Elucidating the Underlying Mechanisms Polyamines Play on Coxsackievirus B3 Infection

Mason Richard Firpo
Loyola University of Chicago Graduate School

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LOYOLA UNIVERSITY CHICAGO

ELUCIDATING THE UNDERLYING MECHANISMS POLYAMINES PLAY ON COXSACKIEVIRUS B3 INFECTION

A DISSERTATION SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

PROGRAM IN MICROBIOLOGY AND IMMUNOLOGY

BY

MASON R. FIRPO
CHICAGO, ILLINOIS
AUGUST 2024
ACKNOWLEDGMENTS

I would first like to acknowledge my wonderful mentor Dr. Bryan Mounce. He took me on as his first Ph.D. student even though I had limited laboratory experience. He always entertained my out-of-the-box ideas, no matter how unlikely they were, and pushed me to pursue what I found most interesting that month. He greatly fostered my scientific development, making my out-of-the-box ideas a little more plausible. He was always excited to talk about interesting data and discuss how it impacted the on-going projects in the lab. His mentorship has made me into the independent scientist I am today and I will always remember my time in his lab fondly.

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<td>ACBD3</td>
<td>Acyl-CoA-binding domain-containing protein 3</td>
</tr>
<tr>
<td>AdoMet</td>
<td>Adenosylmethionine</td>
</tr>
<tr>
<td>AMD1</td>
<td>S-Adenosylmethionine Decarboxylase</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP activated kinase</td>
</tr>
<tr>
<td>AZIn</td>
<td>Antizyme inhibitor</td>
</tr>
<tr>
<td>Bchol</td>
<td>BODIPY-cholesterol</td>
</tr>
<tr>
<td>CAR</td>
<td>Coxsackievirus and adenovirus receptor</td>
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<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
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<td>CHIKV</td>
<td>Chikungunya Virus</td>
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<td>CoA</td>
<td>Coenzyme A</td>
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<td>CVB3</td>
<td>Coxsackievirus B3</td>
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<tr>
<td>DAF</td>
<td>Decay-accelerating factor</td>
</tr>
<tr>
<td>dcAdoMet</td>
<td>Decarboxylated adenosylmethionine</td>
</tr>
<tr>
<td>DCM</td>
<td>Dilated Cardiomyopathy</td>
</tr>
<tr>
<td>DENSpm</td>
<td>Diethylnorspermidine</td>
</tr>
<tr>
<td>DENV</td>
<td>Dengue virus</td>
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<tr>
<td>DFMO</td>
<td>Difluoromethylornithine</td>
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<tr>
<td>DHPS</td>
<td>Deoxyhypusine synthase</td>
</tr>
<tr>
<td>DOHH</td>
<td>Deoxyhypusine hydroxylase</td>
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<tr>
<td>dsDNA</td>
<td>Double stranded DNA</td>
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<td>EBOV</td>
<td>Ebola virus</td>
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<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
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<tr>
<td>eIF5A</td>
<td>Eukaryotic initiation factor 5A</td>
</tr>
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<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>EV-71</td>
<td>Enterovirus-71</td>
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<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>GC7</td>
<td>N1-guanyl-1,7-diamineheptane</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>GSEA</td>
<td>Gene set enrichment analysis</td>
</tr>
<tr>
<td>GuHCL</td>
<td>Guanidine HCl</td>
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<tr>
<td>HCMV</td>
<td>Human cytomegalovirus</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C Virus</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
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<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
</tr>
<tr>
<td>HMG</td>
<td>3-hydroxy-3-methylglutaryl</td>
</tr>
<tr>
<td>HMGCR</td>
<td>HMG-CoA reductase</td>
</tr>
<tr>
<td>HMGCS</td>
<td>HMG-CoA synthase</td>
</tr>
<tr>
<td>hpi</td>
<td>Hours post infection</td>
</tr>
<tr>
<td>HSV-1</td>
<td>Herpesvirus-1</td>
</tr>
<tr>
<td>HRV16</td>
<td>Human Rhinovirus 16</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IFNAR</td>
<td>IFN alpha/beta receptor</td>
</tr>
<tr>
<td>Insig-1</td>
<td>Insulin-induced gene protein-1</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal ribosome entry site</td>
</tr>
<tr>
<td>ISG</td>
<td>Interferon stimulated gene</td>
</tr>
<tr>
<td>Kir</td>
<td>Inwardly-rectifying potassium channel</td>
</tr>
<tr>
<td>LACV</td>
<td>Lacrosse Virus</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>LDLR</td>
<td>Low-density lipoprotein receptor</td>
</tr>
<tr>
<td>Log2FC</td>
<td>Log2 Fold Change</td>
</tr>
<tr>
<td>MARV</td>
<td>Marburgvirus</td>
</tr>
<tr>
<td>MCS</td>
<td>Membrane contact sites</td>
</tr>
<tr>
<td>MHV</td>
<td>Murine hepatitis virus</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>MVD</td>
<td>Mevalonate diphosphate decarboxylase</td>
</tr>
<tr>
<td>NBCS</td>
<td>Newborn calf serum</td>
</tr>
<tr>
<td>NT</td>
<td>No treatment</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>OAZ1</td>
<td>ODC-1 antizyme</td>
</tr>
<tr>
<td>ODC1</td>
<td>Ornithine decarboxylase</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>OSBP</td>
<td>Oxysterol-binding protein</td>
</tr>
<tr>
<td>PAOX</td>
<td>Polyamine oxidase</td>
</tr>
<tr>
<td>PFU</td>
<td>Plaque forming unit</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PI4Kβ</td>
<td>Phosphatidylinositol 4-phosphate beta</td>
</tr>
<tr>
<td>PI4P</td>
<td>Phosphatidylinositol-4-phosphate</td>
</tr>
<tr>
<td>PLP</td>
<td>Pyridoxal 5’-phosphate</td>
</tr>
<tr>
<td>PTM</td>
<td>Post-translation modification</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RO</td>
<td>Replication organelle</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Real-time quantitative PCR</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>S1P</td>
<td>Site-1 protease</td>
</tr>
<tr>
<td>S2P</td>
<td>Site-2 protease</td>
</tr>
<tr>
<td>SAT1</td>
<td>Spermidine/spermine acetyltransferase</td>
</tr>
<tr>
<td>SCAP</td>
<td>SREBP cleavage-activating protein</td>
</tr>
<tr>
<td>SMOX</td>
<td>Spermine oxidase</td>
</tr>
<tr>
<td>SMS</td>
<td>Spermine synthase</td>
</tr>
<tr>
<td>SRE</td>
<td>Sterol regulatory element</td>
</tr>
<tr>
<td>SREBP2</td>
<td>Sterol regulatory element binding protein 2</td>
</tr>
<tr>
<td>SSD</td>
<td>Sterol-sensing domain</td>
</tr>
<tr>
<td>ssRNA</td>
<td>Single-stranded RNA</td>
</tr>
<tr>
<td>SRM</td>
<td>Spermidine synthase</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin-layer chromatography</td>
</tr>
<tr>
<td>uORF</td>
<td>Upstream open reading frame</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>VACV</td>
<td>Vaccinia Virus</td>
</tr>
<tr>
<td>VP</td>
<td>Viral protein</td>
</tr>
<tr>
<td>ZIKV</td>
<td>Zika virus</td>
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CHAPTER 1
STATEMENT OF PROBLEM

Non-polio enteroviruses cause 10-15 million infections per year with children being the most at risk for serious illness. These viruses are transmitted through the fecal-oral route and can cause a wide range of diseases including gastrointestinal (GI) distress, hand, foot, and mouth disease, aseptic meningitis, flaccid paralysis, pancreatitis, and viral myocarditis. Coxsackievirus B3 (CVB3) is a model virus for non-polio enteroviruses that can infect the GI tract, including the liver and pancreas, but it is most well-known for its ability to infect the heart (viral myocarditis).

CVB3 is a non-enveloped, positive sense, single-stranded RNA (ssRNA) virus within the Picornaviridae family and is transmitted through the fecal-oral route in which contaminated water or food is consumed. Upon consumption, CVB3 first infects the intestine where it can rapidly disseminate, in about 20 minutes, to nearby organs such as the liver and pancreas. From here, CVB3 can infect cardiac fibroblasts and myocytes; causing extensive necrosis and apoptosis during the acute phase of infection. After about 18 days post-infection, infectious virus cannot be isolated from murine hearts; however, viral RNA is still present, and the impact this has on disease is unknown. CVB3 infection of the heart is a major cause of dilated cardiomyopathy (DCM), causing about 50% of cases, with males being most susceptible to CVB3-induced DCM. Despite this, there are currently no FDA-approved drugs for CVB3 infection and the only way to treat DCM is a heart transplant.
Cholesterol is an important metabolite required by CVB3 at multiple stages of its replication cycle. To gain access to a cell, CVB3 first binds to decay-accelerating factor (DAF)\textsuperscript{6}. This causes the formation of a cholesterol-dependent lipid raft that is necessary for CVB3 to be transported to Coxsackie and adenovirus receptor (CAR), where the virus can then be endocytosed\textsuperscript{7}. Cholesterol is also required for CVB3 endocytosis\textsuperscript{8}. Upon entry and release of its genome, CVB3 forms replication organelles (ROs) where RNA replication occurs. Cholesterol is then shuttled to ROs by host-cell proteins that are recruited by viral proteins\textsuperscript{9,10}. Cholesterol was found to be important for the formation of ROs and the processing of CVB3’s polyprotein\textsuperscript{11,12}. In vivo work comparing male mice to female mice found that macrophages from the male mice had higher expression of cholesterol synthesis genes, potentially leading to the increased susceptibility of male mice to CVB3 infection and hypercholesterolemic mice have been shown to be more susceptible to Coxsackievirus infection\textsuperscript{13,14}.

Another important metabolite found to be important for CVB3 during binding and replication is a class of metabolites called polyamines. Polyamines are small, positively charged, carbon chains required for many aspects of eukaryotic cell function ranging from RNA/DNA stability, protein synthesis, and protein function. Like cells, viruses require polyamines for a productive infection and use them in a variety of ways. CVB3 requires polyamines for attachment and entry to cells as well as protease function\textsuperscript{15–17}. However, the mechanism behind how polyamines influence CVB3 infection has yet to be fully uncovered.

Despite there being no previously-described metabolic relationship between polyamine synthesis and cholesterol synthesis, mice with increased polyamine
catabolism exhibit decreased serum cholesterol levels and rats treated with a polyamine synthesis inhibitor show a similar phenotype\textsuperscript{18,19}. However, the mechanism behind how polyamines influence cholesterol synthesis remained unknown. Collectively, these knowledge gaps lead to the central hypothesis of this dissertation: polyamines’ underlying influence on cholesterol synthesis impacts CVB3 at multiple stages of infection. The goal of this dissertation is to determine the mechanisms behind how polyamines influence cholesterol synthesis, how this polyamine-cholesterol axis influences CVB3 binding and entry, and how the underlying mechanism impacts CVB3 replication (Fig. 1). The hypothesis was tested via the following three aims:

**Aim 1: Determine the Mechanism Behind Polyamines’ Influence on Cholesterol Synthesis**

Polyamines play a variety of roles within the cell, any of which could lead to a decrease in serum cholesterol previously seen\textsuperscript{18,19}. To first confirm that cholesterol concentration changed within hepatocytes in vitro, cholesterol abundance was measured intracellularly in cells depleted of polyamines and found to be significantly reduced compared to untreated cells\textsuperscript{20}. Due to the diverse roles polyamines play, my working hypothesis is that polyamines globally affect transcription of cholesterol synthesis genes through a specific mechanism called hypusination. To determine if cholesterol synthesis gene transcription was down, cells depleted of polyamines were
analyzed by RNA-sequencing, and the results were confirmed via real-time quantitative PCR (RT-qPCR) and western blot. Next, the role polyamines played on the global cholesterol synthesis gene transcription factor was explored via luciferase promoter assay, western blot, and RT-qPCR.

**Aim 2: Determine if the Polyamine-Cholesterol Axis Affects CVB3 Binding**

Polyamines are important for multiple viruses’ ability to bind and enter cells. We had previously found CVB3 had a reduced ability to bind and enter cells depleted of polyamines\(^\text{16}\). Furthermore, cholesterol is necessary for CVB3 entry and cells depleted of cholesterol had reduced CVB3 endocytosis\(^\text{8}\). To rescue binding, polyamines need to be added to cells before infecting with CVB3. Based on this observation and the role polyamines play on cholesterol synthesis, *my working hypothesis is that polyamines indirectly aid in CVB3 binding through their role in supporting intracellular cholesterol*. Polyamines were first added to polyamine depleted cells at various times to confirm that they could rescue CVB3 binding. To determine if cholesterol could rescue CVB3 binding, cells depleted of polyamines were treated with cholesterol and CVB3 binding and attachment was then measured via plaque assay and RT-qPCR. A CVB3 mutant that does not require polyamines for binding and entry was tested for its ability to infect and bind cells with sequestered cholesterol. Finally, CVB3 binding was measured in cells that lacked hypusinated eukaryotic initiation factor 5A (eIF5A), the modification found to be important for translation of the cholesterol synthesis gene transcription factor in Aim 1\(^\text{20}\).
Aim 3: Resolve how eIF5A-hypusine Impacts CVB3 Infection After Entry

Hypusination is a modification that only occurs to eIF5A, in which the polyamine spermidine is ligated to eIF5A to form eIF5A-hypusine. eIF5A-hypusine is required for the translation of hard-to-read transcripts, most notably di-proline motifs. In cells lacking hypusinated eIF5A, the ribosomes stall on transcripts and fail to translate whole protein. Multiple viruses have been found to require eIF5A-hypusine, including HIV, Ebola virus, and coronavirus. However, the role eIF5A-hypusine has in picornavirus and CVB3 infection is unknown. Inhibiting hypusination results in reduced CVB3 binding and cells treated with a hypusination inhibitor up to 2 hours after infection have decreased titers. CVB3’s polyprotein also contains eight different di-proline motifs in the Nancy strain of the virus, potentially requiring eIF5A-hypusine for its translation. My working hypothesis is that CVB3 requires eIF5A-hypusine for its translation and the formation of replication organelles through cholesterol. To establish the sensitivity of CVB3 to hypusination inhibition and the general stage of infection that is impacted, cells were treated with a hypusination inhibitor at different doses and times followed by infection with CVB3. Viral titers were then measured via plaque assay. To determine if CVB3 requires eIF5A-hypusine for the translation of its polyprotein, CVB3 expressing green fluorescent protein (GFP) was used to infect cells and GFP accumulation was measured at different times during infection via live-cell imaging. Furthermore, to determine if CVB3 was able to overcome hypusination inhibition, CVB3 was passaged in cells treated with a hypusination inhibitor and then tested for resistance via plaque assay. To determine if eIF5A-hypusine is important for RO formation, cells treated with
a hypusination inhibitor were infected with CVB3 and ROs were imaged using transmission electron microscopy (TEM).

These studies investigate a novel role that polyamines have in intracellular cholesterol synthesis and how it relates to the cardiotropic enterovirus CVB3. This work identifies polyamines’ role in translation, through a process called hypusination, as responsible for the decrease in cellular cholesterol. Furthermore, addition of cholesterol rescues CVB3 binding to polyamine depleted cells. Curiously, eIF5A-hypusine is not required for CVB3 translation, but instead formation of ROs through eIF5A-hypusine’s importance for cholesterol synthesis. Additionally, CVB3 is unable to become fully resistant to hypusination inhibition making hypusination a potentially viable antiviral target.
CHAPTER 2

LITERATURE REVIEW

*Parts of this chapter are reprinted with modifications from Firpo et al. 2020*\(^{26}\)

**Section 1: Polyamines Within the Cell**

**Polyamine Metabolism and Regulation**

Polyamines are small, abundant, flexible, biomolecules that consist of carbon chains containing amino groups that give them a positive charge at cellular pH. Eukaryotes have three polyamines that are synthesized in a stepwise process from arginine or can be transported into the cell from the environment in an ATP dependent manner\(^{27,28}\). Arginine is converted to ornithine, which is decarboxylated via ornithine decarboxylase 1 (ODC\(_1\)) to the first polyamine, putrescine. Putrescine is then converted to spermidine via spermidine synthase (SRM), which can then be further processed to spermine via spermine synthase (SMS). In order to convert putrescine to spermidine and spermidine to spermine, adenosylmethionine (AdoMet) is decarboxylated via S-adenosylmethionine decarboxylase (AMD1) into decarboxylated AdoMet (dcAdoMet)\(^{29}\). dcAdoMet is used as the aminopropyl donor by SRM and SMS. Both ODC1 and AMD1 are rate-limiting enzymes and are tightly controlled by the cell. Spermine and spermidine can be catabolized back to putrescine by addition of an acetyl group by spermidine/spermine acetyltransferase (SAT1) followed by polyamine oxidase (PAOX). Acetylated spermidine and spermine can also be exported out of the cell. Spermine can
also be catabolized back to spermidine via spermine oxidase (SMOX). The overview of this pathway can be seen in Figure 2.

**ODC1**. ODC1 is a decarboxylase that requires pyridoxal 5'-phosphate (PLP), a derivative of vitamin B6, as a cofactor and functions as a dimer. ODC1 binds to PLP through K69 forming an internal Schiff base. ODC1 then uses PLP's aldehyde group to form an external Schiff-base structure with ornithine’s amino group. From here, the electrophilic Schiff base nitrogen acts as a sink for the free electron from the carboxylate group of ornithine. This releases CO₂ and a proton is donated by C360 of the other ODC1 monomer to the Schiff base nitrogen, forming putrescine. This then causes the Schiff base nitrogen of PLP to displace putrescine, causing putrescine to
release, and be covalently linked to ODC1’s K69 through transamination to finish the catalytic cycle.

ODC1 is tightly controlled through degradation and has a rapid turnover rate of about 11 minutes, which can be accelerated by high levels of polyamines. Interestingly, ODC1 is degraded by the proteasome in a ubiquitin-independent mechanism. ODC-1 antizyme (OAZ1) binds to ODC1, inactivating it and targeting it to the proteasome. OAZ1 synthesis is regulated by polyamines through a frameshifting mechanism. OAZ1 contains two overlapping open reading frames (ORF), ORF1 and ORF2. ORF2 translation requires a +1 frameshift at ORF1’s last codon, and ORF1 contains a premature stop codon and pseudoknot 3’ of the shift site. Higher concentrations of polyamines are hypothesized to interact with a 5’ element about 20 nucleotides from the premature stop codon in ORF1 to stimulate the frameshift and allow for complete translation of OAZ1. OAZ1 also inhibits polyamines by decreasing their transport, but the mechanism remains unknown.

