically, the 3’ and 5’ UTR are the ones being expressed. The SLiCE reaction itself is also made from the addition of several agents. The basis is 2 microliters of insert, 1 microliter of 10x SLiCE buffer, 2 microliters SLiCE extract, 0.25 microliters of a dilution of a vector, and up to 10 microliters of RNase free water. Each of these amounts is subject to change depending on the concentration of the

insert. Overall, the reaction should be about 10 microliters. All of this is also added to a PCR tube and is then incubated at 37 degrees Celsius for 1-2 hours.

Figure 3 shows the vector with the sites of restriction and digestion utilized during the SLiCE reaction. The enzymes specific to these sites are ECORI and NOT1. An example of an UTR being joined with the vector through a ligation reaction is seen in Figure 4.

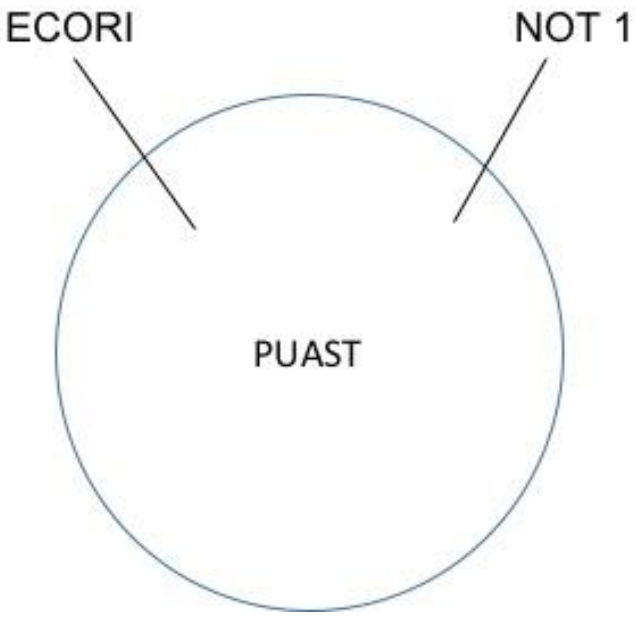


**Figure 3.** Restriction and digestion sites of vector.

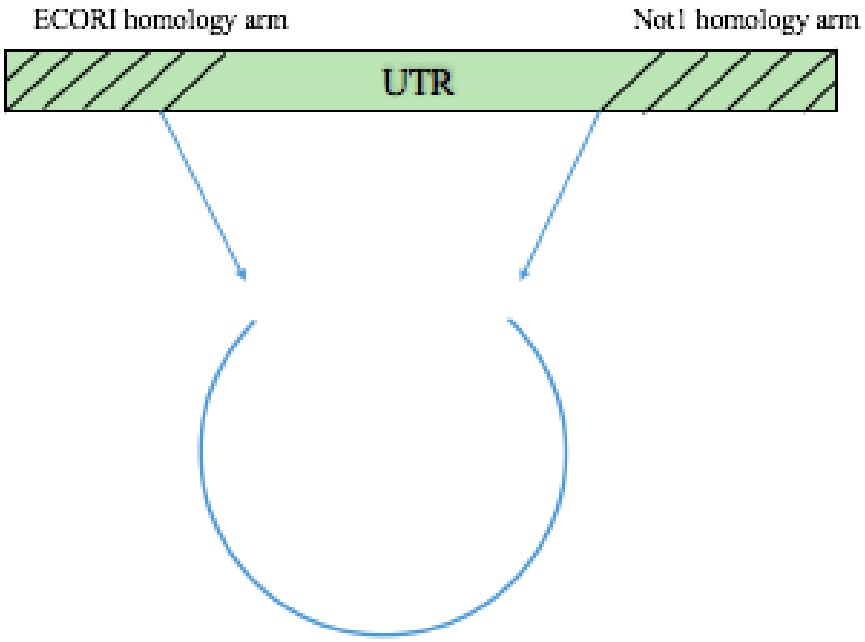


**Figure 4.** Example of protein binding to vector in ligation reaction.

The protein is then inserted into a drosophila expression vector. The S2 cell of drosophila is transfected. About 18-24 hours before transfection, the cells are to be plated in 2.5 ml complete growth medium per well in a 6-well plate. The cells should be around 80% or greater confluent prior to transfection. The cells are then incubated in cell cultures overnight. Right before transfection, the TransIT-LT1 Reagent is warmed to room temperature and vortexed gently before using. 250 microliters of Opti-MEM I Reduced-Serum Medium is added in a sterile tube. Then, 2.5 micrograms of plasmid DNA is also added. It is pipetted gently in order to mix completely. 7.5 microliters of TransIT-LT1 Reagent is added to the diluted DNA mixture. It is pipetted gently again until completely mixed. Then, incubated at room temperature for 15-30 minutes. The TransIT-LT1 Reagent: DNA complexes are added drop-wise to different areas of the

wells. The culture vessels are gently rocked back-and-forth and from side-to-side to evenly distribute the TransIT-LT1 Reagent: DNA complexes. They are incubated for 24-72 hours. The cells are then harvested and assayed.

RNA is then extracted from the cell. The first part of the extraction process is referred to as homogenization. For tissues that are snap frozen or slightly in excess, the homogenization of the tissue should be down by mortar and pestle. Simultaneously, at least 1 milliliter of TRIZOL/ 100 milligram tissue is to be transferred to be homogenized in a falcon tube. The tissue is then transferred to the pestle