INFLUENCE OF CELL CYCLE ALTERATION ON VENEZUELAN EQUINE ENCEPHALITIS VIRUS

by

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A Thesis
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DEDICATION

This work is dedicated to my wife Merry and our two children, Isabelle and Jefferson. Merry, it has been a crazy roller coaster of a marriage and things have almost never gone as planned. I would have it no other way. It always worked out in the end. Isabelle, when you possibly read this later on in life, I want you to know every decision I made for nine months was to get back to you. I watched all your first events through pictures and videos. It took me realizing that my actions influenced you to come full circle. Jefferson, you are my buddy and my exact phenotypic copy. I only hope you grow up to be a kind, caring son in the ways your mother and I shape you to be.

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LIST OF ABBREVIATIONS

ATM..... Ataxia telangiectasia mutated
ATR..... Ataxia telangiectasia and Rad3-related protein
Cdk..... Cyclin Dependent Kinase
CPE .....cytopathic effect
DMEM .....Dulbecco's Modified Eagle Medium
DMSO..... dimethyl sulfoxide
FACS.....Fluorescence-activated cell sorting
FBS .....fetal bovine serum
GFP .....green fluorescent protein
HU..... hydroxyurea
hpi .....hours post infection
MOI..... multiplicity of infection
NOCO ..... nocodazole
NT ..... non-treated
NLS ..... nuclear localization sequence
NES .....nuclear export sequence
ORF..... open reading frame
PBS .....phosphate buffered solution
PI.....propidium iodide
PFU ..... plaque-forming unit
Rb..... Retinoblastoma protein
SFV ..... Semliki Forest virus
SINV .....Sindbis virus
TYD ..... thymidine
VEEV .....Venezuelan Equine Encephalitis

ABSTRACT

INFLUENCE OF CELL CYCLE ALTERATION ON VENEZUELAN EQUINE ENCEPHALITIS VIRUS

Jacque L. Fontenot, M.S.

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Venezuelan Equine Encephalitis Virus (VEEV), of the *Togaviridae* family and *Alphavirus* genus, is the causative agent of potentially fatal viral encephalitis. The virus is transmitted to humans via the bite of infected *Culex* mosquitoes that have fed on infected equines. VEEV is an emerging pathogen and with no tangible large scale vaccine available, has the potential to cause epidemics. The virally encoded capsid protein is responsible for causing cytopathic effect at least partially due to inducing a transcriptional shutoff in infected cells. A nuclear localization sequence within the capsid protein allows nuclear import of capsid and blocking of the nuclear pores thereby inhibiting the import and export of cellular proteins. Other nuclear localized viral proteins are known to induce alterations of the cell cycle in order to create favorable conditions for viral replication. Understanding how VEEV manipulates the cell cycle will provide possible intracellular targets for future antiviral strategies. In this study we show that

VEEV infected cells have a delayed cell cycle progression following serum starvation. Western blot analysis showed decreased Cyclin E and A2 levels at 16 and 24 hours post infection (hpi). Phosphorylation of Rb remained low at 16 and 24hpi in infected cells, further confirming the cell cycle delay in infected cells. UV inactivated VEEV did not induce this delay in return to cell cycle, indicating that replication competent VEEV is needed for the induction of this delay. Cdk4/6 and Cdk1 inhibitor treated cells showed significant decreases in VEEV replication. Hydroxyurea, nocodazole and thymidine synchronized cells showed significant decreases in viral replication as well. The results of our study show that VEEV is delaying the cell cycle progression of synchronized cells and that VEEV needs an actively replicating population of cells for optimal viral replication kinetics. Future studies will focus on confirming that capsid's nuclear localization is responsible for these observations through the use of a capsid NLS mutant virus.

CHAPTER ONE: INTRODUCTION

Venezuelan Equine Encephalitis Virus

Biological Significance

Venezuelan equine encephalitis virus (VEEV) is a member of the Alphavirus genus in the *Togavirdae* family of viruses. It is further classified as a New World Alphavirus which includes the viruses eastern equine encephalitis virus (EEEV) and western equine encephalitis virus (WEEV) (Garmashova et al., 2007a). VEEV is transmitted through the bite from an infected Culex subgenus mosquito that has normally fed on an infected equine amplification host that has developed a high viral titer (Weaver et al., 2004). This infected mosquito then feeds on a human and transmits the virus. Humans are normally infected with the epizootic strains, subtype I-A, B and C and classified as incidental host in the cycle of transmission (Weaver et al., 2004). After infection the incubation period of VEEV in humans is approximately 2-5 days with the most common symptoms being fever, chills and headache (febrile illness) with the acute disease subsiding 4-6 days after the start (Weaver et al., 2004). Following clearance of the virus, a general malaise and weakness could persist for weeks with some possible mild neurological symptoms persisting (Steele and Twenhafel, 2010; Weaver et al., 2004). If the infection is not cleared, viral titers increase with virus systemically

distributed in the blood possibly crossing the blood brain barrier in infected lymphocytes, mononuclear cells and neutrophils (Steele and Twenhafel, 2010). This leads to the rarest, but most severe symptoms of the VEEV induced neurological disease, viral encephalitis. Viral histological damage is not limited to the brain and can be found in other parts of the body including the lungs, liver, GI tract, lymph nodes and spleen with necrosis expected in all regions of infection (Steele and Twenhafel, 2010; Weaver et al., 2004). VEEV infection is lethal in approximately one percent of infections, but the neurological symptoms of the disease can be found in upwards of 14% of cases (Garmashova et al., 2007b; Steele and Twenhafel, 2010; Weaver et al., 2004).

Due to its ability to be cultured at high viral titers, aerosolized and delivered at a low infectious dosage, VEEV has been classified as a category B pathogen by the Centers for Disease Control for use as a possible biological weapon (Spurgers and Glass, 2011). There are currently no FDA approved vaccines for VEEV also making it a prime candidate for weaponization. The current vaccine, the TC-83 live attenuated strain is a mutant virus of the epizootic Trinidad donkey strain that was passaged 45 times in fetal guinea pig heart cells (Kinney et al., 1993). The attenuation of the TC-83 strain is attributed to mutations in the 5' non-coding region and in the E2 glycoprotein genes in the viral genome (Kinney et al., 1993). The TC-83 vaccine, while licensed for use in veterinary medicine, remains in an "Investigational New Drug" status by the FDA even after over a fifty years of investigation (Pittman et al., 1996; Seregin et al.,

2010). Humans immunized with TC-83 seroconvert approximately 80% of the time and 40% of individuals experienced reactogenicity making TC-83 an improbable candidate vaccine for large scale vaccinations (Guerbois et al., 2013; Pittman et al., 1996). A major apprehension of using TC-83 as a vaccine is that RNA viruses lack proofreading capability which increases the probability of a reversion of the vaccine phenotype back to a virulent phenotype (Forrester et al., 2011). TC-83 is currently only used to vaccinate personnel at high risk of exposure, such as military and laboratory personnel for these reasons (Forrester et al., 2011).

Molecular Biology

VEEV is an enveloped, spherical virion that has icosahedral (T=4) symmetry and a diameter of 70nm (Weaver et al., 2004). The viral capsid is surrounded by a bilipid envelope and contains the RNA genome of the virus (Weaver et al., 2004). The genome of VEEV is a single-stranded, positive sense RNA, with a 5' methylguanylate cap and a 3' polyadenylation tail and is approximately 11.4kb in length (Figure 1A) (Leung et al., 2011; Weaver et al., 2004). The genome can be divided into two open reading frames (ORF), the nonstructural and the structural. The nonstructural ORF encodes the nsP1, nsP2, nsP3 and nsP4 proteins, which are proteins involved in viral RNA transcription and replication (Leung et al., 2011; Weaver et al., 2004). The structural ORF encodes the capsid (C) protein and the envelope glycoproteins, E1 and E2, which are responsible for production of the structure of the mature virion (Leung et al., 2011; Weaver et al., 2004). The presence of the 5' methylguanylate cap on the viral genomic RNA allows the

nonstructural ORF proteins to be translated immediately upon entry into the host cell cytoplasm while the structural ORF proteins are produced from the 26s subgenomic RNA which is produced via a negative strand replication intermediate (Spurgers and Glass, 2011). A 5' methylguanylate capped and 3' polyadenylated structural ORF mRNA is produced from the transcription of the replication intermediate negative strand viral mRNA (Spurgers and Glass, 2011; Strauss and Strauss, 1994). Capsid, the first protein of the structural polyprotein translated from the 26s subgenomic mRNA (Figure 1A). Capsid is self-cleaved and captures a positive strand viral RNA by a self-packaging signal present in the genome for inclusion into mature virions (Figure 1A) (Kim et al., 2011). The remaining polyprotein protein contains the PE2, E1 and 6k proteins (Figure 1A) (Strauss and Strauss, 1994; Weaver et al., 2004). This polyprotein is initially cleaved to produce PE2, E1 and the 6k proteins and further cleavage by host cell proteinases produces the mature E2 protein and the small E3 protein (Strauss and Strauss, 1994). The two glycoproteins, E1 and E2, are then inserted into the ER membrane and processed for secretion to the cell plasma membrane where they are inserted into the cell plasma membrane (Weaver et al., 2004). The two glycoproteins then interact with a formed nucleocapsid to bud a mature virion out of the cell (Garmashova et al., 2007b; Leung et al., 2011).

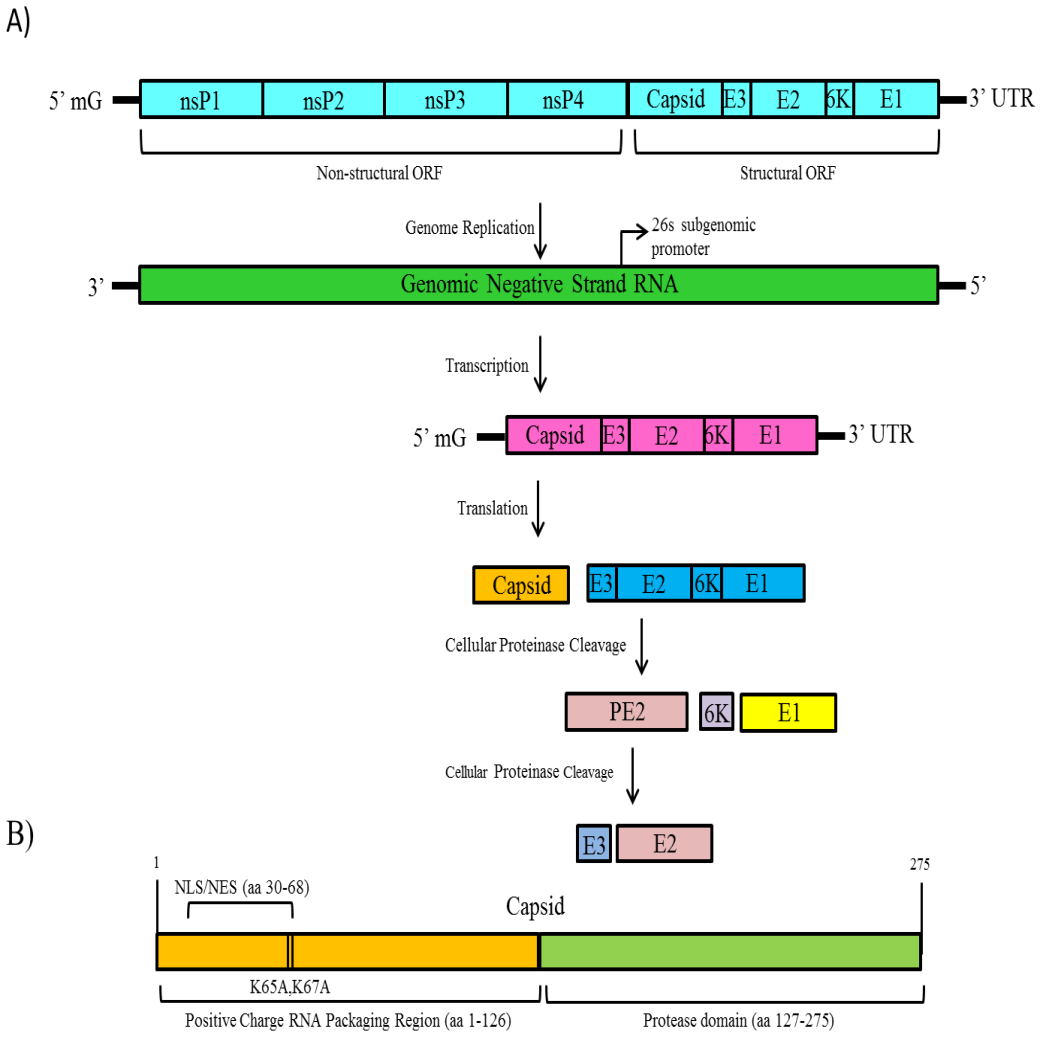

Figure 1: VEEV Genomic Structural ORF protein production. A) VEEV genomic RNA is illustrated with subgenomic RNA intermediate show with structural ORF 26s promoter for transcription of structural ORF mRNA. The structural ORF mRNA is translated to form a polyprotein which capsid is self cleaved. The remaining polyprotein is further processed to produce the PE2, 6K and E1 proteins. The PE2 protein is subsequently cleaved to produce the E3 and E2 proteins. B) Capsid protein is illustrated to show two main domains of the capsid protein and location of NLS and NES . Nuclear localization mutation sites are also shown.

Capsid Protein

VEEV capsid protein is a 275 amino acid long protein encoded by positions 2576-2851 on the viral genome (Figure 1B) (Atasheva et al., 2013; Strauss and Strauss, 1994). There are many distinct domains within the capsid protein sequence including a protease domain (aa 127 to 275) and a highly concentrated region (aa 1 to 126) of positively charged amino acids that play a role in viral RNA packaging during virion assembly (Figure 1B) (Atasheva et al., 2013). The VEEV capsid protein also contains a 39-amino-acid-long sequence (aa 30 to 68) that contains nuclear localization signals (NLS) and nuclear export signals (NES) (Figure 1B) (Atasheva et al., 2010). Mutation in the NLS sequence of lysine residues at positions 65 and 67 to alanine abolished import of VEEV capsid protein into the nucleus (Atasheva et al., 2010).

