Summer 2018

Zika Modulates Arthropod Histone Methylation in Mosquito Cells

Telvin Lee Harrell
Old Dominion University

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ZIKA MODULATES ARTHROPOD HISTONE METHYLATION
IN MOSQUITO CELLS

by
Telvin Lee Harrell
B.S., May 2015, Christopher Newport University

A Thesis Submitted to the Faculty of
Old Dominion University in Partial Fulfillment of the
Requirements for the Degree of

MASTER OF SCIENCE
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August 2018

Approved by:
Girish Neelakanta (Director)
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Emelia Oleszak (Member)
ABSTRACT

ZIKA MODULATES ARTHROPOD HISTONE METHYLATION IN MOSQUITO CELLS

Telvin Lee Harrell
Old Dominion University, 2018
Director: Dr. Girish Neelakanta

Epigenetics is the heritable series of covalent modifications that affect chromatin structure, gene expression, and protein function. Methylation is one such epigenetic modification that involves the addition of chemical modifying entities, such as methyl groups, on nucleic acids or proteins. Recent studies have reported that Zika virus (ZIKV) modulates methylation of human and viral RNA important for its replication in vertebrate cells. However, little is known whether ZIKV exerts methylation in arthropod vectors. In this study, I show that ZIKV modulates S-adenosyl methionine (SAMe) synthase, an enzyme involved in the production of SAMe, and histone methylation for its survival in Aedes albopictus derived C6/36 cell line. Quantitative real-time PCR (QRT-PCR) and immunoblotting analysis revealed increased amounts of the mosquito SAMe synthase at both RNA and protein levels, respectively, in ZIKV-infected C6/36 cells at day 1 post infection (p.i.) in comparison to uninfected controls. Increase in the intracellular SAMe levels was noted in ZIKV-infected C6/36 cells at day 1 p.i. in comparison to uninfected controls. In addition, significant increased amounts of Enhancer of zeste homolog 2 (EZH2) histone methyl transferase-like gene transcripts and histone tri-methylation (H3K27me3) were noted in ZIKV-infected C6/36 cells in comparison to uninfected controls. Exogenous treatment with SAMe showed significant increased viral burden and expression of EZH2 transcripts in ZIKV-infected C6/36 cells at day 1 p.i. in comparison to mock-treated controls. Treatment with DZNep, a known EZH2 inhibitor, revealed a decrease in H3K27me3 and ZIKV burden in ZIKV-infected C6/36 cells. In summary, my data suggests ZIKV-associated modulation of SAMe cycle and H3K27me3, critical for its survival in mosquitoes.
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This thesis is dedicated to “Him” who has made a way despite my doubt.
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I want to acknowledge my family and friends that helped me cope with the stress of getting to this point in my education. Many thanks are extended to my professors and committee members who educated me and provided me with the necessary skill set to produce this work outlined in this thesis. Finally, I want to thank the staff in the Biological Sciences Office for the constant donation of food, entertainment, and graduate counseling they have provided.
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<table>
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<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>CTE</td>
<td>Cytotoxic Effects</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<tr>
<td>DZNep</td>
<td>3- Deazaneplanocin A Hydrochloride</td>
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<tr>
<td>EED</td>
<td>Embryonic Ectoderm Development</td>
</tr>
<tr>
<td>EZH2</td>
<td>Enhancer of zeste homolog 2</td>
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<tr>
<td>H3K27me3</td>
<td>Tri-methylation of Histone H3 at Lysine 27</td>
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<tr>
<td>HAT</td>
<td>Histone Acetyl Transferases</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone De-Acetyltransferases</td>
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<tr>
<td>HRP</td>
<td>Horse Radish Peroxidase</td>
</tr>
<tr>
<td>IPSC</td>
<td>Induced Pluripotent Stem Cells</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of Infection</td>
</tr>
<tr>
<td>NS5</td>
<td>Non-Structural Protein</td>
</tr>
<tr>
<td>PcG</td>
<td>Polycomb Group</td>
</tr>
<tr>
<td>PCR1/2</td>
<td>Polycomb Repressive Complex 1 and 2</td>
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<tr>
<td>p.i.</td>
<td>Post Infection</td>
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<td>RNA</td>
<td>Ribonucleic Acid</td>
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<tr>
<td>SAMe</td>
<td>S-adenosyl methionine</td>
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<tr>
<td>SUZ1/2</td>
<td>Suppressor of zeste 12 homology</td>
</tr>
<tr>
<td>SAH</td>
<td>S- Adenosyl homocysteine</td>
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<td>ZIKV</td>
<td>Zika Virus</td>
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CHAPTER 1

INTRODUCTION

1.1 Purpose

Zika virus (ZIKV) is a mosquito-borne human pathogen that can cause severe neurological complications in newborns and adults. Recent reports provide evidence that ZIKV affects methylation of viral and human RNAs during its interactions with vertebrate cells. However, little is known whether methylation is also evident during its interaction with the vector host. The lack of ZIKV-specific information, in particular the strategies that this virus uses to survive in the arthropod host, has resulted in an inability to prevent or stop the ZIKV epidemic. Studies in understanding ZIKV interactions with its arthropod vector will lead in the development of strategies to treat or control transmission of this virus from mosquitoes. This study will also provide information in understanding interactions of other arboviruses with their vector host. Information generated from this research will help the scientific community to understand ZIKV pathogenesis and infection.¹

1.2 Problem

ZIKV, named after the Zika forest in Uganda, Africa, is a positive sense single stranded RNA virus of the genus Flavivirus. ZIKV was initially isolated in 1947 from a Sentinel Rhesus Monkey, and in 1948 from an A. africanus mosquito (Dick et al., 1952). ZIKV, characterized as enzootic, was largely neglected because of the lack of known human cases. In 1952, neutralizing antibodies against ZIKV were detected in serum of a patient co-infected with dengue (Smithburn, 1952), supporting that ZIKV could infect humans. In 1962, a worker in Uganda developed flu like symptoms after being infected with ZIKV (Simpson, 1964); confirming a ZIKV-disease state. For the next 4 decades, ZIKV remained a minor concern with as few as 14 documented human cases (Olson et al., 1981).

In 2007, ZIKV was identified in an epidemic of the Yap Islands of Micronesia with an estimated 73% of the native population infected (Duffy et al., 2009). Sequence analysis revealed divergent Asian and African ZIKV lineages (Simonin et al., 2017), marking evolutionary differences in infectivity and alterations in cell tropism. From 2013-2015, significant increases in fetal microcephaly (Cao-Lormeau and Musso., 2014) and adults Guillain-Barre Syndrome (GBS) (Besnard et al., 2014) was evident corresponding with peak mosquito season. Clinical diagnostics for chikungunya and dengue virus revealed little results. Test for ZIKV were not performed; due to lack of knowledge and availability of specific clinical test. Ultrasound testing revealed ventriculomegaly and intracranial calcification of

¹ This thesis is formatted for the journal Cell Host & Microbe.
fetuses. In addition, muscle and joint pain was noted in infected individuals supporting a ZIKV infection (Pires et al., 2018). Additional detection methods revealed ZIKV in amniotic fluid and fetal brains that provided further evidence for transplacental and ocular ZIKV infections (Martines et al., 2016). Blood donors screened by polymerase chain reaction (PCR) tested positive, indicating possible ZIKV transmission through blood transfusions (Faria et al., 2016). Overall, combined reports provided evidence that ZIKV was circulating in tropical and subtropical regions and a possible cause for the observed rash, congenital malformations, and GBS. As a result, Brazil declared ZIKV a national public health emergency of international concern on February 2016 (https://www.bbc.com/news/health-35459797).