and grinded until all that is left is a very fine layer of dust. The RNase free spatula is used to transfer the dust to the TRIZOL solution. The mixture is then vortexed thoroughly. Once the mixture is homogenized, the solution is aliquoted to an Eppendorf tube. It is left in TRIZOL at room temperature for 5 minutes. The next steps are the phase separation. Two hundred microliters of chloroform/ 1 milliliter TRIZOL is added then vortexed for 15 seconds and left at room temperature for 2-3 minutes. The samples are centrifuged at 12,000 g for 15 minutes at 2-8 degrees Celsius. The next phase is RNA precipitation. There should be three visible phases in the tube. The top, aqueous layer is transferred to a new tube. In the new tube, 500 microliters of isopropanol/ 1 mL TRIZOL is added and then incubated at room temperature for 10 minutes. The samples are then centrifuged at 12,000 g for 10 minutes at 2-8 degrees Celsius. The next steps are in order to wash and re-suspend the RNA. After centrifugation, the supernatant is removed. The RNA pellet is washed with 80% EtOH/ 1 mL TRIZOL and then vortexed. The samples are centrifuged at 7,500 g for 5 minutes at 2-8 degrees Celsius. Again, the supernatant is removed and the remaining EtOH is allowed to air dry for 2-3 minutes. The pellet is re-dissolved in 81 microliters of

DEPC water. It then undergoes a DNase treatment. Eight microliters of 10X DNase I Buffer and 2 microliters of DNase I Enzyme are added and then vortexed for a quick spin followed by incubation at 42 degrees Celsius for 25 minutes. Then, it undergoes RNeasy column purification. To the solution, 350 microliters of Buffer RLT and 250 microliters of 100% EtOH are added. The entire volume is applied to the RNeasy column and spun at full speed for 1 minute. The entire volume is then reapplied to the RNeasy column and spun again at full speed for 1 minute. The column is then transferred to a new 2 mL collection tube. 750 microliters of Buffer RPE is added and also spun at full speed for 1 minute. The flow-through is discarded and another 750 microliters Buffer RPE is added and spun at full speed for 1 minute. The flow-through is again discarded and spun at full speed for 1 minute. The column is transferred to a new 1.5 mL Eppendorf tube and 56 microliters DEPC H2O is added. After sitting for 2 minutes, it is spun at full speed for 2 minutes. The column is discarded and the tube is transferred to ice. The final phase is quantification and quality control. Each sample is quantified using the Nanodrop. Finally, 5 microliters of each sample is ran on Agarose gel.