VEEV capsid protein is found in the cytoplasm as well as inside of the nucleus for it to carry out three main functions during infection. The first and primary function of the capsid, as in other alphaviruses, is to discriminatingly bind to viral genome prior to the production of viral nucleocapsids and avoid the packaging of cellular and viral subgenomic RNA (Atasheva et al., 2010). The second function of the capsid protein is to assemble into the nucleocapsid. The nucleocapsid is composed of 240 copies of the capsid protein and self-assembles in the cytoplasm with exactly one copy of the plus-stranded RNA genome

(Weaver et al., 2004). The third function of the capsid protein is its ability to inhibit cellular transcription by interaction with the nuclear pore complex thus altering the cell's ability to function in a normal manner and consequently induce high levels of cytopathic effect (CPE) (Atasheva et al., 2008; Garmashova et al., 2007b). Capsid forms complex with the nuclear export receptor CRM1 and the nuclear import receptor importin α/β for translocation in and out of the nucleus to induce these effects (Atasheva et al., 2010). This binding by capsid to importin α/β and CRM1 blocks the nuclear pore and does not allow nuclear import or export of cellular proteins or mRNA and thus shutting down cellular transcription (Atasheva et al., 2010, 2013). This cellular transcription shut off has been directly attributed to the capsid protein of New World alphaviruses (Garmashova et al., 2007b; Garmashova et al., 2007a) but to the nsP2 in Old World alphaviruses such as Sindbis virus (SINV) and Semliki Forest virus (SFV) (Garmashova et al., 2007a). The inhibition of cellular transcription and CPE is attributed to the N-terminus of the capsid protein and directly to the NLS (aa 65 and 67) which interacts with importin α/β (Atasheva et al., 2008, 2013; Garmashova et al., 2007b). VEEV is unable to be imported into mosquito cell nuclei and thus do not induce transcriptional inhibition and CPE in mosquito cells (Atasheva et al., 2008). This allows for establishment of a persistent infection in the mosquito vector (Atasheva et al., 2008).

Cell Cycle Regulatory Proteins

The cell cycle of eukaryotic cells occurs over two distinct periods: interphase and mitotic phase. Interphase is characterized by the G1, S and G2 phases where the cell is preparing to divide. G1 phase is characterized by an enlargement of the cell with high amounts of protein synthesis and organelle production. S phase is characterized by the duplication of the chromosomes in preparation for division. During G2 phase, the chromatin produced in S phase is condensed into chromosomes. After condensation of the chromosomes, the cell moves into the mitotic (M) phase, where the cellular nucleus is duplicated and two daughters cells are produced through cytokinesis. The main proteins controlling this progression through each phase are Cyclin/Cyclin dependent kinase (Cdk) complexes.

Cdk are a family of heterodimeric serine/threonine kinases that control the progression of cell cycle through the regulation of cellular transcription (Pavletich, 1999). They require two subunits for activation, the Cdk and cyclin subunits, and are dependent on the cyclin subunit for activation of the catalytic Cdk subunit (Pavletich, 1999). The Cdks involved in cell cycle progression are Cdk1, Cdk2, Cdk4 and Cdk6 and are held in constant levels in cell throughout the entire cell cycle (Arellano and Moreno, 1997).

The cyclins are a family of related proteins that are required for activation of catalytic Cdk subunits. The cyclin proteins are related through homologous

100 amino acid sequences termed the “cyclin box”, but are otherwise very different in their primary structure (Arellano and Moreno, 1997). The cyclins involved in the direct regulation of eukaryotic cell cycle are cyclin D, E, A and B and are associated with the G1, G1/S transition, S, and G2/M phase of the cell cycle respectively. The Cdk/cyclin complexes formed during each phase are as follows: 1. Cdk4 and 6 complex with Cyclin D and Cdk2 with Cyclin E during G1, 2. Cdk2 complexes with Cyclins A and E during S phase, and 3. Cdk2 complexes with Cyclin A and Cdk1 complexes with Cyclin B during G2/M phase (Figure 2) (Arellano and Moreno, 1997). The cyclins are the limiting factors for the activation of the Cdks as their levels vary as the cell progresses through each phase to ensure that the dividing cell progresses through each phase sequentially (Arellano and Moreno, 1997).

The complexity of the entire cell cycle cannot be covered in this thesis, but a few key proteins are hallmarks at the cell cycle “checkpoints” between the phases in the cell cycle. Signaling is initiated to start replication in the cell by proliferative signals (growth factor receptors, Ras controlled pathways) to move the cell from G0 (resting) to G1 by increasing production of Cyclin D allowing the activation of Cdk4/6 (Downward, 1997). The activation of the Cdk4/6/Cyclin D complex allows the cell to initiate the necessary cellular process to progress in the replication cycle. As the cell approaches mid G1 phase, the levels of Cyclin E rise allowing the activation of Cdk2 which has two vital roles: 1. The promotion of Cyclin A production and 2.The phosphorylation Retinoblastoma (Rb) protein

with the assistance of Cyclin D (Figure 2) (Duronio et al., 1996). The purpose of Cyclin D and Cyclin E phosphorylating Rb is to release the E2F transcription factor which prior to Rb's phosphorylation is bound in its inactive form to Rb (Figure 2). The release and activation of E2F by the phosphorylation of Rb allows E2F to transcribe genes that mediate the cell cycle to transition from G1 phase to S phase (Arellano and Moreno, 1997). As chromosome replication continues in S phase and no DNA damage is detected, the production of Cyclin B reaches a threshold level to allow progression into G2/M phase. In G2/M phase, the phosphorylation of histone H3 at serine 10 is a crucial event that marks the beginning of mitosis in actively dividing cells (Crosio et al., 2002). The completion of the cell cycle is marked by a global dephosphorylation of histone H3 and Rb occurring after cytokinesis (Crosio et al., 2002).

Mechanisms exist to ensure the progression through the cell cycle checkpoints do not allow abnormally replicating cells to keep progressing through cell cycle. Along with the phosphorylation of Rb to allow a cell to progress into S phase, the protein p21 is a global cyclin-dependent kinase inhibitor of cellular Cdks, which allows it to arrest an actively replicating cell in any phase of the cell cycle (Figure 2) (Jung et al., 2010). During times of high cellular stress, such as when DNA damage is detected in S phase, the activation of the ATM/ATR DNA damage response is used in the cell to arrest cells via the p53 dependent expression of p21(Jung et al., 2010). The p21 induced arrest of the cell will regress when the level of cellular stress is reduced and the cell cycle continues or

apoptosis is induced to avoid a possible mutation to be passed along to daughter cells (Jung et al., 2010).

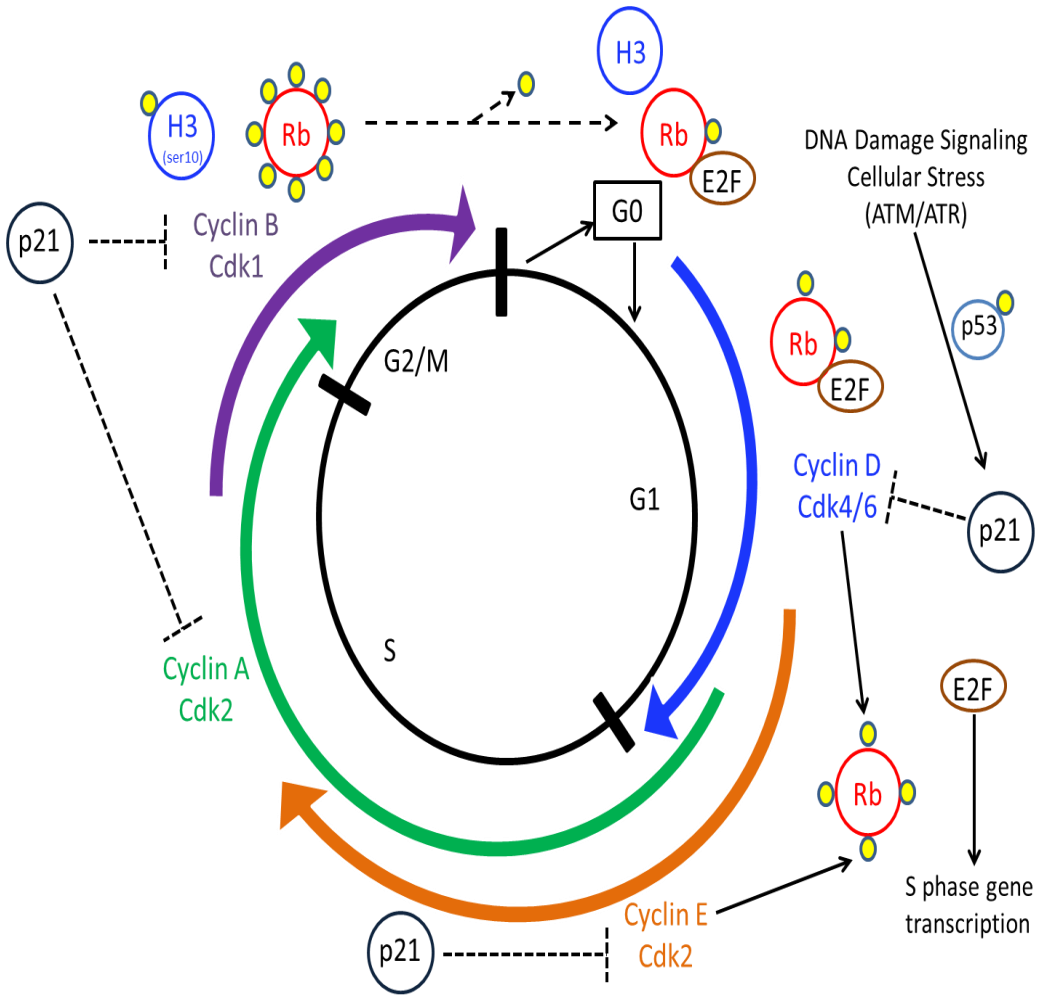

Figure 2: Cell Cycle Regulation Proteins. Major cell cycle checkpoint proteins include Cyclin D, Cyclin E, Cyclin A and Cyclin B. Cyclin substrates Cdk4/6, Cdk2 and Cdk2 are shown with respective activation cyclin. Rb protein is phosphorylated by Cyclin D and Cyclin E in order to release E2F for progression into S phase. p21 production is dependent on p53 activation by induction by the ATM/ATR damage response pathway. pRb and pH3 are dephosphorylated at completion of M phase.

Viral Cell Cycle Regulation

Many viruses manipulate the infected host cell's replication cycle to create favorable conditions for viral replication. Manipulation strategies vary but the subsequent outcome involves the alteration of the levels of key cell cycle proteins by affecting an upstream regulator or by direct interaction with the cell cycle protein. The effects of this are that the infected cell becomes arrested in a particular phase of the replication cycle depending on what cell cycle proteins are affected. Manipulation of the proteins or their regulators has been specifically attributed to a specific viral protein is some instances. The human respiratory syncytial virus (HRSV), a negative sense single-stranded RNA virus, induces a G0/G1 phase arrest by decreasing levels of Cyclin D1/D2 and Cdk4/6 while increasing the level of p21(Wu et al., 2011). Cells blocked by a Cdk4/6 inhibitor in G1 phase and then infected with HRSV enhanced progeny virus production fivefold (Wu et al., 2011). Influenza A's nonstructural protein 1 (NS1) induces G0/G1 cell cycle arrest by primarily suppressing the RhoA/pRb signaling pathway, but also by increasing levels of p21 and decreasing levels of Cyclin D1 (Jiang et al., 2013). Influenza viral titers were higher in G0/G1 synchronized cells than in unsynchronized and G2/M synchronized cells indicating that the G0/G1 phase was favorable for progeny virus production (Jiang et al., 2013). The NS1 viral protein is produced at high levels in infected cells and contains two NLS signals and one NES signal (Hale et al., 2008) The NLS and NES signals allow

NS1 to be actively transported in and out of the nucleus mediated by cellular importin-α (Hale et al., 2008). The M1 virus, a similarly sized (11.7kb) mosquito borne equine amplified alphavirus, induces a S phase arrest in infected cells and decreases the levels of Cyclin D1 and Cdk4, but a specific viral protein cannot be implicated in the induced S phase arrest (Hu et al., 2009).

Aims of the Study

VEEV is a potentially dangerous biological weapon that can be grown to high concentration and easily disseminated by the aerosol route. The current vaccination, the live attenuated TC-83 virus, has numerous issues, including high seroconversion rate and reactogenicity making it a less than ideal large scale vaccine. Discovery of specific cellular proteins being influenced during infection by VEEV can lead to future vaccine development though targeting pathways that VEEV is subverting during the course of infection. Understanding the underlying mechanisms of host cell-virus interaction including how VEEV manipulates the most vital cellular process of replication is key to future studies of not only VEEV, but other alphaviruses. Other viruses alter the cell cycle in infected cells and that a specific viral protein can be ascribed to the induction of the alteration in some instances. My hypothesis is VEEV, through the expression of the capsid protein will alter the cell cycle of infected cells. The following specific aims will address our hypothesis:

Aim 1: Identification of cell cycle alteration induced by and cell cycle regulation proteins being influenced by VEEV infection

Other viruses, such as Influenza A and more importantly, the M1 alphavirus have been shown to induce an S phase arrest in infected cells. VEEV is hypothesized to induce a similar alteration of cell cycle in infected cells. Vero cells were infected with VEEV and collected for cell cycle analysis by propidium iodide (PI) staining for DNA content using flow cytometery and cell cycle analysis software. Following confirmation of cell cycle phase alteration induced by VEEV, whole cell lysates from Vero cells infected with VEEV were prepared for analysis by Western blot. Mock infected lysates were compared to VEEV infected lysates. Western blots were performed against cell cycle regulation proteins Cyclin D1, Cyclin E2, Cyclin A2, p21, pRb (Ser780), p-Histone H3 (Ser10) and VEEV capsid to confirm which cell cycle proteins were being up-regulated, down-regulated or unaffected by VEEV infection. Vero cells were then exposed to UV inactivated VEEV to see if the cell cycle is altered or if the alteration is a cellular response to viral proteins or if an active VEEV virus was needed to induce the cell cycle phase alteration.

