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Zika Virus Inactivates Polyamine Catabolism Via Alternative Splicing to Enhance Infection

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ZIKA VIRUS INACTIVATES POLYAMINE CATABOLISM VIA ALTERNATIVE SPLICING TO ENHANCE INFECTION

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LIST OF ABBREVIATIONS

Dengue virus	DENV
Herpes simplex virus 1	HSV1
Human cytomegalovirus	HCMV
Interferon stimulated genes	ISGs
La Crosse virus	LACV
Messenger RNA	mRNA
Multiplicity of Infection	MOI
N1,N11-diethylnorospermine	DENSpm
ODC1 antienzyme	OAZ1
Ornithine decarboxylase	ODC1
Polyamine oxidase	PAOX
Quantitative Real Time PCR	qRT-PCR
Rift Valley fever virus	RVFV
SAT1 splice variant	SAT1-X
Spermidine synthase	SRM
Spermidine/spermine acetyltransferase	SAT1
Spermine synthase	SMS
Thin layer chromatography	TLC
Truncated SAT1	tSAT1
Uridine-rich small nuclear ribonucleoprotein particles U5	U5 snRNP

Zika virus	ZIKV

ABSTRACT

During viral infection, the virus and host must compete for resources inside the cell. One of these resources is polyamines. Polyamines are small, positively charged molecules that are found in all eukaryotic cells. They play a key role in several cellular functions including growth and proliferation, transcription and translation, and membrane stability. Viruses also rely polyamines for productive replication, utilizing them during DNA/RNA polymerization, nucleic acid packaging, and protein synthesis. In response to a virus infecting a host cell, the host cell will begin to regulate polyamine levels as a way to combat the infection. Polyamine levels are regulated by several different enzymes, including SAT1. SAT1 acetylates polyamines to form acetyl derivatives leading to degradation or excretion of the polyamines and therefore, rendering them useless to the virus. The virus, in turn needs to combat this to continue infection. Recent studies suggest that one way the virus does this is by splicing the polyamine regulatory enzymes' genes before they can be transcribed. We found that Zika virus, a flavivirus, may alter SAT1 splicing and activity. We hypothesize that ZIKV induced alternative splicing of the SAT1 gene generates an enzyme that is nonfunctional allowing robust virus infection by disrupting polyamine depletion.

CHAPTER ONE

BACKGROUND

Review of Literature

Zika Virus: An Overview

Zika virus (ZIKV) has recently gained a lot of attention due to the 2016 outbreak in which the virus spread to over 80 countries becoming the focus of a public health emergency. ZIKV was first isolated in 1947 at Yellow Fever Research Institute in Zika Forest, Uganda. Until 2007, only 14 human cases were identified, and these originated in Southeast Asia and Africa (1). The first outbreak of the virus occurred in Yap (Federal States of Micronesia) in 2007. Due to the similarities of clinical symptoms, the disease was originally diagnosed as chikungunya, dengue, or Ross River disease until specimens from patients revealed the RNA of ZIKV. Approximately 73% of Yap's population was infected in this outbreak (2). Since then outbreaks have occurred in French Polynesia (3), New Caledonia, Easter Island, the Cook Islands, and numerous Latin American countries. By March of 2015, Brazil had between 500,000-1,500,000 ZIKV cases (4). By 2016, the World Health Organization declared ZIKV disease a public health emergency of international concern. As of 2018 a total of 86 countries have reported evidence of ZIKV disease (Figure 1). Since then the number of infections has slowly declined with cases staying persistent at low levels in some areas (5).

Figure 1. Reported Cases of ZIKV.
The map indicates reported cases of ZIKV infection in 2017. The purple represents areas with active transmission in 2017, the orange represents areas affected in 2017, and the yellow represents areas with ZIKV infection reported before 2015 (5).

ZIKV is an arbovirus and is spread primarily by *Aedes* mosquitos, however some reports have found Anopheles, Ertmapodites (6), Culex, and Mansonia (7) species can also be vectors. ZIKV can also be transmitted by blood transfusion, sexual contact, and vertically from mother to child during pregnancy (8) (Figure 2). Most cases present symptoms 3-12 days after infection. Nearly 80% of cases are asymptomatic. Case fatalities and severe illness are rare consequences of ZIKV infection. Symptoms tend to be non-specific, mild, and self-limiting. They include mild fever, dermatological rashes, joint pain, and headache. The most common of these symptoms is the skin rash which is seen in 90% of cases and often lasts 4-5 days. Hospitalization from ZIKV infection is rare (9).

The major complications associated with ZIKV infection is congenital microcephaly and Guillain-Barre syndrome. Guillain-Barre syndrome is a rare disorder

in which the immune system damages nerves resulting in muscle weakness and sometimes paralysis. This is due to high levels of protein in the cerebrospinal fluid (10). In regions affected by the ZIKV epidemic, the incident rate of Guillain-Barre syndrome was 20-fold higher than that of not affected areas (2). Primary microcephaly is defined as a head circumference more than 3 standard deviations below the mean at birth. Symptoms include hearing loss, development delay, seizure disorder, and cerebral palsy. The risk of microcephaly is greatest in the first trimester of pregnancy. Researchers have reported ZIKV in amniotic fluid and in placental cells (11). ZIKV first infects the placenta and then the neural progenitor cells in the developing fetus. This decreases the neural progenitor cells viability and growth through down-regulation of genes involved in cell and organ development and up-regulation of genes involved in immune responses. This ultimately results in inhibited cellular proliferation and differentiation, neuronal apoptosis, and thinning of the cortex (12).

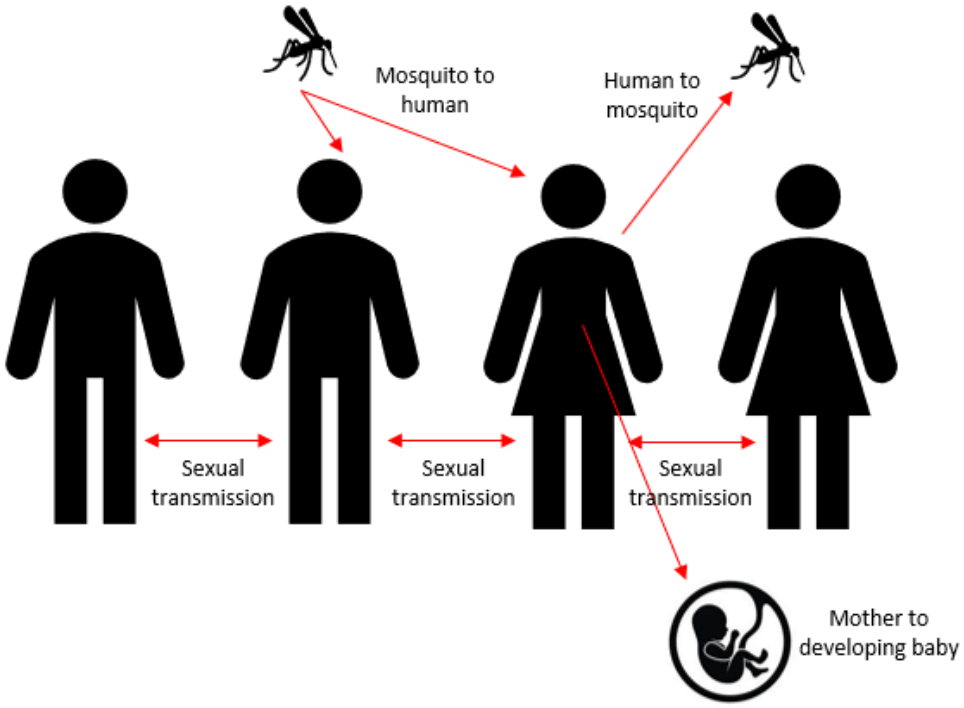

Figure 2. Transmission of ZIKV.
Arrows represent transmission direction. Transmission of ZIKV is primarily by mosquito bites but instances of sexual transmission have been reported as have cases of infection by blood transfusion. ZIKV can also be passed from mother to fetus in the womb.