Declaration of Brazils’ national public health emergency initiated multiple tiers of research to elucidate the risk associated with ZIKV infection. However, many aspects of ZIKV pathogenesis and transmission remained unknown. At the time of writing this thesis, no FDA approved diagnostic test(s) and/or vaccine for ZIKV infection is available. Molecular information regarding risk of ZIKV infection are limited. Specific information on virus life cycle, host reservoirs, and interactions of ZIKV with vertebrate or vector hosts are still poorly understood. The current recommendations to prevent ZIKV infections are to avoid areas where this virus may be circulating in mosquito and/or reservoir hosts or delay pregnancy all together. Therefore, there is an immediate need to understand molecular strategies that ZIKV uses to interact with mosquito host. Understanding vector-pathogen interactions, such as this, will provide significant information in the development of transmission blocking vaccine to treat or prevent ZIKV infections.

1.3 Zika Virus

ZIKV is an arbovirus, primarily spread through the bite of an infected Aedes mosquito. ZIKV transmission through sexual contact (Foy et al., 2011), bodily fluids (including breast milk) (Faria et al, 2016), and human placenta (Martines et al., 2016) have been reported. As ZIKV continues to spread, debate on the competency of different mosquito species to transmit ZIKV and strain specific severity is ongoing (Weger-Lucarelli J et al. 2016). The Asian ZIKV strains have evolved to become more pathogenic to humans causing severe neurological and developmental symptoms. The ZIKV strain MR166 (African, Ugandan Strain) has a more diverse cell tropism and has been reported to replicate at high titers inducing apoptosis in a similar manner to the Asian strains (Simonin et al., 2017). Genome analysis of African ZIKV lineages indicates 99.89% similarity between strains isolated in 1947 and 2007 (Shapshak et al. 2015). In the Americas, the Asian lineage of ZIKV has been detected in both A. aegypti and A. albopictus mosquitoes. The Asian strains of ZIKV, have a higher tropism for human neural stem cells (HNSC), and trophoblast (Miner, J, et al. 2016), believed to contribute to ZIKV-induced fetal
microcephaly (Mlakar et al., 2016), and the establishment of host tissue reservoirs possibly prolonging human infection (Calvet et al., 2016).

ZIKV replicates like other known positive sense single stranded RNA viruses that belong to Baltimore classification system group 4. ZIKV is internalized through receptor-mediated endocytosis. Once inside, ZIKV fuses with the endosomal membrane and releases its genome into the cytoplasm (Ramos da Silva and Gao, 2016). ZIKV RNA is translated into a polypeptide that is later processed into individual viral proteins. In addition, ZIKV genome replicates to form new RNA copies for packaging into new viruses. Assembly of new ZIKV happens on the surface of the endoplasmic reticulum. The immature ZIKV forms are later transported via trans-golgi network for maturation and conversion into infectious viruses. The ZIKV 11kb genome encodes 3 structural and 7 nonstructural proteins (Ramos da Silva and Gao, 2016).

Non-structural protein 5 (NS5) is critical for replication, RNA capping, and interferon suppression (Zhao et al., 2017). Damage to the ZIKV encoded NS5 protein is detrimental for the virus and halts intracellular replication (Zhao et al., 2017). The NS5 protein has 2 domains; an N-terminal methyltransferase domain and a C-terminal RNA polymerase domain (Coutard et al., 2017). The RNA polymerase domain synthesizes positive and negative sense RNA for viral gene expression and replication via de novo synthesis (Kao et al., 2001). ZIKV RNA synthesis requires an RNA template for elongation, but not an initiation primer (Kao et al, 2001). The methyltransferase domain of the NS5 protein is used for the addition of 5’cap on viral RNA, which is critical for initial gene expression (Egloff et al., 2002). The addition of the 5’cap on viral RNA by NS5 requires S-adenosyl methionine (SAMe) from the host cell (Egloff et al, 2002). The importance of ZIKV RNA 5’capping for its survival in the host cells indicates viral dependency on host generated SAMe (Zhao et al., 2017).

1.4 Epigenetics: Histone Modification

Epigenetic regulation comprises both heritable and non-heritable genomic modifications that affect cellular development, differentiation, and gene expression (Berger et al, 2009). Occurring in the form of covalent modifications on nucleic acids and proteins, such as histones, epigenetics has implications in a variety of biological functions (Weinhold, 2006). Genetic diseases such as Prader-Willie Syndrome and some cancers are reported to be epigenetic diseases that exhibit extreme phenotypic expression due to methylation patterns and parental imprinting of genes (Cassidy et al., 2012).

The histone code hypothesis involves the interrelation between histone and DNA modifications. The histone code hypothesis states that genetic information is regulated through posttranslational modifications resulting from alterations on histone amino terminal end (N-terminus) that regulate the attached DNA (Jenuwein and Allis 2001). Lysine acetylation, phosphorylation, and methylation
generally occurring in that order) are the key biological posttranslational modifications occurring on histones (Strahl and Allis, 2000). Lysine acetylation is well characterized and there are many known enzymes that work to maintain its steady state. Histone acetyltransferases (HAT) and histone deacetyltransferases (HDAC) are key acetylation enzymes that regulate gene expression during early cell differentiation, but more importantly during DNA replication (Strahl and Allis, 2000). When a newly synthesized DNA strand is being synthesized, pre-acetylated histones must be simultaneously transcribed and synthesized. Pre-acetylated histones then localize to the new DNA strand providing stability and acting as a scaffold (Turner, 1995). The pre-acetylated state of these histones is inhibited through a dynamic acetylation establishment process involving HAT and HDAC activity (Strahl and Allis, 2000).

Histone phosphorylation is implicated in mitosis for chromosome condensation (Van Hooser et al., 1998), and can be associated with transcriptionally active genes (Halegoua and Patrick, 1980). Phosphorylation of histones has been indicated with induction of immediate early genes; *c-Jun*, *c-Fos*, and *c-Myc* (Strahl and Allis, 2000). Expression of these immediate early genes initiate the transcription and translation of genes essential for cellular development (Zhao et al., 1989). Research supports phosphorylation of histones to be critical for both gene regulation and transcription during mitosis and chromatin de-condensation (Zhao et al., 1989).

The methylation of histones is the least studied posttranslational modification (Strahl and Allis, 2000). Electrostatically acetylation neutralizes a positive charge, while phosphorylation imparts a negative charge on histone tails (Strahl and Allis, 2000). Together, acetylation and phosphorylation can indicate combined activity supporting de-condensation of chromatin (Roth and Allis, 1992). However, histone methylation on arginine and lysine residues does not imply a large shift in the electrostatics of histone tails (Strahl and Allis, 2000). The slight electrostatic shift and lack of known participating enzymes complicates research on histone methylation (Strahl and Allis, 2000). Histone methylation is believed to be associated with transcriptional repression or activation depending on the context in which it occurs (Gupta et al., 2010).