Aim 2: Determine if altered cell cycle promotes VEEV viral replication.

Since other viruses manipulate the cell cycle for the purpose of enhancing the production of progeny viruses, we wanted to see if VEEV also was inducing altering cell cycle for the purpose of enhancing progeny virus production. Three approaches were used to induce cycle arrest in Vero cells: 1. Serum starvation to synchronize in the G0/G1 phase, 2. Cdk inhibitors to induce phase arrest at early

G1, late G1 and G2/M and 3. Drug inhibitors to arrest in G1, S and G2/M phases. Cells were treated with respective treatments and infected with VEEV for 16 and 24 hours. Cells were then collected and stained with PI for cell cycle analysis to profile if the treatments were effective in accomplishing their intended effect and also to see if the virus could overcome the induced block. Supernatants were collected from all drug treated cells and virus replication was assessed by plaque assay.

Aim 3: Determine if capsid protein is responsible for cell cycle alteration in infected cells

Capsid protein is the expected viral protein to be responsible for alteration of cell cycle in infected cells due to its cellular localization in and outside of the nucleus, its ability to block the nuclear pore and the ability to shutdown cellular transcription. A DNA plasmid expressing the TC-83 capsid protein coupled with GFP was cloned and transfected into Vero cells. GFP expression coincided with production of capsid protein. Cells were fixed and stained with PI and assessed by flow cytometry for DNA content and GFP expression. The cell cycle of GFP positive cells was used to determine if cell cycle alteration was induced by expression of the capsid protein by cell cycle analysis software.

CHAPTER TWO: MATERIALS AND METHODS

Cell Culture, Viral Infection

Vero cells were grown in Dulbecco's Modified Minimum Essential Medium (DMEM) augmented with 10% heat-inactivated Fetal Bovine Serum (Thermo Scientific), 1% penicillin streptomycin (Life Technologies) and 1% L-glutamine (Life Technologies). Vero cells were grown in DMEM augmented only with 1% penicillin streptomycin and 1% L-glutamine for serum starvation experiments to synchronize them into the G0/G1 phase of cell cycle. Cell growth conditions are 37^{o}C in 5% carbon dioxide (CO_2).

For experiments using the VEEV TC-83 strain, 6 x 10^5 Vero cells were cultured in 6-well plates, 1.5 x 10^5 Vero cells were cultured in 12-well plates, and 1.5 x 10^4 Vero cells were cultured in 96-well plates unless otherwise noted. VEEV was suspended in supplemented DMEM at MOIs of 1 and 10. Viral suspension was then overlaid onto cells at the volumes of 400 µl per well in a 6-well plate, 200 µl of suspension in a 12-well plate, and 50 µl of suspension in a 96-well plate for all experiments. Cells were then incubated with viral suspension for one hour at 37^{o}C at 5% CO_2.

VEEV ultraviolet light (UV) inactivation was accomplished by placing 1ml of VEEV in a 1.5ml microcentrifuge tube into a UV Stratalinker 1800

(Stratagene). The micorcentrifuge tube was exposed to 120,000 microjoules/cm^2 for four separate cycles. The tube was inverted after the second exposure to ensure complete inactivation. UV viral inactivation was then confirmed by plaque assay as described in the "Plaque Assay" subheading later in the materials & methods. Absence of viral plaques indicated complete viral inactivation of VEEV.

Western Blot Cell Lysate Preparation and Analysis

Western Blot Cell Lysate Preparation

Cultured cells were infected with VEEV suspended in DMEM at MOIs of 1 and 10 by overlaying 400 µl of virus suspension in a 6-well plate. Cells were incubated with viral suspension for one hour at 37^oC at 5% CO_2. The viral suspension was then removed and all cells were washed with Phosphate Buffer Saline (PBS) pH 7.4 (Gibco) and replaced with supplemented DMEM until the desired time point of infection. At the desired time point, the supplemented DMEM was removed and cells were washed with PBS, lysed with 200 µl of lysis buffer and boiled for 10 minutes prior to storage at -80^oC. The lysis buffer consist of a 1.25:1 mixture of tissue protein extraction reagent (Invitrogen) and 2x Tris-Glycine SDS Sample Buffer (Novex), 2.6% 1M dithiothreitol (DTT), and cOmplete, Mini Protease Inhibitor Tablets (Roche), 0.16% 0.1M NaF and 0.8% 0.1 M Na_3VO_4.

Western Blot Analysis

Cell lysates were boiled for three minutes prior to separation. Thirty μl of cell lysate per sample (15 μl for VEEV capsid protein) were loaded into and separated by electrophoresis on a NuPAGE 4-12% Bis-Tris Gel (Novex) in an XCell SureLock Electrophoresis Cell (Invitrogen). Proteins were then transferred overnight at 70 mA to an Immobilon polyvinylidene difluoride (PVDF) membrane (Millipore). The PVDF membrane was cut to the approximate size of the protein gel and placed on top of the protein gel. Chromatography paper (Whatman) was then placed on both sides of the PVDF membrane and the protein gel. The complete paper/PVDF/protein gel/paper composite was then placed between sponges and placed inside an XCell II Blot Module (Invitrogen) and then into an XCell SureLock Electrophoresis Cell. Transfer buffer consisting of 10% tris-glycine transfer buffer (Quality Biological) and 20% methanol in deionized water was added to XCell II Blot Module and run at 70 mA for approximately 24 hours.

The PVDF membrane was then blocked in 3% boiled non-fat milk in a solution of 1x PBS with 0.1% Tween-20 (Promega) for one hour at room temperature. Primary antibodies were diluted in the 3% non-fat milk/PBS-Tween solution at a ratio of 1:1000 and then incubated with the PVDF membrane for 24 hours at 4^{o}C. β-actin was diluted at a 1:10,000 ratio and incubated for 1 hour at room temperature. VEEV capsid protein was diluted at a 1:4000 ratio prior to 24 hour incubation at 4^{o}C. The membrane was then washed four times at five

minutes per wash with PBS-Tween and then incubated with secondary horseradish peroxidase (HRP)-coupled anti-rabbit or anti-mouse antibody at a 1:1000 ratio (1:5000 for anti-goat) in 3% non-fat milk/PBS-Tween solution for two hours. The membrane was then washed four times at five minutes per wash in PBS-Tween. The membranes were then imaged by chemiluminesene using SuperSignal West Femto Maximum Sensitivity Substrate kit (Thermo Scientific) in a Bio-Rad Molecular Imager ChemiDoc XRS System (Bio-Rad) to obtain western blot image.

The primary antibodies used were: Cyclin D1, rabbit (#2978, Cell Signaling); Cyclin E2, rabbit (#4132, Cell Signaling); Cyclin A2, mouse (#4656, Cell Signaling); p21, rabbit (#2947, Cell Signaling); phospho-Rb (Ser780), rabbit (#9307, Cell Signaling) phospho-Histone H3(Ser10), rabbit (#9701, Cell Signaling); HRP conjugated β-actin (#ab49900-100, Abcam); and VEEV capsid protein, goat (NR 9403, BEI Resources).

Plaque Assay

Virally infected cell supernatants were collected at specified post-infection time points and stored at -80^{o}C. Vero cells were plated in 12-well plates at 1.5 x 10^5 cell per well. Cell monolayers were confirmed to be 90-100% confluent at 24 hours post plating. Viral supernatants were serial diluted at a 1:10 ratio in supplemented DMEM with dilutions ranging from 10^{-1} to 10^{-12}. Two hundred microliters of each dilution was used to infect an individual well in the 12-well plate. Each infection was performed in duplicate per dilution. The infected cell

plates were then incubated for 1 hour at 37^{o}C in 5% CO_2. After infection, a 1 ml overlay was added to each well consisting of a 1:1 mixture of 0.6% agarose in deionized water with 2 x Minimum Essential Medium Eagle (EMEM, Quality Biological) that was supplemented with 5% FBS, 1% L-glutamine, 1% penicillin streptomycin, 1% nonessential amino acids, and 1% sodium pyruvate. After allowing the agarose/EMEM to solidify, the plates were incubated at 37^{o}C in 5% CO_2 for 48 hours. A 10% formaldehyde solution (in deionized water) solution was added to each well and left at room temperature for two hours. The agarose plug was then removed and a 1% crystal violet, 20% ethanol (in deionized water) was added to each well and left for one hour at room temperature to stain the preserved cell monolayer and visualize the viral plaque formation with it. Average counts of the duplicates were calculated and applied to the below equation for the calculation of the viral titer in pfu/ml:

$$\text{titer (pfu/ml)} = \text{average count of duplicates} \times \text{dilution} \times 5.$$

Cell Viability Assay

To assess the viability of cells infected with VEEV over the course of infection, the CellTiter-glow Cell Luminescent Viability Assay (Promega) was used in accordance with the vendor's instructions. The cultured Vero cells were allowed to equilibrate to room temperature. An equal volume of CellTiter-Glo reagent was mixed with an equal amount of DMEM that was present in the cell culture plate. The plate was then shaken on an orbital shaker for the prescribed two minutes and then incubated at room temperature for another 10 minutes.

Viability was assessed by luminescence detection using the DTX 880 multimode detector (Beckman Coulter).

Cyclin-dependent Kinase Inhibitors

Cyclin-dependent kinase (Cdk) inhibitors were obtained from EMD Millipore Chemicals. Inhibitors used are Cdk4/6 Inhibitor IV (Calbiochem 219492), Cdk1 Inhibitor (Calbiochem 217695) and Cdk2 Inhibitor III (Calbiochem 238803). Stock solutions of all inhibitors were prepared by resuspension in DMSO. The Cdk4/6 and Cdk 4 inhibitors were resuspended at 15mM and the Cdk2 inhibitor was resuspended at 5mM. Fifty microliters of stock inhibitors were aliquoted into microcentrifuge tubes for storage at -20^{o}C. Dilutions of all inhibitors were prepared in supplemented DMEM with final concentrations of 10 μM.

Cells were plated 24 hours prior to treatment with Cdk inhibitors. Original media was removed from cell culture plates and replaced with DMEM containing Cdk inhibitors one hour prior to infection with VEEV. At time of infections, Cdk containing media was removed from cell culture plates, washed once with 1x PBS and then replaced with virus containing media at an MOI of 1. The infected cell plates were then incubated for 1 hour at 37^{o}C in 5% CO_2. Infectious media was then removed and DMEM containing respective Cdk inhibitor was re-added to the cell culture plates and allowed to incubate at 37^{o}C in 5% CO_2 for 16 and 24 hours. Two hundred microliters of supernatant from each

sample was removed and stored at -80 ^{o}C for the completion of plaque assays. Fifty microliters per sample of supernatant was used in plaque assays.

Drug Induced Cell Cycle Arrest

Hydroxyurea, nocodazole and thymidine were obtained from Sigma-Aldrich. Hydroxyurea and thymidine were diluted in supplemented DMEM to final concentrations of 3mM and 2mM, respectively. Nocodazole was suspended in DMSO at a stock concentration of 50μg/ml. Stock nocodazole was diluted in supplemented DMEM to obtain a concentration of 60ng/ml.

Cells were plated 24 hours prior to treatment with drugs. Original media was removed from cell culture plated and replaced with supplemented DMEM containing respective drug treatments. After completion of drug treatment, drug containing media was removed from cell culture plates and the plates were washed once with 1x PBS prior to infection with VEEV. Infectious media was placed on cell culture plates at MOI of 1. The infected cell plates were then incubated for 1 hour at 37^{o}C in 5% CO_2. Infectious media was then removed and DMEM containing respective drug was re-added to the cell culture plates and allowed to incubate at 37^{o}C in 5% CO_2 for 16 and 24 hours. Two hundred microliters of supernatant from each sample was removed and stored at -80 ^{o}C for the completion of plaque assays. Fifty microliters per sample of supernatant is used in plaque assays.

Hydroxyurea was diluted in supplemented DMEM for a final concentration of 3mM. Cell culture plates were treated for 24 hours with the

3mM hydroxyurea in DMEM prior to infection with VEEV. Thymidine was diluted to a final concentration of 2mM. Cell culture plates were treated for 16 hours with 2mM thymidine in DMEM. The 2mM thymidine media was then removed, cell culture plates washed once with 1x PBS, then supplemented DMEM was placed on cell culture plates for 8 hours. After 8 hours, the supplemented DMEM was removed and cell culture plates washed once with 1x PBS. New DMEM containing 2mM thymidine media was then placed on the cell culture plates for 16 hour prior to infection with VEEV. Cell culture plates were treated for 24 hours with 60ng/ml of nocodazole in supplemented DMEM prior to infection with VEEV.

Cell Cycle Analysis

Cell cycle analysis was accomplished through propidium iodide (PI) staining for DNA content. Cells were trypsinized with 1x 0.25% Trypsin-EDTA (Gibco) and collected in a 1:1 ration of DMEM/PBS (1x). Cells were pelleted by centrifugation (2,000 RPM for 5 minutes at 4^{o}C) followed by washing 1x in PBS. Cells were fixed by addition of 1ml of ice cold 70% ethanol and allowed to set for at least 24 hours at 4^{o}C. Cells were then pelleted by centrifugation (5,000 RPM for 5 minutes at 4^{o}C) and resuspended in 1ml PI stain. The PI stain solution consist of 1x PBS, 25 ug/ml of PI (Sigma-Aldrich) and 100 ug/ml of RNAse A (Qiagen). Cells were allowed to incubate in the PI stain at 37^{o}C for 30 minutes in the dark. Microcentrifuge tubes were then transferred to ice and protected from the light until data was acquired on the Accuri C6 Flow Cytometer (BD

Biosciences). DNA histogram analysis was conducted using FCS Express 3 (De Novo Software) for determination of cell cycle populations.