From there, the RNA undergoes the northern blot test. Northern blotting analyzes a sample of RNA in order to measure the RNA expression of specific genes. A 12% sequence gel is made of Sequagel Diluent, Sequagel Concentrate, and Sequagel Buffer. Then, 200 microliters of 10% APS and 10 microliters TEMED is added. The RNA sample is prepared. 20 micrograms per lane of total RNA are precipitated with 1/10 the volume 3M NaOAc and 2.5 vol EtOH. The pellet is dissolved with 5 microliters 2X Sample Buffer (Ambion). The gel is pre-run under 250V 30mA for 30 minutes in 0.5X TBE. After the samples are applied and the gel is run at 250V 30mA for 3 hours. The gel

is stained with EtBr so a picture can be taken. It is transferred onto Nylon membrane in 0.5X TBE under 10V 300mA for 1 hour in a cold room. It is crosslinked with an UV-crosslinker. The membrane is dried at 80 degrees Celsius for 30 minutes. It is then prehybridized with a hybridization Buffer at 45 degrees Celsius. The radio-labeled probe is prepared with 2 microliters of 1.5 micro-molar LNA oligonucleotide, 3 microliters of 10X T4 PNK buffer, 21 microliters water, 3 microliters Gamma-ATP (6000Ci/mmol), and 1 microliter of T4 PNK. It is incubated for 1 hour at 37 degrees Celsius. The reaction is stopped by adding 20 microliters 20mM EDTA. The probes are cleaned with a Roche spin column. The column caps are discarded and the column set on a waste tube. The buffer is drained by gravity and discarded. The column in centrifuged at 2000 rpm for 4 minutes. The eluate is collected into a new tube. It is eluted with 50 microliters TE. Then, it is again centrifuged at 2000 rpm for 4 minutes. Most of the labeled probe should be eluted in second fraction. The Radio-labeled probe is added to pre-hybridization solution. It is washed with 2X SSC, 0.1% SDS or 0.2X SSC, 0.1% SDS for 15 minutes, 3 times, at 45 degrees Celsius. It is then exposed to a phosphoimager.

## Chapter 3: Results

Saldana et al. confirmed in a study that ZIKV is controlled by RNAs in Aedes aegpyti mosquitoes (2017). Figure 5 shows that most of the RNA is 21 nucleotides, which would most likely be small interfering RNA (siRNA). Figure 6, derived from the same study, shows that the siRNAs are integrated in the positive and negative strand of the ZIKV genome. This study suggests that ZIKV is controlled by siRNA.

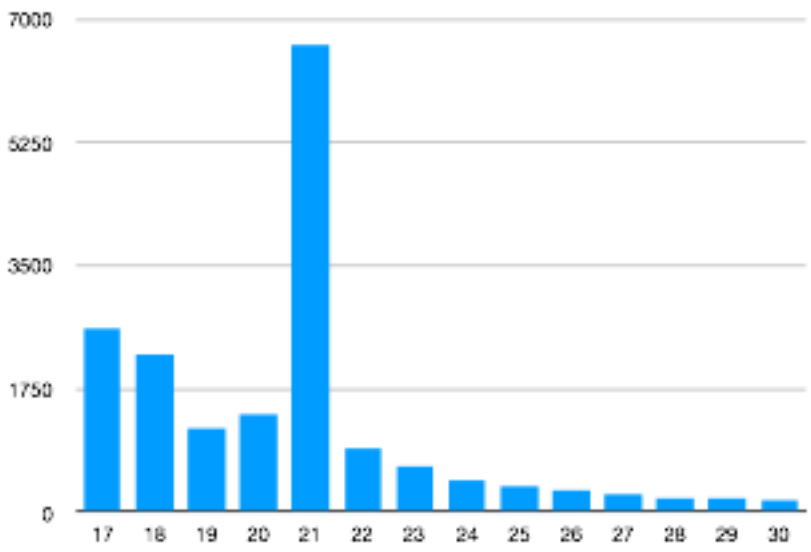


Figure 5. Sizes of RNA found in *Aedes aegypti* mosquitoes.