ZIKV is a member of the Flaviviridae family. Other members include dengue virus, West Nile virus, and yellow fever virus. Like all members of this family, ZIKV has a positive sense single stranded RNA genome approximately 11 kb in size. ZIKV is spherical in shape with an icosahedral like arrangement of surface proteins and is approximately 50nm in diameter. The RNA of the virion is infectious and acts as viral mRNA and viral genome (13).

The lifecycle of ZIKV follows that of most flaviviruses. The virus enters the cells when the virion attaches to the cell membrane of the host via an envelope protein that induces endocytosis. Once inside the host cell, the viral membrane fuses with the endosomal membrane and the RNA is released into the cytoplasm. This RNA is then

translated into a polyprotein which is involved in the formation of all structural and non-structural proteins. Replication of the RNA then occurs in the cytoplasmic viral factories of the ER which produces double stranded RNA. This double stranded RNA then undergoes transcription to form additional single stranded RNA. This new single stranded RNA is then assembled in the ER to form new virions. These virions are ultimately transferred to the Golgi and released and go on and infect new cells (14) (Figure 3). ZIKV, as for all viruses, relies on host machinery and resources during its replication.

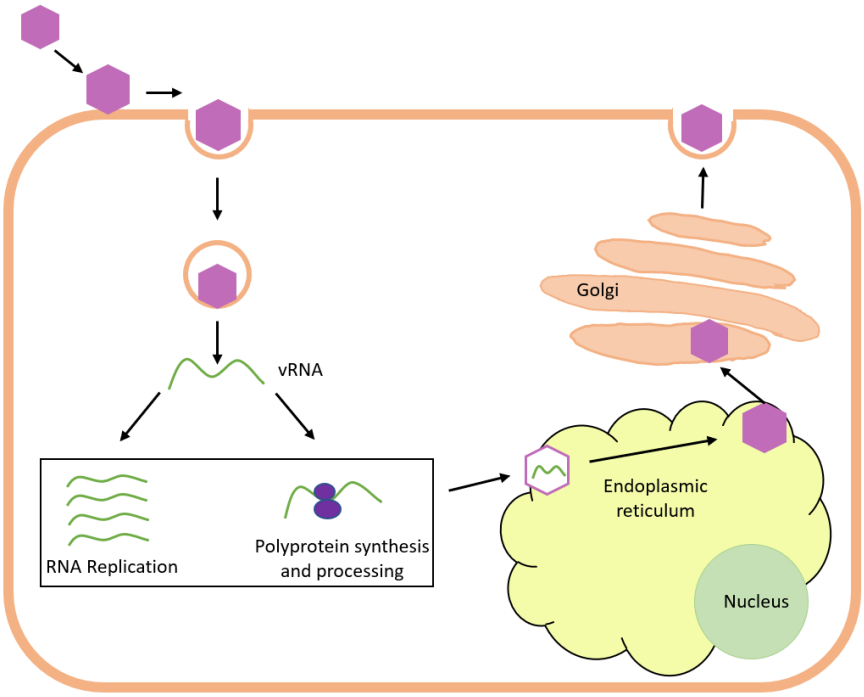

Figure 3. Flavivirus Replication Cycle.
The virus enters the cells when the virion attaches to the cell membrane of the host via an envelope protein that encourages endocytosis. The viral membrane fuses with the endosomal membrane and the RNA is released into the cytoplasm. This RNA is then translated into a polyprotein which is involved in the formation of all structural and non-structural proteins. Replication of the RNA then occurs in the cytoplasmic viral factories of the ER which produces double stranded RNA. This double stranded RNA then undergoes transcription to form additional single stranded RNA. This new single stranded RNA is then assembled in the ER to form new virions. These virions are ultimately transferred to the Golgi and released and go on and infect new cells (14).

Polyamines

Polyamines are small, positively charge molecules found in all cells. They consist of flexible carbon chains with positively charged amino groups. Polyamines are involved in diverse cellular processes including protein synthesis, RNA folding, membrane interactions, protein-RNA interactions, DNA structure, and gene expression(16, 17). Mammalian cells synthesize three biogenic polyamines: putrescine, spermidine, and spermine. All three polyamines are synthesized through the same pathway. The first step occurs when arginine is converted to ornithine. Ornithine is then converted into putrescine via the gatekeeping enzyme of the pathway, ornithine decarboxylase 1 (ODC1). Putrescine is then converted to spermidine by the enzyme spermidine synthase (SRM). Spermidine is ultimately converted to spermine via the enzyme spermine synthase (SMS)(18). The cell exerts a significant amount of energy in maintaining polyamine homeostasis. This is done through synthesis, degradation, import, and export of polyamines. This homeostasis is maintained by the enzymes polyamine oxidase (PAOX), ODC1 antizyme (OAZ1), and spermidine/spermine acetyltransferase 1 (SAT1) in a tightly controlled feedback mechanism (19) (Figure 4).

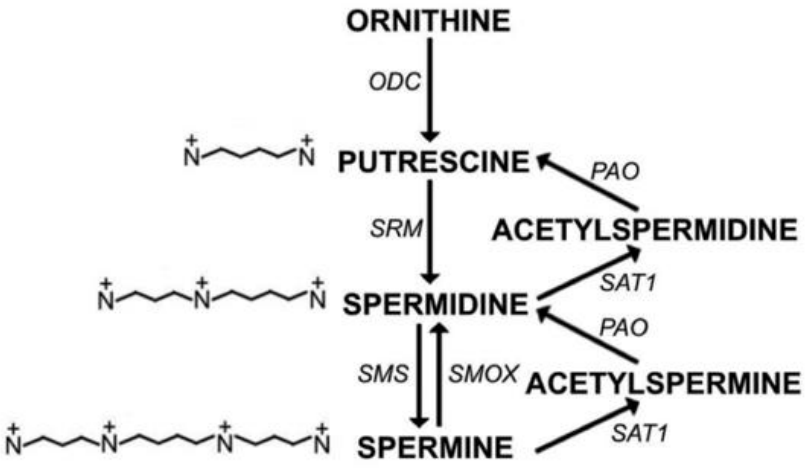

Figure 4. Biosynthesis Pathway of Polyamines.
Polyamine synthesis begins with arginine being converted into ornithine. ODC1, the rate-limiting enzyme, converts ornithine to putrescine. Putrescine is converted to spermidine via SRM. Spermidine is converted to spermine via SMS and converted back via SMOX. Spermine and spermidine can be acetylated by SAT1 and converted back to putrescine via PAO. Image adapted and modified from(20).

Considering the importance of polyamines in cellular processes, it is not surprising that viruses also utilize polyamines during their lifecycle (18). Viruses rely on polyamines for numerous stages in the viral life cycle including genome packaging, DNA-dependent RNA polymerization, genome replication, and viral protein translation. DNA viruses like herpes simplex 1 (HSV1) and human cytomegalovirus (HCMV) have been shown to rely on polyamines for replication (21, 22). Vaccinia virus has also been shown to rely on polyamines in a late step of its viral lifecycle (23). The role of polyamines in RNA viruses has been demonstrated in several different virus families including alphaviruses, enteroviruses, bunyaviruses, and flaviviruses (24, 25). Bunyaviruses, La Crosse virus (LACV) and Rift Valley fever virus (RVFV), have been shown to generate noninfectious viral particles when polyamines are deleted from the cell(26). Replication of these viruses was impacted to various degrees when polyamines

were depleted from the cell making the polyamine pathway a possible target for limiting viral infections(18).

SAT1

Spermidine/spermine acetyltransferase (SAT1) is the rate-limiting enzyme in the interconversion of polyamines. SAT1 works by acetylating spermidine and spermine which results in their degradation or excretion from the cell. SAT1 uses acetyl-CoA to acetylate the aminopropyl end of these higher level polyamines. In the presence of high levels of polyamines, transcription and translation of SAT1 are increased. SAT1 has been shown to be induced in response to many different agents and pathophysiological conditions including polyamines analogs such as N^1, N^1 -diethylnorspermine (DENSpm) and the interferon response (24, 27). The interferon response, which is the innate immune response to viral infection, initiates a series of signaling events that results in the expression of interferon-stimulated genes (ISGs), including SAT1, to counteract viral infection. Studies have shown that SAT1 is upregulated with interferon beta treatment of cells, which results in the depletion of polyamines from the cell and limits viral infection (24).