Plasmid Construction

Two VEEV capsid expression plasmids, pcDNA3.1-Capsid-eGFP (termed C-eGFP) and a nuclear localization sequence (NLS) capsid mutant, pcDNA3.1-Capsid(K65A,K67A)-eGFP (C_{NLS}-eGFP), were constructed by PCR overlap extension. Two separate PCR reactions amplify the capsid coding region from the pTC83 plasmid (a gift from Dr. Elena Frolov, University of Alabama-Birmingham) and the eGFP coding region from pcDNA3.1-eGFP (gift from Dr. Cynthia de la Fuente). The capsid 5' forward primer contained the BamHI restriction enzyme, Kozak sequence, beginning of capsid coding region while VEEV capsid 3' reverse primer contained the a linker region and the beginning of the eGFP open reading frame (ORF). The primers used for the amplification of the VEEV capsid are [VEEV Capsid 5': CGG GCC CG GAT CCA CCG GTC GCC ACC ATG TTC CCG TTC CAG CC] and [VEEV Capsid 3': TCT CCG GAG TAT ACT T]. The primers used for the amplification of the eGFP contain the following sequence: overlap 3' end of the capsid ORF, linker sequence, beginning of the eGFP ORF while the reverse primer contained the 3' end of the eGFP OFR, a stop codon, and a NotI restriction enzyme (RE) site. The primers used for the amplification of the eGFP are [eGFP 5': GCT CAC CAT TCC CCC GCC ACC TTG CTC GCA GTT CTC CGG AGT ATA CTT] AND [eGFP 5': GAC TCG AGC GGC CGC TTT ACT TGT ACA GCT CGT CCA T]. Using the

Platinum PCR Supermix (Invitrogen) the amplification conditions for the reactions are: Step 1. Denature template at 94^{o}C for 4 minutes, Step 2. 30 repeat cycles: 94^{o}C for 20 seconds, 56^{o}C for 25 seconds, 71^{o}C for 2 minutes and 30 seconds, and Step 3. final extension at 71^{o}C for 10 minutes. PCR products were then separated on a 1% TAE agarose gel (containing 0.5ug/ml ethidium bromide). Bands containing the appropriate sized PCR products were then gel purified by MinElute Gel Extraction Kit (Qiagen). A final PCR reaction was done to create a large insert contain the capsid-eGFP using the capsid and eGFP PCR products from the first round of reactions as the template. PCR conditions for the reaction are: 1. 94^{o}C for 4 minutes, 2. 30 cycles of: 94^{o}C for 20 seconds, 56^{o}C for 25 seconds, 71^{o}C for 3 minutes, and 3. 71^{o}C for 15 minutes. The capsid-eGFP PCR product was then separated on a 1% TAE agarose gel (with 0.5ug/ml EtBr) and purified by MinElute Gel Extraction Kit. The capsid-eGFP insert and pcDNA3.1-eGFP (vector) were cut with BamH1 and NotI restriction enzymes (New England Biolabs) and separated on a 1% TAE agarose gel. The appropriate size bands were cut and purified by MinElute Gel Extraction Kit. Gel-purified pcDNA3.1 vector was shrimp alkaline phosphatase (SAP) treated with rAPid Alkaline Phosphatase (Roche). Ligation of pcDNA3.1 and capsid-eGFP was accomplished with Rapid DNA Dephos & Ligation Kit (Roche) according to manufacturer's directions, for the formation of pcDNA3.1-capsid-eGFP plasmid (referred now as to C-eGFP).

Competent DH5 alpha *Escherichia coli* were transformed with C-eGFP by heat shock method. In brief, C-eGFP plasmid was added to DH5a bacteria and allowed to incubate at 4^{o}C for 30mins. Bacteria were then heated to 42^{o}C for 60 seconds. LB broth without antibiotics was added to bacteria and allowed to incubate at 37^{o}C for 30 minutes. Bacteria were then plated on LB Agar (InvivoGen) petri dishes supplemented with 100ug/ml ampicillin overnight at 37^{o}C. Five colonies were selected from the petri dish and incubated in 10ml of LB broth and placed in an incubator shaker overnight at 37^{o}C at 250 rpm. Plasmids from individual bacterial cultures were extracted using PureLink Quick Plasmid Miniprep Kit (Invitrogen). Individual plasmids were digested with BsaA1 and separated on a 1% TAE agarose gel to confirm correct clone fragment sizes. Two clones with the correct digestion fragment size were the sequenced. The clone with the correct pcDNA3.1-Capsid-eGFP sequence was then prepared using the previously described DH5 transformation protocol, except for only one colony was selected for growth in 500ml of LB broth and plasmid was extracted using a Plasmid Maxi Kit (Qiagen).

The capsid NLS double mutation (K65A, K67A) was generated from the pcDNA3.1-Capsid-eGFP plasmid. The amplicon targets for the two sets of primers are: 1. T7 promoter, BamH1 site, Kozak sequence and capsid until just after mutation induction site using primers : [(1)VEEV Capsid K65A, K67A 5': TAA TAC GAC TCA CTA TAG G] [(1)VEEV Capsid K65A, K67A 3': GGC TGG ACT ACG ACA TAG TCT AGT CCG CCA AGA TG] and 2. Capsid prior

to the mutation induction site, linker region and part of 5'end of eGFP using primers: [(2) VEEV Capsid K65A, K67A 5': TCGC GCG AGG CCT CCT TTG CCG GAG CCT TAG CGG AT] [(2) VEEV Capsid K65A, K67A 3': TCT CCG GAG TAT ACT T]. Similar PCR conditions as before were used for the reactions and PCR products were then gel purified as before.

To generate the larger insert the purified products were then used as templates for the final PCR reaction using the (1) 5'K65A, K67A and the (2) 3' K65A, K67A primers to produce the $Capsid_{NLS}$-eGFP for insertion into pcDNA3.1-eGFP backbone. After gel purifying the PCR production the $Capsid_{NLS}$-eGFP insert and pcDNA3.1-eGFP were cut with AflII restriction enzymes (New England Biolabs) and separated on a 1% TAE agarose gel. The appropriate size bands were cut and purified by MinElute Gel Extraction Kit. Purified pcDNA3.1 vector gel product was SAP-treated with rAPid Alkaline Phosphatase (Roche). Ligation of pcDNA3.1 and $Capsid_{NLS}$-eGFP was accomplished with Rapid DNA Dephos & Ligation Kit (Roche) for the formation of pcDNA3.1- $Capsid_{NLS}$-eGFP plasmid (referred now as to C_{NLS}-eGFP). RE digestion with BsaA1 and sequencing were used to identify the correct clone.

Plasmid Transfection

Vero cells were plated in six wells plates at 3e5 cells/well in supplemented DMEM. Three micrograms of plasmid DNA was prepared using TransIT-LT1 Transfection Reagent (Mirus) in accordance with manufacturer's instructions. Two hundred fifty microliters of transfection reagent/DNA plasmid mixture was

placed on Vero cells. Cells were then allowed to cultivate at 37^{o}C in 5% CO_2 for 24 hours. DMEM was replaced with fresh supplemented DMEM after 24 and 48 hours. At 72 hours post transfections, cells were viewed and photographed for GFP expression on an EVOS digital inverted microscope (AMG).

Propidium Iodide Staining and measurement of GFP

Cells are trypsinized with 1x 0.25% Trypsin-EDTA (Gibco) and collected in a 1:1 ration of DMEM/PBS (1x). Cells are washed once by centrifugation (2,000 RPM for 5 minutes at 4^{o}C) in 1x PBS. One milliliter of ice cold 2% paraformaldehyde solution (in 1x PBS) was then added to cell microcentrifuge tubes and allowed to incubate at 4^{o}C for 10 minutes. Cells are washed once by centrifugation (5,000 RPM for 5 minutes at 4^{o}C) in 1x PBS. Cells are fixed by addition of 1ml of ice cold 70% ethanol and allowed to set for at least 24 hours at 4^{o}C. Cells are then pelleted by centrifugation (5,000 RPM for 5 minutes at 4^{o}C) and resuspended in 1ml PI stain. The PI stain solution consist of 1x PBS, 25 ug/ml of PI (Sigma-Aldrich) and 100 ug/ml of RNAse A (Qiagen). Cells are allowed to incubate in the PI stain at 37^{o}C for 30 minutes in the dark. Microcentrifuge tubes are then transferred to ice and protected from the light until data was acquired on the Accuri C6 Flow Cytometer (BD Biosciences) for GFP expression and DNA content. DNA histogram analysis is conducted using FCS Express 3 (De Novo Software) for determination of cell cycle populations.

CHAPTER THREE: RESULTS

VEEV Delays Return to Cell Cycle

To ascertain if VEEV TC-83 was delaying return to cell cycle during the course of infection, Vero cell lines were infected at MOI 1 and 10 (Figure 3). Vero cells were used to as they are very permissive to infection. Vero cells were synchronized in G1/G0 phase by FBS deprivation for 48 prior to infection. Two sets of time points in infection were established, early and late. Early time points are 4 and 8 hours post infection and late time points are 16 and 24 hours post infection. Cells were fixed and stained with PI at 4, 8, 16 and 24 hours post infection for cell cycle analysis using flow cytometry.

Vero cells were tolerant to FBS deprivation and synchronized at G0/G1 prior to infection (Figure 3). Mock infected cells took 16 hours to begin cycling again and 24 hours to return to a normal cell cycle distribution. Infected cells retained the same cell cycle profile until 24hpi, where an increase of 21% (MOI 1) and 8% (MOI 10) in S phase populations is seen over the 16hpi populations. The delay in return to a cell cycle is seen from comparing the 16hpi and 24hpi MOI 1 profiles. The 24hpi MOI 1 profile is similar in profile to the 16hpi mock profile indicating an 8 hour delay in return to cell cycle in the infected cells. The 24hpi MOI 10 cell populations are just beginning to show a change in

distribution, possibly indicating that the high MOI used is further delaying the return to cycling.

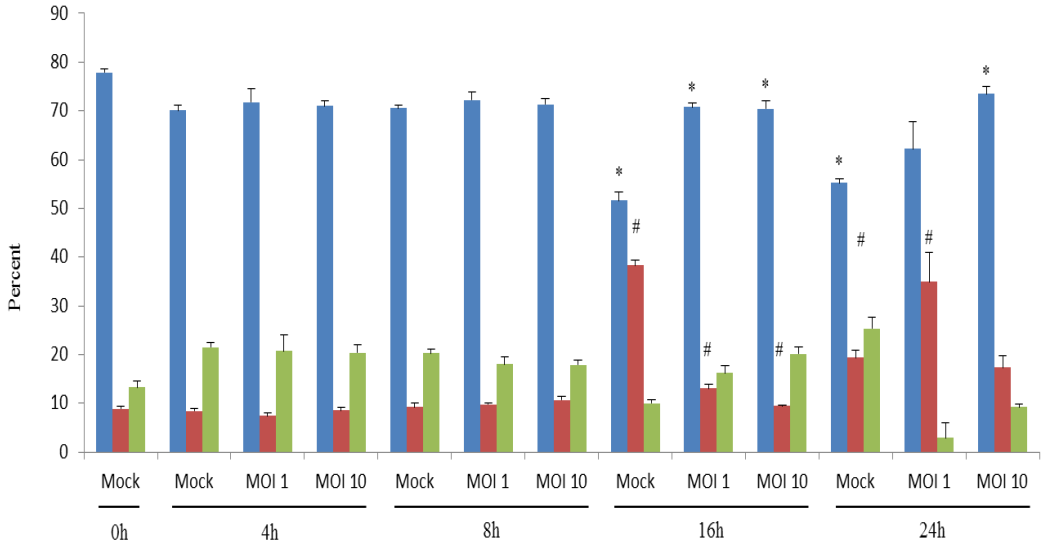

Figure 3: VEEV delays return to cell cycle in infected cells. Vero cells were serum-starved for 48 hours prior to infection. Vero cells were infected with VEEV (MOI 1 and 10). Following infection, cells were released into full media (10% FBS). Cells were collected at 4, 8, 16 and 24 hours post-infection. P-values were calculated for mock populations versus MOI 1 and MOI 10 populations independently. Significant p-values for G1 populations are denoted by *($p < 0.001$). Significant p-values for S phase populations are denoted by # ($p<0.001$) Propidium Iodide staining was used to analyze cell cycle on an Accuri C6 flow cytometer using BD FCS Express software from BD Biosciences.

Capsid's ability to induce CPE is well documented for VEEV infection and a time point analysis for determining optimal experimental conditions needed to be established. The cell cycle profiles for serum starved Vero cells indicated that maximal cell cycle alteration was occurring at 16 and 24hpi (Figure 3). A cell viability assay was conducted for infected Vero cells at MOIs 1 and 10 at 16 and 24hpi to identify if CPE is playing a role in the decrease in levels of G2/M

cell populations seen in the cell cycle analysis (Figure 4). Cell viability was not significantly different between the MOIs at both time points. A significant decrease in viability was seen between mock populations and infected populations at both time points and MOIs. At 16hpi, there was an approximately 22% decrease in viability at both MOIs from mock ($p<0.001$ for MOI 1 and $p=0.0014$ for MOI 10). At 24hpi, there was a 35% decrease in viability at both MOIs from mock ($p<0.001$ for both MOIs). The low populations of infected G2/M populations in cell cycle analysis data coupled with cell viability data indicates that cells were either dying prior to progressing to G2/M.