Vertebrate histone methylation is accompanied by specific signaling cascades and associated proteins that govern “placement” in relation to other epigenetic modifications. Although there is still much to be discovered, some aspects of epigenetic methylation has been explored to give some insight into the mechanistic function. H3K27 (histone 3, lysine residue 27) methylation is one of the simpler mechanisms of histone methylation. Currently there is one known methyltransferases responsible for methylation at this location (Hyun et al., 2017).
H3K27 can be either mono-, di-, or tri-methylated and is a marker for transcriptionally repressed genes (Hyun et al., 2017). H3K27me3 (H3K27 tri-methylation) leads to heterochromatin formation and gene repression through evolutionarily conserved mechanisms in many species (Hyun et al., 2017). H3K27me3 has a role in the regulation of HOX genes, various epigenetic alteration in cancers and is critical in the development and differentiation of Induced Pluripotent Stem Cells (iPSC) (Xiaolei et al., 2014). Large multi-subunit Polycomb Group (PcG) of proteins known as Polycomb Repressive Complex 1/2 (PRC1/2) carries out regulation of methylation at H3K27. These proteins are key regulators of cell development and chromatin structure (Hyun et al., 2017). PRC1 is composed of 5 subunits (Bmi1, Ring1 A/B, Cbx, Mel18, and Mph) and PRC2 is composed of 3 subunits (EZH2, Eed, and Suz12) (Xiaolei et al., 2014).

PRC2 is responsible for marking the target genomic regions for silencing. Its binding to H3K27 can affect neighboring nucleosomes leading to the formation of heterochromatin within the nearby histone complexes (Shen et al., 2008). The Enhancer of zeste homolog 2 (EZH2) subunit is the SET domain containing catalytic methyltransferase of PRC2 (Shen et al., 2008). It is directly responsible for the methylation of H3K27. EZH2 requires the Embryonic Ectoderm Development (EED) and Suppressor of zeste 12 homology (SUZ12) proteins to function efficiently (Pasini et al., 2004). SUZ12 is responsible for nucleosome recognition and along with EED provides stability that link EZH2 to its substrate for subsequent methylation (Pasini et al., 2004).

1.5 S-adenosyl methionine (SAMe) and the SAMe-Cycle

S-adenosyl methionine (SAMe), synthesized by methionine adenosyl transferase from methionine and adenosine triphosphate (ATP) (Cantoni, 1952), is the central methyl-donor for methylation, aminopropylation, and transsulfuration pathways (Bottiglieri, 2002). SAMe is used in the reactions involving RNA and protein methylation. Regulation of intracellular SAMe levels are maintained through a continued cycle involving its use by methyltransferases and conversion into s-adenosyl homocysteine (SAH) (Kerr, 1972, Finkelstein and Martin, 2000). S-adenosyl homocysteine hydrolase rapidly converts SAH into homocysteine (Kerr, 1972), which is highly toxic to the cell (Shea and Rogers, 2014). Homocysteine is then excreted or retracted back into the SAMe cycle by S-adenosyl methionine synthase (Shea and Rogers, 2014).

SAH is a powerful inhibitor of methylation and acts as a transitional regulator molecule within the SAMe cycle (Kerr, 1972). The balance of SAMe and SAH in the cell is usually denoted as the SAMe-SAH ratio (Cantoni et al., 1980). Regulation of this balance allows the cell to control the SAMe cycle restricting the intracellular level of homocysteine (Catoni et al., 1980). Intracellular maintenance of the SAMe-SAH
balance is essential to prevent methionine or homocysteine toxicity (Shea and Rogers, 2014). Imbalances can cause neurological disorders, psychiatric disorders, and cancer metastasis (Cantoni et al., 1980).

1.6 Hypothesis
ZIKV induces methylation of viral and human RNA in vertebrate cells. So far, studies have not elucidated whether ZIKV induces methylation of vector molecules. Due to the role of SAMe as an important methyl donor, it is not surprising to hypothesize that viruses depend on this molecule for its replication and survival in the host. The objective of this study is to explore the effect of ZIKV-induced methylation of vector host proteins on its replication and survival in mosquito cells. Using A. albopictus cell line C6/36, I intend to explore ZIKV-associated modulation of arthropod SAMe cycle and histone tri-methylation in these cells. I hypothesize that ZIKV exerts methylation of proteins, such as histones, within arthropod host that support its survival. Information generated in this study will provide substantial knowledge to the scientific community in understanding the molecular mechanism(s) of ZIKV survival in arthropod vectors.

1.7 Limitations of Existing Studies
Arthropods are major vectors for the spread of many arboviruses and diseases across the globe. However, there is a lack of information regarding many aspects of vector-pathogen interactions and disease transmission. ZIKV is a clear example of arboviruses, where we know little about its interactions with the arthropod vector. In just a few years ZIKV progressed from minor tropical virus to international health concern affecting countless people and spreading in countries in tropical and subtropical regions.

ZIKV research is progressing because of its continued threat to human health. But many aspects of ZIKV pathogenesis, especially the arthropod perspective, is severely under researched. My research focuses on ZIKV modulation of molecular signaling in the vector host with a focus on epigenetics, the SAMe cycle and ZIKV pathogenesis. With the exception of Drosophila melanogaster, epigenetics of insects is not a major focus for the scientific community. This is mainly because of its lack of relation to humans. However, studies in the field of human epigenetics have made great strides in recent years. Recent advances in molecular biology and diagnostic methods has provided important discoveries in cancer research and a greater understanding of epigenetic diseases.

The SAMe cycle has been well studied in humans. But little has been done to explore its effects and mechanisms within arthropods. In humans, the SAMe cycle has been shown to have a role in cancer metastasis, depression, and arthritis (Bottigleri, 2002). Treatment with the SAMe molecule has been reported to improve the prognosis of liver diseases (Bottigleri, 2002) prompting SAMe to be available as a supplement with/without a prescription. The importance of SAMe in many diverse biological functions
has been characterized including its essential role in basic liver function. However, the role of SAMe in arthropods has not been specifically explored. I believe that any information discovered about SAMe in humans are conserved in other creatures as well; including arthropods.
CHAPTER 2

METHODS

2.1 Cell Culture, Cell Harvesting, and Infections

*Aedes albopictus* C6/36 Cells were cultured in Gibco media by Life Technologies Minimum Essential Media Alpha 1X + GlutaMAX with the addition of 10% Fetal Bovine Serum (FBS) with 1% Penicillin, Streptomycin, and Glutamine (PSG). Cell culture media was changed once every 5-7 days. Cells were then placed in an incubator set at 29ºC with 5% CO₂. *Aedes aegypti* Aag2 cells were cultured in Gibco media by Life Technologies Schneider’s Media with the addition of 10% Fetal Bovine Serum (FBS) and 1% Penicillin, Streptomycin, and Glutamine (PGS) with media changed once every 5-7 days. The Aag2 cells were then placed in an incubator at 29ºC with 5% CO₂.

For experimental trials, C6/36 or Aag2 cell media was removed from culture flask. Cells were treated with 1 ml 0.05% trypsin for 1-5 min or until cells were no longer adhered to the bottom. Trypsin was neutralized, at a 1:5 ratio, with fresh culture media. Cells along with trypsin and fresh media were then centrifuged at 800rpm in a sterile conical Eppendorf tube. After centrifugation, neutralized trypsin and media was removed leaving the cell pellet behind. Collected cells were re-suspended in fresh culture media. Resuspended cells were counted in a hemocytometer and 1e5 cells were plated in triplicates for allotted time points depending on experimental design. An acclimation period of 24hrs was allotted prior to proceeding with experimental procedures. Post acclimation period, all cells were infected with PRVABC59 ZIKV at 1 MOI. The *in vitro* infection experiments were performed based on the approved Old Dominion University Institutional biosafety committee protocol number 15-014.