OAZ1 itself is inhibited by antizyme inhibitor (AZIn). AZIn is structurally very similar to ODC1 but lacks catalytic activity and is monomeric. AZIn has a higher affinity to OAZ1 compared to ODC1, allowing it to sequester OAZ1 and prevent ODC1 degradation. AZIn is expression is regulated by various growth factors and polyamines have been shown to affect AZIn transcription and splicing.

ODC1 expression can be upregulated by a variety of stimuli. These include hormones, tumor promoters, and growth factors. Most notably, ODC1 expression is upregulated by the oncogene, c-myc. It has also been shown that the Ras oncogene also targets ODC1 by altering mRNA stability. ODC1 is translationally regulated as
well. Its long 5’ untranslated region (UTR) contains an internal ribosome entry site (IRES) which allows for cap-independent translation, but can also undergo cap-dependent translation\(^4^9\). Ras, which is regulated by the MEK/Erk and phosphatidylinositol 3-kinase pathway, promotes the cap-dependent translation of ODC1\(^5^0\).

**AMD1.** AMD1 is a homodimer that decarboxylates AdoMet. Its decarboxylation activity differs from that of ODC1 through its cofactor. AMD1 is part of a limited class of decarboxylases that use covalently bound pyruvate as the prosthetic group to form dcAdoMet\(^5^1,5^2\). This pyruvate is formed through an autoprotease reaction with an internal serine residue and the AMD1 proenzyme, splitting AMD1 into α and β subunits with the pyruvate on the N-terminus of the α chain\(^2^9,5^3\). Despite AMD1 not requiring vitamin B\(_6\) as a cofactor, the transamination that must occur with the internal pyruvate can sometimes result in an irreversible reaction making AMD1 catalytically inactive. The dcAdoMet formed by AMD1 is then used as the aminopropyl donor for SRM and SMS as stated above.

AMD1 is the other rate-limiting enzyme in the polyamine synthesis pathway and as such, is tightly regulated. Upon decarboxylation, dcAdoMet is no longer able to be used for methyl transfer reactions and is committed to being the aminopropyl donor for polyamine biosynthesis\(^5^4\). Due to this and SRM/SMS activity only being regulated by substrate availability, dcAdoMet concentration is kept at 1-2% of AdoMet within the cell\(^5^5\).

Putrescine is a key regulator of AMD1 and is responsible for activating it. Putrescine activates the auto processing of the proenzyme into its α and β subunits and
enhances the decarboxylation reaction by binding to an acidic pocket away from the active site but close to the dimer interface\textsuperscript{56–58}. Mutation of amino acids within putrescine’s binding pocket greatly affects AMD1 activity, and without putrescine binding, AMD1 activity is significantly decreased\textsuperscript{59,60}.

AMD1 is also negatively regulated by spermidine and spermine at the transcriptional, translational, and protein degradation level. Spermidine and spermine have been shown to downregulate transcription of AMD1 potentially through a polyamine-responsive element upstream of the gene; however, this is poorly understood\textsuperscript{61,62}. Polyamines control the translation of AMD1 through its 5’ UTR. Within AMD1’s 5’ UTR, there is a small upstream ORF (uORF) that encodes a \textit{cis}-acting element, MAGDIS\textsuperscript{63}. Polyamines have been shown to reduce MAGDIS synthesis by stabilizing the nascent peptide-tRNA complex at the stop codon, causing the ribosome to stall, preventing it from accessing the ORF encoding for AMD1\textsuperscript{29,63,64}. The ubiquitin-dependent degradation of AMD1 is also controlled by the concentration of polyamines within the cell. When polyamines are at high abundance, there is a significant increase in the amount of ubiquitinated AMD1, specifically the catalytic inactive form\textsuperscript{38,65}. However, the exact mechanism polyamines play on the increased ubiquitination is unknown.

ODC1 and AMD1 can also be inhibited by nitric oxide (NO) production through nitrosylation of a cysteine in the active site\textsuperscript{66,67}. Overall, polyamine synthesis is a highly regulated process, and the cell expends considerable energy in maintaining homeostatic polyamine levels.
**Polyamine catabolism.** Spermidine and spermine can undergo catabolism as well. Catabolism is an important means of controlling polyamine concentration within cells since putrescine is more easily exported than other forms. Spermine can be catabolized back to spermidine by two different processes. One is directly through SMOX, which produces aldehyde and H$_2$O$_2$. There are multiple splice variants of SMOX that exist in the nucleus, but their roles are unknown. Spermine can also be converted to spermidine (and spermidine to putrescine) through acetylation by SAT1 followed by oxidation through PAOX. Like SMOX, PAOX also produces reactive aldehydes and H$_2$O$_2$; however, PAOX is located within peroxisomes, limiting the damage caused by oxidation. Acetylated polyamines have also been found to be exported out of cells, but to a lesser extent compared to putrescine.

Polyamine catabolism is primarily regulated through SAT1 expression, mRNA processing, and degradation. Upstream of SAT1, there is a polyamine response element that enhances SAT1 transcription when polyamines are high through the transcription factors Nrf-2 and polyamine-modulated factor-1. SAT1 transcription has also been shown to increase through a number of different stimuli including a viral induced interferon (IFN) response. SAT1 pre-mRNA splicing is also a regulated step in polyamine catabolism. SAT1’s pre-mRNA’s intron 3, which is normally spliced out of mRNA under high polyamine conditions, contains a premature stop codon. SAT1 mRNA that includes intron 3 results in a truncated form of SAT1 and results in non-sense mediated RNA decay. The truncated form of SAT1 was found at higher abundance in cells depleted of polyamines and it was found that polyamines and their analogs increase the stability of SAT1. Furthermore, viruses have been shown to promote
the inclusion of intron 3, discussed in more detail later\textsuperscript{76}. Polyamines also enhance SAT1 translation; nucleotides 1-45 and 492-504 in the coding region of \textit{SAT1} mRNA mediated polyamine-stimulated translation\textsuperscript{77}. Polyamines also bind to SAT1, stabilizing it and preventing polyubiquitination and degradation\textsuperscript{78,79}.

**Cellular Functions of Polyamines**

Polyamines are at high abundance (millimolar range) within cells and play key roles in a wide variety of cellular processes\textsuperscript{80,81} (Fig. 3). Due to their charge, they bind nucleic acids as well as affect chromatin structure\textsuperscript{82}. They also impact ion channels, gap junctions, protein function, cell signaling, translation, and even cell proliferation and differentiation\textsuperscript{29,81}.

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**Figure 3. Functions of Polyamines Within the Cell.** Polyamines are required for a wide variety of cellular functions including, but not limited to nucleic acid function, protein translation, membrane rigidity, and chromatin structure. The diverse functions of polyamines stem from their positive charge which allows them to interface with negatively charged molecules.

**Nucleic acids and gene expression.** Due to polyamines’ positive charge, they can interact with DNA and RNA, affecting their conformation. Polyamines can compact both naked DNA and chromatin\textsuperscript{83–86}. Spermidine and spermine have been associated
with highly compacted mitotic chromosomes\textsuperscript{87,88}. In brain tumor cells depleted of polyamines, chromatin condensation was negatively affected\textsuperscript{89}. Polyamines also promote the left-handed Z-DNA conformation and stabilize the DNA quadruplex conformation of \textit{c-myc}, resulting in the overexpression of \textit{c-myc}\textsuperscript{90}. This acts as a positive feedback loop due to the ODC1, SRM, and SMS genes being transcriptional targets of \textit{c-myc}\textsuperscript{91,92}. Interestingly, cGAS, a double stranded DNA (dsDNA) sensor involved in innate immunity and triggering an IFN response, was found to have decreased binding affinity to polyamine induced Z-DNA compared to B-DNA (right-handed conformation)\textsuperscript{93}. Furthermore, SAT1 facilitated cGAS activation by decreasing spermidine and spermine and Z-DNA accumulation\textsuperscript{93}.

Polyamines’ ability to bind to nucleic acids also affects polymerase function and DNA repair. Both spermidine and spermine stimulate DNA and RNA polymerases, at concentrations of 0.5-1.0mM for spermidine and 2-10\textmu M spermine for a bovine DNA polymerase\textsuperscript{94,95}. It was found that addition of polyamines to DNA polymerases extracted from human sera reduced the concentration of Mg\textsuperscript{2+} to physiologically relevant levels\textsuperscript{96}. However, at high polyamine concentrations, polymerase activity was inhibited, potentially through their role in DNA condensation\textsuperscript{97,98}. Cells depleted of polyamines are also more sensitive to genotoxic conditions like ultraviolet light and ionizing radiation\textsuperscript{99,100}. This is due to polyamines role in DNA double-strand break repair. Polyamines were found to be important for RAD51 recombinase’s DNA binding ability and enhanced the capture of homologous duplex DNA\textsuperscript{101}. This is one of the main reasons why targeting polyamines in cancer cells is an active area of research.
Polyamines also play an important role in estrogen receptor signaling\textsuperscript{102–104}. When the estrogen receptor is activated, it is translocated into the nucleus where it binds to estrogen response elements upstream of specific genes and activates their transcription\textsuperscript{105}. Spermidine, was shown to enhance DNA bending within these estrogen response elements and promote estrogen receptor binding affinity\textsuperscript{103}. However, it is unknown if polyamines are involved in binding to other signaling response elements.

**Hypusination.** Besides polyamines’ role in frameshifting, found only to occur with OAZ1 (stated above) and the yeast retrotransposon Ty1, spermidine is also used as a specific post-translation modification (PTM) of eIF5A in a process called hypusination (Fig. 4)\textsuperscript{22,106,107}. Spermidine is first ligated to lysine 50 of eIF5A via deoxyhypusine synthase (DHPS) forming eIF5A-deoxyhypusine. Next, deoxyhypusine hydroxylase (DOHH) adds a hydroxyl group to the deoxyhypusine moiety, forming eIF5A-hypusine\textsuperscript{108}. eIF5A is the only known mammalian protein to be hypusinated and the hypusine moiety was first discovered in 1971 as a new amino acid occurring in bovine brain\textsuperscript{109}. In 1981, polyamines were discovered to be the precursor of

![Figure 4. Hypusination of eIF5A. Spermidine is first ligated to eIF5A’s lysine 50 by DHPS, forming eIF5A-deoxyhypusine. DOHH then adds a hydroxyl group to the deoxyhypusine moiety, creating eIF5A-hypusine. eIF5A-hypusine aids in ribosomal translation of poly-proline motifs, as well as translation initiation.](image-url)
hypusine by Park and colleagues noticing $[^3]H$putrescine and $[^3]H$spermidine was incorporated into a single protein, later discovered to be eIF5A (previous nomenclature of eIF-4D), and was formed through post-translational modification$^{110,111}$. eIF5A is also a highly conserved protein found in all eukaryotes, archaea (aIF5A), and bacteria (EF-P); with eIF5A and aIF5A being hypusinated and EF-P’s lysine being conjugated with a hydroxylated $\beta$-lysine$^{112-115}$.

eIF5A’s role in translation was first found to be important for global elongation in yeast$^{116,117}$. Depletion of eIF5A in yeast resulted in an increase in the polysome fraction, clusters of ribosomes held together by a single mRNA that each ribosome is translating, and ribosome transit time. eIF5A was later found to be important for alleviating ribosomal stalling of long polypeptides in yeast$^{118}$. eIF5A was also found to be important for translation termination by increasing the rate of peptidyl-tRNA hydrolysis$^{119}$. Despite previous evidence suggesting that eIF5A is not involved in translation initiation$^{116}$, it was found that eIF5A is important in maintaining correct start codon selection$^{120}$. Within human cells, eIF5A is required for translation of specific mRNAs as well$^{20,121-123}$.

The EIF5A transcript has an additional upstream start codon, resulting in eIF5A having two isoforms, eIF5A1 and eIF5A2, both of which can be hypusinated$^{124-126}$. eIF5A1 is the canonical eIF5A (referred to as eIF5A throughout this text), which is ubiquitously expressed within all cell types. eIF5A2 contains 30 additional amino acids at its N-terminus and its function is distinct from eIF5A1. Previous work with eIF5A2 suggested that it promoted cancer development and depletion of eIF5A2 in mice had no difference in weight or survival compared to control mice$^{127}$. Interestingly, it was found that eIF5A2 did not regulate protein synthesis in the SW480 colon cancer cell line$^{128}$.
Instead, knockout of eIF5A2 led to decreased intrinsic expression of antiviral genes and cells were more permissive to RNA viral infection compared to WT. However, much remains unknown about eIF5A2 and how hypusination affects its function.

Hypusinated eIF5A was found to specifically aid in the translation of polyproline motifs, containing at least a di-proline\textsuperscript{21}. It does this by binding between the exit-tRNA (E-site) and peptide-tRNA site (P-site). Here, the hypusine-containing domain is presented to the P-site and allosterically allows for the formation of a proline-proline bond by stabilizing the peptide chain of the tRNA, preventing ribosome stalling (Fig. 5)\textsuperscript{129–131}. Besides aiding the ribosome in translating polyproline motifs, hypusination of eIF5A is also important for its subcellular localization within the cytoplasm\textsuperscript{132}. Hypusine is bound by nuclear export factor exportin 4, which aids in eIF5A-hypusine’s
localization\textsuperscript{133}. \textit{In vivo} studies of hypusinated eIF5A remain difficult due to the small moiety of hypusine and that the use of tagged eIF5A (such as a FLAG tag) prevents its hypusination and affects its cellular localization\textsuperscript{132}.

eIF5A-hypusine is involved in a variety of cellular processes, most notably cellular differentiation and proliferation. The silencing of eIF5A by the use of shRNA in HeLa cells found that multiple cellular processes were upregulated including vesicular trafficking, proteolysis, mRNA processing, protein folding and ER stress, and metabolic processes\textsuperscript{134}. Rat stem cells from skeletal muscles treated with N\textsuperscript{1}-guanyl-1,7-diamineheptane (GC7), a reversible, competitive inhibitor specific to DHPS, failed to differentiate to skeletal muscle cells\textsuperscript{135}. Furthermore, polyamine metabolism, and specifically hypusination of eIF5A was found to be critical for CD4+ T cell differentiation\textsuperscript{136}. The authors found that T cell-specific deletion of DOHH in mice resulted in T cell dysregulation via improper regulation of cytokine expression and transcription factors. B cells are also impacted by eIF5A-hypusine. Decreased levels of hypusinated eIF5A results in the reduced translation of TFEB, a critical protein in autophagy, leading to B cell senescence\textsuperscript{123}. This process may be in part why the elderly, in which spermidine is often low, have decreased memory B cell function\textsuperscript{137}.

eIF5A-hypusine is also involved in metabolism. Treatment of kidney cells with GC7 resulted in reduced translation of GLUT1, a glucose transporter, pushing the cells towards using glucose as their primary energy source and allowing the cells to be oxygen independent and promoting kidney transplant outcomes\textsuperscript{138,139}. These cells also had reduced anoxia-induced generation of reactive oxygen species (ROS). However, GLUT1 does not contain any polyprolines and its mRNA does not contain the
consensus sequence required to link eIF5A. In line with this, insulin-resistant mice depleted of DHPS specifically in islet-beta cells had impaired proliferation and induced overt diabetes, revealing an important role for hypusination in glucose homeostasis. Likewise, hypusination was shown to decrease the expression of mitochondrial proteins in macrophages.

Despite all the work done on hypusination and how it impacts cells, little is known about how it is regulated. Only recently was it found that DHPS directly binds to, and is phosphorylated at serine 233, ERK1/2. ERK1/2 is an important kinase involved in signaling cascades, and once activated, promotes cell proliferation, survival, and differentiation. Knockdown of DHPS resulted in reduced cellular proliferation that was rescued by the phosphomimic DHPS mutant S233D. DOHH expression is also downregulated by the microRNAs miR-331-3p and miR-642-5p, which bind to the 3'-UTR of DOHH. SAT1 can also regulate hypusination by binding to eIF5A. SAT1 acetylates lysines 47 and 68, causing eIF5A to be localized within the nucleus and preventing it from being hypusinated. These studies have provided insights into how hypusination is regulated; however, much remains unknown. It is unknown how DHPS and DOHH transcription are regulated, if they are at all. Upon searching an IFN transcriptomic database, DHPS expression was shown to either be up or down regulated approximately 1.2 fold in cells treated with type I and type II IFN while DOHH expression was primarily downregulated by 1.4 fold in cells treated with type I or type II IFN. The differences seen between expression could be a result of the cell type used for the transcriptomic data, the time of collection, or the concentration of IFN used.
Membrane functions. Polyamines play an important role in membrane function, including ion channel regulation. Inwardly-rectifying potassium (Kir) channels are an important subset of potassium channels that help maintain membrane potential by importing potassium, thereby maintaining and regulating the action potential in electrically excitable cells like neurons and cardiac muscle\textsuperscript{148}. This means that Kir channels remain active when the cell is at its resting potential. When the cellular membrane undergoes depolarization, Kir channels’ activity decreases. When these channels were first discovered and characterized in the 90s, it was found that the Kir channels’ ability to inwardly rectify depended on an extrinsic factor\textsuperscript{149}. It was later found that polyamines, specifically spermine, are responsible for Kir channels’ ability to maintain membrane potential. Polyamines bind to specific pockets within Kir channels’ transmembrane and cytoplasmic pore domains, giving the channels their ability to be voltage-dependent\textsuperscript{150}. Glutamate receptors are ion channels that mediate excitatory synaptic signals within the brain. Like Kir channels, polyamines have been shown to bind within the pores and limit calcium influx, altering the excitability threshold at synapses\textsuperscript{29}.

Gap junction proteins are an important class of cellular proteins that form pores between neighboring cells, allowing for cell-to-cell communication. The gap junction protein connexin40, was found to be inhibited by spermine; however, spermine increases gap junction communication of connexin43\textsuperscript{151,152}. Interestingly, polyamines have also been found to decrease membrane fluidity in plant cells and human blood cells by binding to negatively charged phospholipids\textsuperscript{153–155}. Polyamine binding to
phospholipids also protect vesicles from lipid peroxidation\textsuperscript{156}. Altogether, polyamines are vital for the function of cellular membrane proteins and reducing membrane fluidity.