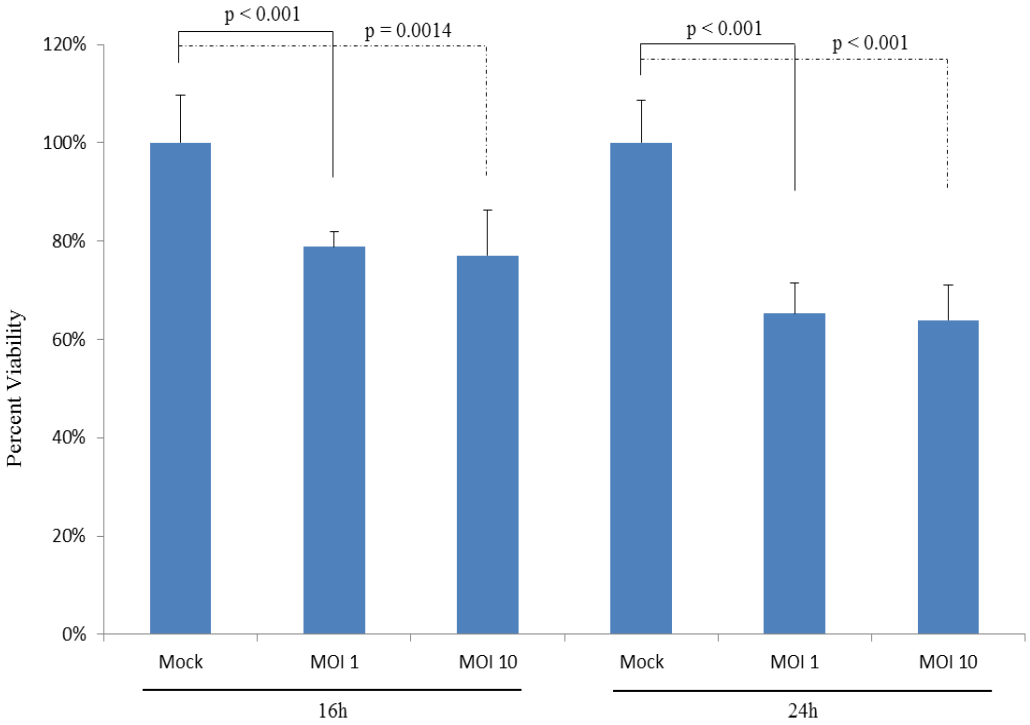

Figure 4: VEEV induces CPE in Vero cells. Serum starved Vero cells were mock infected or infected at MOIs 1 and 10 then released into full media following infection. Cell viability was assessed following VEEV infection using CellTiter-Glo Luminescent Cell Viability Assay per manufactures instructions at 16 and 24 hours post infection.

VEEV Induces Downregulation of G1 and S Phase Cell Cycle Proteins

The multifactorial cascade of the cell cycle covers many proteins, gene expression and signal cascades whose even minor alteration could lead to cell cycle arrest. As VEEV is delaying the cell cycle we investigated next the effect of infection on cell cycle checkpoint proteins responsible for the progression through the cell cycle by western blot. Levels of Cdks are held in constant levels throughout all phases of the cell cycle and it is assumed that production of cyclin indicates the activation of their respective Cdk. Levels of Cyclin D1, E2 and A2 were measured to see if infected cells were expressing differential levels.

Western blot analysis of serum starved Vero cells showed VEEV induced alteration of cell cycle by changing the dynamics of cell cycle protein expression (Figure 5). Cyclin D1 protein levels in infected cells were consistent in mock and infected samples at 4 and 8 hour time points (lanes 1-7). A decrease of Cyclin D1 in mock samples at 16 hours (lane 8) indicates that cells were returning to a cycling profile, but levels of Cyclin D1 in infected cells remained at similar levels to their earlier infection time points (compare lane 8 with 9 and 10). This is in concurrence with the cell cycle analysis data in Figure 3 of a delayed return to cell cycle of infected cells. By 24hpi, levels of Cyclin D1 begin to decrease in infected cells indicating that infected cells are progressing from early G1 to late G1 (lane 12 and 13). The expression of Cyclin E2 was unchanged at early time points in both mock and infected samples. At later time points, both Cyclin E2

expression in and Cyclin A2 expression was decreased in infected cells (lanes 9, 10, 12 and 13) as compared to mock cells (lanes 8 and 11). The lack of Cyclin A2 expression in infected cells indicates that a majority of infected cells were not progressing into S phase by 16 and 24hpi (lanes 9, 10, 12 and 13). The lack of expression of p-Histone H3 further confirms that few infected cells were in G2/M phase (lanes 9, 10, 12 and 13).

A delay in return to cell cycle during the course of infection is reinforced by the downregualtion of expression of pRb(Ser780) at late timepoints (lanes 9, 10, 12 and 13). The downregulation of pRb, represses the release of E2F and in turn does not allow progression past the G1/S transition point. Protein levels for Cyclin D1, Cyclin E2, Cyclin A2 and pRb were quantified by densitometry for 16 and 24 hour time points (Figure 5B). The downregulation of the production of Cyclin E2, Cyclin A2 and pRb were confirmed as was varying levels of Cyclin D1 in infected cells (Figure 5B). The cell cycle repressor p21, shows very little variation during the course of infection (lanes 8-13). The p21 protein may not be a factor in the regulation of cell cycle in Vero cells lines in VEEV infection as the levels were generally not changed during the course of infection. Collectively, these results demonstrate that VEEV is inducing a delay in production of G1/S phase proteins, possibly by the production of large amounts of capsid protein.

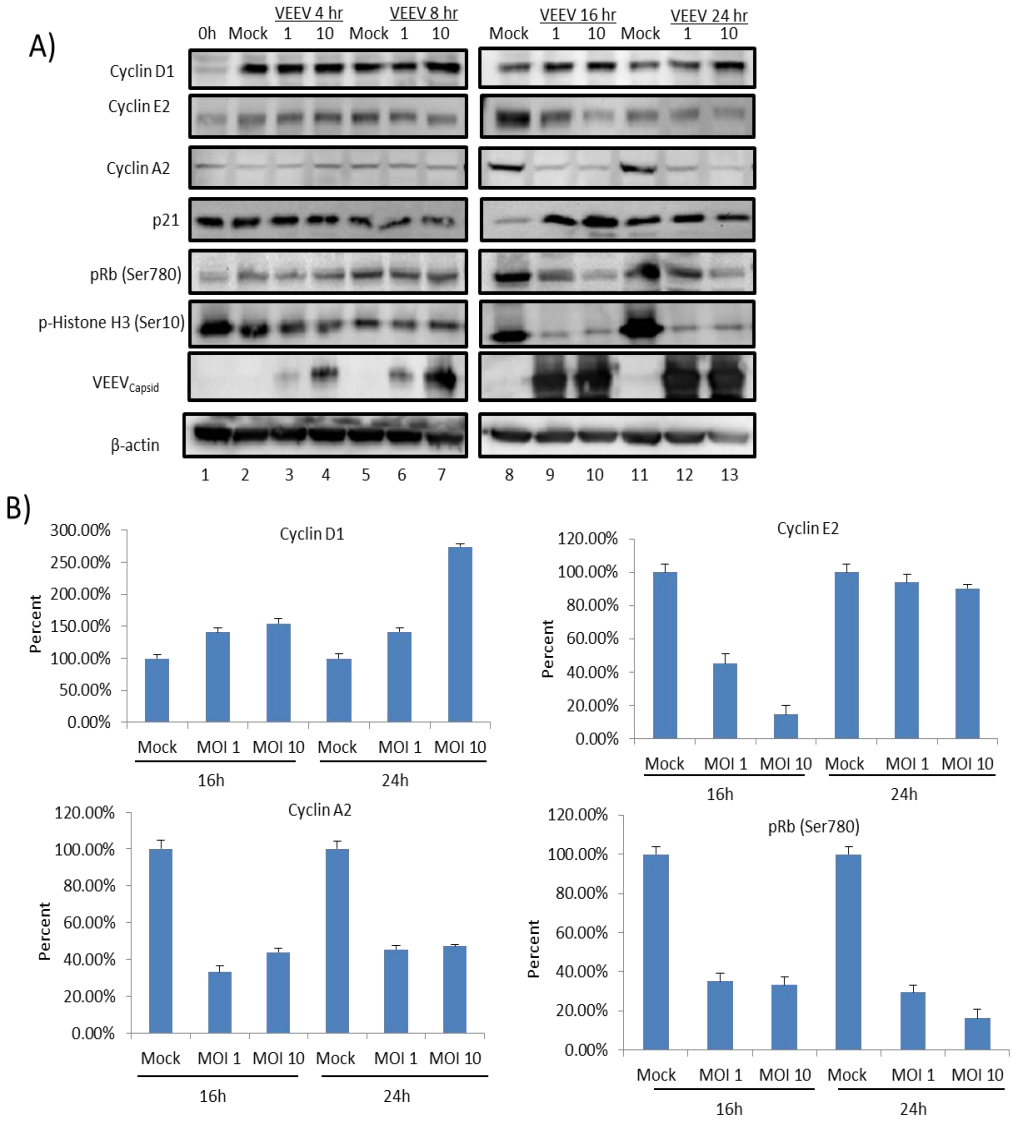

Figure 5: Downregulation of G1/S phase checkpoint proteins following VEEV infection. (A) Serum starved Vero cells were mock infected or infected at an MOIs of 1.0 and 10 with VEEV, and then collected at 4, 8, 16 and 24h postinfection. Whole cell lysates were separated by gel electrophoresis and examined using antibodies against Cyclin D1, Cyclin E2, Cyclin A2, p21, pRb and p-histone H3. VEEV capsid protein is also shown. β-actin is used as a loading control. (B) The bar graphs represent protein levels at 16 and 24hpi by densitometry. Each time point is compared as a percentage to their respective mock sample. Samples were normalized to loading control.

UV Inactivated VEEV Does Not Induce Alterations in the Cell Cycle

UV inactivated VEEV was used to address if the delay in return to cell cycle would be induced only by the presence of replicating virus. Serum starved Vero cells were infected with UV inactivated VEEV or replication competent VEEV and cell cycle profiles were assessed at 16 and 24hpi. Vero cells infected with UV inactivated VEEV had similar profiles to mock cells (Figure 6). Replication competent VEEV infected cells showed a delayed return to cell cycle when compared to mock and UV inactivated samples (Figure 6). The lack of a delay in cell cycle in the UV inactivated VEEV infected cells indicates that live, replicating VEEV is needed to induce specific events in Vero cells and is not a non-specific response to the presence of viral proteins.

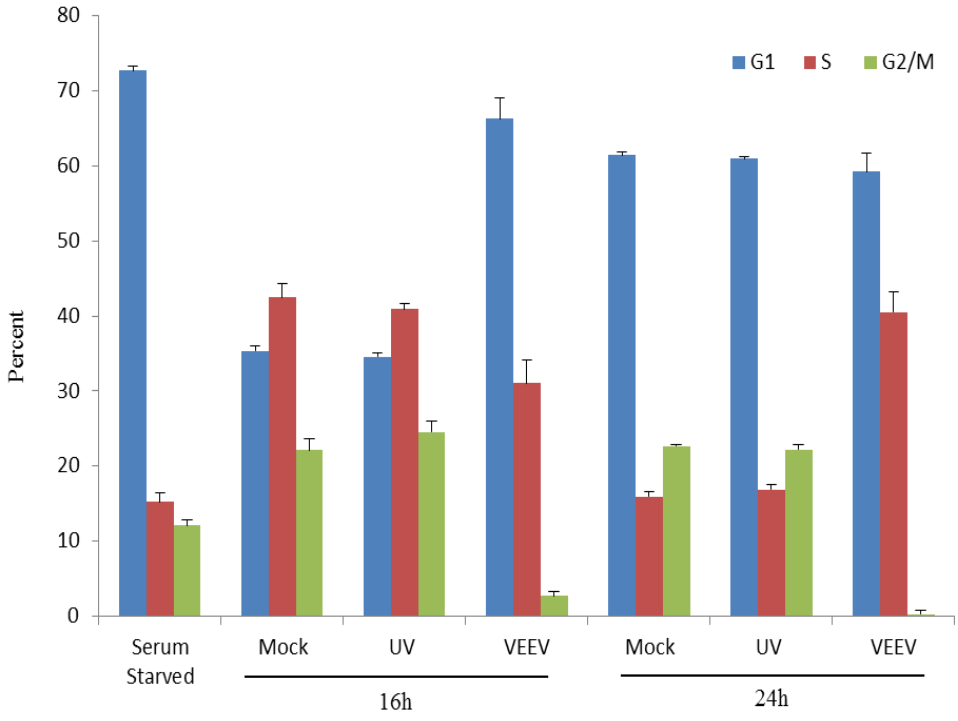

Figure 6: UV inactivated VEEV does not induce S-phase arrest in Vero cells. Serum starved Vero cells were infected at MOI 1 with either UV inactivated VEEV or VEEV. Following infection, cells were released into full media (10% FBS). Cells were collected at 16 and 24 hours post-infection. Propidium iodide staining was used to analyze cell cycle on an Accuri C6 flow cytometer using BD Accuri C6 software from BD Biosciences. Absence of viral plaques in plaque assays confirmed UV inactivation of VEEV (not shown).

G1/G0 Arrested Cells Reduce Viral Titers

VEEV infection caused a delay of serum starved Vero cells to return to a normal cell cycling distribution (Figure 3). We wanted to investigate if the virally induced delay was advantageous for VEEV replication. Vero cells were infected with VEEV (MOI 1) that were either held in G1/G0 by FBS depravation for the course of infection (SSS), FBS deprived for 48 hour then released into full media after infection (SSR) or an asynchronous population (ASYN) (Figure 7). Viral

titers were then assessed by plaque assay for the 16 and 24hpi. At 16hpi, there was not a noticeable difference in viral titers in all three populations, but by 24hpi, there was a significant decrease ($p=0.0128$) in infected cells held in G1/G0 (SSS) over the serum starved and released cells (SSR) (Figure 7). This implies that VEEV requires actively replicating cells to achieve maximal viral titers.