For harvesting cells at different time points, experimental cell culture media was discarded or collected depending on experimental procedures. If collected, cell culture media was stored undiluted at -20ºC for short term analysis or -80ºC for long term storage. For quantitative real-time PCR (QRT-PCR) analysis supernatant was discarded and cells were collected using 500 μL of RNA lysis buffer. Cells were stored suspended in RNA lysis buffer at -20ºC for short term or -80ºC for long term storage. For immunoblots, cells were collected in 500 μL RIPA lysis buffer and stored at -20ºC for short term or -80ºC for long term storage.

2.2 Quantitative real-time PCR (QRT-PCR)

For QRT-PCR, C6/36 cells stored in RNA lysis buffer were allowed to warm to RT prior to processing for total RNA. Total RNA was generated using the Aurum Total RNA mini kit (Bio-Rad, USA) following the manufacturer’s spin protocol. Isolated total RNA was subsequently converted to cDNA using iScript
cDNA Synthesis Kit (BioRAD, USA) following the manufacture’s protocol. For QRT-PCR analysis, mosquito β-actin was used as an internal control to normalize the QRT-PCR reaction. QRT-PCR was performed using CFX96 QPCR machine (BioRad, USA) with iQ-SYBR Green Supermix (BioRad, USA). PCR mastermix contained 4 µL SYBR Green Mastermix, 1 µL primer, 2 µL cDNA, and 1 µL nuclease free water. QRT-PCR initial denaturation step was at 95°C for 30 sec. QRT-PCR cycling continued for 47 cycles with denaturation at 95°C for 10 sec, annealing at 54°C for 10 sec, elongation at 72°C for 30 sec. ZIKV, SAMe synthase expression, and EZH2 expression in mosquito cells was quantified from total RNA extracts from ZIKV-infected or uninfected cells. In QRT-PCR reactions, 10-fold serial dilutions (1 ng to 0.00001 ng) of known β-actin, ZIKV, SAMe synthase, and EZH2 transcripts were used to generate a standard curve. A complete list of oligonucleotides used in this study is listed in Table 1.

### Table 1: Oligonucleotides used in this Study

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<tr>
<th>Sequence (5‘-3’)</th>
<th>Purpose</th>
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<td>CCATGTACGTCGCCATCCA</td>
<td>actin, QPCR</td>
</tr>
<tr>
<td>GCGGTGGCCATTTCCTG</td>
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</tr>
<tr>
<td>AARTACACATACCARAACAAAGTGGT</td>
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</tr>
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<tr>
<td>CTTGATGACCTTGTCATGATTTTC</td>
<td>SAMe synthase, QPCR</td>
</tr>
<tr>
<td>CCCACGCAGACGACTTTATG</td>
<td>EZH2 methyl transferase, QPCR</td>
</tr>
<tr>
<td>CCTTGGTGCTATCGTTGCTGCT</td>
<td>EZH2 methyl transferase, QPCR</td>
</tr>
</tbody>
</table>

### 2.3 SAM Bridge-It-S-adenosyl methionine Fluorescence Assay Kit

The SAM Bridge-It-S-adenosyl methionine fluorescence assay kit (Mediomics LLC, USA), is a fluorescence assay kit used for the quantification of SAMe levels in biological fluids, tissue, and cell cultures. The kit utilizes two specific DNA fragments, labeled with biosensors Oyster 645 and Flouricin, and a MetJ protein. In the presence of S-adenosyl methionine the binding affinity of MetJ for the labeled DNA fragments increases binding to form a DNA-MetJ protein complex that will produce measurable fluorescence at absorption.
For SAMe level quantification, collected cells and supernatant were allowed to warm to RT. Cells were pelleted by centrifugation and washed between spins with 1X PBS. PBS was removed, and cells were treated with CM buffer (included in the kit) for cell lysis and SAMe release. Collected supernatant was treated with Buffer S (included in the kit). For analysis, all samples were mixed with the Bridge-it Kit Assay solution (included in the kit), placed in a 96 well-black microplate (included in the kit), and immediately incubated at 25°C for 30 min. Post incubation, fluorescent intensity was measured using a Tecan Infinite M200PRO fluorescence microplate reader (TECAN, USA) with settings excitation at 485 nm and emission at 665 nm. ZIKV-infected samples were compared to uninfected samples for all time points. Standard 10-fold serial dilutions (1 µL to 0.000000001 µL) were generated of known amounts of pure SAMe (provided in the kit) according to manufactures protocol. SAMe serial dilutions were used to quantify fluorescence results.

2.4 Immunoblotting Analysis

Amount of total protein in collected samples were measured using the BCA Protein Assay Kit (Pierce/ThermoScientific) following the manufacturer’s protocol. Briefly, a total of 25-35 µg of protein samples stored in RIPA buffer was boiled in Laemmlli buffer for protein denaturation. Samples were removed immediately from boiling, incubated on ice for 5 min and then centrifuged at 800 rpm. Samples were then loaded into a 12% SDS page gel. The gels were run with at ~110 Volts for 45-75 min. SDS page gel containing samples was treated with a Ponceau stain for 1 min. and imaged using a ChemiDoc MP Imaging System (BioRad). The images were processed using Image Lab software (BioRad). Transfer reaction was run overnight at 4°C to migrate protein to a nitrocellulose membrane. Nitrocellulose membrane was blocked for 24 hrs in 3-5% milk buffer and then treated with primary rabbit monoclonal Anti-MAT1A (Abcam, USA) or mouse monoclonal Anti-H3K27me3 (Abcam, USA) antibodies. After an additional 24 hrs, solution containing primary antibody was removed and membranes were treated with secondary anti-rabbit HRP conjugated (SantaCruz Inc., USA) or anti-mouse HRP conjugated (SantaCruz Inc., USA) antibodies at RT for 4 hrs. Bound antibodies were detected using WesternBright ECL kit (Advansta, BioExpress). Final images contain Ponceau stained picture, showing total protein profiles, and immunoblot image. The immunoblot image was acquired using ChemiDoc MP Imaging System (BioRad).

2.5 Exogenous S-adenosyl methionine Treatment and DZNep Treatment

For exogenous SAMe or DZNep treatment experiments, 1e5 mosquito cells were plated in 12 well plates and allowed to acclimatize for 24 hrs. Post acclimation period, cells were treated with SAMe (Mediomics LLC, USA) at a 10 µM final concentration or with 1 mM DZNep (Abcam, USA) to 5 µM or 20 µM final
concentration 4 hrs prior to PRVABC59 ZIKV infection. Equal volume of 10 mM 2-mercaptoethanol or sterile water was used as mock solution for SAMe-treatment experiments or DZNep-treatment experiment, respectively. Cells were collected at day 1 p.i. and processed for RNA extraction followed by QRT-PCR to determine EZH2 gene expression or ZIKV loads. Although DZNep is commonly used as cancer treatment to induce apoptosis, no CTE/CPE of C6/36 cells was observed.

2.6 Fluorescent Microscopy

Microscopic (Phase contrast/fluorescence) analysis was performed using the EVOS Fluorescence System (Invitrogen/ThermoScientific). A total of 1e5 C6/36 cells were plated on 18 mm coverslips in complete MEM medium and allowed to acclimatize for 24 hrs. Post acclimation period, cells were infected with 1 MOI PRVABC59 ZIKV. Coverslips were removed at day 1 p.i., fixed with 4% paraformaldehyde, permeabilized with 0.2 % Triton X-100 and processed for blocking with 3% BSA. Primary antibody (Anti-H3K27me3) and secondary antibody (Anti-mouse IgG -Alexa 488 conjugated) were added for staining and coverslips were later mounted on slides using Clearmount mounting solution (Thermo Fisher Scientific, USA). Coverslip slides were dried for 24 hrs and imaged using EVOS Fluorescence System (Invitrogen/ThermoScientific).