**Polyamination.** A lesser known and poorly understood use of polyamines within the cell is the processes of polyamination in which a polyamine is used as a PTM. The use of polyamines as a PTM is unique, since most PTMs impart a negative or neutral charge compared to polyamines’ positive charge. This positive charge gives proteins increased stability and insolubility as well as increased resistance to proteolysis\textsuperscript{157}. The Ca\textsuperscript{2+}-dependent transglutaminase 2 (TG2) is responsible for the polyamination of glutamine residues present on proteins with putrescine, spermidine, and spermine\textsuperscript{158}. Polyamination of tau, a protein associated with Alzheimer’s disease, resulted in resistance to proteolysis\textsuperscript{159}. TG2 has been found to be upregulated in Alzheimer tissue as well. Within the same line of increasing stability, polyamination is important for the increased stability needed for neuronal microtubules in axons\textsuperscript{160}. Without polyamination, microtubules are unable to form the long chains needed for axons and negatively impact neuronal architecture. In contrast, polyamination activates phospholipase A2, a protein responsible for releasing fatty acids from membrane glycerophospholipids and overactive phospholipase A2 has been implicated in inflammatory diseases such as rheumatoid arthritis\textsuperscript{161}. Polyamination also plays a role in regulating transcription of specific genes\textsuperscript{162}. How the overarching polyamination plays a role in cellular function and disease states, as well as its regulation, has yet to be identified.
Section 2: Polyamines During a Viral Infection

Viral Use of Polyamines

From their key roles in mammalian cells, polyamines also play important roles in viral infection. Their importance and roles for viruses are diverse, with some viruses requiring polyamines for a productive infection, while others are unbothered by a lack of polyamines\textsuperscript{163,164}. To date, roles for polyamines in mammalian viruses include viral entry, transcription, replication, and virion packaging (Fig. 6).

![Figure 6. Polyamines in Cellular and Viral Processes.](image)

Cells rely on polyamines for transcription, translation, nucleic acid metabolism and structure, chromatin and DNA packaging, and membrane fluidity. Viruses similarly utilize polyamines for much of the same things as cells; however, they also use polyamines in binding to cells and for enhancing viral enzyme activity.

Polymine in virions. While the earliest reports of polyamines in bacteriophages was in the late 1950s\textsuperscript{165}, it was not until 1971 that polyamines were found in a human virus. This virus was the herpes simplex virus (HSV-1), and it was found to contain spermine in the nucleocapsid and spermidine in the viral envelope\textsuperscript{166}. Later, it was shown that human cytomegalovirus (HCMV) stimulated ODC1 activity and increased polyamine uptake, presumably to enhance cellular polyamine levels\textsuperscript{167}. The
inhibition of polyamine metabolism after the eclipse phase did not affect viral replication, suggesting that polyamines may not play a role late in infection but may have roles early in infection\textsuperscript{168,169}.

The initial descriptions of spermidine and spermine in the virions of HSV-1 suggested that mammalian viruses may package polyamines, as observed for bacteriophages and plant viruses. HSV-1 was shown to produce comparable amounts of viral particles in cells depleted of polyamines with the specific ODC1 inhibitor difluoromethylornithine (DFMO) and control cells, but those produced from polyamine-deficient cells had different abundance of DNA fragments and DFMO-treated cells produced lower viral titers\textsuperscript{170,171}. Similarly, the bunyaviruses, Rift Valley Fever virus, and Lacrosse virus (LACV) produced non-infectious particles in polyamine-depleted cells\textsuperscript{172}. Rift Valley Fever virions that lack polyamines have significantly reduced infectivity and are more temperature sensitive compared to virions containing polyamines, specifically spermidine\textsuperscript{173}. Polyamines are present to varying degrees in capsids of diverse viruses. Vaccinia virus (VACV), a double-stranded DNA virus with a large (1 Mb) genome, packages polyamines putatively to neutralize DNA’s negative charge\textsuperscript{174}. In contrast, poliovirus and Coxsackievirus package negligible amounts of polyamines\textsuperscript{175}. The precise roles of these packaged polyamines are unclear.

**Polymerase activity.** Early work in bacteriophages suggested a role for polyamines in polymerase activity and viral genome synthesis. In vitro genome synthesis assays demonstrated that polyamines enhanced viral polymerase activity of bacteriophage T7 polymerase\textsuperscript{176}. Work with mammalian viruses also showed that polyamines contribute to these functions, specifically in the alphavirus chikungunya
virus (CHIKV) and the flaviviruses Zika virus (ZIKV), hepatitis C virus (HCV), the herpesvirus HSV-1, and the DNA-dependent RNA polymerase of VACV\textsuperscript{73,177–179}. Addition of polyamines stimulated \textit{de novo} RNA replication of the alphaviruses by about 4-fold compared to untreated cells\textsuperscript{73}.

**Translation.** In addition to roles in polymerase activity, CHIKV, ZIKV, HCV, and other RNA viruses' translation rely on polyamines\textsuperscript{180}. DFMO-treated cells exhibited reduced viral protein translation\textsuperscript{73,181}. Further work in Ebola virus (EBOV) and Marburgvirus (MARV) highlighted that polyamines function in transcription of viral genomes, but the translation of these transcripts relied on polyamines through eIF5A hypusination\textsuperscript{24,182}. The knockdown of eIF5A or inhibition of DHPS resulted in significant decreases in the accumulation of viral protein \textsuperscript{30}. Further, hypusination inhibition reduced EBOV and MARV infectious titers. Kaposi’s sarcoma-associated herpesvirus also requires eIF5A-hypusine for the translation of the viral protein latency-associated nuclear antigen, which contains polyprolines\textsuperscript{183,184}. The model coronavirus, murine hepatitis virus (MHV) A59, had significantly reduced viral titers when cells were treated with hypusination inhibitors\textsuperscript{25}. Due to the large RNA genome of coronaviruses (~30kb), eIF5A-hypusine may be required to enhance ribosome stability and translation of proteins containing poly-prolines. The use of ciclopirox and deferiprone, non-specific inhibitors of DOHH, inhibited HIV gene expression\textsuperscript{23}. Treatment of human peripheral blood mononuclear cells with either of these inhibitors reduced transcription initiation of HIV. However, the mechanism behind how eIF5A-hypusine affects transcription initiation is unknown and is most likely an indirect effect of blocking hypusination.
**Viral binding and protein function.** In addition to roles in transcription and translation, polyamines affect additional viral enzymes and processes to enhance virus replication. The enterovirus, Coxsackievirus B3 (CVB3), requires polyamines for multiple stages in its infectious life cycle. CVB3 exhibited reduced ability to bind polyamine-depleted cells, a phenotype shared with additional enteroviruses. Interestingly, CVB3 passaged in the presence of DFMO accumulated a mutation in the capsid protein VP3 at site Q234R, which increased cellular attachment of CVB3 to polyamine-depleted cells\(^{185}\). Polyamines added directly to CVB3 rescued binding; however, polyamines added to cells only rescued viral binding when cells were given polyamines 16 hours before binding. This may hint to the polyamination of VP3, but more evidence is needed. Coronavirus also require polyamines for binding and entry. Cells treated with DFMO had reduced MHV-A59 attachment and fusion, with SARS-CoV-2 pseudo-particles exhibiting a similar phenotype\(^{25}\). How polyamines impact CVB3 binding is one of the questions explored in this dissertation.

Additionally, CVB3 proteases 2A and 3C exhibit sensitivity to polyamines, as polyamine depletion precludes their activity both \textit{in vitro} and in the context of infection. CVB3 also showed resistance to polyamine depletion through mutations in its 2A and 3C proteases, which are responsible for cleaving host and viral proteins\(^{186}\). Curiously, mutations observed here and in a similar experiment with CHIKV changed a negatively charged amino acid to a positively charged residue (either lysine or arginine), as if to substitute for the loss of the positively charged polyamines. Polyamines were also shown to stimulate the proteinase activity of several cellular enzymes\(^{187,188}\). However, the mechanisms behind how polyamines influence protease activity remain unknown.
Interestingly, the ORF47 kinase of varicella-zoster virus, the causative agent of chickenpox, is also stimulated by polyamines\textsuperscript{189}. Specifically, polyamines enhance the autophosphorylation of ORF47. The proposed mechanism behind this is that polyamines stabilize the structure of ORF47 and potentially aid in dimer formation.

**Figure 7. Viral Manipulation of Polyamines in Infected Cells.** The herpesviruses EBV, HSV, bovine herpesvirus (BoHV), and HCMV induce polyamine levels through ODC1 and AMD1. The flavivirus HCV induces both ODC1 and SAT1 while Dengue virus promotes alternative splicing and subsequent degradation of SAT1 by inhibiting RBM10. PBCV-1 is a large algae virus that encodes its own polyamine biosynthetic pathway. Mechanisms that are not fully understood are represented by dashed lines.

**Viral manipulation of polyamines.** Polyamines are important resources for viruses and cells, and viruses have evolved mechanisms to maintain, enhance, or manipulate polyamine metabolism to support virus infection (Fig. 7). Perhaps the most extreme example is PBCV-1, a large DNA algae virus, which encodes an entire polyamine biosynthesis pathway in its dsDNA genome\textsuperscript{190}. This implies that polyamines are vital for these viruses to infect hosts; however, little is known about polyamines’
exact roles in infection. Epstein-Barr virus (EBV) has also been shown to manipulate polyamine levels in cells by decreasing expression of SAT1\textsuperscript{191}. Additionally, EBV is able to stabilize c-myc through its nuclear antigen 3C, resulting in overexpression of polyamine synthesis proteins\textsuperscript{192}. This overexpression of c-myc could be the cause of the down regulation of SAT1, but whether polyamines are directly involved in this process is unknown.

HSV-1 was also shown to up regulate expression of AMD1 mRNA in infected cells\textsuperscript{193} and HCMV, another herpesvirus, stimulates ODC1 activity\textsuperscript{167}. A distantly related virus, bovine herpes virus, encodes an ODC-like protein that shares around 55% amino acid homology with mammalian ODC1 and contained all the amino acids necessary for decarboxylase activity\textsuperscript{194}. Interestingly, the viruses described above that manipulate polyamine levels are DNA viruses, and, in the case of herpesviruses, result in permanent infection of the host.

RNA viruses are also able to manipulate polyamines within cells. Recent work has demonstrated that HCV induces polyamine metabolic genes, including \textit{ODC1}, \textit{SAT1}, and \textit{SMOX} during infection\textsuperscript{195}. This could be the result of HCV triggering an IFN response or the generation of ROS, but the mechanism behind polyamine gene upregulation remains unknown. The flavivirus dengue virus (DENV) also manipulates intracellular polyamines. It was recently discovered that DENV promotes the alternative splicing of \textit{SAT1} resulting in the truncated version of \textit{SAT1}, which undergoes non-sense mediated decay\textsuperscript{196}. DENV’s NS5 protein was later found to bind to RBM10, a component of the spliceosome and involved in intron 3 skipping of \textit{SAT1}, causing RBM10 to be degraded by the proteasome\textsuperscript{197}. This degradation of RBM10 enhances
viral replication partially through its role in SAT1 splicing. It is currently unknown if other flaviviruses affect SAT1 splicing. Conversely, cells persistently infected with CVB3 had reduced ODC1 translation, but titers were still negatively impacted when treated with polyamine inhibitors. Whether other acute RNA viruses manipulate polyamines to their favor is unknown; regardless, viruses have evolved strategies to interface with polyamines in infected cells.

**Polyamines During an Interferon Response**

Cells have an innate immune response that allows them to sense and limit viral infections. Upon infection, viruses activate the IFN response, alerting the cell to their presence, and triggering the expression of hundreds of interferon-stimulated genes (ISGs). Several of these genes deplete key metabolites required by viruses for a productive infection, including polyamines. Upon signaling through IFN α/β, SAT1 is induced and depletes cellular polyamines. In fact, SAT1 knockout cells replicate virus to higher titers than wildtype cells when treated with type I interferon. Prior work also highlighted that ODC1 activity decreases with IFN α, β, or γ treatment, though the effect on polyamine levels was not investigated. In a separate study, intraperitoneal delivery of IFN α and β inhibited ODC1 activity in mice. Thus, polyamine depletion is a strategy by which mammalian cells can reduce viral infection.

**Polyamines in Metabolic Pathways Key to Virus Infection**

Cellular metabolic pathways are interconnected and complex. Polyamines play a wide role in key metabolic processes including nucleotide metabolism, formation of ROS, and lipid metabolism among many other metabolic pathways in mammalian cells
Thus, polyamines likely affect virus replication through modulation of these distinct cellular metabolic pathways.

Polyamines play a role in nucleotide pools due to the requirement of dcAdoMet to act as the aminopropyl group donor. When cells were treated with DFMO, dcAdoMet was synthesized at normal levels leading to higher levels of cellular adenine pools\textsuperscript{201}. This is due to dcAdoMet containing adenine, leaving it trapped in the pathway unable to be used. Polyamine depletion via DFMO also induces thymidine depletion in colon tumors\textsuperscript{202}. Another link between polyamines and nucleotides was demonstrated in a study with the broad-spectrum antiviral ribavirin, which resembles guanosine\textsuperscript{203}. Treated cells exhibited higher levels of SAT1 and decreased polyamine levels; when guanosine was added exogenously to these treated cells, polyamine levels were restored, and viral

\textbf{Figure 8. Polyamines Affect Diverse Metabolic Pathways in Mammalian Cells.} Polyamine synthesis consumes decarboxylated S-adenosyl methionine, resulting in reduced nucleotide pools. These reduced nucleotide pools induce SAT1, which depletes polyamines, resulting in a feedback loop. The interconversion of polyamines via SMOX and PAOX accumulates ROS, specifically hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}). Polyamines also are described to neutralize ROS due to their abundance and charge. Additionally, polyamines maintain AMPK in its unphosphorylated, non-activated state to maintain lipid homeostasis. AMPK activation induces polyamine synthesis through c-myc mediated ODC1 induction.
titers were partially recovered\textsuperscript{204}. This partial recovery of viral titers suggests that a portion of ribavirin’s antiviral activity is through polyamines and highlights the connectedness between nucleotide and polyamine synthesis.

The AMP activated kinase (AMPK) is a broad regulator of cellular metabolism that, upon activation, decreases cellular lipid synthesis, enhances β-oxidation, increases glucose uptake, and activates autophagy. Cells treated with DFMO exhibit reduced AMPK activation\textsuperscript{205}. Furthermore, spermidine increased the phosphorylation of AMPK, thus limiting lipogenic gene expression\textsuperscript{206}. When AMPK was knocked down in cardiomyoblasts in the presence of isoproterenol, a transcriptional activator of ODC1, ODC1 levels increased above those seen with treatment of isoproterenol alone suggesting that AMPK is able to downregulate transcription of ODC1\textsuperscript{207}. Conversely, a recent study showed that activated AMPK facilitated ODC1 expression in a cancer cell line by phosphorylating the transcription factor CNBP\textsuperscript{208}. The differences seen are most likely due to the different cell types used in each study.

Polyamines and their catabolism play a part in the generation of ROS as well as sequestering ROS. The turnover of spermidine and spermine via PAOX results in the generation of hydrogen peroxide in cells. However, both spermidine and spermine have been shown to neutralize ROS and are important for prevention of oxidative damage\textsuperscript{209,210}. Interestingly, the SAT1 gene is targeted by p53 and SAT1 expression causes an increase in lipid peroxidation, stimulating cells to undergo ferroptosis\textsuperscript{211}. Many viruses, including HCV, HSV-1, and influenza virus induce ROS formation during infection\textsuperscript{212–215}. Polyamines may play a role in neutralizing these ROS since their generation can cause a ferroptotic response and potentially draw unwanted attention
from immune cells to the infected area. However, polyamines may not be beneficial for all viruses and may inhibit some stages of infection. The M2 protein of influenza, which plays a role in viral uncoating, can be inhibited by polyamines\textsuperscript{216}. Influenza also causes an increase in NO, through interferon $\gamma$, which inhibits ODC1. Whether these processes aid influenza virus in subsequent cellular entry has yet to be explored.

**Targeting Polyamines as an Antiviral Therapy**

![Figure 9. Polyamine Modulating Molecules with Antiviral Activity. Several molecules target polyamine biosynthesis to alter polyamine levels and hypusinated eIF5A within the cell. Difluoromethylornithine (DFMO), MDL 72527, diethylnorspermidine (DENSpm), $N^1$-guanyl-1,7-diamineheptane (GC7), ciclopirox (CPX), and deferiprone (DEF) target distinct metabolic enzymes and exhibit antiviral activity.]

Given the importance of polyamines to diverse aspects of viral infection, polyamine synthesis inhibitors have gained attention as potential antivirals. Initially, targeting polyamines gained traction as a potential cancer therapeutic\textsuperscript{217}. Given polyamines' roles in promoting cell cycle, it was not surprising that several types of cancers enhanced polyamine synthesis. However, anti-cancer therapies targeting polyamines were largely unsuccessful initially. However, continued work on polyamines in the development and progression of cancer remains an active area of research.
Importantly, several potential polyamine-targeting molecules (Fig. 9) received extensive testing in animal models and clinical trials, providing a wealth of information on their toxicity and in vivo effects. Thus, these molecules may hold promise when repurposed as antivirals.

Perhaps the best known and characterized molecule for polyamine synthesis inhibition is the FDA approved drug DFMO, which is an irreversible inhibitor of ODC1. DFMO binds to the active site of ODC1 where ODC1 decarboxylates DFMO. The decarboxylation of DFMO leads to an irreversible modification within ODC1’s active site rendering it unable to release DFMO. DFMO treatment results in significant reductions in putrescine and spermidine levels in a time- and dose-dependent manner in multiple cell types. Clinically, DFMO is effective against trypanosomiasis, or African sleeping sickness, with mild side effects including reversible ototoxicity. The trypanosomal ODC1 is highly sensitive to DFMO, resulting in reduction of polyamines in the parasite and clearance by infected individuals. In fact, DFMO is a frontline drug in the treatment of trypanosomiasis and is on the list of the World Health Organization’s essential medicines. DFMO can be taken orally, topically, or intravenously. Treated individuals experience mild side effects, including thrombocytopenia, though these effects are reversible upon cessation of treatment. DFMO inhibits infection by several viruses, both in vitro and in vivo. Thus, DFMO administration may be a promising route to quell virus replication. Virus infection is best reduced with DFMO pretreatment, and treatment post-exposure requires significant additional investigation. Nonetheless, prophylactic DFMO treatment may be reasonable
in certain instances, such as to protect healthcare workers, uninfected contacts of infected patients, or immunocompromised individuals.