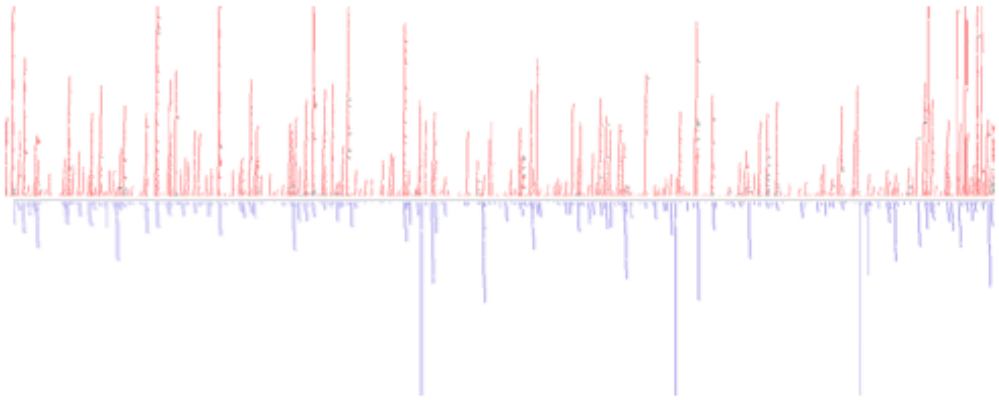

**Figure 6.** Small RNAs derived from Zika virus positive and negative strand.

The computational data compared RNA of Mock and ZIKV cells from Drosophila at a particular locus. Figure 7 displays expression of siRNA in the mock and ZIKV cells. There is less siRNA in ZIKV than in the mock cells. That same locus was plotted with many other loci to show siRNA expression with ZIKV versus without it. The green line in the middle of figure 8 shows that these loci express the same amount of siRNA

whether it is infected with ZIKV or not. All of the dots under the line shows a lesser amount of siRNA in ZIKV infected cells. The transposable element loci from figure 7 can be found below the line.

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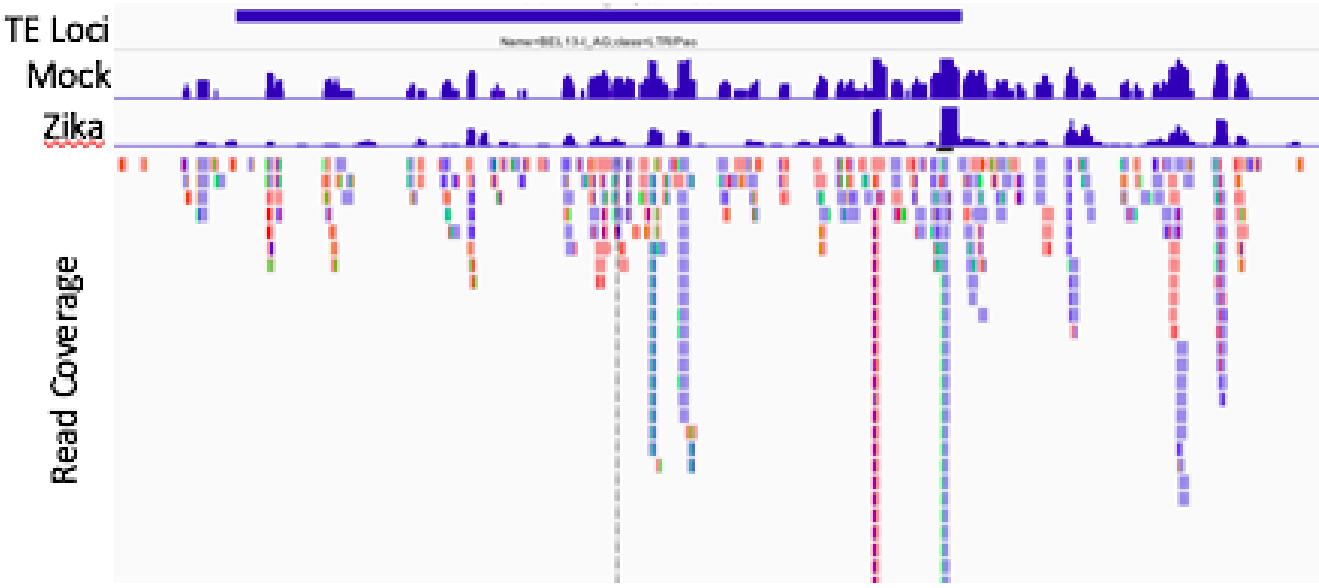