2.7 GenBank Accession Numbers used in this Study

Stated below are the GenBank accession numbers used in this study: A. albopictus S-adenosyl methionine (SAMe) synthase-like gene (GenBank acc. no. XM_019702033), A. albopictus encodes EZH2-like gene in its genome (GenBank acc. no. XM_020075762), A. albopictus histone H3 (GenBank acc. no. XP-019933535), A. aegypti histone H3 (GenBank acc. no. XP_021711172), Culex quinquefasciatus histone H3 type 2 (GenBank acc. no. EDS37386), Anopheles gambiae histone 3A (GenBank acc. no. AAK61362), Drosophila melanogaster histone H3 (GenBank acc. no. NP_001027387) and Homo sapiens histone H3 (GenBank acc. no. CAB02546)

2.8 Statistics

GraphPad Prism6 software and Microsoft Excel 2016 were used to analyze the statistical significance in all data sets. Non-paired t-test was performed to compare two means. P values less than 0.05 (P<0.05) were considered significant in all analysis. Horizontal lines seen in some graphs represent the mean of the treatment readings. P values are indicated at the relevant places (above represented data sets) in the figures.
CHAPTER 3

RESULTS

3.1 ZIKV induces S-adenosyl methionine (SAMe) Synthase Expression in A. albopictus C6/36 cell line

To understand the infection kinetics of ZIKV in mosquito cells, I infected C6/36 cell line with PRVABC59 strain of ZIKV at multiplicity of infection (MOI) 1. Cells were harvested at different time points (days 1, 3, 5, 7 post infection (p.i.)). Quantitative real-time PCR (QRT-PCR) data show that C6/36 cell line can be infected with PRVABC59 strain of ZIKV with peak infection at day 3 p.i. (Figure 1A). The A. albopictus genome encodes a SAMe synthase-like gene (GenBank acc. no. XM_019702033) that is expressed and shows homology to human S-adenosyl methionine synthase. In humans S-adenosyl methionine synthase is known to synthesize methionine for a variety of biological functions, used as a primary methyl donor (Cantoni, 1952). I analyzed C6/36 SAMe synthase-like gene through QRT-PCR for expression changes during ZIKV infection. QRT-PCR results revealed a significant (P<0.05) increase in SAMe synthase transcripts at day 1 p.i. when compared to uninfected samples (Figure 1B). No significant (P>0.05) difference was observed at later time points (day 3, 5, 7 p.i.) (Figure 1B). To determine if SAMe synthase QRT-PCR results are evident at the protein level, I performed an immunoblotting analysis using an anti-MAT1A antibody (Abcam, USA) that targets human MAT1A S-adenosyl methionine synthase enzyme. Immunoblotting analysis revealed a band at the expected size (~50kDA), indicating a two-fold increase in the S-adenosyl methionine synthase (Figure 1C) at day 1 p.i. The anti-MAT1A immunoblotting analysis supported the QRT-PCR data revealing no significant changes on day 3 and 5 (Figure 2A-B); although a slight decrease in protein levels was observed at day 7 p.i. (Figure 2B). Figure 1C shows the data from uninfected or ZIKV-infected cells collected at day 1 time point obtained with other time points (days 3, 5, 7 p.i.) shown as Figure 2. Collectively, the data indicate that ZIKV modulate the expression of S-adenosyl methionine synthase in C6/36 cells in vitro at day 1 p.i.
Figure 1. ZIKV Upregulate SAMe Synthase in Mosquito Cells

(A) QRT-PCR analysis showing levels of ZIKV in C6/36 cells at indicated time points (day 1, 3, 5, 7 p.i.).

(B) QRT-PCR analysis showing levels of SAMe synthase (GenBank acc. no. XM_019702033) transcripts in C6/36 cells at indicated time points (day 1, 3, 5, 7 p.i.). The levels of ZIKV NS5 and SAMe synthase transcripts were normalized to mosquito actin levels. In both A and B each circle indicates data from one independent culture well. Open circles indicate data from uninfected cells and closed circles indicate data from ZIKV-infected cells. Horizontal lines indicate mean of the values. P value from non-paired t-test is shown.

(C) Immunoblotting analysis showing levels of MAT1A (alias name for SAMe synthase) at day 1 p.i. Arrow indicates the SAMe synthase band at the expected size. Ponceau stained membrane image serves as loading control. The mass of protein marker is indicated in kDa. In all panels UI indicates uninfected cells and I indicates ZIKV-infected cells.
Figure 2. MAT1A Immunoblot Analysis
Immunoblot analysis showing MAT1A at day 3, 5, 7 p.i. time points. Ponceau stained image is shown as loading control. Arrow indicates the SAMe synthase band at expected size (~50kDa). UI indicates uninfected cells and I indicates ZIKV-infected cells.

3.2 ZIKV Infection Increases SAMe Levels in Mosquito Cells

The S-adenosyl methionine synthase enzyme catalyzes the rate limiting step (conversion of homocysteine into methionine) within the SAM Cycle (Markham and Pajares, 2009). The newly synthesized methionine is converted into S-adenosyl methionine which functions as a methyl donor (Figure 3A) (Cantoni, 1952). I hypothesized that an upregulation of S-adenosyl methionine transcripts and protein would lead to an increase in SAMe levels in C6/36 mosquito cells. To test this hypothesis, I measured the SAMe levels in uninfected and ZIKV-infected cell lysates and supernatants using a SAM Bridge-It-S-adenosyl methionine fluorescence assay kit (Mediomics LLC, USA). At day 1 p.i. I observed a significant (P<0.05) increase in SAMe levels in ZIKV-infected cell lysates when compared to uninfected cell lysates (Figure 3B). However, no differences in SAMe levels was observed at day 3 p.i. in cell lysate samples (Figure 3B), or in cell culture supernatant at day 1 and 3 (Figure 3C). But, in cell culture supernatant an upward trend was observed (Figure 3B) at both tested time points. This data suggests that increase in intracellular SAMe levels could be due to an increase in SAMe synthase RNA transcripts and protein levels in ZIKV-infected mosquito cells (Figure 1B and C).
Figure 3. ZIKV Induces SAMe Levels in Mosquito Cells
(A) Schematic representation of SAMe pathway. SAMe synthase participates in the synthesis of methionine from homocysteine. Methionine is then converted to SAMe by methionine adenosyl transferase. SAMe provides a methyl group for methylation of nucleic acids and proteins and results in the formation of S-adenosyl homocysteine (SAH). SAH hydrolase converts SAH to homocysteine.
(B) Measurement of SAMe concentrations in uninfected (UI) and ZIKV-infected (I) cell lysates is shown. (C) Measurement of SAMe concentrations in supernatants collected from uninfected (UI) and ZIKV-infected (I) cells is shown. Each circle represents SAMe level from one independent assay well. Open circles indicate data from samples generated from UI cells and closed circle represents data from samples generated from I cells. Horizontal lines indicate mean of the values. P value from non-paired t-test is shown.
3.3 Exogenous Addition of SAMe Increases ZIKV Loads and Histone Methyltransferase Transcript Levels in Mosquito Cells