SAT1 is regulated, by polyamines and their analogs, at many different levels of gene expression including transcription and stabilization of messenger RNA (mRNA). The *Sat1* gene contains six exons that encode mRNAs of 1.3 and 1.5 kb. SAT1 pre-mRNA has been shown to undergo alternative splicing to yield a longer variant (SAT1-X). Studies have shown that inclusion of this exon is inhibited by polyamines and their analogs resulting in a stable and correct mRNA. This longer variant has an additional 110bp exon between exon 3 and exon 4. This exon introduces three in-frame premature

termination codons which marks it for nonsense mediated mRNA decay (28) (Figure 5). Previous studies have shown that SAT1-X mRNA is accumulated upon various factors including X-ray irradiation (29), iron chelation, hypoxia (30), and infection with Venezuelan equine encephalitis virus and tick-borne encephalitis virus (31). However, it is unknown if this longer SAT1 variant is induced upon ZIKV infection.

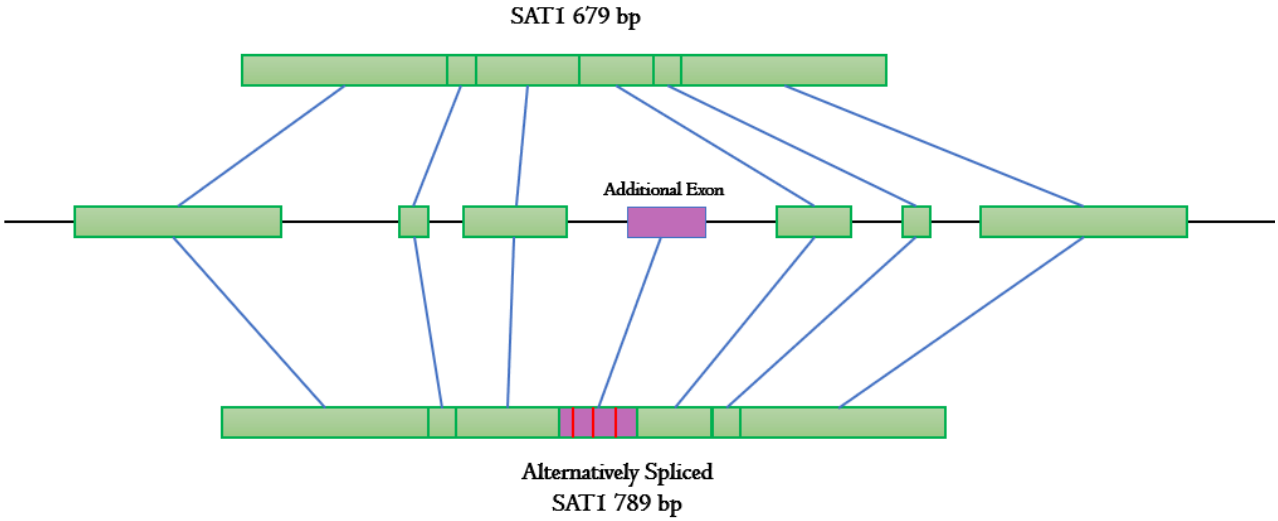

Figure 5. Alternatively Spliced SAT1.
SAT1 has been shown to be alternatively spliced to yield a longer variant. This is due to the addition of an exon about 110 bp long between exons 3 and 4 indicated in purple. This additional exon introduced three premature termination codons indicated in red.

The normal SAT1 transcript encodes a protein of about 171 amino acids. Due to the presence of three premature stop codons, the alternatively spliced variant is only capable of producing a 71 amino acid polypeptide (31) (Figure 6). It has been reported that 68 amino acid residues of the N-terminus of the tSAT1 are identical to the full-length SAT but due to a shift in the reading frame from the addition 110 bp, tSAT1 has a new C-terminus. This reconstructed C- terminus of tSAT1 lacks both the acetyl-CoA binding and catalytic motifs which are essential for polyamine regulation (30). Thus, it is likely tSAT1 is not antiviral though that has not been reported.

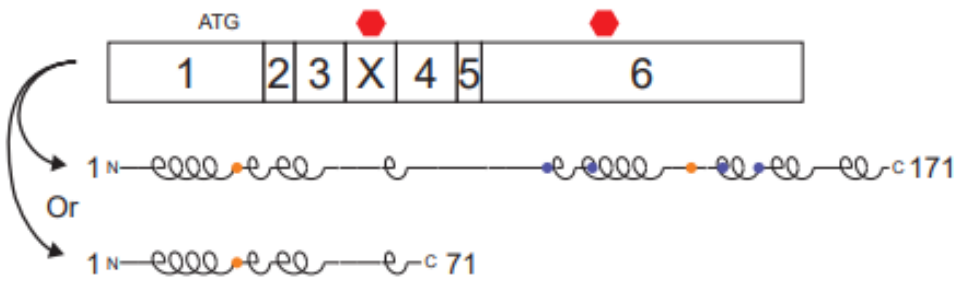

Figure 6. Truncated SAT1 Protein
The normal SAT1 transcript encodes a protein of about 171 amino acids, SAT1-X is only capable of producing a 71 amino acid polypeptide. This truncated polypeptide (tSAT1) is the result of a reading frame shift that introduces three premature stop codons (31).

Hypothesis and Aims

Studies have shown that RNA viruses, such as ZIKV, rely on polyamines for replication and depletion of these polyamines results in decreased viral replication. It has also been shown that SAT1 is induced upon the interferon response to viral infection. SAT1 reduces polyamine levels in the cell by acetylating spermidine and spermine, marking them for degradation or excretion. This ultimately restricts viral replication by depleting the cell of polyamines (24). Furthermore, studies have shown that SAT1 can be alternatively spliced in response to polyamine analogs and other pathophysiological agents including some RNA viruses (31). This alternative splice variant introduces three premature termination codons which results in nonsense mediated mRNA decay (28). We hypothesized that ZIKV induces alternative splicing of SAT1, generating an enzyme that is nonfunctional and thus allowing robust virus infection by disrupting polyamine depletion.

In AIM 1, we measured SAT1 induction and its effects on polyamine levels during ZIKV infection. I hypothesized that ZIKV infection would cause SAT1 induction and

there would be a decrease in polyamine levels due to SAT1 activation. The results from this aim determined how viral infection and polyamine catabolism interrelate.

In AIM 2, we determined the mechanism of SAT1 inactivation during viral infection. I hypothesized that ZIKV was inducing alternative splicing of SAT1, creating the longer variant carrying three premature termination codons. The results from this aim determined how ZIKV may be inhibiting SAT1 function.

In AIM 3, we measured SAT1 splicing in multiple cell types and with different viruses. I hypothesized that that immune signaling would play an important role in the induction of SAT1 and that this would be a conserved mechanism for viruses from different families. The results from this aim determined if SAT1 alternative splicing is induced through interferon signaling.

The data generated from these aims will define a novel interaction between host cells and ZIKV. We see that ZIKV does induce alternative splicing of SAT1 which prevents the cell from regulating polyamine levels during a viral infection.

CHAPTER TWO

MATERIALS AND METHODS

Cell Culture

Cells were maintained at 37ºC in 5% CO_2, in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) with bovine serum and penicillin-streptomycin. Vero cells (BEI Resources) were supplemented with 10% new-born calf serum (NBCS; Thermo-Fisher) and Huh7 cells, kindly provided by Dr. Susan Uprichard, were supplemented with 10% fetal bovine serum (FBS; Thermo-Fisher). THP1 cells were donated by Dr. Makio Iwashima and maintained in RPMI (Thermo-Fisher) supplemented with 2% FBS and beta-mercaptoethanol (BME, 50mM; Thermo-Fisher). THP1's were differentiated with phorbol 12-myristate 13-acetate (PMA, 100 pg/ mL; Thermo-Fisher).

Drug Treatment

N1,N11-Diethylnorspermine (DENSpm; Santa Cruz Biotechnology) were diluted to 100x solution (100mM and 10mM, respectively) in sterile water. Cells were treated with 100uM of DENSpm 24 hours before, 2 hours after, and 4 hours after infection. During infection, media was cleared and saved from the cells. The same medium containing DENSpm was then used to replenish the cells following infection. Cells were incubated at the appropriate temperature for the duration of the infection.