**Figure 7.** Differential expression of Mock versus ZIKV infected cells.

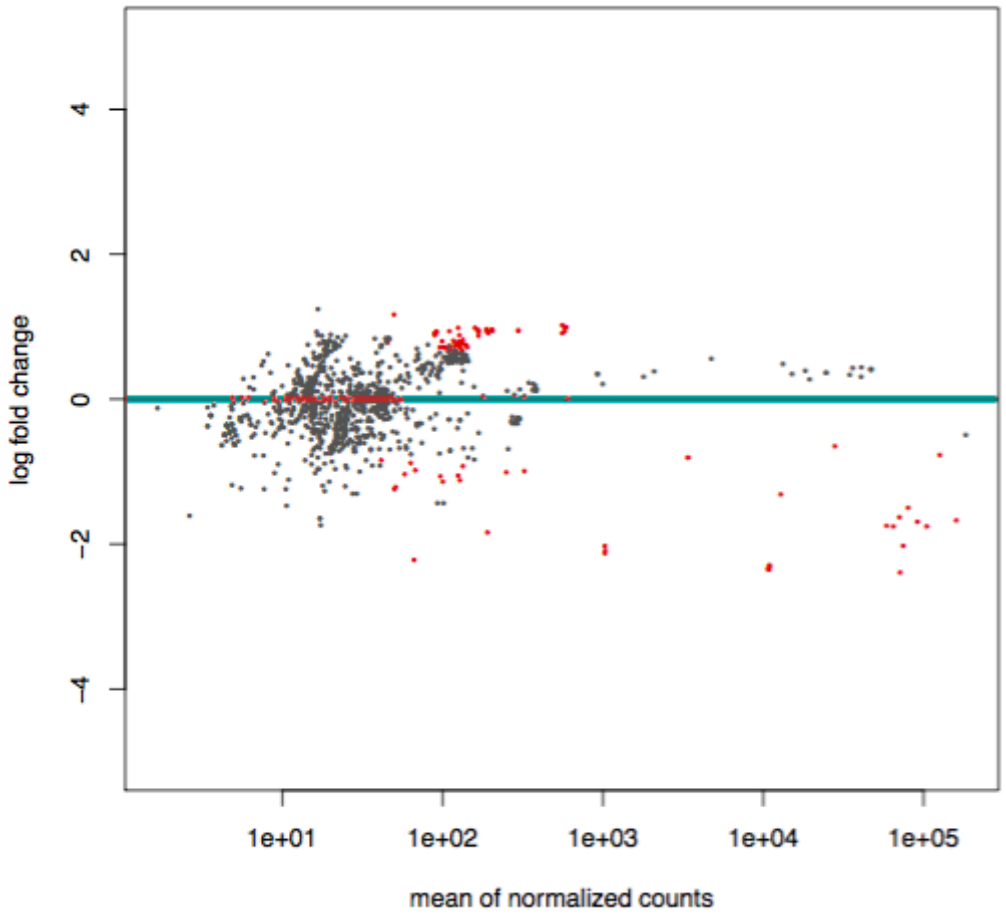


**Figure 8.** Scatter plot of siRNA at various loci.

There was also a generous amount of wet lab research done. The RNA from ZIKV infected cells, received from our collaborators in Dr. Bai's lab, was from a neuroblast cell line of a mouse, n2a. Reverse transcription was performed in order to derive DNA from the infected cells.