Some viruses modulate histone methylation to suppress antiviral gene expression for its survival in host cells (Ramasubramanyan et al., 2011). Histone 3 lysine 27 tri-methylation (H3K27me3) is a distinct methylation hallmark of silenced promoters leading to transcriptional repression (Hyun et al., 2017). Methylation at this location, and specific residue, is carried out by the Enhancer of zeste homolog 2 (EZH2) protein (Shen et al., 2008). *A. albopictus* mosquitoes contain an EZH2-like gene within the genome (GenBank acc. no. XM_020075762). I performed QRT-PCR to determine if expression of the EZH2 is increased at day 1 p.i. upon ZIKV infection. QRT-PCR results revealed a significant (P<0.05) increase in EZH2-like gene transcripts in ZIKV infected C6/36 cells at day 1 p.i. when compared uninfected cells (Figure 4A). Based on the findings from SAMe synthase expression and SAMe levels quantified in supernatant and cell lysate (Figure 3A and B), additional time points were not explored, and focus was placed on day 1 p.i. To determine if the EZH2 expression and ZIKV replication are altered because of increased intracellular SAMe levels, I added 10 μM exogenous SAMe into cell culture for 4 hrs followed by ZIKV infection. No cytotoxic effect was observed after addition of exogenous SAMe (Figure 4B) indicating no cellular methionine toxicity at this concentration. QRT-PCR results revealed significant (P<0.05) increase in EZH2 (Figure 4C) and ZIKV loads (Figure 4D). Taken together these results support that SAMe effects ZIKV replication, possibly through the EZH2 enzyme at day 1 p.i. in C/36 cells.
Figure 4. ZIKV and SAMe Induces EZH2-like Methyl Transferase Gene Expression in Mosquito Cells.

(A) QRT-PCR analysis showing levels of EZH2-like methyl transferase (GenBank acc. no. XM_020075762) transcripts in C6/36 cells at day 1 p.i. Open circles indicate samples generated from uninfected cells and closed circle indicates samples generated from ZIKV-infected cells.

(B) Phase contrast microscopic images of ZIKV-infected C6/36 cells treated with either mock or SAMe (10 μM) is shown. Scale bar indicates 200 μm.

(C) QRT-PCR analysis showing levels of EZH2-like methyl transferase transcripts in ZIKV-infected C6/36 cells at day 1 p.i. treated with either mock (open circles) or SAMe (10 μM, closed circles).

(D) QRT-PCR analysis showing viral loads in ZIKV-infected C6/36 cells at day 1 p.i. treated with either mock (open circles) or SAMe (10 μM, closed circles). In panels A, C, D, the levels of EZH2-like methyl transferase transcripts or ZIKV loads were normalized to mosquito actin levels. In panel A, C, D, horizontal lines indicate mean of the values. P value from non-paired t-test is shown.
3.4 Comparison of *A. albopictus* Histone H3 with other Mosquito H3 Orthologs

EZH2 is the only methyl transferase enzyme known to methylate H3K27me3 in humans (Cha et al., 2005). Histone H3, H2, and H4, play a crucial role in gene expression. Each contributes directly to the structure of DNA supporting either repression or induction in response to diverse epigenetic signaling cascades (Barski et al., 2007). Both, EZH2 and histone H3 are highly conserved across many species (Figure 5 and 6). *A. albopictus* EZH2 (GenBank acc. no. XP_019931321) shows 97.7% identity with *A. aegypti* EZH2 (GenBank acc. no. XP_001663394) and 61.2% identity with *H. sapiens* EZH2 (GenBank acc. no. NP_004447) (Figure 5). To represent the sequence homology of histone H3 across species, I aligned the amino acid GenBank annotated sequences of *A. albopictus* histone H3 (GenBank acc. no. XP-019933535), *A. aegypti* histone H3 (GenBank acc. no. XP_021711172), *Culex quinquefasciatus* histone H3 type 2 (GenBank acc. no. EDS37386), *Anopheles gambiae* histone 3A (GenBank acc. no. AAK61362), *Drosophila melanogaster* histone H3 (GenBank acc. no. NP_001027387) and *Homo sapiens* histone H3 (GenBank acc. no. CAB02546). These sequences were downloaded from National Center for Biotechnology Information (NCBI) and analyzed through DNASTAR. All sequences exemplify a high degree of conservation (Figure 5, Figure 6). *A. albopictus* H3 showed 100% identity with H3 from *A. aegypti*, *C. quinquefasciatus*, and *D. melanogaster* and a high degree of conservation with *A. gambiae* (97.1%) and *H. Sapiens* (98.5) (Figure 6B).
Figure 5. Amino Acid Sequence Alignment of *A. albopictus* EZH2 Methyl Transferase with other Orthologs

The *A. albopictus* EZH2 methyl transferase amino acid sequence alignment with *A. aegypti* and human orthologs using ClustalW program in DNASTAR (Lasergene Genomics Suite) is shown. Residues that match are shaded in black. GenBank accession numbers for *A. albopictus*, *A. aegypti*, and *H. sapiens* sequences are shown. Total length and percent identities of the amino acid sequences are provided at one end of each sequence.
3.5 ZIKV Induces H3K27me3 Methylation in Mosquito Cells

With an increase in EZH2 transcripts, I hypothesized an increase in H3K27me3. To test this hypothesis, total protein extracts were prepared for immunoblotting analysis and probed with anti-H3K27me3 antibody. Immunoblot image revealed a significant increase in H3K27me3 at day 1 p.i. (Figure 7A). To observe this phenomenon on the cellular level I performed immunofluorescence. Using fluorescent microscopic images, I show an increase in H3K27me3 staining in C6/36 cells at day 1 p.i. when comparing uninfected samples to ZIKV infected samples at the same timepoint (Figure 7B).
together, immunoblotting analysis and fluorescent microscopy support that upon ZIKV infection there is an increase in H3K27me3 in C6/36 mosquito cells. Figure 7A shows the data from uninfected or ZIKV-infected cells collected at day 1 time point obtained with other time points (days 3, 5, 7 p.i.) shown as Figure 8. No difference in H3K27me3 levels was observed at later time points (Figure 8).

Figure 7. ZIKV Modulates H3K27me3 Levels in C6/36 Cells
(A) Immunoblotting analysis showing levels of H3K27me3 in uninfected or ZIKV-infected cells at day 1 p.i. Arrow indicates the H3K27me3 band at the expected size (~15 kDa). Ponceau stained membrane image serves as loading control. The mass of protein marker is indicated in kDa. In both panels UI indicates uninfected cells and I indicates ZIKV-infected cells.

(B) Microscopic images showing levels of H3K27me3 staining in uninfected or ZIKV-infected cells at day 1 p.i. Cells were permeabilized and treated with anti-H3K27me3 antibody followed by fluorophore-tagged conjugated secondary antibody. Phase contrast images for both groups are shown for comparison. Scale bar indicates 200 μm.
Figure 8. H3K27me3 Levels in C6/36 Cells at Different Days Upon ZIKV Infection
Immunoblotting analysis showing levels of H3K27me3 in uninfected or ZIKV-infected cells at days 3, 5, and 7 p.i. Arrow indicated the H3K27me3 band at the expected size (~15 kDa). Ponceau stained membrane image serve as loading control. The mass of protein marker is indicated in kDa. In both panels UI indicates uninfected cells and I indicates ZIKV-infected cells.

3.6 Treatment of Mosquito Cells with 3-Deazaneplanocin A Hydrochloride (DZNep), an Inhibitor of EZH2 Inhibits H3K27me3 and ZIKV Replication in Mosquito Cells

The induction of H3K27me3 upon ZIKV infection in C6/36 cells prompted me to explore the effect of inhibition of EZH2 on this methylation and viral burden. I hypothesized that inhibition of EZH2 affects H3K27me3 that would subsequently have a negative effect on ZIKV survival in C6/36 cells. To inhibit EZH2, I used 3-Deazaneplanocin A Hydrochloride (DZNep) a known cancer drug that has been reported to inhibit EZH2 in mammalian cells (Tan et al., 2007). Initial treatment with DZNep at 5 μM showed no cytotoxic effects on the C6/36 cells (Figure 9A) and showed no effects on ZIKV levels (Figure 9B). A concentration of 20 μM also revealed no cytotoxic effects in C6/36 mosquito cells (Figure 10A).