In addition to DFMO, other compounds have received attention as potential antivirals. Diethylnorspermidine (DENSpm), another potential anti-cancer therapeutic, enhances polyamine catabolism and rapidly depletes polyamines. Though not as extensively tested as DFMO, DENSpm has been explored in clinical trials\textsuperscript{229,230}. DENSpm also exhibits broad antiviral activity and a shorter pretreatment time, making it potentially more functional than DFMO in certain instances\textsuperscript{17}. Additionally, molecules such as MDL 72757, targeting polyamine interconversion\textsuperscript{231}, exhibit antiviral activity \textit{in vitro}\textsuperscript{182}.

Another highly promising category of molecules targeting polyamine metabolism are the hypusination inhibitors GC7, deferiprone, and ciclopirox\textsuperscript{232}. Both deferiprone and ciclopirox are FDA approved drugs used to topically treat fungal infections. They are broad iron chelators that limit cellular proliferation and have also been shown to limit cancer growth\textsuperscript{233–235}. DOHH requires iron for its hydroxylation activity and is inhibited by deferiprone and ciclopirox\textsuperscript{236}. However, these are non-specific inhibitors and using them as antivirals may cause unforeseen side effects. GC7 on the other hand, is a specific inhibitor of DHPS. It directly binds to DHPS’s active site blocking its function\textsuperscript{237}. Studies by Olsen and colleagues\textsuperscript{24,182} demonstrated efficacy of these inhibitors against Ebola virus infection, and prior work showed that HIV-1 is similarly sensitive to this group of inhibitors\textsuperscript{23}. Additionally, the model coronavirus MHV-A59 is sensitive to GC7 treatment\textsuperscript{25}. In sum, several polyamine-targeting molecules show activity against
viruses, and further work into their mechanisms of action, toxicity, and in vivo activity is required for the possibility of targeting polyamines to treat or prevent viral infection.

**Section 3: Cholesterol Metabolism and Function**

**Cholesterol Synthesis and Regulation**

Cholesterol is a vital metabolite found in most cells, with hepatocytes (liver cells) responsible for maintaining whole-body cholesterol\(^ {238} \). It is the major sterol found in animal tissues and consists of four carbon rings (3 hexamers and 1 pentamer), a hydrophilic hydroxyl headgroup, and a flexible carbon chain that branches off the carbon pentamer (Fig. 10). The carbon body is hydrophobic, making the molecule amphipathic and able to interact with lipid bilayers. Cholesterol can be obtained through diet or synthesized *de novo* within cells\(^ {239} \). The *de novo* synthesis consists of 22 enzymes, but can involve multiple pathways once the first sterol, lanosterol, is formed requiring more or fewer enzymes\(^ {240-242} \).

Starting from acetoacetyl-coenzyme A (CoA), 3-hydroxy-3-methylglutaryl (HMG)-CoA synthase (HMGCS) combines acetoacetyl-CoA with acetyl-CoA to form HMG-CoA within the cytosol. Next, HMG-CoA reductase (HMGCR) reduces HMG-CoA into mevalonate\(^ {242} \). HMGCR is a transmembrane protein located within the endoplasmic reticulum (ER) and is one of the two rate-limiting enzymes involved in cholesterol
synthesis; thus, is highly regulated. Mevalonate is then phosphorylated and undergoes a series of modifications at the ER membrane, eventually forming squalene. The second rate-limiting enzyme, squalene monooxygenase (SM), converts squalene to monooxidosqualene. From here, several more steps occur, eventually leading to the formation of cholesterol within the ER membrane (Fig. 11).

**Figure 11. Cholesterol Synthesis Pathway.** The cholesterol synthesis pathway consists of 22 enzymes and starts with the conversion of acetoacetyl-CoA and acetyl-CoA to HMG-CoA by the enzyme HMGCS. HMG-CoA is then converted to mevalonate by the first rate-limiting enzyme in the pathway, HMGCR. Mevalonate then undergoes a series of modifications, eventually forming squalene. Squalene is then converted to monooxidosqualene by the second rate-limiting enzyme SM. Monooxidosqualene is then converted to the first sterol, lanosterol, and inserted into the ER membrane. From here, lanosterol is modified by multiple enzymes, eventually leading to the synthesis of cholesterol.

**SREBP2.** Regulation of cholesterol synthesis starts at the transcriptional level. Sterol regulatory element binding protein 2 (SREBP2) binds to sterol regulatory elements (SRE) upstream of cholesterol synthesis genes such as *HMGCS, HMGCR, Low-density lipoprotein receptor* (LDLR), *SM*, and many others, including itself (*SREBF2*). When SREBP2 binds SREs within the promoter region, this activates the transcription of those genes. SREBP2 processing and translocation from the ER to the nucleus is regulated by cholesterol and other oxysterols and is summarized in Figure 12.
Figure 12. SREBP2 Pathway. Full length SREBP2 is in the ER where it interacts with SCAP and Insig. When SCAP is not bound by cholesterol, it prevents Insig from binding, and allows COPII proteins to bind and form vesicles. These vesicles are then transported and fused to the Golgi. Once SREBP2 is in the Golgi membrane, it is cleaved first by S1P then S2P. The cleavage by S2P releases the N-terminal domain of SREBP2 where it then translocates to the nucleus and binds to SREs to upregulate transcription of cholesterol synthesis genes.

SREBP2 initially resides in the ER membrane and is a two-helix transmembrane protein with both the N- and C- terminal domains facing the cytosol. The N-terminus contains the basic-helix-loop-helix leucine zipper active domain which allows it to recognize SREs within the genome\textsuperscript{245,246}. This region also has multiple di-prolines present, potentially requiring eIF5A-hypusine for translation. In the ER, SREBP2 localizes with the SREBP cleavage-activating protein (SCAP) and the Insulin-induced gene protein-1 (Insig-1). Under normal conditions, cholesterol is bound by SCAP with its sterol-sensing domain (SSD), causing Insig-1 to bind. Insig-1 binding causes
conformational changes in SCAP, preventing COPII coat proteins from binding to the SREBP2-SCAP complex and forming vesicles to transport SREBP2-SCAP to the Golgi. When the ER cholesterol level is below 5% of ER lipids, SCAP undergoes conformational changes, causing Insig-1 to dissociate and be degraded, and SREBP2-SCAP is transported to the Golgi. Once in the Golgi, SREBP2 is first cleaved by site-1 protease (S1P), which then causes site-2 protease (S2P) to cleave and release the N-terminal of SREBP2. SREBP2 is then transported to the nucleus where it upregulates cholesterol synthesis genes. SREBP2 is also able to activate Insig-1 transcription, which acts as a regulatory-feedback loop preventing over-activation of SREBP2.

**HMGCR.** The first rate-limiting enzyme in cholesterol synthesis is HMGCR, and as such, its activity is tightly controlled. As previously stated, HMGCR transcription is regulated by SREBP2; however, HMGCR is also post-translationally regulated. Like SCAP, HMGCR also contains a SSD where cholesterol and other sterol intermediates bind. Sterol binding by HMGCR allows for Insig to bind to HMGCR. This triggers membrane-bound E3 ubiquitin ligases associated with Insig to polyubiquitinate HMGCR’s K248, causing HMGCR to be degraded. Conversely, HMGCR can also be deubiquitinated by the mTORC1 activated protein USP20, preventing HMGCR’s degradation. HMGCR can also be phosphorylated by AMPK, disrupting its activity. Phosphorylation of HMGCR acts as a quick stop to prevent production of mevalonate, but does not impact its degradation. HMGCR is also the target of statins, a class of drugs commonly prescribed to lower cholesterol within the blood. They work by partially binding to the catalytic site of HMGCR, blocking substrate binding.
**Squalene monooxygenase.** SM is the other rate-limiting enzyme involved in cholesterol synthesis and is regulated similarly to HMGCR. SM contains multiple SRE sites within its promoter that activate its transcription upon sterol depletion. Other transcription factors also regulate its transcription such as c-Myc\textsuperscript{258}. SM contains a different cholesterol-sensitive domain compared to HMGCR, but it acts in a similar way. When cholesterol is bound, the conformation of SM changes, causing a disordered sequence and allowing an E3 ligase to bind to SM and trigger its ubiquitination and subsequent degradation\textsuperscript{259,260}. Alternatively, squalene can bind to the cholesterol-sensitive domain of SM and stabilize it, preventing its degradation\textsuperscript{261}.

**Intracellular Cholesterol Transport**

Trafficking of cholesterol within the cell is a highly complex process that differs between the organelles involved and from where the cholesterol is coming. Cholesterol is also distributed unevenly within cellular membranes; with the plasma membrane containing about 40% of intracellular cholesterol\textsuperscript{262}. Upon cholesterol synthesis in the ER membrane, cholesterol is rapidly transported to other organelles to maintain the lower cholesterol concentrations found within the ER\textsuperscript{263}. The transport of cholesterol happens through either vesicular transport or at membrane contact sites (MCS) where opposing membranes are about 10-30 nm apart\textsuperscript{264–266}. Most of the newly synthesized cholesterol is transported directly to the Golgi where it can be sorted and transported to the plasma membrane.

The transport of cholesterol to the Golgi occurs at MCS and is primarily mediated by oxysterol-binding protein (OSBP). OSBP bridges the gap between the Golgi and the ER by binding to phosphatidylinositol-4-phosphate (PI4P) on the Golgi membrane and
the vesicle-associated membrane protein-associated protein (VAP) on the ER membrane\textsuperscript{267,268}. OSBP facilitates transport of cholesterol to the Golgi by countertransporting PI4P to the ER, where PI4P concentration is relatively low\textsuperscript{269}. The ER transmembrane phosphatase Sac1 dephosphorylates PI4P to phosphatidylinositol (PI) to maintain the low concentration of PI4P to allow for the continued export of cholesterol to the Golgi. On the Golgi, the ACBD3 protein, as well as others, help to localize PI-4 kinase IIIβ (PI4Kβ) which phosphorylates PI to PI4P\textsuperscript{269}. This process is also important for a variety of viruses, including CVB3, which will be discussed later. Mitochondria also receive a majority of their cholesterol through direct membrane contact with the ER\textsuperscript{270}.

Late endosomes/lysosomes also have MCS with the ER that allows for the ER to transport cholesterol to them, or for the endosomes to transport cholesterol to the ER or other organelles. When cells endocytose LDL, cholesterol-esters are broken down within lysosomes/endosomes to free cholesterol which can be recycled directly to the plasma membrane or to the ER in the form of lipid droplets, where it is re-esterfied\textsuperscript{271}. Niemann-Pick Type C1 protein (NPC1) within these lipid droplets is vital for the transport of cholesterol to the ER. This protein is named after the fatal disease that is a result of a mutation making NPC1 non-functional, causing cholesterol accumulation within endosomes and lipid droplets\textsuperscript{272}. NPC1 is also the receptor used by EBOV and MARV to infect cells\textsuperscript{273}. The inhibitor U18666A binds to the SSD of NPC1, blocking the movement of cholesterol out of the endosomes as well as inhibiting EBOV membrane fusion with the endosome\textsuperscript{274,275}.

These are just some examples of cholesterol transport within the cell and the ones pertinent to this dissertation. Overall, cholesterol transport is a highly complex
process and occurs in all human cells. Likewise, many viruses utilize different cholesterol transport pathways for their benefit, including CVB3.

**Cholesterol Functions**

Cholesterol is used in a variety of ways within the cell, either as itself or as a precursor for a variety of molecules including bile salts used to break down lipids in the stomach, vitamin D, hormones, and even an antiviral to block enveloped virus fusion\(^{276}\). Dysregulation of cholesterol has also been implicated in cancer, promoting tumorigenesis and suppressing immune responses\(^{277}\).

**Lipid rafts.** The main role of cholesterol within the cell is to promote membrane fluidity. As previously stated, most of the cholesterol within the cell is found in the plasma membrane and determines its fluidity and structure. Cholesterol is also found in high concentrations within microdomains of the plasma membrane called lipid rafts. Lipid rafts are heterogeneous and highly dynamic regions that selectively recruit membrane proteins such as GPI-anchored proteins, a variety of different kinases, receptors, etc\(^{278}\). These lipid rafts serve as a scaffold for signal transduction. Membrane proteins can be recruited or excluded from lipid membranes depending on if the signal needs to be enhanced or dampened. The basis of protein recruitment to these domains is a PTM called palmitoylation\(^{279}\). The lipid palmitate is ligated to cysteine residues of the transmembrane proteins and allow them to be transported to lipid rafts. Proteins can also contain a lysine-rich region that also acts as a signal to target the protein to lipid rafts\(^{280}\). However, the exact mechanisms for how proteins are taken out of lipid rafts is unknown.
Section 4: Coxsackievirus B3

Coxsackievirus B3 Pathogenesis

CVB3 is a non-enveloped, positive single-stranded RNA (ssRNA) virus that is in the *picornaviridae* family and is classified as a non-polio enterovirus. These non-polio enteroviruses are estimated to infect 10-15 million people and hospitalize tens of thousands each year in the United States alone, with children and elderly being the most susceptible. Coxsackieviruses were first identified in 1947 in the town of Coxsackie, New York during an outbreak of polio-like symptoms. However, this virus was distinct from polio as it was able to infect mice and was not neutralized by polio antiserum. Later, Coxsackievirus was split into two groups, A and B, due to differences in pathology in suckling mice. Currently, there are 23 different Coxsackievirus A serotypes and 6 Coxsackievirus B serotypes that have been described.

CVB3, like other enteroviruses, infects new hosts through fecal-contaminated food and water. Once past the stomach, CVB3 infects intestinal epithelial cells or potentially M cells (cells within the intestinal tract that present antigens to immune cells), where it can be released into the lymphatic system. From here, the virus can spread to bloodstream where it can rapidly disseminate. However, the mechanism of CVB3 dissemination or if it can infect M cells is unknown. In mice lacking the IFN alpha/beta receptor (IFNAR), CVB3 is able to rapidly disseminate, ~20 minutes from oral inoculation, to local tissues including mesenteric lymph nodes, pancreas, liver, spleen, and even the heart. Dissemination of CVB3 in immunocompetent mice was not seen by 20 minutes; however, more work is needed to determine if/when CVB3 spreads and if this depends on mouse and/or virus strain.
CVB3 is most well-known for its ability to infect the heart, pancreas, and liver and has been implicated in a number of different diseases. CVB3 infection of differing organs and tissues is mostly due to the levels of CAR and DAF expression, hence why CVB3 is not found to infect lung tissues. However, receptor expression alone is not enough since the kidney is most often not infected by CVB3. Symptoms and diseases are thought to arise from inflammation caused by CVB3 lysing cells so it can be released and infect new cells.

Most infections with CVB3 are asymptomatic or result in GI distress. However, it can cause more severe disease if it escapes the GI tract. CVB infection has been implicated in the development of type 1 diabetes. Both in vivo and in vitro data show that CVB3 can infect the pancreas, and despite its cytolytic release, can develop persistent infection within pancreatic cells. CVB infection of pancreatic cells in mice does lead to autoimmunity towards islet cells and biopsy samples from patients newly diagnosed with type 1 diabetes had persistent enterovirus infections with a low proportion of infected cells. Several antiviral therapies are currently being developed and proposed to prevent CVB infection, including a multivalent vaccine, as well as drugs to treat those already with a persistent CVB infection. However, most antivirals that are in clinical trials directly target the virus. CVB is able to mutate rapidly and has been shown to gain resistance to many of these proposed drugs, including enviroxime. The development of type 1 diabetes by CVB infection may also be dependent on the serotype. One study showed that in children with a genetic risk of developing type 1 diabetes, infection with CVB3 or CVB6 early in life actually protected those from
developing islet autoimmunity\textsuperscript{285}. However, no other studies have further supported or disproven this finding.

While CVB3 does infect the pancreas and the liver, it is mostly thought of as cardiotropic virus. Once CVB3 reaches the heart, most likely through the bloodstream, it binds to and enters cardiac fibroblasts and myocytes\textsuperscript{5}. Once in these cells, CVB3 shuts down cellular translation, alters host cell metabolism, and disrupts intracellular transport and structure. These alterations, as well as the presence of CVB3 proteins and immunogenic intermediates cause an inflammatory response within infected cells that lead to cell death via apoptosis, pyroptosis, ferroptosis, or necrosis\textsuperscript{286}. This damage to cells can lead to an autoimmune disease called myocarditis, the inflammation of heart muscle. This broad inflammation is triggered by immune cells infiltrating heart muscle and continually producing inflammatory cytokines and an inability to clear the infection\textsuperscript{287,288}.

It is currently not well understood how acute myocarditis progresses to chronic myocarditis and DCM, which is characterized by enlargement of the left ventricle due to remodeling. Current mouse models of CVB3 induced myocarditis show that the progression from acute to chronic myocarditis depends on the strain of mouse, the sex of the mouse (males being more susceptible), and the strain of CVB3. Some studies attribute a more prolonged inflammatory Th1 response in contributing to the progression of myocarditis while the Th17 response may contribute to the development of persistent infection and DCM\textsuperscript{287}. Some hypothesize that progression of disease is also related to differing expression of certain host-cell proteins such as viral myocarditis susceptibility gene (\textit{vms1}) and genes relating to the inflammatory response\textsuperscript{289,290}. Significantly more
work is needed to understand how CVB3 infection within the heart leads to chronic myocarditis and DCM.

The incidence of myocarditis and DCM has been significantly increasing in developing countries and is thought to be due to viral infection. Viral myocarditis mortality in young people is as high as 21% and sudden deaths in children due to viral myocarditis or arrhythmias is 20%.286,291 Unfortunately, once the disease progresses to DCM, a heart transplant must be performed. With CVB3 being a major driver of myocarditis, it is crucial that we continue to study this virus and develop treatment options.