The plasmid we utilized in our lab work was pU6-Bbsl-chiRNA. Figure 9 depicts the plasmid. It is 3458 base pairs. The restriction enzyme we utilized was Bbs1. The site of restriction was downstream of the U6 promoter. The promoter region allowed for RNA polymerase to bind to it and transcribe the UTR into RNA form. Once the insert was sliced into the plasmid, it was sent off for sequencing. The sequencing confirmed the new

plasmid design was successful. The plasmid was then transfected into drosophila S2 cells. During transfection, there was a growth period for the cells. The cells, preferably, should be about 80% confluent. For cells that were adherent, the range of density is $1.6 - 3.2 \times 10^5$ cells/ml. A hemocytometer was used to check the confluency of the cells throughout the growth period.

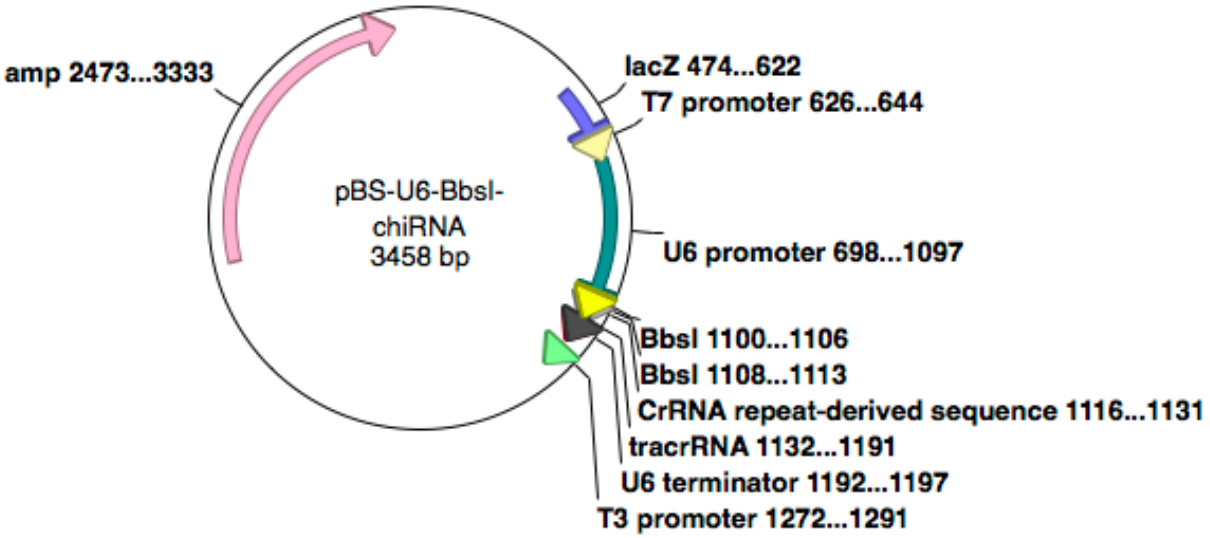


**Figure 9.** Graphic of plasmid design.

The extracted RNA underwent a Northern blot test. The test included a RNA ladder, three samples of the 3' UTR region, and three of the 5' UTR region. Each set of three samples increased in size. Figure 10 shows they all had the same band, exhibiting the same amount of siRNA.

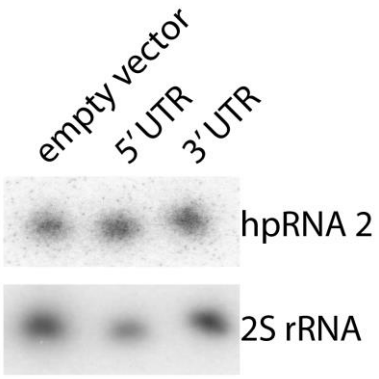


**Figure 10.** Northern blot of 3' and 5' UTR of ZIKV

## Chapter 4: Conclusions

Flaviviviruses are a part of the family of Flaviviridae that are positive-sense, single-stranded, enveloped RNA viruses. They are primarily found in ticks and mosquitoes and include WNV, Dengue, ZIKV, and more. It is known that subgenomic flavivirus RNA (sfRNA) plays a role in the transmission of these viruses to their host. GP et al. demonstrated that sfRNA inhibited the RNAi within the 3' UTR regions of the WNV genome (2016). The purpose of this study was to determine if the same conclusion could be made for ZIKV.