Immunoblotting analysis data after treatment with 20 μM DZNep revealed significant reduction in H3K27me3 levels in ZIKV infected cells when compared to mock treated cells at day 1 p.i. (Figure 10B). QRT-PCR revealed a significant (P<0.05) decrease in ZIKV replication upon treatment with 20 μM
DZNep in C6/36 cells at day 1 p.i (Figure 10C). Collectively, these results indicate that H3K27me3 mediated by EZH2 and SAMe is critical for ZIKV replication in C6/36 cells mosquito cells.

Figure 9. Treatment of DZNep (EZH2-Methyl Transferase inhibitor) at 5 µM has no Effect on ZIKV Replication in Mosquito Cells
(A) Phase contrast microscopic images of ZIKV-infected C6/36 cells treated with either mock or DZNep (5 µM) is shown. Scale bar indicates 200 µm.
(B) QRT-PCR analysis showing viral loads in mock- (open circles) or DZNep (5 µM, closed circles)-treated ZIKV-infected C6/36 cells at day 1 p.i. The ZIKV loads were normalized to mosquito actin levels. Horizontal line indicates mean of the values and P values from non-paired t-test is shown.
Figure 10. Treatment with DZNep (EZH2-Methyl Transferase Inhibitor) Affects H3K27me3 Methylation and ZIKV Burden in Mosquito Cells
(A) Phase contrast microscopic images of ZIKV-infected C6/36 cells treated with either mock or DZNep (20 μM) is shown. Scale bar indicates 200 μm.
(B) Immunoblotting analysis showing levels of H3K27me3 in mock or DZNep-treated ZIKV-infected cells at day 1 p.i. Arrow indicates the H3K27me3 band at the expected size (~15 kDa). Ponceau stained membrane image serves as a loading control. The mass of protein marker is indicated in kDa.
(C) QRT-PCR analysis showing viral loads in mock- (open circles) or DZNep (20 μM, closed circles)-treated ZIKV-infected C6/36 cells at day 1 p.i. The ZIKV loads were normalized to mosquito actin levels. Horizontal line indicates mean of the values. P value from non-paired t-test is shown.
CHAPTER 4

DISCUSSION

4.1 Overview of Findings

My research displays ZIKV-associated modulation of the SAMe cycle, SAMe, and H3K27 trimethylation in C6/36 *Aedes albopictus* mosquito cells. Although not much is known about the SAMe cycle in mosquito cells, my data provides evidence that ZIKV infection induces the expression of S-adenosyl methionine synthase enzyme facilitating increased levels of SAMe and H3K27me3 in the *A. albopictus* C6/36 cell line.

Based on the findings, I have created a model chronologically outlining the modulation of H3K27me3 methylation in C6/36 cells upon ZIKV infection (Figure 11). Upon internalization, ZIKV upregulates SAMe synthase enzyme and histone methyl transferase enzyme, EZH2. Increase in the SAMe synthase levels permits an increased production of intracellular SAMe, a prominent methyl donor. In addition, upregulation of EZH2 with an increase availability of SAMe facilitates methyl group transfers from SAMe to H3K27 residue to facilitate H3K27me3. Increased tri-methylation at H3K27 suggests repression of the mosquito factors that inhibit ZIKV replication and survival.

Within the scientific community there is discrepancy among researchers on the permissiveness of different mosquito cell lines to ZIKV infection (Simonin et al., 2017). In my experiments, I was able to show that C6/36 *A. albopictus* cells are readily permissible to PRVABC59 strain of ZIKV and these cells can maintain infection. Based on the infection kinetics, I noted that peak of ZIKV infection in C6/36 cells is at day 3. However, day 1 p.i. is an important time point that facilitates ZIKV initial replication and establishment in the cells. On day 5 and 7 p.i., I observed a continuation of infection as the ZIKV persisted in cell culture. At the analyzed time points (day 1 to 7 p.i.) no cytotoxic effects were observed as a result on ZIKV infection in C6/36 cells.

The SAMe cycle has not been characterized in mosquito vectors. As a result, the importance of SAMe for the pathogenesis of viruses has not yet directly been explored in arthropods. Previous reports state the importance of flaviviral NS5 methyltransferase protein, and the ability of ZIKV to preferentially methylate its own genome (Coutard et al., 2017). The viral and host RNA methylation and RNA structure remodeling was evident upon ZIKV infection of vertebrate cells (Lichinchi et al, 2016). The mosquito EZH2-like enzyme is a methyl transferase. In humans, EZH2 is known to preferentially methylate histone H3 at lysine residue 27; which can me mono-, di-, or tri-methylation (Pasini et al., 2004). Tri-methylation at this location is commonly associated with silencing of gene expression (Pasini et al., 2004). In this
study, I noted an increase in H3K27me3 upon ZIKV infection at day 1 p.i. I hypothesize that increased H3K27me3 levels will lead to the suppression of host factors that inhibit ZIKV replication in mosquito cells. This study provides evidence that ZIKV can modulate methylation of mosquito histones for its survival in arthropod cells. The observation of reduced mosquito H3K27me3 levels and viral loads upon treatment of C6/36 cells with 20 µM DZNep suggests that this inhibitor could be considered as a strong therapeutic candidate molecule to treat or control ZIKV infections.

Figure 11. Schematic Model Showing Roles of SAMe and H3K27me3 Signaling in ZIKV Survival in Mosquito Cells
ZIKV up-regulates SAMe-synthase (a) and EZH2-methyl transferase (b) gene expression upon entry into mosquito cells. The increased levels of SAMe (c) and EZH2-methyl transferases (d) lead the later enzyme to acquire methyl group for transfer to H3. EZH2-methyl transferase methylate H3 (e) that subsequently lead to transcriptional silencing (f) of mosquito anti-viral genes. The absence of anti-viral response (g) leads ZIKV successfully to replicate and establish in mosquito cells.
4.2 Research Implications

The data presented in the study exemplifies the role of epigenetics, SAMe/SAMe cycle, and ZIKV pathogenesis in arthropods. The goal of this in vitro study was to understand ZIKV pathogenesis in arthropod cells. However, findings from this study may also facilitate to understand interactions of ZIKV with vertebrate cells.

My data shows the dependency of ZIKV on SAMe, which can provide insight into the symptoms observed during infection. Permissiveness of cells to ZIKV infection may depend on the SAMe levels. Where, cells with higher SAMe level may be more preferential targets for ZIKV infection. Because very high levels of SAMe are known to be present in the liver, this organ could function as a reservoir for ZIKV infection. High SAMe levels could allow enhanced ZIKV survival in different tissues and prolong infection.

In addition to other effects reported to be influenced by the SAM cycle, SAMe and Homocysteine are known to have differential effects on neurons (Shirafuji et al., 2018). In early development, neurons require increased amounts of SAMe for gene expression and proper differentiation. ZIKV was detected in amniotic fluid of pregnant mothers (Brasil et al., 2016), and became an international health concern when a link between microcephaly and infection was established. The manifestation of microcephaly was due to a delayed neurogenesis causing abnormalities within the corpus callosum, ventriculomegaly, collapse and folding of the meninges (Cugola et al., 2016). My data suggests that in an arthropod host ZIKV modulates SAMe cycle and H3K27me3 resulting in the repression of host factors that are lethal for its survival. Similar studies on the modulation of the SAMe cycle in response to ZIKV replication in vertebrate cells could lead in understanding the cause of birth defects observed in ZIKV-infected pregnant mothers.