**CVB3 Infection**

CVB3’s +ssRNA genome is roughly 7.5kb in length and the ORF consists of 11 viral proteins. There are roughly 800 nucleotides in the 5’ UTR and 100 nucleotides in the 3’ UTR. Viral proteins (VP) 4-1 make up CVB3’s icosahedral capsid that contains its genome (Fig. 13A). The other 7 proteins play important roles in replication and altering the host cell for replication. Interestingly, CVB3’s polyprotein contains 8 di-proline motifs, one in VP3, three in VP1, two in 2C, and two in 3A (Fig. 13B).

![Figure 13. Coxsackievirus B3.](image)

(A) representative CVB3 capsid with +ssRNA genome. (B) CVB3’s polyprotein. Orange lines denote di-prolines.
**Binding and entry.** Efficient CVB3 infection first starts with CVB3’s ability to bind and enter cells. The icosahedral capsid is made up of 60 protomers, with each protomer containing VP1, VP2, VP3, and VP4. VP1-3 make up the outer layer of the capsid while VP4 is internal. In polarized cells, such as epithelial cells in the intestines, CVB3 first attaches to DAF (also called CD55)\(^{292}\). DAF is a GPI-anchored protein that is present on the apical side of cells in lipid raft microdomains and forms clusters upon CVB3 binding\(^{293}\). When lipid raft formation is inhibited with cholesterol inhibitors like MβCD, DAF clustering is altered and CVB3 binding and entry was inhibited\(^{293}\). DAF clustering then triggers actin rearrangements within the cell that causes cell surface translocation to the tight junction\(^{294}\). Once at these tight junctions in polarized cells, CVB3 binds to its entry receptor, CAR. Under normal circumstances, CAR is an immunoglobulin-like transmembrane protein that aids in cell-to-cell adhesion and maintaining tight junctions. Interestingly, in non-DAF (i.e. CVB3 Nancy) and DAF binding strains of CVB3, lipid rafts were still required for CVB3 entry in the nonpolarized HeLa cell line\(^8\). Despite CVB3 Nancy being defined as a non-DAF binding strain, its VP3 protein still has a glutamine at 234 (Q234). Clinical isolates of CVB3 primarily have VP3 Q234 and have the highest binding affinity to DAF compared to other isolates that differed at VP3 amino acid 234\(^{295}\). VP1-T271 and VP2-D138 have also been shown to aid in DAF binding, with CVB3 Nancy instead having VP2-Q138, which may account for its decreased affinity for DAF\(^{296,297}\). Interestingly, when our group passaged CVB3 Nancy in cells treated with DFMO, one of the mutations that occurred was VP3 Q234R\(^{185}\). This virus had significantly higher binding to cells treated with DFMO compared to WT virus. The Q234R mutation changes the amino acid charge from negative to positive, potentially
counteracting the lack of polyamines and might be a site where polyamines directly bind to CVB3. This mutation may be able to arise due to CVB3 Nancy not requiring DAF to attach to cells and it solely alters its binding affinity to CAR. However, more work is needed to unravel how this mutation confers resistance. There is also a strain of CVB3, called CVB3-PD, that does not require either DAF or CAR, but instead uses heparin sulfates to bind and enter cells \(^{298}\).

The capsid of enteroviruses often "breath," in which the capsid alters between expanded and closed conformations. When CVB3 binds to CAR, this triggers conformational changes and locks the capsid in the expanded state, termed the A particle, and allows for endocytosis, capsid dissociation, and genome release \(^{299,300}\) (Fig. 14A). This process is hypothesized to be triggered by VP1 which is in the "canyon" of the icosahedral capsid and is where CAR binds \(^{301}\). VP1 contains a hydrophobic pocket that contains a host-cell lipid called "pocket factor," which is most often palmitate in enteroviruses \(^{301,302}\). When CAR binds, this triggers the pocket factor to be ejected and the formation of the expanded uncoating intermediate A particle. The A particle was thought to form at a pH of 6 when CVB3 was bound to CAR \(^{303}\). However, a recent study found that the pocket factor was ejected from CVB3 VP3 Q234 after 20 mins at pH of 7.4 when bound to CAR and looked similar to CVB3 VP3 Q234 bound to CAR at a pH of 5.5 for 20 mins \(^{295}\). However, it was not tested whether the pocket factor was ejected quicker at the lower pH. This same study also found that VP3 E234 seemed to be less stable and was able to eject the pocket factor after 10 min and a more acidic pH enhanced genome release \(^{295}\). Just how VP3 234 contributes to pocket factor release is unknown.
After CVB3 is endocytosed and the hydrophobic pocket collapses, it undergoes further conformational changes to form pores and release its genome into the cell (Fig. 14B). However, the mechanism behind this is poorly understood not just for CVB3 but the enterovirus genus as a whole. It is thought that low pH causes the release of VP4 which then forms pores within the endosome/liposome. At the same time, the hydrophobic pocket collapse in VP1 triggers the N-terminus of VP1, which contains an amphipathic helix, to be externalized and bind to the endosomal/liposomal membrane. This A particle is also no longer bound to CAR because of VP1 N-terminal exposure. A recent study with poliovirus suggest that the virion is directly associated with the pores formed by VP4 due to the observation of long, umbilical connectors between the capsid and the pores, protecting the RNA genome. This is further supported by the fact that RNA genome degradation of polio does not happen...
when RNases are present within the endosome/liposome\textsuperscript{306}. Since genome release does not occur right after receptor binding, and only after a period of time inside the cell, it is hypothesized that there is another uncoating cue\textsuperscript{307–309}. A potential uncoating cue is PLA2G16, a phospholipase found to be a universal host factor for enteroviruses that facilitates genome displacement\textsuperscript{310}. It is hypothesized that PLA2G16 is involved directly or indirectly with the pores formed in the endosomal membrane; however, its exact role remains unknown\textsuperscript{300}.

**CVB3 genome translation and replication.** Upon release of CVB3’s +ssRNA genome into the cytosol, it undergoes translation by host cell machinery. Since CVB3’s genome does not have a typical 5’ cap to recruit the ribosome, it instead relies on its IRES. This area within the 5’ UTR is highly structured with high GC content\textsuperscript{311}. With the aid of other host factors, the ribosome is recruited and starts translation of CVB3’s genome. The result of CVB3 translation is a polyprotein containing all 11 viral proteins. The polyprotein then undergoes a series of autocatalytic cleavages. The first is cleavage of the polyprotein into P1, P2, and P3. P1-P2 is cleaved by the 2A protease while P2-P3 is cleaved by the 3C protease\textsuperscript{312}. P1 is then cleaved into the 4 structural proteins by 3C and 3CD. P2 and P3 are cleaved into their respective proteins by 3C.

Besides cleavage of the polyprotein, the chymotrypsin-like proteases 2A and 3C cleave a reported 211 host proteins\textsuperscript{313}. One of the most well-known proteins that is cleaved by 2A is eIF4G. eIF4G is crucial for cap-dependent translation of mRNA, and cleavage of it shuts down cap-dependent translation. Furthermore, the cleaved c-terminal aids in the IRES-dependent translation of CVB3\textsuperscript{314}. It has also been reported that 2A is able to activate cholesterol and lipid synthesis through cleavage of an
unknown protein involved in the ERK1/2 pathway. 3C, besides cleaving a majority of the viral proteins, also cleaves host-factors such as STING and MAVS which senses the dsRNA intermediate that is formed during RNA virus infection. CVB3 has also been shown to mutate these proteins in response to polyamine depletion. Depletion of polyamines with DFMO treatment results in 2A Q29R and 3C Q52K, both of which have increased proteolysis activity in the absence of polyamines compared to WT virus. Interestingly, these mutants are not resistant to DENSpm treatment. However, CVB3 develops the S35G mutation in 2A which rescues eIF4G cleavage and 2A proteolytic activity during polyamine catabolism activation.

Replication of CVB3’s RNA genome involves the viral RNA-dependent RNA polymerase 3D, 3CD, 3AB, and 2C, which is a RNA helicase and RNA chaperone. 3B (also VPg) acts as a primer for the initiation of negative strand synthesis by 3D and binds to the 5’ end of +RNA genome. Negative charges at the lipid bilayer as well as 3B aids in the membrane recruitment of 3D. The 3D polymerase is also responsible for transcribing more +RNA genomes from the negative strand. VPg is hypothesized to translocate to the 3’-end of the negative strand with 3CD/3D to initiate transcription of +RNA. Host cell proteins also aid in transcription of both + and – strand RNA including PCBP2 and PABP which circularize the + RNA genome and hRNP C is thought to stabilize the dsRNA intermediate used to create more + RNA genomes.

The replication of CVB3’s genome occurs within ROs, which are specialized, fully enclosed, structures formed by CVB3 and act as a scaffold for replication on their exterior. It is hypothesized that these ROs prevent detection of viral RNA by innate immunity sensors. However, this is not the case for CVB3. There was no increased
activation of the IFN response with a mutant virus that does not form ROs. Other enteroviruses, such as polio, do seem to require ROs to evade host-cell sensors.

CVB3 RO formation first occurs at the ER membrane and then at the Golgi later in infection. The viral proteins 2B, 2BC, 2C, and 3A aid in the formation of ROs through direct action and by recruiting cellular proteins involved in the secretory and autophagy pathway, and recruiting lipid droplets. 2BC binds to lipid droplets and tethers them to ROs and in conjunction with host lipolysis proteins, 2BC transfers lipids to ROs to aid in their biogenesis. The precise role of 2B in RO biogenesis is unknown, but poliovirus 2B has been shown to have viroporin activity, which permeabilizes the ER and Golgi and is hypothesized to reduce apoptosis. Enterovirus-71’s (EV-71) 2C binds reticulon 3, which promotes ER membrane curvature; however, just how this interaction promotes RO biogenesis is unknown. The above proteins have also been shown to trigger esterified LC3 liposomes and promote autophagy. During CVB3 infection, there is an accumulation of these autophagosomes within cells, most likely due to 3C cleavage of SNAP29 and PLEKHM1, which promote autophagosome fusion. The role of these autophagosomes has yet to be elucidated; however, it is hypothesized that they are used to generate RO scaffolds or are involved in the non-canonical release of virus due to the prevention of autophagosome-lysosome fusion.

CVB3’s 3A protein is a membrane protein and is responsible for maintaining the optimal lipid and cholesterol content in the RO and altering the secretory pathway. It contains a proline-rich region which binds to GBF1, thereby blocking Arf1 activation and preventing ER-to-Golgi transport. This in-turn prevents reconstitution of the Golgi and
the CVB3 ROs having to compete with the Golgi for resources. 3A also promotes cholesterol transport to the ROs (Fig. 15). It does this by first binding acyl-CoA-binding domain-containing protein 3 (ACBD3)\(^ {327}\). ACBD3 is then able to recruit PI4Kβ to the ROs where it phosphorylates PI to PI4P\(^ {328}\). PI4P is then used by OSBP to exchange PI4P on the RO for cholesterol in the ER membrane as described in cholesterol transport\(^ {12}\). Cholesterol can also be transported to ROs via clathrin-mediated endocytosis in which 3A and PI4Kβ bind to Rab11 on recycling endosomes, preventing cholesterol from being transported back to the plasma membrane\(^ {12}\).

Cholesterol is important for proper replication, possibly through viral polyprotein processing\(^ {284}\). The inhibition of PI4Kβ resulted in reduced polyprotein processing, specifically at the 3A-3B junction, and delayed generation of ROs; however, the precise mechanism of how cholesterol influences polyprotein processing is unknown\(^ {284,329}\). CVB3 passaged in cells treated with the PI4Kβ inhibitors, enviroxime or GW5075, develop the mutation H57Y in 3A\(^ {330}\). This mutant was able to enhance the processing of
3A-3B in PI4Kβ inhibited cells to levels similar to those with WT virus in untreated cells\textsuperscript{284}. Since this mutation is close to the C-terminal hydrophobic domain, Melia, et. al, hypothesized that the mutation results in an altered conformation of 3AB that increases the accessibility of the 3AB cleavage site\textsuperscript{284}. Furthermore, this mutant no longer requires ROs during the exponential phase of viral RNA replication. PI4Kβ inhibited cells infected with the 3A\textsuperscript{H57Y} virus still formed ROs, but it was significantly delayed. Overall, it appears that cholesterol is required for proper replication, but PI4P is not needed for RO formation and is primarily used to acquire cholesterol.

**Assembly and release.** The assembly and release of enteroviruses is poorly understood. The host cell protein, heat shock protein 90, along with 3CD cleaves P1 into VP0 (VP2 and VP4), VP1, and VP3 creating a particle that self-oligomerizes\textsuperscript{300}. The capsid proteins then condense around replicating viral RNA, followed by cleavage of VP0, which is mediated by the viral RNA, and resulting in the formation of the mature virion\textsuperscript{331}. Current literature suggests that there is no package signal in the viral RNA and instead packaging is mediated by interactions between 2C and VP3\textsuperscript{332}.

CVB3 is a cytolytic virus, meaning that it is released through the death and lysis of the host cell. This allows for progeny virions to infect neighboring cells and start the replication process over again. However, CVB3 has also recently been shown to be released in extracellular vesicles (exosomes)\textsuperscript{333}. These exosomes are hypothesized to expand the tissue tropism of CVB3 and that CVB3 coupled to exosomes had increased mortality in mice\textsuperscript{333}. However, significantly more work is needed to determine just how exosomes enhance tissue tropism and mortality since CVB3 was not found inside the
exosomes. Other enteroviruses have been found within exosomes and this could account for the expanded tropism of CVB3 *in vivo*.
CHAPTER 3

METHODS

Cell Culture

Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies) with bovine serum and penicillin-streptomycin at 37°C and 5% CO₂. Huh7 cells were supplemented with 10% fetal bovine serum (FBS; Thermo-Fischer). Vero cells were obtained through Biodefense and Emerging Infections (BEI) Research Resources. Vero cells were supplemented with 10% new-born calf serum (NCBS; Thermo-Fischer).

Virus Infection

CVB3 (Nancy strain) was derived from the first passage of virus in Vero cells after rescue from an infectious clone. CVB3-GFP<sup>334</sup> (provided by Frank J.M. van Kuppeveld) was rescued from infectious clones and propagated in Veros. LaCrosse virus (LACV, strain NR-540) was obtained from BEI Research Resources. Human Rhinovirus 16 (HRV16) was provided by William T. Jackson and were rescued from infectious clones and propagated in Vero cells. Cells were infected at a multiplicity of infection (MOI) of 0.1 plaque forming units (PFU) unless otherwise noted. Viral stocks were maintained at -80°C. Viral titers were enumerated via plaque assay.
**Plaque Assay**

Vero cells were seeded into 24-well plates with fresh medium supplemented with 10% NBCS at a density to reach 100% confluency on the day of infection. Infected cell supernatant was serially diluted in serum-free DMEM and directly added onto the monolayer of Vero cells for 10-15 min at 37°C. Cells were overlain with 0.8% agarose in DMEM containing 2% NBCS (CVB3, HRV14, LACV) or serum-free DMEM (VACV). CVB3 and HRV14 samples were incubated for 2 days. LACV was incubated for 5 days. VACV was incubated for 1 day.

**Drug Treatments**

DFMO (TargetMol) was diluted to a 100 mM solution in sterile water. For DFMO treatments, cells were trypsinized and reseeded with fresh medium supplemented with 2% FBS (Huh7) or 2% NCBS (Vero). Cells were treated with 1 mM DFMO unless otherwise indicated. Cells were incubated with DFMO for 96h to allow for depletion of polyamines. GC7 (Caymen Chemical) was diluted to a 100 mM solution in sterile water. Cells were trypsinized and reseeded with fresh medium supplemented 2% FBS (Huh7s) or 2% NBCS (Vero) for 16-24h. Cells were then treated with GC7 and 500 µM aminoguanidine at indicated times. Cholesterol was diluted to 10 mg/ml in ethanol then added to adherent cells for 16-24h. GW5074 (TargetMol) was diluted to a 100 mM solution in DMSO. Ribavirin (Caymen Chemical) was diluted to a 200 mM solution in DMSO. Difluoromethylornithine (DFMO; TargetMol) was diluted to a 100 mM solution in sterile water.
RNA Purification and cDNA Synthesis

Media were cleared from cells, followed by 1 PBS wash, and 200 µL of Trizol reagent (Zymo Research) was added directly. Lysate was then collected, and an equal amount of 100% molecular grade ethanol was added to the lysate. RNA was purified through a Zymo RNA extraction kit according to manufacturers protocol. RNA was eluted in 20 µL of sterile water. Purified RNA was subsequently used for complementary DNA (cDNA) synthesis using High Capacity cDNA Reverse Transcription Kits (Thermo-Fischer), according to the manufacturer’s protocol, with 10–100 ng of RNA and random hexamer primers.

Thin-layer Chromatography of Polyamines

Huh7 cells were plated at a density of 8 x 10^4 in a 6-well plate in 2% FBS DMEM and treated with the indicated doses of DFMO for 96h. Cells were trypsinized and then spun down at 5000 rpm for 5 min. Pellets were washed with PBS and then resuspended in 200 µL 2% perchloric acid. Samples were then incubated overnight at 4°C. Supernatant or 1 µM of putrescine, spermidine, and spermine standards were combined with 5 mg/mL dansyl chloride (Sigma Aldrich) in acetone and saturated sodium bicarbonate. Samples were incubated in the dark overnight at room temperature. Excess dansyl chloride was cleared by incubating the reaction with proline (Sigma Aldrich). Dansylated polyamines were extracted with toluene (Sigma Aldrich) and centrifuged. Sample was added in spots to silica gel matrix TLC plates (Sigma Aldrich) and exposed to ascending chromatography with 1:1 cyclohexane: ethyl acetate. The plate was dried and visualized via exposure to UV.
**RNA Sequencing**

RNA was purified and prepared as described from Huh7 cells treated for 96h with DFMO or infected for 24h with CVB3. Libraries were prepared by the University of Chicago Genomics Facility and analyzed by Illumina NovaSeq 6000. Read quality was evaluated using FastQC (v0.11.5). Adapters were trimmed in parallel to a quality trimming (bbduk, sourceforge.net/projects/bbmap/). All remaining sequences were mapped against the human reference genome build 38 with STAR (v2.5.2b)\(^{335}\). HTseq (v0.6.1) was used to count all reads for each gene and set up a read count table\(^{336}\). Differential gene expression analyses were performed using the DESeq2 Bioconductor package (v1.30.1)\(^{337}\). The default “ashr” shrinkage (v2.2-47)\(^{338}\) set up was used for our analysis. Gene set enrichment analysis (GSEA) was performed with the fgsea Bioconductor package\(^{339}\), using Hallmark gene sets downloaded from the Molecular Signatures Database\(^{340}\).