Infection and Enumeration of Viral Titers

RVFV and LACV were derived from the first passage of virus in Huh7 cells. ZIKV (MR766) was derived from the first passage of virus in Vero cells. ZIKV and LACV were obtained from Biodefense and Emerging Infections (BEI) Research Resources. For all infections, DENSpm was maintained throughout infection as designated. Viral stocks were maintained at -80ºC. For infection, virus was diluted in serum-free DMEM for a multiplicity of infection (MOI) of 5 on Huh7 cells, unless otherwise indicated. Viral inoculum was overlain on cells for 10 to 30 minutes, and the cells were replenished with media. Supernatants were collected from MP-12, LACV, and ZIKV 24 hpi. Dilutions of cell supernatant were prepared in serum-free DMEM and used to inoculate confluent monolayer of Vero cells for 10 to 15 min at 37ºC. Cells were overlain with 0.8% agarose in DMEM containing 2% NBCS. MP-12, ZIKV, and LACV samples incubated for 3 days at 37ºC. Following incubation, cells were fixed with 4% formalin and revealed with crystal violet solution (10% crystal violet; Sigma-Aldrich). Plaques were enumerated and used to back-calculate the number of plaque forming units (pfu) per milliliter of collected volume.

Thin Layer Chromatography Determination of Polyamines

Polyamines were separated by thin-layer chromatography as previously described (Madhubala, 1998). For all samples, cells were treated as described prior to being trypsinized and centrifuged. Pellets were washed with PBS and then resuspended in 200 uL 2% perchloric acid. Samples were then incubated overnight at 4ºC. 200 uL of supernatant was combined with 200 uL 5 mg/ml dansyl chloride (Sigma Aldrich) in acetone and 100 uL saturated sodium bicarbonate. Samples were incubated in the dark

overnight at room temperature. Excess dansyl chloride was cleared by incubating the reaction with 100 µL 150 mg/mL proline (Sigma Aldrich). Dansylated polyamines were extracted with 50 µL toluene (Sigma Aldrich) and centifuged. 5 µL of sample was added in small spots to the TLC plate (silica gel matrix; Sigma Aldrich) and exposed to ascending chromatography with 1:1 cyclohexane: ethylacetate. Plate was dried and visualized via exposure to UV.

RNA Purification and cDNA Synthesis

Cells were collected in Trizol reagent (Zymo Research). RNA was purified with a phenol-chloroform extraction, DNase treated (Invitrogen), and used for cDNA synthesis using Multiscribe reverse transcriptase (Applied Biosystems), with 500 ng RNA and random hexamer primers.

Gene Expression Analysis

RNA from cells was collected and purified. Gene expression was analyzed by quantitative real-time PCR with SYBR Green (DotScientific) using a one-step protocol using QuantStudio 3 (ThermoFisher Scientific). Relative gene expression was calculated using the ΔC_T method, normalized to GAPDH qRT-PCR control. Primers were verified for linearity using eight-fold serial diluted cDNA and checked for specificity via melt curve analysis following by agarose gel electrophoresis.

Splicing Analysis

RNA from cells was collected and purified. Splicing was analyzed by real-time PCR with DreamTaq (DotScientific) using SimpliAmp Thermal Cycler (Applied Biosystems by Life technologies). PCR product was analyzed by agarose gel electrophoresis.

Table 1. Primers Used in This Study

Primers	Forward	Reverse
SAT1	5’-GAAGAGGTGCTTCTGATCTGTC-3’	5’-CTCACTCCTCTGTTGCCATTT-3’
tSAT1	5’-GTCTCTAGCTTCGCCATGTA-3’	5’-CTAGGAAATGTGTTATTTCATC-3’
ZIKV	5’-CCCTCAAGTATAGCAGCAAGAG-3’	5’-TGAGTTGGAGTCCGGAAATG-3’
β-Actin	5’-CACTCTTCCAGCCTTCCTTC-3’	5’-GTACAGGTCTTTGCGGATGT-3’

Western Blots

Samples were collected with Bolt LDS Buffer and Bolt Reducing Agent (Invitrogen) and run on polyacrylamide gels. Gels were transferred using the semi-dry transfer method. Membranes were probed with primary antibody for GAPDH (1:5000, proteintech) and SAT1 (1:100, Santa Cruz Biotechnology). Membranes were treated with SuperSignal West Pico PLUS Chemiluminescent Substrate (ThermoFisher Scientific) and visualized on ProteinSimple FluorChem E imager.

Polyamine Luciferase Reporter Assay

To measure free polyamine levels in cells, a dual-luciferase vector containing the wild-type -1 frameshift antizyme OAZ1 (pC5730), were transfected into cells with LipoD293 (SignaGen). Free polyamines modulate OAZ1 mRNA frameshifting and these constructs can measure relative endogenous polyamine concentrations via a dual-luciferase reporter as previously described (32). Huh7 cells were seeded with 2% media and drug treated as described above. Cells were transfected with 62.5 ng of reporter plasmid of and after 24 hours of incubation, luminescent signal was measured using the Dual-Luciferase Reporter Assay System (Promega) by measuring both firefly and

Renilla luciferase with the Veritas Microplate Luminometer (Turner Biosystems). Firefly luciferase was normalized to *Renilla* and the wild-type values and subsequently normalized to untreated controls.

Transfection of Plasmids

Primers were designed to target SAT1 with overlapping ends. Gibson assembly was used to generate plasmid with tetracycline promoter.

Quantification and Statistical Analysis

Prism 6 (GraphPad) was used to generate graphs and perform statistical analysis. For all analyses, one-tailed Student's t test was used to compare groups, unless otherwise noted in figure legends, with $a = 0.05$. For tests of sample proportions, p values were derived from calculated Z scores with two tails and $a = 0.05$. Correlations between SAT1 expression and viral titer were calculated using either the linear regression or exponential growth curve fitting function of Prism. Statistical details are noted in individual figure legends. Mean and standard error of the mean are shown in figures. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, NS $p > 0.05$.

CHAPTER THREE

RESULTS

SAT1 is Induced Upon Viral Infection

The interferon response, triggered by viral infection, leads to a series of signaling events that results in the expression of interferon stimulated genes (ISGs). These ISGs counteract the viral infection in several different ways, including degrading viral mRNA, altering membranes, and inducing apoptosis. Studies have shown that SAT1 is upregulated in response to interferon beta treatment of cells (24). To investigate if SAT1 is induced upon viral infection, we infected PMA derived macrophages (THP1) with ZIKV, LACV, or the vaccine strain of RVFV at a multiplicity of infection (MOI) of 5 plaque forming units (pfu) per cell. Samples were collected after 24 hours and SAT1 induction was measured via qRT-PCR with SAT1 specific primers. We observed a significant increase in SAT1 gene expression in cells infected with virus (Figure 7A). This experiment was also done in huh7 cells. We observed a similar phenotype in which SAT1 gene expression was significantly increased in cells infected with virus (Figure 7B). This suggests that SAT1 is induced in response to infection with ZIKV, LACV, and RVFV.

SAT1 is the rate-limiting enzyme in the interconversion of polyamines. SAT1 works by acetylating spermidine and spermine, which results in their degradation or excretion from the cell (18). Since SAT1 is induced upon viral infection, we sought to determine polyamine levels during a viral infection. Considering that SAT1 is induced,

we hypothesized that the higher-level polyamines (spermine and spermidine) would not be present in infected cells. We infected THP1 cells with ZIKV, LACV, or RVFV at a MOI of 5 and collected after 24 hours and performed thin-layer chromatography (TLC) on polyamines. We found that, compared to our positive control of DENSpm treated cells, which showed no spermine or spermidine, virus infected cells still had these higher-level polyamines present (Figure 7C). This suggests that while SAT1 is induced transcriptionally upon viral infection, it is not functioning to acetylate spermine or spermidine for degradation or excretion.