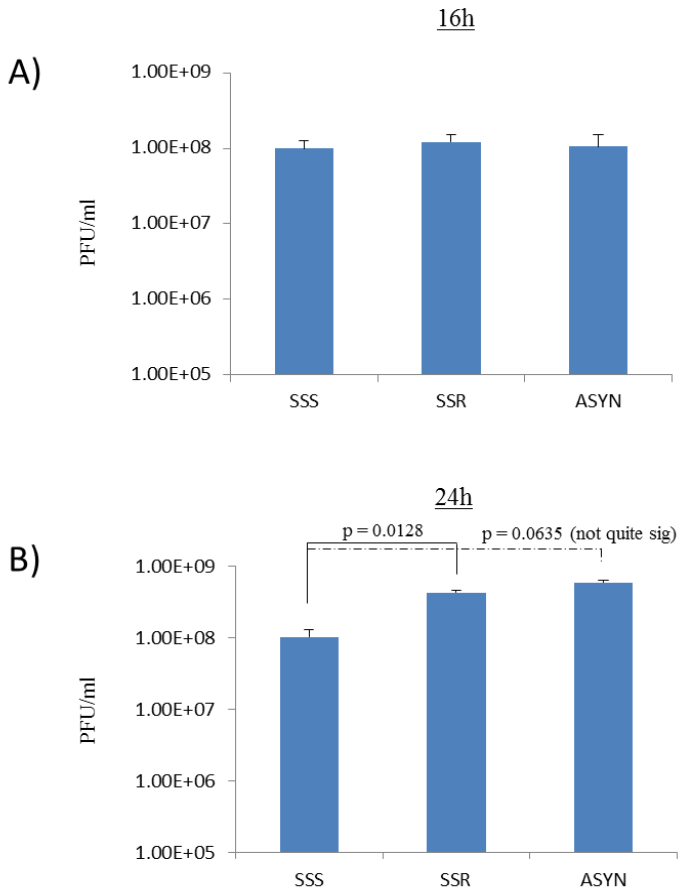

Figure 7: G1/G0 arrested cells reduce viral titer. Vero cells were serum starved for 48 hours prior to infection or maintained in media containing 10% FBS. Cells were then infected at MOI 1 with VEEV. After infection, previously serum starved cells were released into FBS deficient media (SSS) or media containing 10% FBS (SSR). Asynchronous (ASYN) cells were cultivated in media containing 10% FBS for duration of infection. Viral supernatants were collected at 16 and 24h postinfection. The viral supernatants were used to perform plaque assays and average viral titers are shown (pfu/ml) for (A) 16h supernatants and (B) 24h supernatants. Significant values are shown

Inhibition of Cdks Reduces VEEV Replication

In order to further investigate if VEEV required actively replicating cells to achieve maximum viral titers, Vero cells were chemically arrested in G1, late G1/early S or G2/M by inhibition of Cdk4/6, Cdk2 or Cdk1, respectively. Cdk

inhibitor concentrations were selected based on a literature search and cell viability assays were performed to verify that they were non-toxic (data not shown). Based on these experiments, a working concentration of 10μM was chosen for these studies. Cells were pretreated for one hour prior to infection with Cdk inhibitor and treatment continued after infection with inhibitor. Cells were collected at 16 and 24hpi for analysis by FACS and MOI 1 supernatants were used to assess viral titer by plaque assay.

Cdk4/6 Inhibitor Reduces Viral Titers

Cell cycle analysis of VEEV infected Vero cells treated with a selective Cdk4/6 inhibitor (Calbiochem 219492) was conducted to determine if holding cells in G1would influence viral replication. The G1 population of infected treated cells remains constant across both time points at both MOIs and in mock cells, indicating a successful inhibition of Cdk4/6 (Figure 8A). There is a larger S phase population of infected inhibitor treated cells over mock at 16 hpi at both MOIs with an accompanying increase in G2/M population of infected inhibitor treated cells at 24hpi (Figure 8A). The increase in G2/M population suggests that cells in S phase at 16hpi were still cycling and continued to cycle into G2/M by 24hpi, but then unable to progress into G1 phase due to inhibition of Cdk4/6. Infected DMSO treated cells show higher S phase populations than inhibitor treated cells at both time points and MOIs. Plaque assays showed a significant decrease in viral titers at 16hpi ($p=0.0061$) and a noticeable decrease at 24hpi

(Figure 8B). The inhibition of Cdk4/6 in unsynchronized Vero cells reduces viral titers significantly at 16hpi and not significantly at 24hpi.

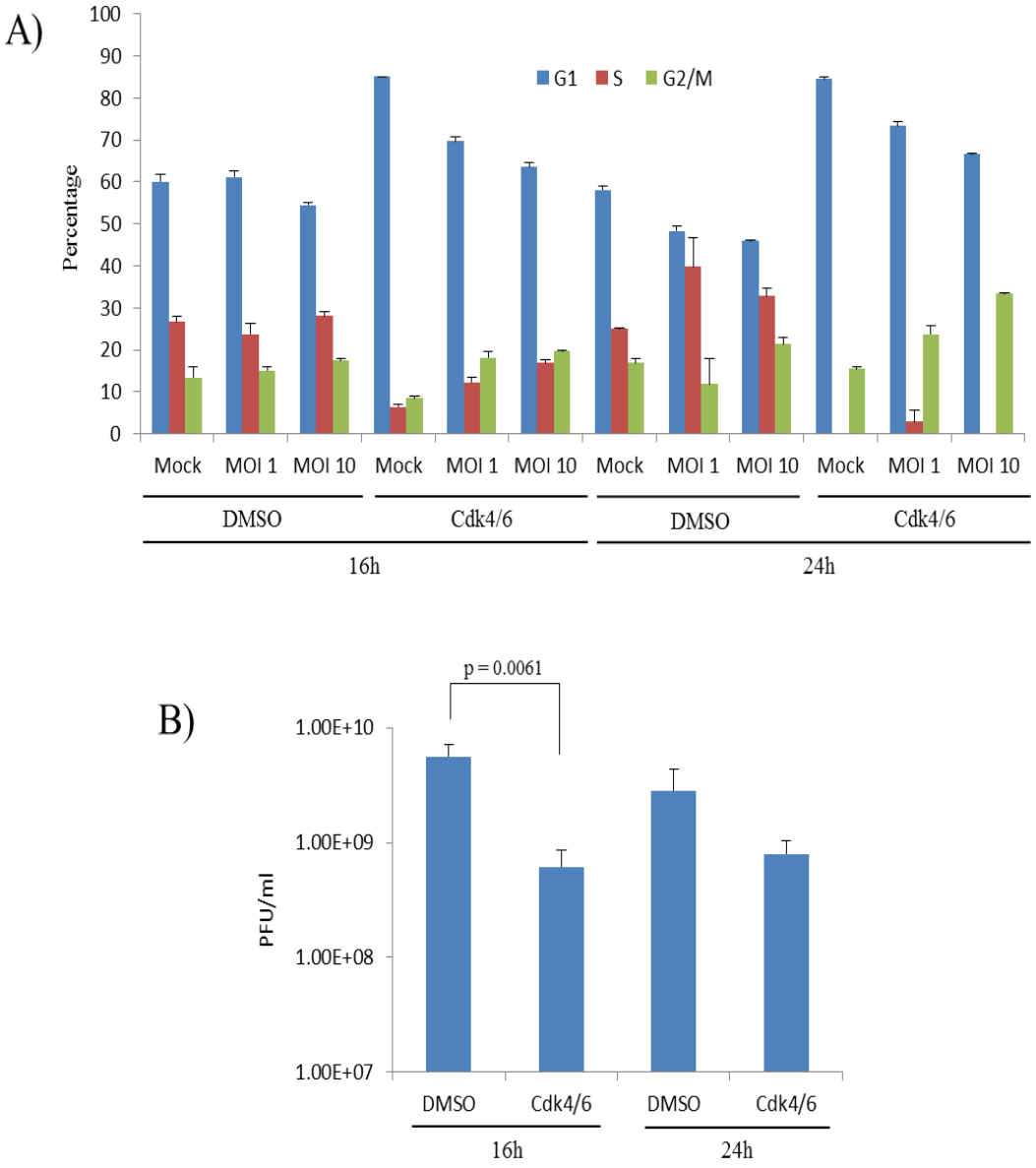

Figure 8: Cdk 4/6 inhibitor effect on viral replication. (A) Vero cells were pretreated with DMSO or 10uM Cdk 4/6 inhibitor (Calbiochem 219492) for one hour prior to infection with VEEV at MOIs 1 and 10. After infection cells were released into full media containing DMSO or Cdk4/6 inhibitor. At 16 and 24 hours post infection viral supernatants were collected and cells were PI stained for cell cycle analysis using flow cytometery (Accuri C6). (B) The MOI 1 supernatants were used to perform plaque assays and average viral titers (n=3) are shown (pfu/ml) with significant values.

Cdk2 Inhibitor Reduces Viral Titers

The selective Cdk2 (Calbiochem 238803) was used to block Vero cells in late G1/S transition point to assess if inhibition at this point would alter viral kinetics during this phase. FACs analysis show similar cell cycle profiles in both infected DMSO treated and inhibitor treated cells at both MOIs at both time points (Figure 9A). Successful synchronization of Cdk2 inhibitor treated cells was not accomplished until the 24 hour time point, possibly due to asynchronous cells being used. The asynchronous cells that were in S and G2/M phase would take 12-24 hours to return to the late G1/early S phase, thereby not giving a true inhibition of Cdk2 during infection. At 24hpi, there is a decrease in S phase populations in infected cells from 16hpi without an accompanying increase in G2/M possibly due to CPE between the 16 and 24 hour time points. Plaque assays show a significant decrease ($p=0.006$) in viral titers at 16hpi, but similar viral titers at 24hpi (Figure 9B). Since the Cdk2 inhibitor is targeting both Cyclin E2 and A2, the effect of inhibiting Cdk2 may have off target effects to effectively arresting cells in G1/S transition. The plaque assay data is inconclusive due to the multiple targets and the inability to synchronize the cells early enough during infection.

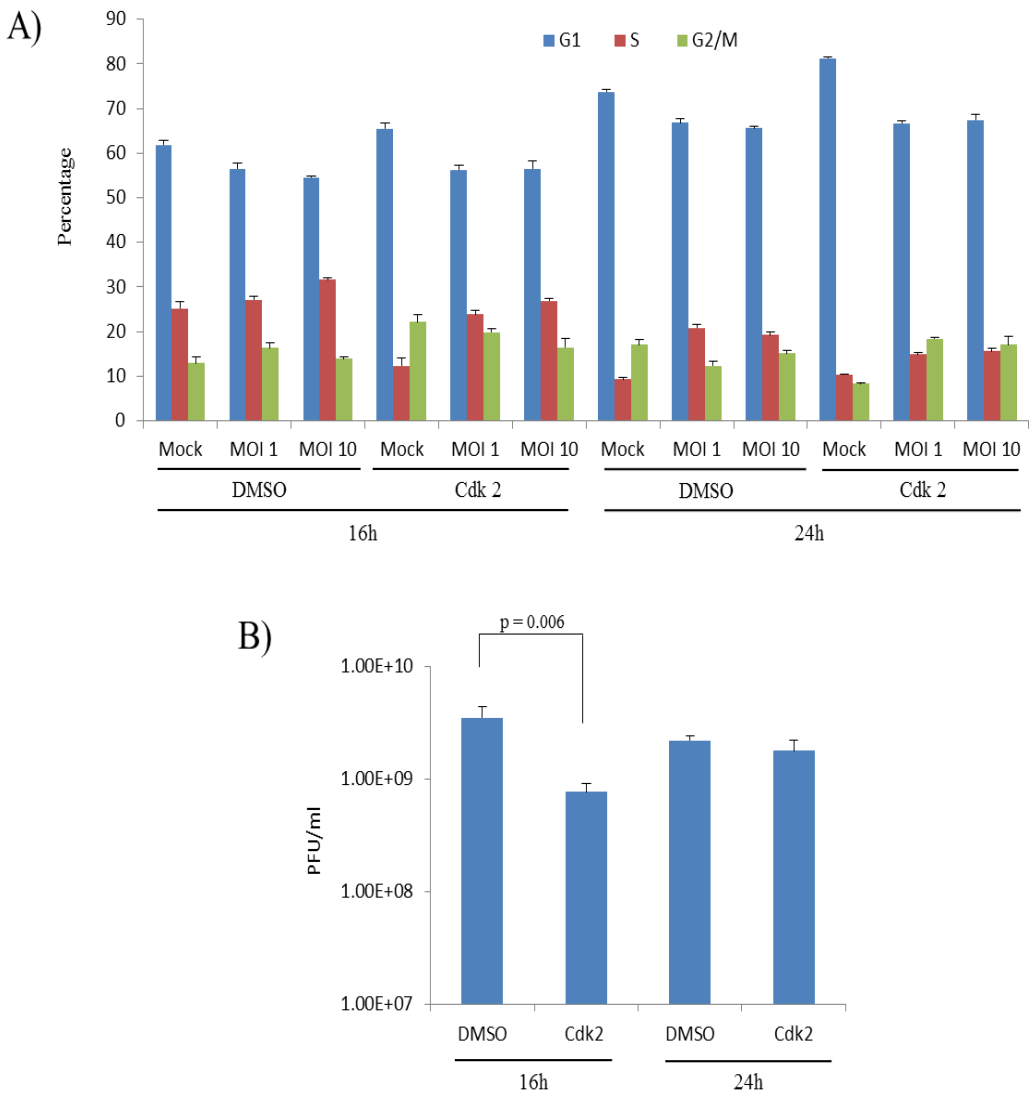

Figure 9: Cdk 2 inhibitor effect on viral replication. (A) Vero cells were pretreated with DMSO or Cdk2 inhibitor (Calbiochem 238803) for one hour prior to infection with VEEV at MOIs 1 and 10. After infection cells were released into full media containing DMSO or Cdk2 inhibitor. At 16 and 24 hours post infection viral supernatants were collected and cells were PI stained for cell cycle analysis using flow cytometery (Accuri C6). (B) The MOI 1 supernatants were used to perform plaque assays and average viral titers (n=3) are shown (pfu/ml) with significant values.

Cdk1 Inhibitor Reduces Viral Titers

Vero cells were arrested in G2/M by treatment with a selective Cdk1 inhibitor (Calbiochem 217695) and infected with VEEV to assess cell cycle profile and viral titers. Inhibitor treated cells have majority G2/M populations at both time points and MOIs in infected cells (Figure 10A). The S phase populations are equal between infected DMSO treated and inhibitor treated cells at both MOIs and at both time point (Figure 10A). As infection progresses from 16 to 24hpi, the population of G1 cells increases in inhibitor treated populations, implying that the G2/M block is being overcome by VEEV to progress the infected cell into G1 (Figure 10A). Plaque assays show significant decreases in viral titers at 16hpi ($p=0.0226$) and at 24hpi ($p<0.001$) indicating that high populations of G2/M cells are not conducive to viral replication kinetics (Figure 10B).