**Cell Viability**

Huh7 cells (1 x 10\(^4\) cells/well) were plated in a 96 well plate with 2% FBS DMEM, then treated for 24h with aminoguanidine alone or in combination with increasing concentrations of GC7. After 24h, viability was measured using the CytoTox-Fluor Cytotoxicity Assay (Promega) according to the manufacturer’s protocol.

**Intracellular Cholesterol Abundance Assay**

Huh7 cells were plated at a density of 5000 cells/well in a 96 well plate in 2% FBS DMEM. Cells were treated with DFMO for 96h or after 72h, treated with DEF or GC7 for 24h. The following day, the media was removed from cells followed by a PBS wash. To measure total intracellular cholesterol abundance, we used the
Cholesterol/Cholesterol Ester-Glo Assay (Promega) in accordance to manufacturer’s protocol.

**qPCR Gene Expression Assay**

Huh7 cells were seeded at 4 x 10^4 (DFMO) or 1 x 10^5 (GC7) cells per well in 24-well plates in DMEM with 2% FBS. Cells were treated with varying concentrations of DFMO for 96h or GC7 and aminoguanidine for 16-24h. The media was aspirated off cells, washed 1x with PBS, and then, 200 µL of Trizol was added to the cells. The RNA was extracted with the Zymo RNA extraction kit, converted to cDNA, and quantified by real-time PCR with SYBR Green (DotScientific) using the one-step protocol QuantStudio 3 (ThermoFisher Scientific). Relative expression was calculated using the ΔΔC\textsuperscript{T} method, normalized to the β-actin qRT-PCR control, and calculated as the fraction of the untreated samples. Primers were verified for linearity using 8-fold serial diluted cDNA and checked for specificity via melt curve analysis. The primer sequences are in Table 1.

**Table 1. List of qPCR Primers.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer set (5’-3’)</th>
</tr>
</thead>
</table>
| HMGCR | F: GAGACAGGGATAAACCAGAAAG  
R: GGAGGAGTTACCAACCACAAA |
| HMGCS | F: CCTGCCAAGAAAGTACCAAGA  
R: GTCTTGCACCTCACAGATATC |
| MVD   | F: TGGTTCTGCCCATCAACT  
R: GGTGAAGTCCTTGCTGATGA |
| SREBP2| F: CTGTAGCGTCTTTGATTCTCTCC  
R: CCTGGCTGTCCTGTGTAATAA |
| β-Actin| F: CACTCTTGCAGCCTTCCTTC  
R: GTACAGGTCTTTGGCAGATGT |
| CVB3  | F: GTGCTGTGTATACCCTTGATAAG  
R: GCTGGGACCACATGTGTGTTG |
Western Blot

$8 \times 10^4$ (DFMO) or $4 \times 10^5$ (GC7) Huh7 cells per well were plated in 6-well plates in 2% FBS DMEM 16-24h before treatment. Samples were collected with Bolt LDS Buffer and Bolt Reducing Agent (Invitrogen, Waltham, MA, USA) and run on 10% polyacrylamide gels. Gels were transferred using the iBlot 2 Gel Transfer Device (Invitrogen). Membranes were blocked with either 5% BSA or 5% milk in TBST depending on the antibody used (Table 2) and incubated overnight at 4°C. Membranes were then rinsed 3x in TBST followed by 3 10 min TBST washes at RT. Secondary antibody was then added to the membrane for 1h at RT. Membranes were then rinsed 3x with TBST followed by 3 5 min TBST washes. Membranes were treated with
SuperSignal West Pico PLUS Chemiluminescent Substrate (ThermoFisher Scientific) and visualized on Fluorchem E imager (Protein Simple, San Jose, CA, USA) for blots in Chapter 4 or on BioRad ChemiDoc XRS+ with Image Lab Software for blots in Chapter 6. Quantification of western blots were done by using ImageJ and normalizing to NT and relative to actin density.

**qPCR CVB3 Relative Genome Assay**

Huh7 cells were seeded at 4 x 10^4 cells per well in 24-well plates in DMEM with 2% FBS. Cells were treated with 500 µM GC7 and aminoguanidine at the time of infection. CVB3 was added directly to cells (0h) or into medium (2, 4, 8, 10h) at an MOI of 1 (CVB3-GFP) or an MOI of 5 (CVB3 WT/2A3A). RNA was then purified as described above. Relative expression was calculated using the ΔΔCT method, normalized to the β-actin qRT-PCR control, and calculated as the fraction of the input virus (0h).

**SRE Promoter Luciferase**

Complimentary primers were made containing SRE consensus sequence were ordered flanked by SfiI cut site overhangs (FWD: 5’-CGGCCATCACCCACGGCCTCGG-3’; REV 3’-GCCGCCGGTAGTGGGGTGCCGGAGA-5’). Primers were phosphorylated and annealed at 37°C for 30 minutes then 95°C for 5 minutes and were allowed to cool to 25°C. pGL4.10 (Promega) was digested with SfiI in Fast Digest buffer for 15 min at 50°C. The cut plasmid was purified with Zymo DNA clean up kit. The annealed primers were then ligated into the cut plasmid using T4 ligase followed by transformation into chemical competent E. Coli. Colonies were picked and grown up followed by sequencing to confirm the SRE sequence was present.
**Promoter Luciferase Assay**

7000 Huh7 cells/well were plated in a 96 well plate with 2% FBS DMEM then treated with 1 mM DFMO for 96h or after 96h, treated with 500 µM GC7. Cells were transfected with SRE-pGL4.10, 5’ HMGCS-Fluc (Addgene #60444), or pLDLR-Luc (Addgene #14940) after cells had been plated for 96 h. All cells were transfected with the Renilla control plasmid (siCheck, Promega). 100 ng of plasmid were transfected with LipoD293 according to manufacturer’s protocol. 24h after transfection, media was removed followed by one wash with PBS. Cells were then lysed with gentle lysis buffer for 15 min.

**SREBP2 Luciferase Assay**

SREBP2 fragment 1 contained nucleotides 3348-3798 and fragment 2 contained nucleotides 3348-4400 from Addgene #32018 (pLKO-puro FLAG SREBP2, murine SREBP2) were cloned into pLenti-CMV-Luc by Applied Biological Materials Inc. Huh7 cells were plated at 5000 cells/well in a 96 well plate and treated with 500 µM GC7 and aminoguanidine for 24h. Cells were transfected with 100 ng of plasmid were transfected with LipoD293 according to manufacturer’s protocol. 24h after transfection, media was removed followed by one wash with PBS. Cells were then lysed with gentle lysis buffer for 15 min.

**Plaque Formation Attachment Assay**

Vero cells were seeded in 6-well plates at 2.5 x 10^5 and grown to 100% confluence in DMEM with 2% NCBS and treated for 96h with the indicated concentrations of DFMO or GC7. After 96h of DFMO treatment or 16h of GC7, cells were placed on ice and the media aspirated from the cells. 500 µL of serum free media
containing 1000 PFU CVB3 was added to cells on ice for 5 min. Cells were washed 3x with PBS and then overlaid with 0.8% agarose containing DMEM with 2% NCBS. The plates were then incubated at 37°C for 2 days for plaques to develop. The cells were fixed with 4% formalin, and the plaques were visualized with crystal violet staining. For the cholesterol rescue, cells were washed 3x with PBS before infecting with CVB3.

**Crystal Violet Viability Assay**

Vero cells were seeded at a density of $5 \times 10^4$ per well in a 96-well plate with 2% FBS DMEM. Cells were treated with the indicated doses of Atorvastatin or Lovastatin for 24h. The media was then removed, and the cells were fixed with 4% formalin for 15 min. The formalin was then removed, and crystal violet was added directly to the wells and allowed to sit for 5 min followed by a quick diluted bleach and water wash. The plate was then allowed to dry overnight. The next day, 100 µL of 10% acetic acid was added to each well to solubilize the crystal violet. The absorbance at 590 nm was then read.

**qPCR Attachment Assay**

Vero cells were seeded at $4 \times 10^4$ per well in a 24 well plate with 2% NCBS DMEM. Cells were treated with GC7 for 24h or with DFMO for 96h. Cholesterol was added to indicated samples for 24h. Cells were placed on ice, media removed, and CVB3 at MOI 5 in 100 µL DMEM was then added directly to cells for 5 min. Input samples were then collected in TRIzol. Bound samples were washed 3x in PBS then collected in TRIzol. RNA was then extracted, purified, then reverse transcribed as described. cDNA was then quantified by real-time PCR with SYBR Green as described.
Relative genomes were calculated using the ΔΔCT method with actin and normalized to input virus.

**CVB3 Replicon Assay**

Huh7 cells were plated in a 96 well plate with 2% FBS DMEM 16-24h before treatment. Cells were treated with 500 µM GC7 and aminoguanidine, 2 mM guanidine HCL, or 400 µM Ribavirin 15 min before transfection. Cells were transfected with either 100ng pCB3-T7-RLuc replicon341 (provided by Frank J.M. van Kuppeveld) and 200ng T7 or with 300ng Renilla control plasmid (siCheck, Promega). With LipoD293 according to manufacturer's protocol. 24h after transfection, media was removed, and cells were lysed with gentle lysis buffer for 15 min at 37°C.

**CVB3 Passages**

Huh7 cells were plated in 24 well plates with 2% FBS DMEM. Half of the plate was left untreated, and the other half was treated with 500 µM GC7 and aminoguanidine 16h before infection. The first row of cells were infected at an MOI of 0.1. 24h later, 10-30 µL of cell supernatant was used to infect the corresponding well in the next row. This was repeated until passage 23.

**CVB3 Sequencing**

200 µL of passage 23 supernatant was added to 200 µL TRizol and RNA was extracted with Zymo RNA extraction kit and converted to cDNA. cDNA was sent to GENEWIZ for sequencing with primers spaced 1000bp apart. Sequences were aligned to CVB3 (Nancy) in Benchling.
**CVB3 Mutagenesis**

CVB3 2A^{Q85L} and CVB3 3A^{H57Y} mutagenesis was done by GENEWIZ. CVB3 2A^{Q85L}3A^{H57Y} was cloned by restriction enzyme digest using BssHII and BstEII on CVB3 2A^{Q85L} (backbone) and CVB3 3A^{H57Y} (fragment). Cut plasmid and fragment were purified using Zymoclean gel DNA recovery kit (Zymo Research) according to manufacturer’s protocol. 75ng of 3A^{H57Y} purified fragment was ligated to 25ng of purified 2A^{Q85L} cut plasmid using T4 ligase in a 10 µL reaction for 2h at room temperature. The ligation reaction was then directly transformed into STABL3 Mix & Go (Zymo Research) cells according to manufacturer’s protocol.

**Live Cell Imaging and Quantification**

Huh7 cells were plated on µ-Slide 8 well high ibiTreat (ibidi) in 2% FBS Optimum for 24h. Cells were then treated and infected with CVB3-GFP at an MOI of 1. Images were captured on a Nikon Ti2-E inverted microscope equipped with X-Cite XYLIS XT720S Broad Spectrum LED Illumination System (modified with OD 1.5 neutral density filter), Nikon Perfect Focus, Okolab stage-top incubation chamber, DS-Qi2 CMOS camera, and CFI Plan Apochromat Lambda D 40X air NA 0.95 and Plan Apo Lambda 20x air NA 0.75 objectives. Time lapse fields were configured immediately after inoculation at 37°C and 5% CO₂. 8 fields per condition were imaged every 30 minutes until 16 hours post infection. Time-lapse images were analyzed using a custom macro written for the FIJI package of Image J, which has been made available in a GitHub repository: https://github.com/abbykroken/GFP-cell-accumulation. In brief, the macro segments GFP-positive cells using a global threshold over the entire stack, and measures the GFP-positive area and mean intensity in each frame of the time-lapse.
**Immunofluorescence**

Huh7 cells were seeded in 6-well plates containing cover slips in 2% FBS DMEM for 16h. Cells were then left untreated or treated with 500 μM GC7 and aminoguanidine. Right after treatment, cells were infected with CVB3 at an MOI of 5. At 6 hpi, media was removed, and the cells were washed 1x with PBS. Cells were then fixed with 4% formalin for 15 min then washed 3x with PBS. Cells were permeabilized with 0.2% Triton X-100 and 2% BSA (blocking buffer) for 1h at room temperature (RT). Cells were then incubated with 1:500 primary mouse anti-dsRNA (Nordic-MUbio, K1) in blocking buffer for 2h at RT. Cells were then washed 3x with PBS followed by incubation with 1:500 GtαMs conjugated to Alexa Fluor™ 488 in blocking buffer for 1h at RT in the dark. Cover slips were placed on glass slides with Vectashield Plus antifade mounting medium with DAPI. Samples were imaged with Zeiss Axio Observer 7 with Lumencor Spectra X LED light system and a Hamamatsu Flash 4 camera using appropriate filters using Zen Blue software with a 60x objective.

**Transmission Electron Microscopy**

Huh7 cells were plated in a 6-well plate in 2% FBS DMEM for ~16h. Cells were then treated with 500 μM GC7 and aminoguanidine or left untreated. At the same time, cells were infected with CVB3 at MOI 5. After 6h, the media was removed, and cells were washed 1x with PBS. Cells were then trypsinized at 37°C for 10 min. The trypsin was neutralized with 2% FBS and spung down at 800g for 10 min at 4°C. The supernatant was removed and 200 μL of 4% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 (fixative buffer) was slowly added to the pellet and fixed for 10 min. Then 200 μL of fixative buffer was slowly added to the pellet and the pellet was dislodged and
fixed for an addition 40 min. The samples were then spun down again and stored at 4°C before being sent to Indiana University Electron Microscopy Center for imaging.

**Statistical Analysis**

Prism 6 (GraphPad) was used to generate graphs and perform statistical analysis. Data are presented as means ± SEM. For all analyses, a two-tailed Student’s *t* test was used to compare groups, unless otherwise noted. All experiments were done in triplicate.
CHAPTER 4
POLYAMINE DEPLETION REDUCES CHOLESTEROL SYNTHESIS THROUGH TRANSLATION OF SREBP2

Parts of this chapter are reprinted with modifications from Firpo et al. 2023²⁰

Introduction

Polyamines are involved in diverse roles throughout the cell including gene transcription and translation, genome organization, cell signaling, etc. Due to this, we hypothesized that depleting polyamines would significantly alter genes or pathways that would negatively affect CVB3 infection through a yet unknown mechanism. To determine how polyamines’ cellular roles affect CVB3, we first had to unravel which gene(s) and pathway(s) are most affected by polyamine depletion and the mechanism behind how polyamines alter them.

Results

Polyamine Depletion Significantly Alters Cellular Gene Expression

To determine how polyamines influence cellular gene transcription, we first confirmed that Huh7 cells treated with DFMO had reduced polyamines via thin-layer chromatography (TLC) (Fig. 16A). DFMO treatment resulted in the complete loss of spermidine and the partial loss of spermine. This partial loss of spermine due to DFMO treatment has been seen in multiple studies²⁹,3⁴². Huh7 cells were then treated with 1 mM DFMO for 96h. After, total cellular RNA was extracted and analyzed by Illumina paired-end reading. After the alignment of reads against human genome,
differential gene expression analysis was conducted to identify significant changes in expression. Compared to no-treatment cells (NT), cells treated with DFMO had 4,413 genes whose expression was significantly changed ($p < 0.05$). We plotted these genes on a volcano plot with significance and Log$_2$Fold Change (Log$_2$FC) and overlayed the top gene hits (Fig. 16B). The top, downregulated genes include the RAN like binding protein $RANBP3L$ (-3.756 Log$_2$FC), $HID1$ (-2.011 Log$_2$FC), $carbonic anhydrase 5B$ (-1.872 Log$_2$FC), and $SAT1$ (-1.819 Log$_2$FC). The top, upregulated genes include $AMD1$ (2.278 Log$_2$FC), the zinc finger protein $ZBTB20$ (1.789 Log$_2$FC), and $hyaluronan synthase 2$ (1.768 Log$_2$FC). The top five up- and downregulated genes are represented in Table 3. The downregulation of $SAT1$, and the upregulation of $AMD1$, further confirms that polyamines were depleted since the cells were trying to compensate for the lack of polyamines.
Figure 16. Polyamine Depletion Alters Cellular Gene Expression. (A) TLC of Huh7 cells treated with increasing doses of DFMO for 96h. Putrescine, spermidine, and spermine standards are shown on the left. (B) Volcano plot indicating \( \log_{2}FC \) for genes from differential gene expression analysis comparing DFMO treated cells relative to untreated cells. Significant changes in gene expression are plotted in purple for genes: \( p\text{-adj}<0.05, -1<\log_{2}FC>1 \), in orange for \( p\text{-adj}<0.05, -1<\log_{2}FC<1 \) and in yellow for \( p\text{-adj}>0.05, -1<\log_{2}FC<1 \). P-values were adjusted for false discovery rate using Benjamini Hochberg method. Results are based on duplicates.

Table 3. Top Five Up- and Downregulated Gene Hits.