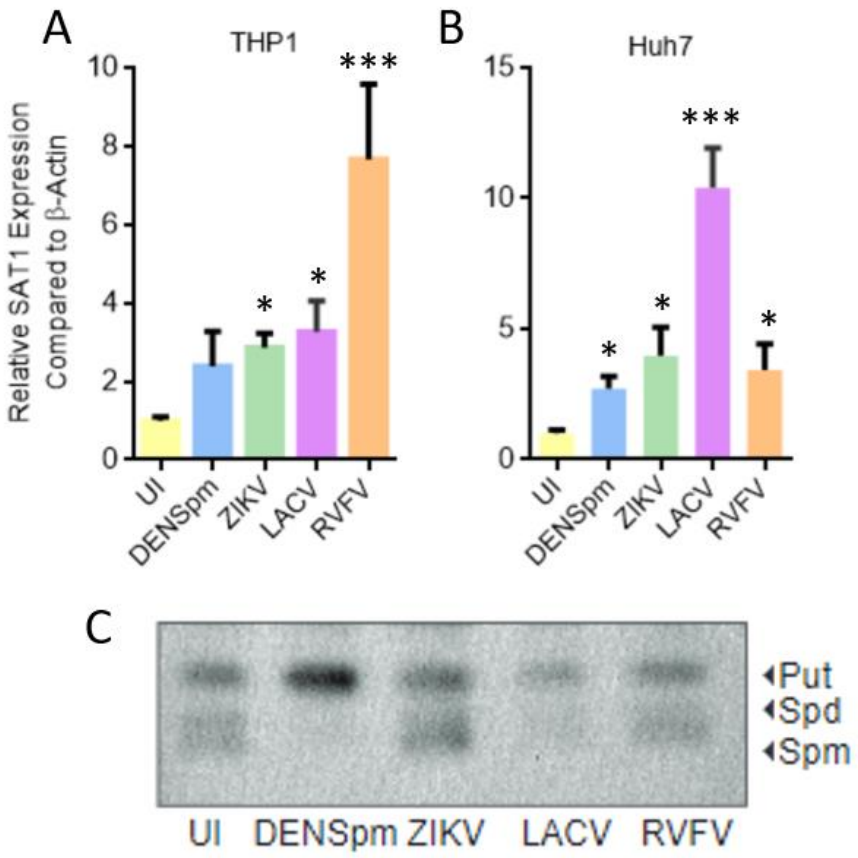

Figure 7. SAT1 is Induced Upon Viral Infection.
(A) THP1 cells were infected with ZIKV, LACV, or RVFV at a MOI of 5. Samples were collected after 24 hours. Following RNA purification, SAT1 gene expression was measure via qRT-PCR and normalized to cellular β-Actin. (B) Huh7 cells were infected with ZIKV, LACV, or RVFV at a MOI of 5 and collected after 24 hours. Following RNA purification, SAT1 gene expression was measured via qRT-PCR. (C) Thin layer chromatography on THP1 cells infected as in (A) to measure biogenic polyamine levels. Values provided above data bars represent the fold change compared to untreated conditions. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ using Student's t test ($n \geq 3$) comparing infection to uninfected controls. Error bars represent ± 1 SEM. Statistical comparison were performed between infected and uninfected conditions.

DENSpm-mediated SAT1 Induction Does Not Alter Viral Replication After Infection Initiates

Previous studies have shown that flaviviruses, including ZIKV, rely on polyamines for a productive infection (25). Our data suggests that, even though SAT1 is induced during a viral infection, the enzyme is not acetylating the higher-level

polyamines marking them for excretion or degradation. We sought to see the effects on viral replication if we forced the expression of SAT1 using DENSpm. DENSpm is a polyamine analog and induces SAT1. We treated THP1 cells with 100 μM DENSpm either 24 hours before, 2 hours after, or 4 hours after infection with ZIKV at a MOI of 5. After 24 hours, we titered the samples on Vero-E6 cells. We found that, compared to our not treated samples, the samples treated with DENSpm 24 hours before infection had a decrease in viral replication indicating the antiviral effects of SAT1. However, when cells are treated with DENSpm 2- or 4-hours after infection, the viral titers have not decreased when compared to not treated samples (Figure 8A). This was repeated in Huh7 cells and the same phenotype was observed, when cells were treated before infection, the viral titers were decreased but when the cells were treated at either time point after infection, the viral titers did not decrease (Figure 8B). To visually confirm that it is the polyamine levels being affected by the DENSpm treatment, we performed a TLC on samples from THP1 cells. The cells were once again treated with 100 μM DENSpm 24 hours before, at the time of infection, 2 hours after, or 4 hours after infection with ZIKV. The samples were collected after 24 hours and TLC on the polyamines was performed. We found that when cells are treated with 100 μM DENSpm 24 hours before infection, there is no spermidine or spermine in the samples. When cells are treated at or after infection however, spermidine and spermine are present in the samples (Figure 8C). This is suggesting that if SAT1 is induced after viral infection has initiated, it is not acetylating spermidine and spermine, marking them for excretion or degradation.

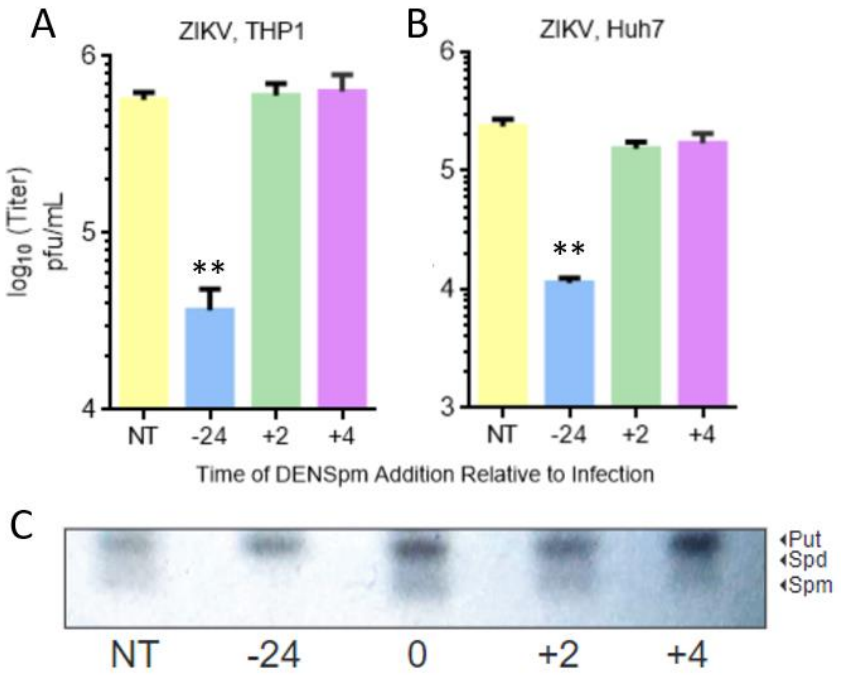

Figure 8. DENSpm-mediated SAT1 Induction Does Not Alter Polyamine Levels After Infection Initiates.
(A) THP1 cells were treated with DENSpm 24 hours before, 2 hours after, or 4 hours after infection with ZIKV. Samples were collected after 24 hours and titered via plaque assay. (B) Huh7 cells were treated with DENSpm 24 hours before, 2 hours after, and 4 hours after infection with ZIKV. Samples were collected after 24 hours and titered via plaque assay. (C) Thin layer chromatography on cells treated and infected as in (A) to measure biological polyamine levels. Values provided above data bars represent the fold change compared to untreated conditions. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ using Student's t test ($n \geq 3$) comparing DENSpm treated to untreated controls. Error bars represent ± 1 SEM. Statistical comparison were performed between treated and untreated conditions.

To ensure that SAT1 is being induced in these samples, we performed the same time of DENSpm addition as before on THP1 cells and collected the samples 24 hours after infection with ZIKV. We then measured SAT1 induction via qRT-PCR using SAT1 specific primers. We found that in all samples treated with DENSpm, there was an induction of SAT1 compared to not treated samples (Figure 9A). This indicates that, regardless of when samples are treated with DENSpm relative to viral infection, SAT1 is being induced. We also looked at protein levels via western blot analysis using the

same experimental setup. We found that when cells are treated with DENSpm before infection there is SAT1 present but after infection the amount of SAT1 protein seems to decrease with time (Figure 9B). The cause and effect of this is unclear at this time and further investigation is needed. Together, these data suggests that, once virus infection is initiated, SAT1 is induced, yet its induction does not result in the exportation or degradation of spermidine and spermine and therefore, is not antiviral.