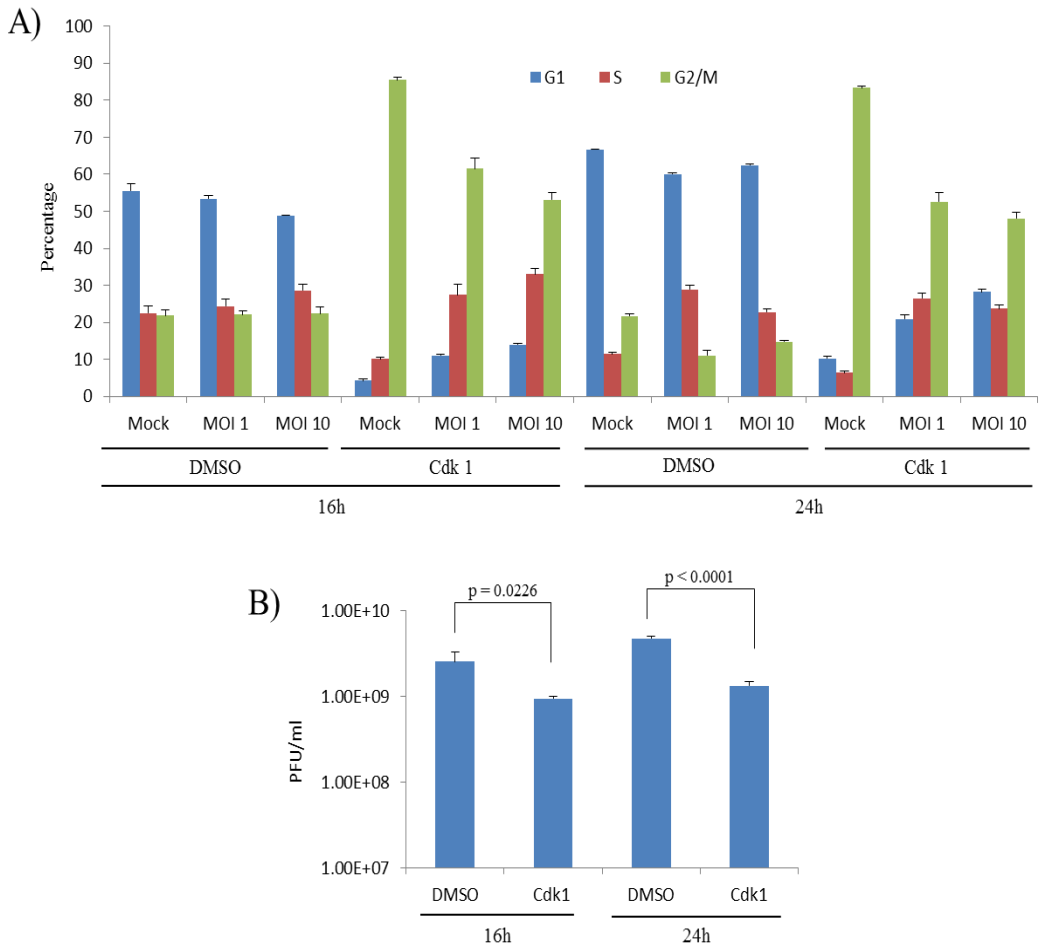

Figure 10: Cdk 1 inhibitor effect on viral replication. (A) Vero cells were pretreated with DMSO or 10uM Cdk1 inhibitor (Calbiochem 217695) for one hour prior to infection with VEEV at MOIs 1 and 10. After infection cells were released into full media containing DMSO or Cdk1 inhibitor. At 16 and 24 hours post infection viral supernatants were collected and cells were PI stained for cell cycle analysis using flow cytometery (Accuri C6). (B) The MOI 1 supernatants were used to perform plaque assays and average viral titers (n=3) are shown (pfu/ml) with significant values.

Drug Induced Cell Cycle Arrest Impedes Viral Replication Kinetics

VEEV is influencing the regulation of cyclins in serum starved Vero cells and the inhibition of the catalytic Cdk subunits decreased viral replication. Next, we wanted to take another approach to assess the importance of cell cycle phase on VEEV replication. Vero cells were arrested in late G1/early S phase by treatment with hydroxyurea (HU), in S phase with a double thymidine (TYD) block treatment or in G2/M by treatment with nocodazole (NOCO). After infection, cells were fixed and stained with PI for FACS analysis and viral supernatants were used to assess viral titers.

Hydroxyurea Reduces Viral Titers

Vero cells were treated with 3mM HU for 24 hours prior to infection at MOI 1 and collected for cell cycle analysis at 16 and 24hpi (Figure 11A). Cell cycle analysis show a 10% higher S phase population in infected HU treated cells over mock infected cells and a total absence of G2/M populations (Figure 11A). By 24hpi, a 20% decrease in S phase cells is seen in infected HU treated cells from mock infected cells (Figure 11A). Viral replication was assessed by plaque assays at both time points with a significant reduction in viral titers at 16hpi ($p=0.007$) and at 24hpi ($p=0.005$) (Figure 11B). This gives further reinforcement that VEEV requires actively replicating cells for maximum virion production.

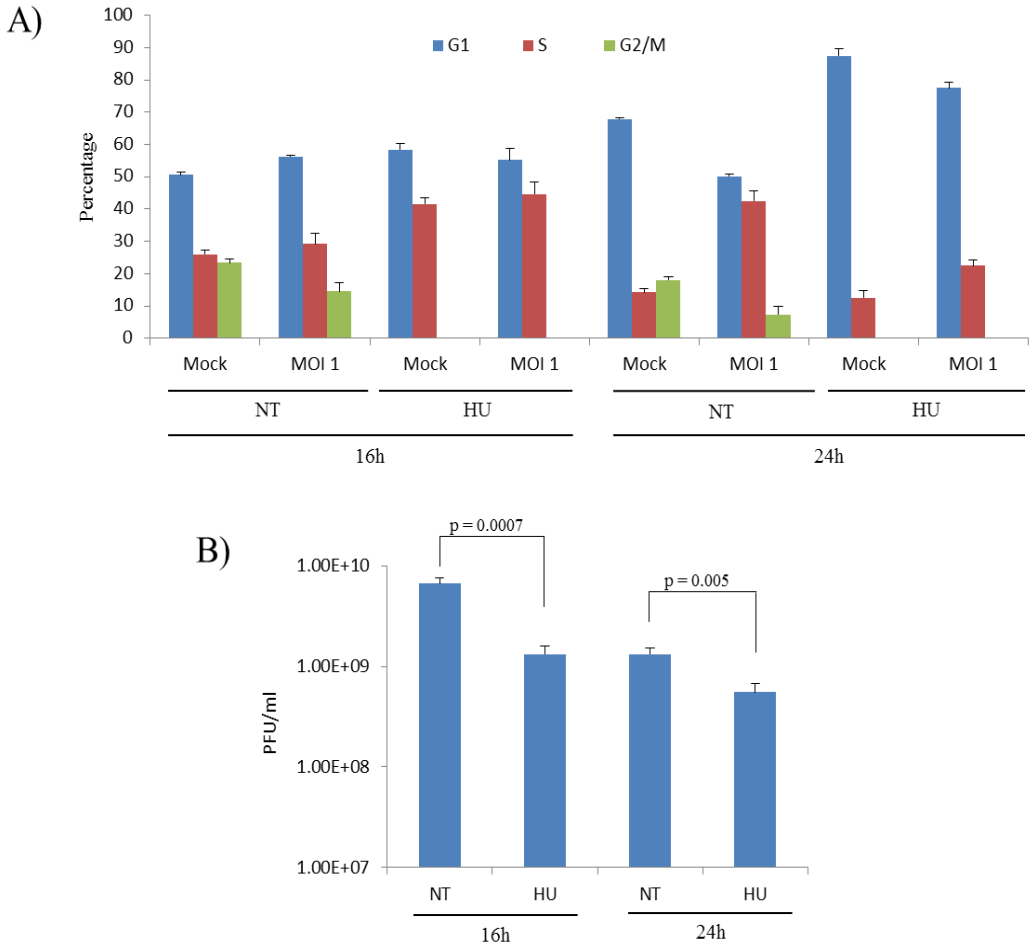

Figure 11: Drug induced G1 cell cycle arrest affects viral replication kinetics. (A) Cell cycle profiles of cells arrested in G1 phase using HU (3mM). Following 24 hours of drug treatment, cells were infected at MOI 1 and then incubated in DMEM containing HU for 24 hours. At 16 & 24 hours post infection viral supernatants were collected and cells were PI stained for cell cycle analysis using flow cytometery. (B) The MOI 1 supernatants were used to perform plaque assays and average viral titers (n=3) are shown (PFU/ml) with significant values.

Nocodazole Reduces Viral Titers

To reassess the impact of G2/M arrested cells on VEEV viral replication, Vero cells were treated with nocodazole (60ng/ml) for 24 hours prior to infection with VEEV (Figure 12A). Cells were successfully arrested in G2/M at 16hpi, with over 80% of infected NOCO treated cells being arrested in G2/M (Figure 12A). By 24hpi, cell cycle profiles for NOCO treated cells were beginning to return to a normal distribution possibly due to the exhaustion of NOCO in the media (Figure 12A). Viral replication was assessed by plaque assay and show a significant decrease ($p=0.0003$) in viral replication at 16hpi in NOCO treated samples but no decrease at 24hpi in NOCO treated samples (Figure 12B). The absence in reduction in viral replication at 24hpi may be attributed to the NOCO being exhausted by this time point in the media as indicated by the cell cycle analysis.

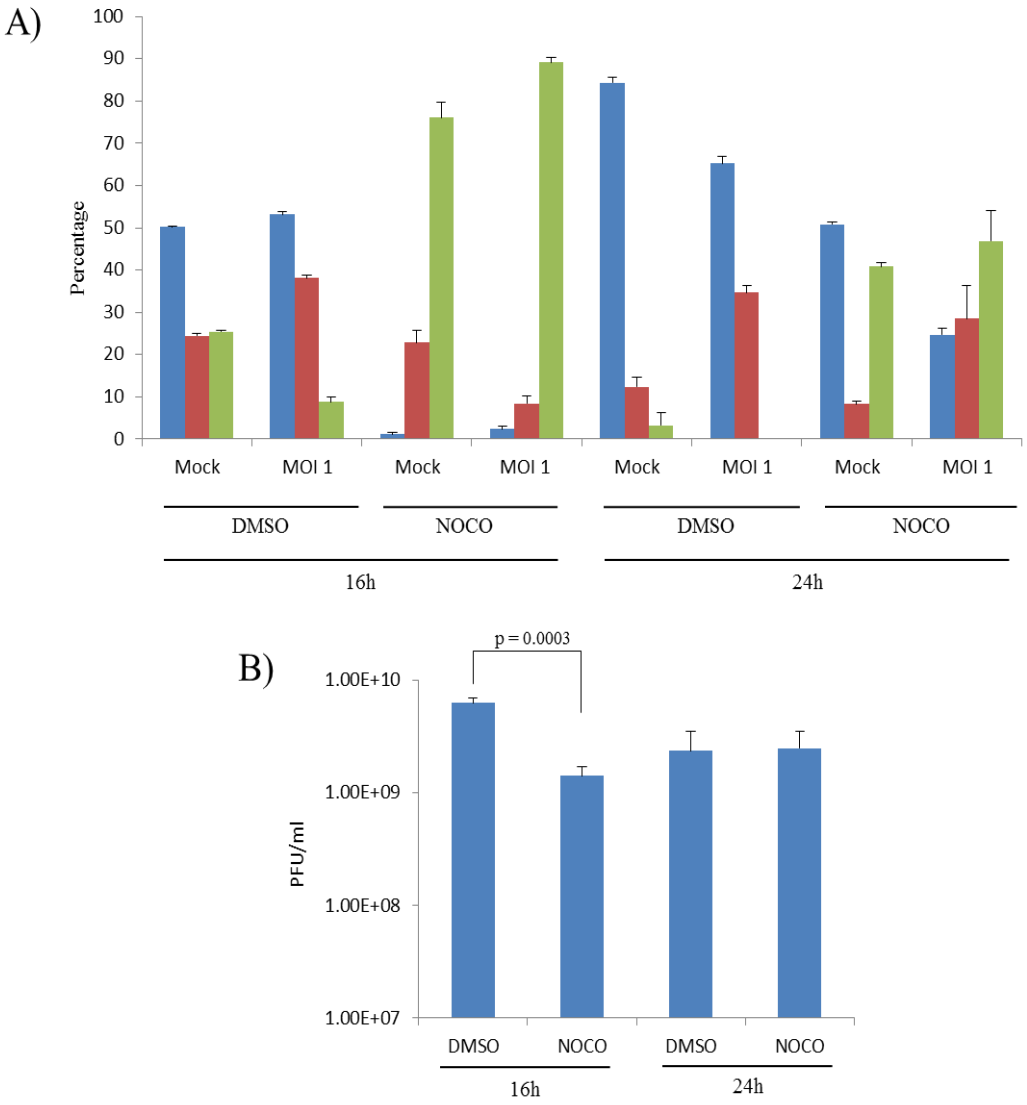

Figure 12: Drug induced G2/M cell cycle arrest affects viral replication kinetics. (A) Cell cycle profiles of cells arrested G2/M phase using NOCO (60ng/ml). Following 24 hours of drug treatment, cells were infected at MOI 1 and then incubated in DMEM containing NOCO drug for 24 hours. At 16 & 24 hours post infection viral supernatants were collected and cells were PI stained for cell cycle analysis using flow cytometery. (B) The MOI 1 supernatants were used to perform plaque assays and average viral titers (n=3) are shown (PFU/ml) with significant values.