<table>
<thead>
<tr>
<th>Gene</th>
<th>( \log_{2}FC )</th>
<th>Gene</th>
<th>( \log_{2}FC )</th>
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<tbody>
<tr>
<td>AMD1</td>
<td>2.28</td>
<td>RANBP3L</td>
<td>-3.76</td>
</tr>
<tr>
<td>ZBTB20</td>
<td>1.78</td>
<td>HID1</td>
<td>-2.01</td>
</tr>
<tr>
<td>HAS2</td>
<td>1.77</td>
<td>IL11</td>
<td>-1.96</td>
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<tr>
<td>ZNF460</td>
<td>1.53</td>
<td>VNN2</td>
<td>-1.94</td>
</tr>
<tr>
<td>CASC19</td>
<td>1.48</td>
<td>CA5B</td>
<td>-1.87</td>
</tr>
</tbody>
</table>
Cholesterol Homeostasis is Altered by Polyamine Depletion

To uncover the underlying biological processes that were most affected by polyamine depletion, we performed a gene set enrichment analysis (GSEA). This showed multiple pathways that were significantly enriched for decreased gene expression by polyamine depletion, including mTORC1 signaling, cholesterol homeostasis, and the p53 pathway (Fig. 17A). Since mTORC1 signaling acts as a global regulator of cellular metabolism and growth, we decided to further explore cholesterol homeostasis due to its importance for CVB3 infection343. In order to investigate specific cholesterol genes affected by DFMO, cholesterol genes involved directly in cholesterol synthesis were overlayed in a volcano plot (Fig. 17B). Multiple genes were down regulated, including HMGCS, HMGCR, and mevalonate diphosphate decarboxylase (MVD) (Fig. 17C). Thus, the transcriptomic analysis of polyamine depleted cells revealed that cellular metabolic processes and, specifically, cholesterol biosynthesis are significantly decreased.
Figure 17. Cholesterol Homeostasis is Down During Polyamine Depletion. (A) GSEA was conducted on genes differentially expressed by 1 mM DFMO treated Huh7s versus untreated Huh7 cells. The top positively and negatively enriched Hallmark pathways were plotted (p.adj<0.05, pink, p.adj >0.05, purple). (B) Volcano plot indicating Log₂FC for genes from differential gene expression analysis comparing DFMO treated cells relative to untreated cells. Significant changes in gene expression are plotted in purple for genes: p.adj<0.05, -1<Log₂FC<1, in orange for p.adj<0.05, -1<Log₂FC<1 and in yellow for p.adj>0.05, -1<Log₂FC<1. P-values were adjusted for false discovery rate using Benjamini Hochberg method. (C) Cholesterol synthesis pathway with the representation of down-regulated genes HMGCS, HMGCR, and MVD. Values indicated fold change in gene expression. Results are based on duplicates.
Polyamine Depletion Decreases Cholesterol Gene Expression, Protein Levels, and Intracellular Cholesterol

To test polyamines’ effect on the transcription of cholesterol synthesis genes and confirm the RNA-seq data, cells were treated with increasing concentrations of DFMO. RNA was then extracted, and RT-qPCR was performed using optimized and specific primers. HMGCR, HMGCS, and MVD showed moderate but significant reductions in expression (Fig. 18A-C), aligning with the RNA-sequencing data.

To determine if this reduction in transcription affected protein synthesis, we examined total cellular levels of HMGCR and MVD by western blot. Both proteins showed reduced levels with polyamine depletion (Fig. 19A). Finally, to determine if reduction of transcription and translation of cholesterol synthesis proteins affected...
intracellular cholesterol, cells were treated with DFMO, and total cellular cholesterol was measured via a luciferase-based cholesterol assay. We found that total cellular cholesterol was significantly reduced with DFMO treatment, consistent with a decrease in expression of cholesterol synthesis genes (Fig. 19B). Thus, cellular cholesterol synthesis relies on polyamines through the expression of cholesterol synthetic proteins.

Figure 19. Cholesterol Synthesis Proteins and Intracellular Cholesterol are Decreased during DFMO Treatment. (A) Western blot of Huh7 cells treated with increasing doses of DFMO. Actin was used as a loading control. Protein abundance is normalized to NT and relative to actin. (B) Intracellular cholesterol abundance in DFMO treated Huh7 cells. **** p < 0.0001 by the Student's t test. Data from at least three independent experiments. Error bars represent standard error.
Polyamine Depletion Decreases SREBP2 Translation and Activity but not Gene Expression

Having observed that an array of cholesterol synthetic enzymes were reduced in transcription and translation by polyamine depletion, we considered that polyamines may affect a master regulator of their expression, rather than each gene individually. We hypothesized that polyamines were affecting the transcription factor SREBP2, one such regulator. To test the impact polyamines have on SREBP2, Huh7 cells were treated with increasing doses of DFMO followed by qPCR (Fig. 20A). Unlike the cholesterol synthetic genes, we observed no significant change in $SREBP2$ transcripts. However, examining SREBP2 protein levels by western blot revealed that polyamine depletion caused a reduction of SREBP2 protein (Fig. 20B), though this reduction appeared to level off despite increasing doses of DFMO. To determine if this reduction of SREBP2 translation was sufficient to impact the expression of cholesterol synthesis genes, we measured the activity of SREBP2 binding to its promoter, the SRE. We transfected cells with or without polyamines with a construct encoding firefly luciferase driven by distinct cellular SREs (Fig. 20C). We used the $HMGCS$ SRE, the $LDLR$ SRE, and a generalized SRE created with the SRE consensus sequence. Additionally, we transfected Renilla luciferase to control for the effects of polyamine depletion on transfection efficiency. When we measured SRE activity, we noted a significant reduction in activity in polyamine depleted cells for all SREs tested, suggesting that polyamine depletion impacts SRE promoter activity, likely due to a reduction in SREBP2 synthesis. Thus, polyamines facilitate translation and activity but not transcription of SREBP2.
A well-described mechanism by which polyamines support cellular translation is through the post-translational modification of eIF5A, in which spermidine is conjugated and hydroxylated, forming a unique amino acid called hypusine (summarized in Fig. 4). eIF5A is currently the only known protein to undergo hypusination in eukaryotic cells. Hypusinated eIF5A facilitates translation on a subset of cellular proteins often containing polyproline tracts, of which human SREBP2 contains 5\textsuperscript{21}. We first confirmed that DFMO treatment reduced levels of hypusinated eIF5A (Fig. 21A). To determine if SREBP2 is included in the subset of cellular proteins sensitive to hypusination, we
treated cells with the specific DHPS inhibitor, GC7. GC7 treatment significantly reduced SREBP2 protein levels, though no change in total elf5A levels was observed (Fig. 21B). In concurrence with this, 500 µM GC7 resulted in a significant decrease in SRE promoter activity (Fig. 21C). SREBP2 mRNA expression with GC7 treatment resulted in no significant changes in gene expression (Fig. 21D) as seen with DFMO and consistent with a role for hypusination in translation. Further, we observed no significant change in viability with GC7 treatment (Fig. 21E).

**Figure 21. GC7 Inhibits SREBP2 Translation.** (A) Western blot of Huh7 cells treated with increasing doses of DFMO for 96h and analyzed for hypusinated elf5A. (B) Western blot of Huh7 cells treated with increasing doses of GC7 probed for SREBP2, hypusine, and total elf5A. SREBP2 abundance relative to actin and hypusine abundance relative to total elf5A. (C) Huh7 cells treated with 500 µM GC7 for 24h followed by transfection with the SRE promoter luciferase constructs and Renilla luciferase as a transfection control. Results were normalized to NT and relative to Renilla luciferase activity. Two-way ANOVA was used to analyze statistical significance. (D) Huh7 cells were treated with increasing doses of GC7 (50, 300, 700 µM) and SREBP2 transcription measured by RT-qPCR. (E) Cells were treated as in (D) and analyzed for viability after 24h of treatment. *p < 0.05, **p < 0.01, and ***p < 0.001 by the Student's t test. Data representative of at least three independent experiments.

To determine if cholesterol synthesis genes were affected by hypusination inhibition, we treated cells with GC7 or the non-specific DOHH inhibitor DEF and measured HMGCR and MVD via western blot (Fig. 22A and B). However, there was no
change in MVD protein levels. To confirm that these changes in SREBP2 and cholesterol synthesis gene expression affected cellular cholesterol, we measured total cellular cholesterol in cells treated with increasing doses of GC7 (Fig. 22C) and DEF (Fig. 22D). Similar to our results with DFMO, we observed a significant, dose-dependent reduction in cellular cholesterol when hypusination was inhibited, suggesting that polyamines facilitate cholesterol synthesis through hypusination.

**Figure 22. Cholesterol Synthesis is Impacted by Hypusination Inhibition.** (A, B) Western blots of Huh7 cells treated with GC7(A) or DEF(B) for 24h and analyzed for HMGCR and MVD. Actin was used as a loading control. (C, D) Intracellular cholesterol abundance in GC7(C) or DEF(D) Huh7 cells treated for 24h. *p < 0.05, **p < 0.01, ****p < 0.0001 by the Student’s t test.

**SREBP2’s di-prolines Require eIF5A-hypusine for Translation**

SREBP2 contains di-proline motifs within its N-terminal domain, which are a known motif sensitive to eIF5A hypusination. To determine if these di-prolines are responsible for the decrease in SREBP2, we designed mouse SREBP2 fragments,
stopping before (construct 1) or containing di-proline motifs (construct 2), in pLenti-CMV-Luc (summarized in Fig. 23A). We transfected these constructs into cells treated with 500 µM GC7 and measured luminescence 24h later and normalized to untreated cells (Fig. 23B). We observed a significant reduction in luminescence for the polyproline-containing fragment (construct 2) compared to construct 1, lacking the di-proline motif. There was no significant difference in luminescence between fragment 1 and the vector, suggesting that the reduction in translation with fragment 2 was specific to the sequence containing the di-proline motif.

![Diagram of constructs](image)

**Figure 23. SREBP2’s di-prolines Limit Translation in the Absence of eIF5A-Hypusine.** (A) Constructs of SREBP2 fused to luciferase used in (B) to measure translation after treatment with 500 µM GC7. Firefly luciferase activity was measured and normalized to Renilla luciferase activity, comparing NT to GC7 treatment conditions. ****p < 0.0001 by the Student’s t test. Data from three independent experiments.
Discussion

Polyamines function in diverse ways within the cell, and their connections to distinct metabolic pathways are still being discovered\textsuperscript{26,81,131}. Here, we establish a novel connection between polyamines and cholesterol synthesis. Prior work has described how animal models treated with DFMO exhibit altered serum lipid profiles, though the molecular mechanisms remained unexplored\textsuperscript{18,19}. We found that polyamines support intracellular cholesterol levels through the hypusination of eIF5A. eIF5A-hypusine is required for the translation of SREBP2, a key regulator in cholesterol homeostasis. When cells were depleted of polyamines with DFMO, or hypusination was directly inhibited, SREBP2 translation was significantly down compared to untreated cells. This reduction of SREBP2 causes a significant reduction of sterol synthesis gene expression, culminating in reduced cellular cholesterol (Fig. 24).

\textbf{Figure 24. Model of Polyamines’ Influence on Cholesterol Synthesis.} Polyamines facilitate hypusination of eIF5A, which promotes SREBP2 synthesis. SREBP2 binds to SREs in the promoters of cholesterol synthesis genes, leading to their expression and cholesterol synthesis.

Due to polyamines’ diverse roles within the cells, a large number of genes exhibited significant changes upon polyamine depletion. Individual genes that were involved in various aspects of RNA processing were significantly down. However, the molecular pathway that had the most overall decrease in gene expression was the mTORC1 pathway. The mTORC1 signaling pathway is responsible for relaying extracellular and intracellular information to the cell and eliciting changes in metabolism, proliferation, and survival. mTORC1 is involved in lipid synthesis, autophagy, protein
synthesis, and even microtubule organization. This piece of data shows that polyamines are involved in multiple pathways and have broad effects on the cell. It is possible that the genes in this pathway were downregulated due to the long treatment time of DFMO. Serum used in cell culture contains polyamines, and due to the presence of polyamine transporters used to import polyamines, cells must be treated with DFMO for at least 72h to deplete extracellular polyamines and intracellular polyamine pools. However, the long treatment time and lack of polyamines could result in a decreased ability of the cell to uptake nutrients from the serum, leading to the decrease in expression of genes in the mTORC1 signaling pathway. This could also explain the increased expression of genes involved in the G2 checkpoint. A lack of polyamines, which are crucial for cellular proliferation, would signal to the cell that it should not undergo mitosis. Likewise, decreased nutrients within the serum could also cause cells to become senescent.

The next gene pathway that exhibited significantly altered gene expression was the cholesterol homeostasis pathway. Unlike the mTORC1 pathway, cholesterol homeostasis encompasses a narrower set of genes and is a specific pathway compared to the broad signaling pathway of mTORC1. Furthermore, polyamines have been shown to affect serum cholesterol and cholesterol is important for CVB3, which is further explored in Chapter 5. Despite the cholesterol homeostasis pathway exhibiting the second most reduced expression, cholesterol synthesis genes had marginal decreases in expression. HMGCR had only a -0.25 Log₂FC and SREBP2 had a 0.19 Log₂FC. This could be a result of the sensitivity of RNA sequencing and the inability to perform more independent repeats due to cost. Regardless of the modest change in cholesterol
synthesis genes, RT-qPCR showed significant decreases in HMGCS, HMGCR, and MVD when cells were treated with DFMO. Furthermore, we also saw HMGCR protein levels drop to 24% in cells treated with 1 mM DFMO compared to NT. Conversely, HMGCR expression dropped by about 50% in cells treated with 1 mM DFMO. This difference between expression and translation is most likely the result of HMGCR mRNA being translated multiple times. However, it is possible that HMGCR itself requires eIF5A-hypusine for its translation. HMGCR does not have any di-prolines, but it does have the 3 amino acid motif GGT at position 807 (UniProt #P04035), which has been shown to require eIF5A-hypusine. This potential sensitivity to hypusination inhibition could also explain why HMGCR protein is down but not MVD (Fig. 22A/B).

MVD protein levels were shown to decrease with DFMO treatment (Fig. 19A), but not GC7 (Fig. 22A) or DEF (Fig. 22B) treatment. A potential reason why we may not see a decrease in MVD could be due to its half-life. If MVD was relatively stable, then we would not expect it to decrease much over 24h even if SREBP2 is down. However, the exact half-life of MVD in human cells is unknown. Curiously, MVD has two di-prolines at positions 272 and 331 (UniProt #P53602). This should make it sensitive to hypusination inhibitors on top of it being regulated by SREBP2. It is possible that MVD in Huh7 cells contains a mutation making it more stable, resistant to eIF5A-hypusine, or its expression is not as reliant on SREBP2. More work is needed to determine why there was no visible change in MVD protein levels during hypusination inhibition.

Although we show that SREBP2 relies on polyamines for translation through hypusinated eIF5A, regulation of SREBP2 and its activity is complex. Polyamines play a wide variety of roles within the cell, one of which is stabilizing DNA and promoting
transcription factor engagement. Another potential area of involvement for polyamines within the cholesterol pathway is the stabilization of SREBP2 binding of SRE. It has previously been shown that polyamines maintain the estrogen receptor elements in the correct motif to allow for estrogen receptor to bind, and a lack of polyamines decreased the ability of estrogen receptor to bind to estrogen receptor elements. Polyamines could be required to put SRE into the correct conformation for SREBP2 to access it and promote the expression of itself and other cholesterol synthesis genes.

Polyamines also play a role in protease function. Our lab previously demonstrated that both of CVB3’s proteases develop mutations in response to polyamine depletion, making them resistant to the lack of polyamines, suggesting a role for polyamines in protease activity. SREBP2 processing requires two proteases, S1P and S2P, for its maturation. It is unclear whether SREBP2 cleavage or S1P/S2P protease activity relies on polyamines. A potential assay to determine if S1P or S2P activity requires polyamines would be to insert secreted alkaline phosphatase in between the S1P and S2P sites of SREBP2. Upon S1P and S2P cleavage, secreted alkaline phosphatase would be left in the ER and eventually secreted into the extracellular environment where it could be measured. It is unlikely that hypusine-eIF5A is affecting another protein upstream of SREBP2 as its transcript levels do not significantly change with DFMO (Fig. 20A) or GC7 (Fig. 21D) treatment.

Polyamines may act elsewhere in cholesterol homeostasis and signaling events within the cell. Kir channels, which require polyamines for their function, can be down or up-regulated by cholesterol depending on the channel. Cholesterol was shown to
impair vasodilation by suppressing Kir channels, but just how the polyamine-cholesterol axis affects this is an area that needs more research\textsuperscript{349}. Polyamines have also been linked with decreased ageing and longer lifespans while high cholesterol is associated with decreased lifespans\textsuperscript{350}. There are likely many reasons for the increased longevity of human diets that contain high polyamines, but it is possible that an increased ratio of cholesterol to polyamines would have an ill-effect on Kir channel function and thus, cell function.

In order to measure SREBP2 translation with or without di-prolines, we made two different constructs containing segments of SREBP2 fused with luciferase (Fig. 23). For this, we used mouse SREBP2 due to its availability on Addgene. Cloning of SREBP2 directly from Huh7 cells was attempted but was not successful, likely due to the size of the transcript encoding for SREBP2 (3,423 nucleotides). This leads to a higher likelihood of at least some of the mRNA to be degraded during RNA extraction and the cDNA reaction. The addition of luciferase adds an element of complication to the system as it could be affected by a lack of eIF5A-hypusine. Unfortunately, to determine which di-proline motifs are responsible for SREBP2’s sensitivity to GC7, we would have had to mutate each motif separately and in pairs and transfected the altered SREBP2 into cells and see if translation was restored and cholesterol abundance remained unchanged. However, mutating these motifs was not a viable strategy since prolines are crucial to protein structure\textsuperscript{351}. Mutating them could cause improper protein folding and lead to the degradation of the mutated SREBP2. Furthermore, three of the five di-proline motifs are present within human SREBP2’s (UniProt #Q12772) functional N-terminal domain where it binds to DNA. Prolines are crucial for the protein structure of
DNA-binding domains and are also in close proximity to where SREBP2 binds to importin-β\textsuperscript{352,353}. Mutating these could lead to SREBP2 being unable to enter the nucleus due to an inability to bind to importin-β.

The discovery that polyamines influence cholesterol metabolism through hypusination could change the way we treat certain disease states. Many cancers exhibit increased cholesterol metabolism and have been shown to require eIF5A-hypusine\textsuperscript{354,355}. This polyamine-cholesterol relationship may allow for more targeted therapies to better prevent metastasis and limit tumor growth. These findings could also have important implications on how polyamines may influence high or low serum cholesterol levels and its impact on aging. However, significantly more work is needed to determine if polyamines impact on cholesterol is involved in human disease states and aging. Viruses also require both polyamines and cholesterol for a productive infection. The impact of the polyamine-cholesterol axis on CVB3 infection is the focus of Chapters 5 and 6.
CHAPTER 5

THE POLYAMINE-CHOLESTEROL AXIS IS CRUCIAL FOR CVB3 BINDING

Parts of this chapter are reprinted with modifications from Firpo et al. 2023

Introduction

We have previously shown that polyamines are vital for CVB3 binding. Without polyamines, CVB3 is unable to attach to or enter cells effectively and CVB3 can overcome this binding inefficiency through the mutation Q234R within VP3. However, the mechanism behind why polyamines affect CVB3 binding is unknown. CVB3 also requires cholesterol for productive infection. Without cholesterol present at the plasma membrane, lipid rafts are unable to form and CVB3 is unable to bind and enter cells. With our finding that polyamines help maintain cholesterol homeostasis through supporting translation of SREBP2, we hypothesized that polyamines indirectly affect CVB3 binding through cholesterol synthesis.