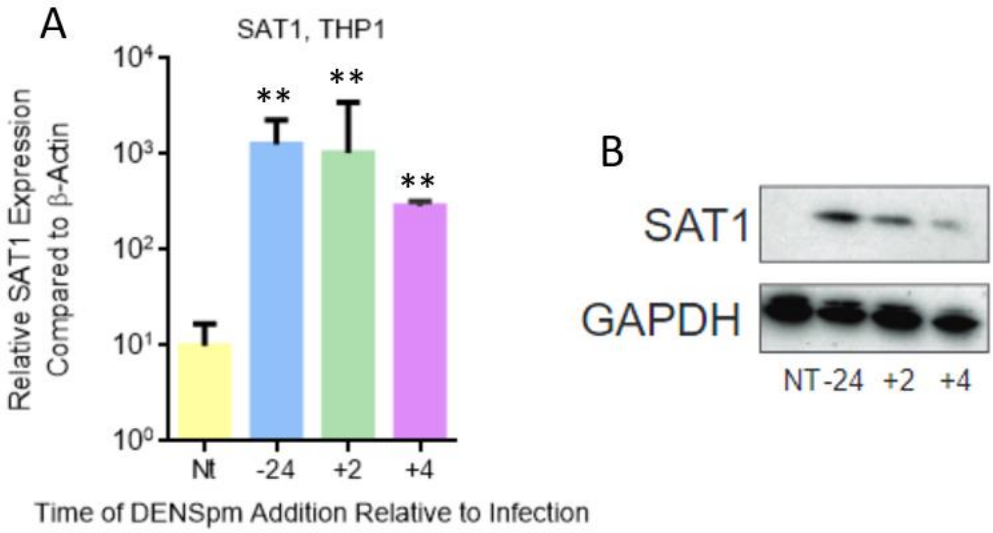

Figure 9. SAT1 is Induced Upon DENSpm Treatment After Infection is Initiated. (A) THP1 cells were treated with DENSpm 24 hours before, 2 hours after, or 4 hours after infection with ZIKV. Samples were collected after 24 hours and gene expression was measured via qRT-PCR and normalized to cellular β-Actin. (B) Western blot analysis of samples treated and infected as in (A). Values provided above data bars represent the fold change compared to untreated conditions. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 using Student's t test (n ≥ 3) comparing DENSpm treated to untreated controls. Error bars represent ± 1 SEM. Statistical comparison were performed between treated and untreated conditions.

SAT1 Splicing is Altered with Infection

SAT1 is regulated by polyamines at many different levels of gene expression, including transcription and stabilization of mRNA. Previous studies have shown that SAT1 pre-mRNA can undergo alternative splicing. This alternative splicing produces a

longer variant (SAT1-X) by insertion of an additional exon between exons 3 and 4. The additional exon carries three pre-mature stop codons, marking it for nonsense-mediated RNA decay (Figure 5) (28). This variant has been shown to be induced by tick-borne encephalitis virus and Venezuelan equine encephalitis virus, both of which are RNA viruses (31). We hypothesized that ZIKV also induces the alternative splicing of SAT1 to generate a nonfunctional enzyme and prevent the degradation of polyamines. To test this hypothesis, we performed a time course of ZIKV infection. We infected THP1 cells and collected samples 2 hours, 4 hours, 8 hours, 16 hours, and 24 hours after infection followed by RNA purification and qRT-PCR using primers specific to SAT1 and SAT1-X, the alternatively-spliced variant. We found that, as expected, as infection progressed there was an increase in SAT1 expression (Figure 10A). We also found that as infection progressed, there was also an increase of SAT1-X expression (Figure 10B). To visualize this, we also performed RT-PCR with primers specific for SAT1 on these samples. We found that all samples had SAT1, however as the infection progresses, we found the presence of an additional SAT1 band (Figure 10C). This band is about 110 bp larger than the normal SAT1 band suggesting it is SAT1-X. This suggests that ZIKV infection does result in alternative splicing of SAT1 to produce a larger variant by insertion of an additional exon.

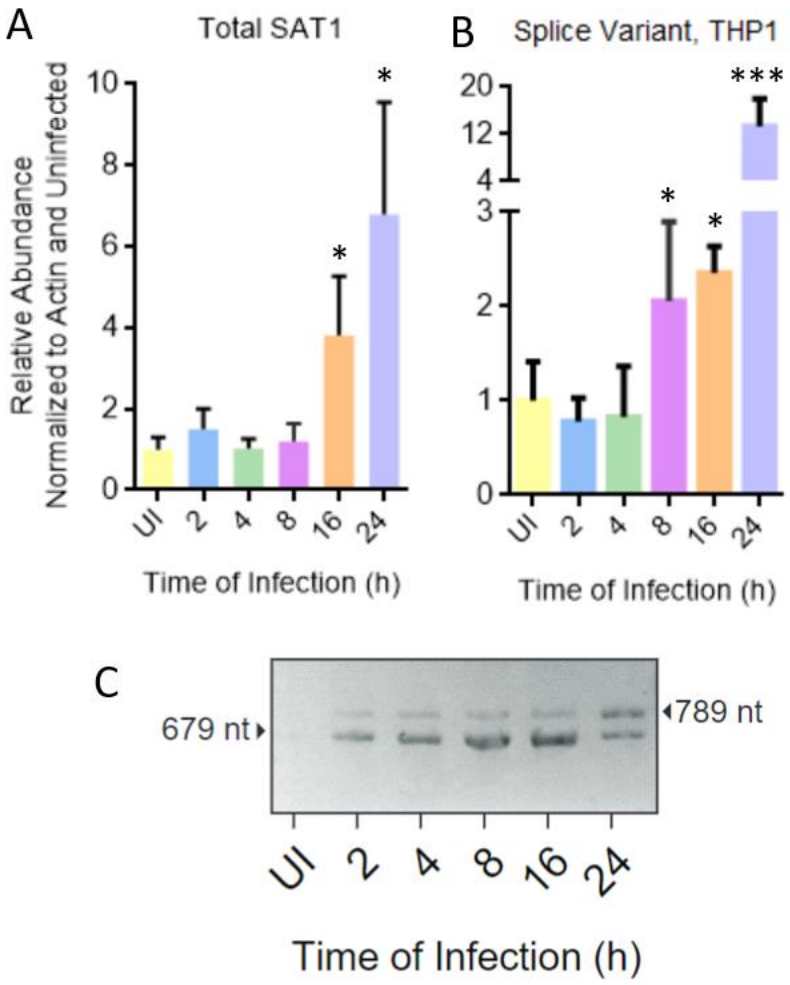

Figure 10. SAT1 Splicing is Altered with ZIKV Infection.
(A) THP1 cells were infected with ZIKV and samples were collected after 2 hours, 4 hours, 8 hours, 16 hours, and 24 hours. RNA was purified and total SAT1 gene expression was measured via qRT-PCR and normalized to β-Actin and uninfected cells. (B) qRT-PCR with primers specific to SAT1-X was performed on samples from (A). (C) RT-PCR was performed on samples from (A) and run on an agarose gel. Values provided above data bars represent the fold change compared to untreated conditions. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 using Student's t test (n ≥ 3) comparing infection to uninfected controls. Error bars represent ± 1 SEM. Statistical comparison were performed between infected and uninfected conditions.

We further explored this phenotype in LACV and RVFV as well as Huh7 cells. Our previous data showed that SAT1 is induced in LACV and RVFV infections, but the polyamine levels were not altered by expression. We hypothesized that infection with these viruses also results in the alternative splicing of SAT1. We infected Huh7 or THP1

cells with LACV or RVFV and collected samples after 24 hours. We purified RNA and performed qRT-PCR using SAT1-X specific primers. We found that both viruses induced the expression of SAT1-X in THP1, specifically RVFV had a high induction of this variant (Figure 11A). We found infection with these viruses also results in the expression of SAT1-X in Huh7 cells as well (Figure 11B). To visualize this, we performed RT-PCR on these THP1 samples and ran them on an agarose gel. We found the presence of an additional band about 110 bp larger than the standard SAT1 band, indicating the presence of the additional exon (Figure 11C). These data suggests that this phenotype is not specific to ZIKV and that RVFV and LACV infections also results in the alternative splicing of SAT1 to generate a nonfunctional enzyme that does not alter polyamine levels in the cell.