The computational data was done to make sure that ZIKV does interact with the RNAi mechanism. Figure 7 and figure 8 show the repression of siRNA production in ZIKV. Short interfering RNA play a role in the RNAi pathway. The computational data supports that ZIKV does inhibit RNAi.

The Northern blot allowed us to infer that the UTRs we were investigating do not have a role in inhibiting the RNAi pathway. We investigated the UTRs that inhibit RNAi

in West Nile Virus. If there had been inhibition, the bands of the Northern blot would have exhibited less siRNA.

In conclusion, ZIKV has been confirmed to downregulate siRNA in order to evade the RNAi pathway, allowing it to invade the host. However, the 3'and 5' UTR region of the ZIKV is not involved in the repression of RNAi of its host. This could mean that is in a region other than the UTR, the mechanism could be mosquito specific, or ZIKV does not try to evade RNAi. In the future, a different region within the ZIKV genome could be tested for RNAi repression. For example, the nonstructural proteins could be expressed and tested.

**References:**

Anders, Simon, and Wolfgang Huber. "Differential expression analysis for sequence count data." *Genome biology* 11.10 (2010): R106.

Campbell, Grant L., Marfin, Anthony A., Lanciotti, Robert S., Gubler, Duane J. West Nile virus. The Lancet Infectious Diseases. 2002 Aug.

Duffy, Mark R., et al. "Zika virus outbreak on Yap Island, federated states of Micronesia." *New England Journal of Medicine* 360.24 (2009): 2536-2543.

Faria, Nuno Rodrigues, da Silva Azevedo, Raimunda do Socorro, Kraemer, Moritz U. G., Souza, Renato, Cunha, Mariana Sequetin, et al. 2016. Zika virus in the Americas: Early epidemiological and genetic findings. Science. 352: 345-351.

Filipowicz, Witold. "RNAi: the nuts and bolts of the RISC machine." *Cell* 122.1 (2005): 17-20.

Gitlin, Leonid and Andino, Raul. 2003. Nucleic Acid-Based Immune System: the Antiviral Potential of Mammalian RNA Silencing. Journal of Virology. 77: 7159-7165.

GP, Goertz, JJ, Fros, P., Miesen, CB, Vogels, ML, van der Bent, C., Geertsema, CJ, Koenraadt, RP, van Rij, MM, van Oers, GP, Pijlman. 2016. Non-coding subgenomic flavivirus RNA is processed by the mosquito RNAi machinery and determines West Nile virus transmission by Culex pipiens mosquitoes. Journal of Virology. 1-49.

Lanciotti RS, Kosoy OL, Laven JJ, Velez JO, Lambert AJ, Johnson AJ, et al. Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007. Emerging Infectious Diseases. 2008 Aug.

Langmead, Ben, and Steven L. Salzberg. "Fast gapped-read alignment with Bowtie 2." *Nature methods* 9.4 (2012): 357.

Okamura, Katsutomo, et al. "The Drosophila hairpin RNA pathway generates endogenous short interfering RNAs." *Nature* 453.7196 (2008): 803.

Quinlan, Aaron R., and Ira M. Hall. "BEDTools: a flexible suite of utilities for comparing genomic features." *Bioinformatics* 26.6 (2010): 841-842.

Saldaña, Miguel A., et al. "Zika virus alters the microRNA expression profile and elicits an RNAi response in Aedes aegypti mosquitoes." *PLoS neglected tropical diseases* 11.7 (2017): e0005760.

Zhang, Yongwei, Uwe Werling, and Winfried Edelmann. "Seamless ligation cloning extract (SLiCE) cloning method." *DNA Cloning and Assembly Methods*. Humana Press, Totowa, NJ, 2014. 235-244.