Thymidine Block Reduces Viral Titers

Excess TYD in cell culture inhibits DNA synthesis and arrest cells in late G1/S transition (Galgano and Schildkraut, 2006). Cells were treated with a 2mM TYD double block (See M&M) and then infected with VEEV for 16 or 24 hours (Figure 13A). At 16hpi, a majority of TYD treated cells were in S phase compared to non-treated (NT) cells. Infected cells show a 10% higher S phase population than NT infected cells at 16hpi (Figure 13A). By 24hpi, almost all infected TYD treated cells were in S phase as compared to only 35% of infected NT cells (Figure 13A). Plaque assays were conducted to confirm if the double TYD block had any effect on viral replication (Figure 13B). At 16hpi, a significant reduction of viral titers was observed ($p=0.005$) in TYD treated cells versus NT cells and no significant difference in viral titers observed at 24hpi (Figure 13B).

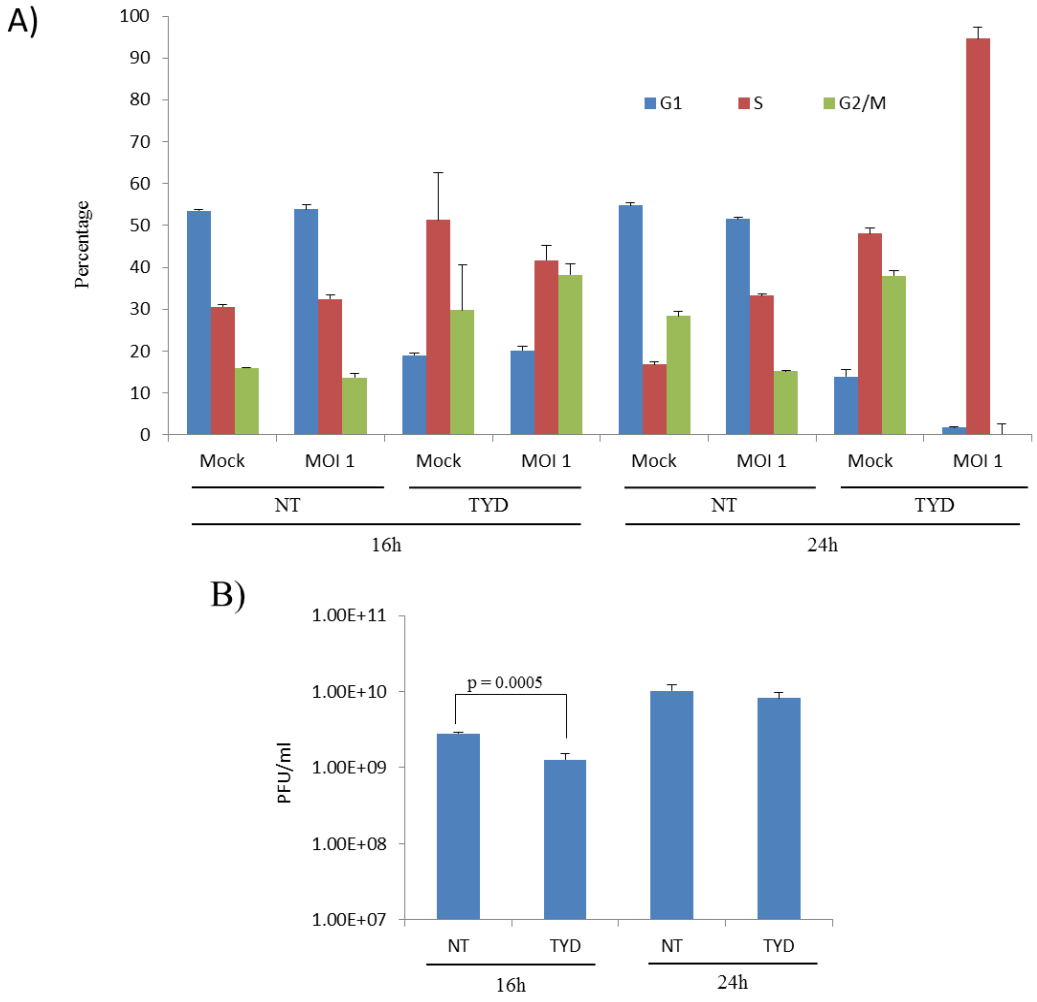

Figure 13: Induced S phase arrest affects viral replication kinetics. (A) Cell cycle profiles of cells arrested in S phase by double TYD (2mM) block. Following treatment, cells were infected with VEEV at MOI 1 and then incubated in TYD until 16 or 24 hpi. At 16 & 24 hours post infection viral supernatants were collected and cells were PI stained for cell cycle analysis using flow cytometery. (B) The MOI 1 supernatants were used to perform plaque assays and average viral titers (n=3) are shown (pfu/ml) with significant values.

VEEV Capsid NLS Mutant Does Not Localize in the Nucleus of Transfected Vero Cells

The third function of the VEEV capsid protein is to inhibit cellular transcription and induce CPE which is why we believe it is inducing the delayed return to cell cycle. We wanted to address this by constitutive expression of the VEEV capsid protein and VEEV NLS mutant capsid protein in transfected cells. A plasmid containing the VEEV capsid protein linked to a GFP reporter was constructed and a plasmid containing VEEV capsid protein with mutations in the NLS (K65A,K67A). The hypothesis is that the non-mutated capsid protein will induce the observed delayed cell cycle through inhibition of cellular transcription but the NLS mutated capsid protein will be unable to localize in the nucleus of the transfected cells and unable to induce cell cycle alteration.

Vero cells were transfected with 3ug of plasmid containing either VEEV capsid or VEEV NLS mutant capsid for 72 hours. Cells were then viewed for GFP expression and photographed (Figure 14). Cells expressing the VEEV capsid can be seen expressing GPF in both the cytoplasm and in the nucleus (Figure 14A). Cell expressing the NLS mutant capsid primarily have expression in the cytoplasm, giving indication that NLS mutation was effective (Figure 14A). Magnification of Vero cells expressing NLS mutation are shown at 40x magnification in Figure 14B.

Cell cycle analysis was unable to be performed on capsid expressing transfected Vero cells due to the high amount of CPE. This CPE was induced by the non-release of capsid protein from the cells due to lack of VEEV components

necessary for virion formation. A possible solution is to make the NLS mutation in the full VEEV TC-83 virus genome for comparison to WT TC-83 in viral infections.

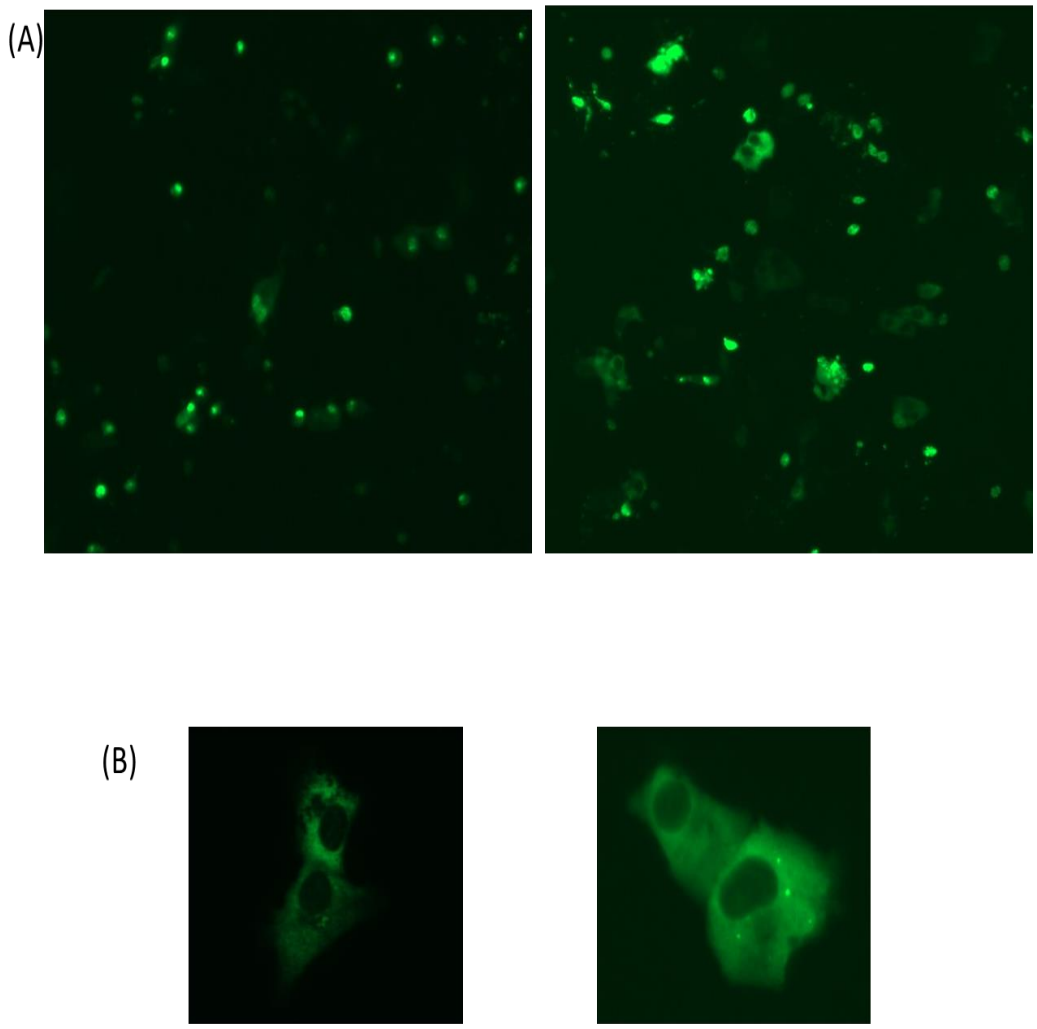

Figure 14: Capsid NLS Mutant Localizes in Cytoplasm of Transfected Vero Cells (A) VEEV-capsid-eGFP (left) localizes in the cytoplasm and nucleus. NLS mutant (right) localizes in cytoplasm. (B) VEEV-capsid-eGFP transfected cells show localization of capsid protein in cytoplasm.

CHAPTER FOUR: DISCUSSION

The purpose of this thesis was to ascertain if the VEEV TC-83 strain was altering the cell cycle of infected cells and if that alteration was impacting cell cycle protein production. We have shown that VEEV is inducing a delayed return to cell cycle by approximately 8 hours in serum starved Vero cells. This delay is seen in western blot analysis by the absence in production of Cyclin A2. Cyclin A2 is a key cell cycle protein indicative of S phase within a replicating cells. The levels of Cyclin A2 do not change over the course of infection and knowing that approximately 35% of infected cells have died by this time point, it can be assumed that infected cells are not progressing pass late G1/early S phase due to CPE induced by the virus. CPE could be induced by the capsid protein's ability to block transcription of S phase genes and subsequent cellular apoptotic programing could induce cell death because of the failure to progress in the cell cycle.

The levels of Ser780 phosphorylated Rb are decreased as well in infected cells over the mock. The mock western blot data at the 16 hour time point shows high expression of pRb(Ser780) and cell cycle analysis show a high percentage of S phase cells. High amounts of pRb(Ser780) correlate with release of E2F allowing transcription of S phase genes, including the production of Cyclin A2,

allowing the mock cells to progress in the cell cycle. The lower pRb(Ser780) at 16 and 24hpi in infected cells further reinforces the lack of production of Cyclin A2 and low population of S phase infected cells. The 16hpi MOI 1 cell populations show a higher level of pRb(Ser780), and this does correlate with a higher S phase population seen in cell cycle analysis, but does not explain the low level of Cyclin A2. Should the course of infection be allowed to continue pass 24hours, there could be an increase of Cyclin A2 in the MOI 1 cells.

The high concentration of capsid protein in the western blot indicates that the cells are saturated with capsid protein at the 16 and 24 hour time points. Since capsid is both localized in the cytoplasm and nucleus and blocks the nuclear pore, it is hard to disregard the possibility that capsid is interfering with the trafficking of the Cyclin D1, E2 and A2 in and out of the nucleus. Cyclin D1 and E2 are needed to phosphorylate Rb and if they are unable to be imported into the nucleus, Rb phosphorylation cannot occur. This would explain the low level of Rb phosphorylation at 16 and 24hpi and that capsid is the main antagonist of the event. Future studies on the cellular localization of the cyclins during the course of infection could provide insight if the localization of the cyclins is being influenced by VEEV infection or if they are not able to enter and exit the nucleus.

The inability to produce higher titers of VEEV in cells arrested in a specific phase of cell cycle proved to be disappointing, but intriguing. Arresting cells by any means interrupts other cell cycle processes that may not be related to cell cycle. Normal processes, such as respiration, metabolism, translation and

transcription are impacted as well during treatments to induce cell cycle arrest. VEEV's use of other cellular pathways has not been studied in detail and this could be the reason lower titers were produced. The hydroxyurea treatment studies showed significant decreases in viral titers at both 16 and 24hpi. Hydroxyurea inhibits the cellular enzyme ribonucleotide reductase, responsible for production of deoxyribonucleotides from ribonucleotides. The interference of this enzyme could induce a negative feedback loop in the production of ribonucleotides with an endstate of a depleted ribonucleotide pool. RNA viruses depend on the host cell's pool of ribonucleotides in order to replicate genomic RNA. The kinetics of viral replication may be slowed by hydroxyurea due to the inability to produce enough viral genomic RNA and both ORF mRNA by the lack of available or exhaustion of the ribonucleotide building block.

We showed in our study that mutation of the NLS (K65A,K67A) abolished import of capsid into the nucleus of transfected cells. The long time needed to establish sufficient expression of the linked GPF was 72 hours, which proved to cause the build-up of high amounts of capsid with the transfected cells. High CPE was seen in transfected cells due to the inability to shed the constitutively expressed capsid protein as it would have in the course of infection with VEEV. Mutation of the VEEV NLS sequence in would allow study of the effect that the cellular localization of capsid protein has on the alteration of cell cycle and if a persistent infection is induced.

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BIOGRAPHY

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