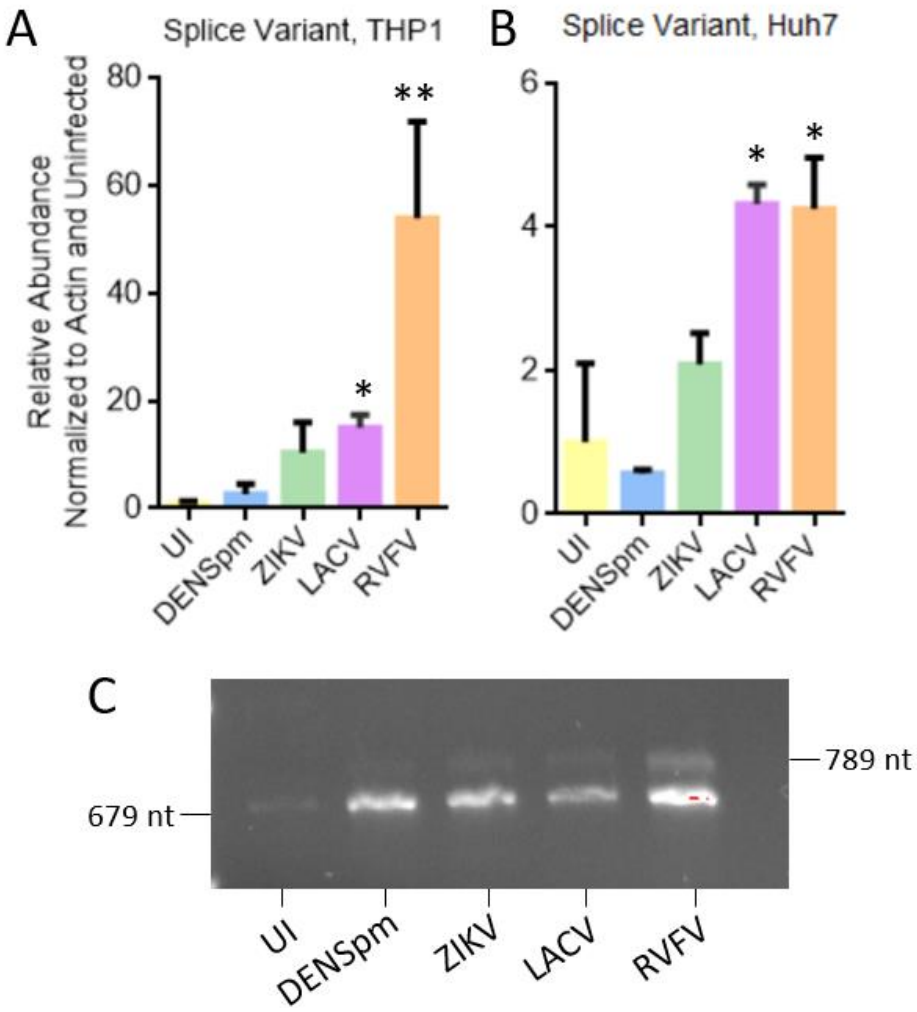

Figure 11. SAT1 Splicing is Altered with LACV and RVFV Infection.
(A) THP1 cells were infected with ZIKV, LACV, or RVFV and collected after 24 hours. RNA was purified and SAT1-X expression was measured via qRT-PCR and normalized to β-Actin and uninfected. (B) Huh7 cells were infected with ZIKV, LACV, or RVFV and collected after 24 hours. RNA was purified and SAT1-X expression was measured via qRT-PCR. (C) RT-PCR was performed on samples from (A) and run on an agarose gel. Values provided above data bars represent the fold change compared to untreated conditions. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ using Student's t test ($n \geq 3$) comparing infection to uninfected controls. Error bars represent ± 1 SEM. Statistical comparison were performed between infected and uninfected conditions.

Alternatively Spliced SAT1 is Not Antiviral

Our previous data has shown that SAT1 is alternatively spliced with viral infection. This splice variant introduces an additional 110 bp exon between exons 3 and 4 which introduces a reading frame shift resulting in three premature termination codons

ultimately marking the mRNA for nonsense-mediated mRNA decay (28). While the N-terminus of tSAT1 is identical to SAT1, the C-terminus lacks both the acetyl-CoA and catalytic motifs required for polyamine regulation (30). Therefore, we hypothesized that tSAT1 would not be antiviral due to its inability to acetylate polyamines marking them for degradation. Using the process of Gibson assembly, we were able to generate a plasmid carrying tSAT1. To investigate tSAT1's effects on polyamine levels, we transfected increasing amounts of tSAT1 plasmid into 293T cells and measured polyamine levels using a quantitative polyamine-sensitive luciferase assay. We found that regardless of how much tSAT1 plasmid we transfected in, there was no reduction in polyamine levels (Figure 12A). This suggests that tSAT1 does not reduce polyamine levels in the cell. We further wanted to look at the tSAT1's effects during virus infection. To do this, we transfected cells with 100ng of tSAT1 along with 100 ng of ZIKV full-length infectious clone plasmids. After 24 hours, we measured ZIKV viral genomes via qPCR. We found that while SAT1 decreased the amount of viral genomes, tSAT1 had no effect when compared to controls (Figure 12B). These data suggest that tSAT1 is not antiviral, though more experiments are necessary to conclude this.

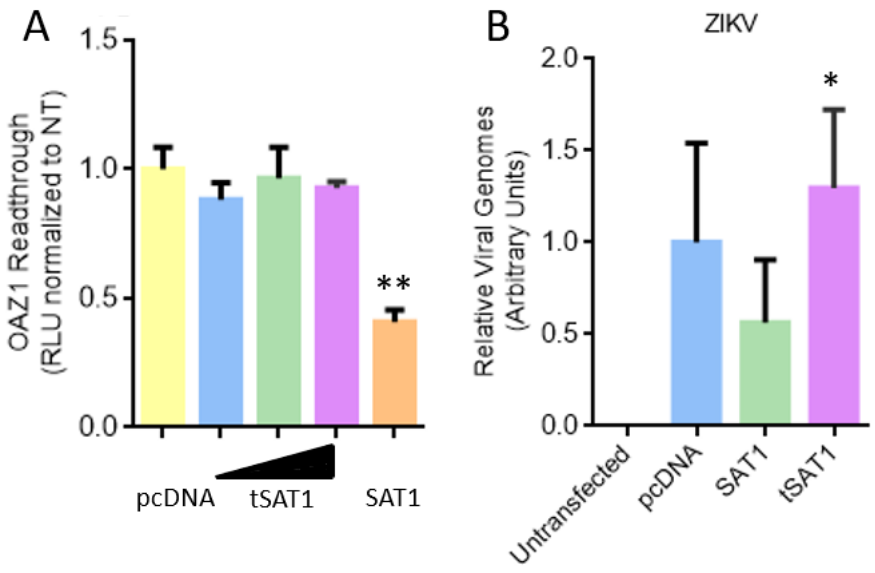

Figure 12. tSAT1 Does Not Reduce Polyamine Levels and is Not Antiviral.
(A) 293T cells were transfected with 100 ng, 500 ng, or 1000 ng of tSAT1 plasmid and intracellular polyamine levels were measured using dual luciferase assay reporter of an OAZ1 transcript construct. Relative luciferase activity was normalized to untransfected cells. (B) Huh7 cells were transfected with 100 ng of tSAT1 plasmid and 100 ng of ZIKV protein plasmids, viral genome was measured with qRT-PCR and normalized to untransfected samples. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ using Student's t test ($n \geq 3$) comparing untransfected cells to transfected cells. Error bars represent ± 1 SEM. Statistical comparison were performed between transfected and untransfected conditions.