Recent reports have presented evidence that ZIKV can establish in tissue reservoirs in human host (Alcendor, 2017). In select tissues, ZIKV can persist after systemic clearance by maintaining a low titer. Establishment of tissue reservoirs allow ZIKV to replicate without pressure from the host immune system that subsequently lead for its persistence within the host for extended periods of time (Ma, W., et al. 2016). The success of ZIKV to establish in tissue reservoirs for extended periods could result from EZH2-mediated H3K27me3 silencing of lethal host factors that inhibit its survival. Understanding the role of H3K27me3 in host-pathogen interactions will provide novel insights on the mechanisms that ZIKV and perhaps other Flaviviruses uses to persist in different vertebrate tissues. Inhibition of EZH2 could be envisioned as a good therapeutic strategy to prevent ZIKV ability to establish in tissue reservoirs and thus allowing the immune system to completely clear the virus.
4.3 Research Limitations

This study is focused on the \textit{in vitro} aspect of viral induced modulation of SAMe Cycle in C6/36 \textit{A. albopictus} cells. Since little has been done to explore the role of the SAMe cycle in vector-virus interactions, any work in this research area would be of great benefit to the research community. Because this is the first report of such a study, most of the information regarding arthropod methyltransferases and enzymes for histone methylation had to be adapted from mammalian species; primarily humans. Although many biological processes that are linked to the SAMe cycle are not completely understood in humans, even less is understood in arthropods vectors. This is a very novel study that provides important information on the role of arthropod SAMe cycle and H3K27me3 in vector-virus interactions.

There is a discrepancy within the ZIKV research community on the permissiveness of certain mosquito species to transmit and maintain infectious titers of ZIKV within the environment (Weger-Lucarelli J et al. 2016). All data conducted in this study was in an \textit{in vitro} \textit{Aedes albopictus} mosquito C6/36 cell line and PRVABC59 strain (Puerto Rican) of ZIKV. Additional ZIKV strains (West African, East African) are additional ZIKV strains that are potential pathogens. However, neither of these strains was used in this study. Additional research needs to be performed to determine the extent to which other strains of ZIKV affect the SAMe cycle. Studies with other ZIKV strains would elucidate whether modulation of SAMe cycle and H3K27me3 is a strain specific phenomenon or a common strategy used by all strains to survive in their vector hosts.

My research is focused on the role of H3K27me3 in ZIKV-mosquito interactions. Although H3K27me3 is a hallmark of genetic repression, many additional protein and nucleic acid regulation events that could occur within the cell were not analyzed in this study. Focus on H3K27me3 was due to the strong findings on the upregulation of SAMe synthase and EZH2 in mosquito cells. I list this methylation site as a limitation because it’s the only methylation event studied in this work. The possibility of other posttranslational events such as acetylation on H3 contributing for ZIKV survival in mosquito cells cannot be excluded. However, reporting the modulation of H3K27me3 upon ZIKV infection suggests that ZIKV has the ability to alter posttranslational modifications such as methylation within \textit{A. albopictus} C6/36 cells.
CHAPTER 5

CONCLUSION

5.1 Primary Contributions of this Study

As ZIKV continues to spread and is disseminated through new environments in response to human and animal migration, there is a need for strategies to target ZIKV-mosquito interactions. Although some researchers speculate that A. aegypti and not A. albopictus is the primary vector responsible for ZIKV spread, both contribute to its spread and human infection. The research presented here can be directly translated for other Aedes species. Through this research, I have presented evidence of ZIKV modulating the SAMe cycle and H3K27me3 during infection in A. albopictus C6/36 cell line for its survival. Information presented in this study will increase the knowledge on ZIKV pathogenesis and help us to develop new methods to combat infection.

Methylation is known to be involved in shaping the RNA transcriptome and influencing RNA structure and function. There are no previous reports of ZIKV modulation of histone methylation in arthropods, including H3K27me3. My data showing the effect of ZIKV on H3K27me3 forms the basis to understand the mechanism(s) of neurological and developmental defects observed in human infection.

In addition to ZIKV modulating mosquito H3K27me3, my research also demonstrates that ZIKV replication can be repressed through inhibition of arthropod EZH2 enzyme using a known cancer drug DZNep. Treatment with this drug could be used in adults infected with ZIKV. The mechanism(s) involving SAMe cycle and H3K27me3 could be further explored to understand the direct cascade that ZIKV induces to suppress the immune response for its survival in the host.

5.2 Widening the Scope and Suggestions for Future Research

This research focused primarily on arthropod side of ZIKV infection. However, the modulation of the SAMe Cycle and histone methylation can be translated to examine the effects imposed during ZIKV infection in humans. ZIKV became an international health emergency when human infection became associated with microcephaly in human fetuses; a condition characterized by a delayed neurogenesis and neural differentiation in the brain. Despite the detection of ZIKV in the amniotic fluid of pregnant mothers, a mechanism by which ZIKV contributes to microcephaly has yet to be discovered. Because ZIKV can interact extensively within the RNA transcriptome and modulate host epigenetic mechanisms, additional research should be carried out in this area of viral pathogenesis. Histone methylation is known to be critical for fetal development while being highly time and site specific. Interruption in histone methylation could be detrimental to proper development of the host organism. The H3K27me3
modulation occurring during ZIKV infection, displayed in my research, may be a possible means by which ZIKV is causing delayed neurogenesis observed in human fetal infection. Exploring the effects of ZIKV in epigenetic modifications and SAMe cycle could illuminate the mechanism(s) of microcephaly in humans.

The observation of increased expression of EZH2 leading to an increase in H3K27me3 methylation suggests a decrease in lethal host factors allowing ZIKV to persist in arthropod cells. This phenomenon could be a means by which ZIKV establishes tissue reservoirs and persist in certain tissues throughout the body. ZIKV infection in humans is known to persist for extended period of time in semen and cause severe ocular infection (Robinson, et al., 2018). It would be interesting to determine if ZIKV preference for infection in certain tissues is moderately dependent on the level of methionine present. If indeed ZIKV prefer to persist in host tissues with high methionine, the liver would be a possible tissue reservoir. It is not surprising to hypothesize that ZIKV and high concentration of methionine in liver could possibly increase expression of methyltransferases subsequently leading to an increase in H3K27me3. The increase in H3K27me3 might lead to silencing of host genes that inhibit ZIKV persistence in liver.

In summary, this data supports that ZIKV infection induces expression of SAMe synthase and EZH2-like histone methyltransferases leading to an increase in H3K27me3. The increase in H3K27me3 leads to suppression of host factors that inhibit ZIKV replication in A. albopictus C6/36 cell line. Characterization of arthropod molecules and signaling pathways that are involved in the mosquito SAMe cycle and H3K27me3 could lead in the development of new strategies to target ZIKV spread and transmission to humans. It is my hope that the current study would not only provide novel information on viral pathogenesis but will also help the scientific community to move forward in the development of strategies to treat or control ZIKV infections.
REFERENCES


Xiaolei, D., Xiaoying, W., Stephanie, S., Jie, Q., Paul, W., Qiong, L., Martin, Z., (2014) The polycomb protein EZH2 impacts on induced pluripotent stem cell generation. Stem Cells Dev. 23, 931-940


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