CHAPTER FOUR

DISCUSSION

ZIKV Induces Alternative Splicing of SAT1

Previous studies have shown that RNA viruses, such as ZIKV, rely on polyamines for replication and depletion of these polyamines results in decreased viral replication. It has also been shown that SAT1 is induced upon the interferon response to viral infection. SAT1 reduces polyamine levels in the cell by acetylating spermidine and spermine, marking them for degradation or excretion. This ultimately restricts viral replication by depleting the cell of polyamines (24). Furthermore, studies have shown that SAT1 can be alternatively spliced in response to polyamine analogs and other pathophysiological agents including some RNA viruses (31). Our goal was to determine if ZIKV induces the alternative splicing of SAT1 to prevent the acetylation of higher-level polyamines. Our data suggests that during ZIKV infection, SAT1 is induced via interferon signaling but does not acetylate spermidine/spermine marking them for degradation or excretion from the cell. Furthermore, our data suggest that SAT1 is alternatively spliced, generating a longer variant with the addition of a 110 bp exon. This newly included exon creates a reading frame shift that introduces three premature stop codons and thus a truncated protein. We further demonstrated that this truncated protein might not be antiviral. We have generated a working model in which ZIKV utilizes polyamines during its infection cycle, as a way to combat this, type 1 interferon signals to activate SAT1. SAT1 then acetylates spermidine/spermine marking them for

degradation or export from the cell. However, ZIKV can induce alternative splicing of SAT1, generating a truncated version which prevents the export of these polyamines, allowing the virus to use them during its lifecycle. (Figure 13).

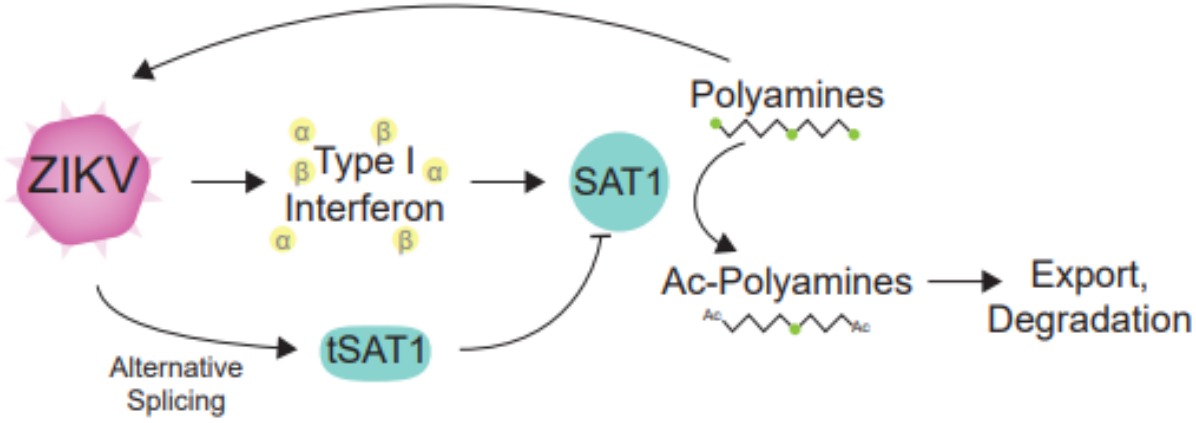

Figure 13. Proposed Model of SAT1 Alternative Splicing during ZIKV Infection. SAT1 is induced during a ZIKV infection through type 1 interferon signaling. SAT1 then acetylated spermidine/spermine marking them for degradation or export from the cell. ZIKV however, induces the alternative splicing of SAT1 generating a truncated version that does not result in the acetylation of these polyamines and thus allows the virus to use them during its lifecycle.

We further observed this phenotype in other RNA virus families, including bunyaviruses such as LACV and RVFV. Both of these also induced alternative splicing of SAT1 during infection. This indicates that this may be a conserved mechanism among viruses. In addition to bunyaviruses, other studies observed this alternative splicing of SAT1 in alphaviruses including Semliki Forest virus and Venezuelan equine encephalitis virus (28, 31).

Proposed Mechanism of Alternative Splicing of SAT1

During a viral infection, viral proteins work to subvert various cellular processes, remodels intracellular membranes, alters metabolic pathways to block the innate immune response. One could suggest that the alternative splicing of SAT1, the key

enzyme in polyamine regulation, could be another way the virus disarms the innate immune response. How the virus does this remains unknown and further investigation would be required to answer this question. However, recent studies have shown the NS5 protein of dengue virus (DENV), another flavivirus very similar to ZIKV, to interact with the spliceosome in infected cells. In this study, the researchers found the DENV NS5 protein to interact with the uridine-rich small nuclear ribonucleoprotein particles U5 (U5 snRNP) to moderate splicing. They revealed differences in splicing of transcripts involved in innate immune responses and cell cycle control, including a few ISGs; however, SAT1 was not on the list of proteins they investigated (33). Another study, which delved into the molecular mechanism underlying SAT1 alternative splicing upon DENV infection found similar results to what our data suggests. The researchers observed lower protein levels of RBM10, a splicing factor responsible for SAT1 exon 4 skipping, during infection. They reported the NS5 DENV protein interacts with RBM10 and triggers RBM10 proteasome-mediated degradation. By over-expressing RBM10 in virus infected cells, they observed limited viral replication and no changes to SAT1 splicing. Whereas RBM10 depletion resulted in increased SAT1 splicing and an increase in viral replication (34). Another study looked at the localization of ZIKV proteins during an infection and found the NS5 protein localizes to the nucleus and promotes trafficking of the splicing factor SC35 (35). This suggests that the ZIKV NS5 protein may play a role in regulating the gene splicing process in host cells. During a viral infection, ZIKV relies on many host machinery and resources during its life cycle (18). We could hypothesize that it would be beneficial to disarm the interferon response within the host cell. By targeting the spliceosome or the components of gene splicing,

the interferon response would be hindered for the benefit of the virus. Our data supports this hypothesis and includes SAT1 among other ISGs. By generating a non-functional SAT1, the polyamine levels, which ZIKV relies on, would not be diminished, allowing the virus to continue to use them and have a robust infection.

Physiological Role of tSAT1

The alternative splice variant of SAT1 has been reported during times of cellular stress, including X-ray irradiation (29), iron deficiency, or hypoxia (30). One study indicated that cells stably over-expressing the splice variant were protected from apoptosis under iron-deficient conditions (30). This brings into question the physiological role of the splice variant. One study showed that the splice variant is a target for non-sense mediated mRNA decay due to the introduction of three premature stop codons (28). This would ultimately prevent any production of new SAT1 protein to decrease polyamine levels. If the splice variant mRNA was not degraded, it would generate a truncated protein (termed here as tSAT1) that is only the first 71 N-terminal amino acids (31). tSAT1 would lack both the acetyl-CoA binding and catalytic motifs, which are essential for polyamine regulation (30). Another possibility of how tSAT1 disrupts polyamine regulation is that active SAT1 is an oligomeric protein (36) and tSAT1 could bind to the enzyme as a defective subunit. This would, in turn, inhibit SAT1 function. Regardless of how tSAT1 prohibits polyamine export and degradation, the alternative splicing of SAT1 may contribute to cell survival. Considering the splice variant has been induced during different unfavorable conditions, it is hypothesized to be a defense mechanism of the cell to prevent apoptosis (30). If this is the case, it is not surprising tSAT1 is induced during a viral infection. Viruses rely on the host for

replication and preventing cellular apoptosis would allow the virus to continue its lifecycle.

It would be interesting to further explore the role of tSAT1 by generating a cell line carrying this truncated protein. This would allow for further investigation into its effects on polyamine levels in the cell. This cell line would also provide insight into if the truncated protein is antiviral or not. It would also be beneficial to generate a cell line carrying a version of SAT1 that cannot undergo alternative splicing. We further want to explore if the NS5 ZIKV protein is responsible for the alternative splicing of SAT1 as it has been suggested in DENV infection.

While there are many avenues to explore involving the alternative splicing of SAT1 during a viral infection, our data has provided insight into the dynamic relationship between virus and host. Understanding how the virus neutralizes the innate immune system can provide insight into both the mechanisms of viral infection and the host response, in addition to highlighting novel therapeutic routes.

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VITA

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