Results

Polyamine Depletion Reduces CVB3 Binding

We previously found that inhibition of polyamine synthesis by the specific inhibitor DFMO significantly decreases CVB3 binding to cells compared to untreated cells. To confirm this phenotype, we treated Vero cells with increasing doses of DFMO to deplete cellular polyamines. We then added CVB3 directly to cells on ice for 5 minutes. Virus was then washed off followed by agar overlay, in media containing polyamines. Thus, in these assays, polyamines are depleted only for attachment. CVB3
readily forms plaques on Vero cells and the Coxsackievirus and Adenovirus receptor (CAR) binding domain has 100% sequence identity compared to human CAR. Plaques generated from successful attachment and entry were enumerated two days later (Fig. 25A). We found that DFMO significantly reduced bound virus in a dose-dependent manner (Fig. 25B), in agreement with prior work and corresponding to a decrease in cellular polyamines. To determine if this polyamine-dependent attachment phenotype relied on cellular factors, such as cholesterol, we treated cells with DFMO to deplete polyamines and subsequently replenished the polyamines (putrescine, spermidine, and spermine) in an equimolar concentration. Adding polyamines to the cells at the time of infection did not rescue viral attachment (Fig. 25C). However, when polyamines were added 24h prior to infection, we observed a full rescue in CVB3 attachment. These data suggest that polyamines rescue viral attachment but rely on an extended incubation period, perhaps because of their role in supporting cholesterol synthesis.
Figure 25. CVB3 Requires Polyamines for Binding. (A) Representative plaques from B. (B) Quantification of a representative CVB3 binding assay on Vero cells treated with indicated doses of DFMO. (C) Vero cells were treated with DFMO for 96h then treated with 10 μM equimolar ratio of polyamines at the indicated times before infection. *\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \) by one-way ANOVA. Data from at least three independent experiments.
Statins Do Not Alter CVB3 Infection

Figure 26. Atorvastatin Does not Decrease CVB3 Binding. (A) Representative CVB3 binding assay on Vero cells treated with the indicated doses of Atorvastatin for 16h. (B) Crystal violet viability assay of Vero cells treated with Atorvastatin for 24h. NS= not significant and ****p < 0.0001 by one-way ANOVA. Data is from two independent experiments.

Statins are class of inhibitors that are prescribed to lower serum cholesterol levels\textsuperscript{257}. They work by directly binding to HMGCR and blocking its function. They also inhibit the replication of a variety of different viruses\textsuperscript{356–358}. We tested two statins, atorvastatin and lovastatin, to see if they were able to reduce CVB3 titers. We treated cells with atorvastatin (Fig. 26A) for 16h before performing a binding assay. Despite a drop in bound virus, it was not significant. We next measured the viability of Vero cells treated with atorvastatin using a crystal violet absorbance assay. We found that atorvastatin was cytotoxic at the doses tested (Fig. 26B) and discontinued working with the drug. We next tested a different statin, lovastatin, on its ability to reduce CVB3 binding (Fig. 27A). Despite it being relatively non-toxic (Fig. 27B), there was little change in bound virus despite the significance at 75 µM. These findings show that statins are not a viable strategy to reduce CVB3 binding and infection with our cell system.
Cholesterol Perturbation Inhibits CVB3 Binding

We next tested if sequestering cholesterol inhibited CVB3 binding. We treated Vero cells with the NPC1 inhibitor U18666A for 16h and measured bound CVB3 (Fig. 28). We found that CVB3 binding to cells treated with U18666A was significantly reduced. This data shows that CVB3 binding does require cholesterol for binding and entry.

eIF5A-hypusine is Required for Efficient CVB3 Attachment and Binding

To start to determine if the polyamine-cholesterol axis impacts CVB3 binding, we tested eIF5A-hypusine’s impact on binding since we
found that eIF5A-hypusine was required for SREBP2 translation and maintaining intracellular cholesterol. Vero cells were treated with varying doses of GC7 and 500 µM of aminoguanidine for 16h followed by a CVB3 plaque binding assay (Fig. 29A). There were significantly fewer PFUs in cells treated with GC7 compared to NT. We also wanted to measure the number of CVB3 genomes bound to cells treated with GC7. We treated Vero cells with 250 µM GC7 and aminoguanidine for 16h. We then removed the media and added CVB3 directly to cells on ice at an MOI of 5 for 5 min. The cells plus virus were then either collected directly with TRIzol or washed three times with PBS before collection. Viral genomes were then analyzed via qPCR (Fig. 29B). The number of relative genomes were significantly down in cells treated with GC7 compared to untreated cells. These data show that hypusination facilitates CVB3 binding and entry.

Figure 29. GC7 Blocks CVB3 Attachment and Entry. (A) CVB3 plaque binding assay on Vero cells treated with increasing doses of GC7. (B) Vero cells were treated with 250 µM GC7 for 24h and viral attachment was measured via qPCR. *p < 0.05 and **p < 0.01 by Student’s t test.
Exogeneous Cholesterol Rescues CVB3 Binding

Since we established that polyamines, cholesterol, and eIF5A-hypusine are important for CVB3 binding, we next sought out to show that polyamines’ influence on cholesterol was responsible for decreased CVB3 binding. To determine if the decrease in cholesterol from polyamine depletion affected CVB3 binding, we attempted to rescue viral binding by adding cholesterol to cells exogenously. Cells depleted of polyamines were treated with increasing doses of cholesterol overnight, followed by washing away excess cholesterol and performing a binding assay as before. When plaques were revealed, we found that cholesterol significantly rescued CVB3 binding in a dose-dependent manner (Fig. 30A). To determine if adding cholesterol was able to rescue the amount of bound genomes, we performed a qPCR binding assay as described above (Fig. 30B). Polyamine depletion significantly decreased the number of bound genomes while the addition of cholesterol was able to significantly rescue bound genomes to polyamine depleted cells. To test if this rescue was due to a direct interaction of CVB3 and cholesterol or if cholesterol had to be incorporated into cellular membranes, cholesterol was added to cells at 22h, 4h, and 0h before CVB3 binding. Only cholesterol added 22h and 4h before binding was able to significantly rescue CVB3 binding to polyamine depleted cells (Fig. 30C), suggesting that viral attachment requires cellular cholesterol incorporation. To test if hypusination’s role in CVB3 binding is due to cholesterol, we treated cells with 100 µM GC7 and cholesterol before measuring bound virus (Fig. 30D). 250 µg/mL of cholesterol was able to significantly rescue the amount of bound virus in GC7 treated cells. We confirmed that adding exogenous cholesterol restored cholesterol to basal conditions in both GC7 (Fig. 30E) and DFMO (Fig. 30F).
Figure 30. Addition of Cholesterol Rescues CVB3 Binding in Polyamine Depleted Cells. (A) Binding assay of Vero cells left untreated or treated with DFMO for 96h. After 72h, cells were treated with 100, 250, or 500 µg/mL cholesterol for 24h prior to performing the binding assay. (B) Vero cells were treated as in A with 250 µg/mL cholesterol 24h prior to performing qPCR attachment assay. (C) Binding assay of DFMO treated Vero cells treated with 500 µg/mL cholesterol at the indicated times. (D) Vero cells treated with 100 µM GC7 were supplemented with 100 or 250 µg/mL cholesterol and viral binding was measured via plaque assay. (E) Cells treated as in (D) were assayed for total cellular cholesterol levels. (F) Cells treated as in (A) were assayed for total cellular cholesterol levels. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 by the Student’s t test. Data from at least three independent experiments.
**VP3^{Q234R} is Resistant to Cholesterol Perturbation**

We previously described a viral mutant that exhibits enhanced viral attachment in polyamine depleted cells via mutation of VP3 at position 234 (VP3^{Q234R})\textsuperscript{185}. This mutant was found when we passaged virus in DFMO-treated cells, suggesting that CVB3 harboring this mutation may be resistant to polyamine depletion by overcoming a block in attachment. To determine if this block was cholesterol-dependent, we tested viral attachment in cells where cholesterol transport is impaired. We found that treatment of cells with U18666A reduced CVB3 attachment in a dose-dependent manner (Fig. 31A), as well as replication (Fig 31B). When we performed an attachment assay on U18666A treated cells using CVB3 VP3^{Q234R}, we observed a modest reduction in viral attachment compared to WT CVB3, suggesting that the mutant may overcome polyamine depletion via bypassing the need for cholesterol (Fig. 31A). Additionally, we measured viral titers in U18666A-treated cells infected with CVB3 VP3^{Q234R} and observed titers significantly higher than WT CVB3 (Fig. 31B). Together, these data suggest that polyamine indirectly promote viral attachment by supporting cholesterol biosynthesis and that CVB3 can overcome polyamine depletion by mutation of VP3, which bypasses the need for cholesterol in viral attachment.
**Discussion**

The roles of polyamines in a viral infection are diverse and appear to be distinct for different families\(^{26}\). They range from stabilizing viral genomes and facilitating polymerase function to aiding protease function and binding\(^{17,177,185,186,228}\). In the case of CVB3, polyamines facilitate the latter. However, the mechanism(s) by which polyamines promote binding was unclear. We show that polyamines rescue CVB3 binding to DFMO treated cells only when they are added at least four hours before infection, suggesting they play an indirect role in binding and attachment. Based off our findings that polyamines support cholesterol synthesis through translation of SREBP2, we hypothesized that polyamines were affecting CVB3 binding indirectly through their role in maintaining cholesterol. We next confirmed that CVB3 was sensitive to altered

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**Figure 31. CVB3 VP3 Mutant is Resistant to Cholesterol Perturbation.** (A) Vero cells were treated with increasing doses of U18666A for 16h prior to WT or VP3\(^{Q234R}\) CVB3 binding. Bound virus was enumerated by counting plaques indicative of attached virus. (B) Vero cells were treated as in (A) and infected at MOI 0.1 with WT or VP3\(^{Q234R}\) CVB3. Viral titers were determined at 24 hpi. *\(p < 0.05\) and **\(p < 0.01\) via Student’s t test from three independent experiments.
intracellular cholesterol by inhibiting NPC1 and that eIF5A-hypusine was important for binding as well. To connect these different components involved in CVB3 binding, we depleted cells of polyamines or hypusine and added back only cholesterol. We found that cholesterol was able to significantly rescue CVB3 binding. To further support our finding that the polyamine-cholesterol axis is important for CVB3, we tested the sensitivity of VP3^{Q234R} to NPC1 inhibition and found that the virus was partially resistant to cholesterol perturbation. These data reveal that polyamines’ support of intracellular cholesterol levels is needed for CVB3 binding.

We have observed that cellular attachment to DFMO-treated cells was reduced for a variety of viruses, including distinct picornaviruses. Thus, it is unlikely that a reduction in receptor expression is sufficient to explain this broad phenotype. Cholesterol is a key molecule in enterovirus attachment and entry, and its association with lipid rafts has been demonstrated to facilitate CVB3 engagement with its receptor CAR\textsuperscript{8}. It stands to reason that a reduction in polyamines reduces the amount of cholesterol within the plasma membrane, thus limiting lipid raft formation and CVB3 entry. Unfortunately, there are limited tools to determine the amount of cholesterol within the plasma membrane during viral binding. One such tool is BODIPY-cholesterol (Bchol). Bchol is a fluorescently tagged cholesterol that is relatively non-polar and can be inserted into lipid bilayers, including the plasma membrane. However, it can self-quench at high concentrations through the formation of dimers\textsuperscript{359}. This could potentially prevent the imaging of lipid rafts. Another issue from the use of Bchol, and other fluorescently tagged forms of cholesterol, is that they tend to alter local lipid structures due to their size and thus limit the receptor clustering needed for CVB3 entry\textsuperscript{359}. The
main method used to observe cholesterol in the plasma membrane is a stain called filipin. Filipin directly binds to the cholesterol present, thus allowing for the direct observation of cholesterol in stained cells\textsuperscript{359}. Unfortunately, filipin emission is in the UV spectrum and we are unable to visualize it.

Cholesterol was able to rescue not just CVB3 binding and entry, but also attachment to DFMO treated cells. The number of bound genomes measured via qPCR significantly increased when cholesterol was added to DFMO treated cells and was even higher compared to cells given exogenous cholesterol only. We hypothesize that the increase in cholesterol in the plasma membrane increases the density of CAR within lipid rafts, increasing the number of available receptors for CVB3 to attach to and prevent it from being washed off. There are a few explanations as to why cholesterol greatly increased CVB3 attachment in DFMO treated cells compared to the marginal increase in untreated cells. Cells depleted of cholesterol through treatment with DFMO may overcompensate for the lack of cholesterol at the plasma membrane, resulting in an increased plasma membrane concentration of cholesterol compared to untreated cells. The addition of excess cholesterol may also affect other cellular factors on the plasma membrane that aid in CVB3 attachment.

The qPCR attachment assay measures the total amount of bound virus, regardless of if that virus would be able to replicate within the cell. The use of the plaque binding assay grants the ability to measure infectious virus that binds to and infect cells. The cholesterol rescue for the plaque binding assay for untreated cells had a markedly different phenotype compared to the qPCR attachment assay. Cholesterol was able to increase PFUs over that of untreated cells. In this case, the addition of
cholesterol could increase cellular energetics and allow for more viruses to infect cells since more cells are in a permissive state. Cholesterol is important for other aspects of CVB3 replication such as RO formation and polyprotein processing\textsuperscript{10,11,330}. The addition of excess cholesterol likely increases cellular cholesterol pools within lipid droplets, which CVB3 can then use to enhance polyprotein processing and genome replication. The downstream effect of this would be more progeny virions in cells that were already permissive to CVB3 infection or allow CVB3 to infect cells that may not have been permissive if excess cholesterol was not administered.

In order to determine if cholesterol depletion affected CVB3 binding we first used a popular class of HMGCR inhibitors called statins. Statins show antiviral properties \textit{in vitro} and have been correlated with a lower risk of viral infections with patients that have hyperlipidemia\textsuperscript{360,361}. The antiviral properties of statins have been attributed to their direct role in limiting cholesterol through HMGCR inhibition as well as its immunomodulatory effects. However, we failed to decrease viral binding or titers (data not shown) of CVB3 with atorvastatin, lovastatin, or simvastatin at doses that were non-cytotoxic for either Vero or Huh7 cells. Furthermore, statins have been shown to reduce the incidence of CVB3 induced myocarditis and improve survival of mice, but how the statins affected CVB3 titers were not measured\textsuperscript{362,363}. Interestingly, Huh7 cells treated with these statins, and others, from 5 – 50 µM decreased EBOV titers and were non-toxic\textsuperscript{356}. This difference between cell viability is most likely due to the cell strain used. We used Huh7.10s while the EBOV study used Huh7.5 derived cells. This could account for the difference in susceptibility between the two cell lines.
CVB3 with the Q234R mutation in VP3 exhibits not just resistance to polyamine depletion, but also cholesterol transport inhibition. The mechanism behind VP3 Q234R’s resistance to polyamine depletion is unknown; however, the mutation is near the binding pocket of CAR. The mutation may alter VP3’s conformation allowing it to better bind to CAR and enter cells. Glutamines have also been shown to undergo polyamination. This PTM stabilizes microtubules within axons, and without it, neuronal cells have significant structural changes\textsuperscript{160}. During virion maturation in the host cell, TG2 could polyaminate VP3 at Q234 and this PTM may stabilize the virion and prevent it from releasing its genome too early or enhance binding to CAR. The positively charged polyamine could give the virion some resistance to protons and prevent the early release of the genome within the extracellular environment. Without polyamines present within the host cell, CVB3 is forced to mutate the glutamine to the positively charged arginine to account for the difference in stability. This mutation may slightly decrease viral fitness in some way compared to WT virus but is necessary when the cell lacks polyamines. The enhanced binding or stability of VP3\textsuperscript{Q234R} could limit its spread or decrease tissue tropism \textit{in vivo}. Significantly more work is needed to determine if polyamination does affect CVB3 virion stabilization and CAR binding.

The addition of cholesterol to polyamine depleted cells completely rescued viral binding, providing evidence that polyamines’ impact on cholesterol homeostasis affects CVB3 binding. However, the addition of cholesterol did not fully rescue viral binding to cells treated with GC7. There was a significant rescue, but to only 55~60\% of that seen in untreated cells. Inhibiting hypusination likely has other effects on the cell that cannot be rescued with exogeneous cholesterol that affect CVB3 binding and infection. The
addition of cholesterol did restore intracellular cholesterol levels, but it is possible that not all the cholesterol is able to localize to the plasma membrane. There could also be decreased translation in other cellular proteins that CVB3 requires for binding and entry. Furthermore, inhibiting hypusination may have a direct effect on CVB3 translation. CVB3 has multiple di-prolines present within its polyprotein, making it potentially require eIF5A-hypusine for its translation. This would prevent CVB3 from establishing an infection and creating more progeny virions, resulting in fewer lysed cells and thus, fewer plaques. Determining how eIF5A-hypusine inhibition affects other aspects of CVB3 infection is the goal of the next chapter. Together, these data show that polyamines’ influence on cholesterol homeostasis affects CVB3’s ability to bind and enter cells, and that binding to polyamine depleted cells can be rescued with exogenous cholesterol.
EIF5A-HYPUSINE IS REQUIRED FOR CVB3 REPLICATION

Introduction

EIF5A is the only known enzyme within the eukaryotic cell to be hypusinated, and it supports the translation of diverse cellular proteins. Prior work showed that Ebolavirus relies on hypusination specifically for the translation of VP30, a viral transactivator that facilitates viral gene expression\(^{182}\). Other work showed that inhibitors of hypusination reduce translation of retroviruses, including HIV-1\(^{23,364}\). The direct roles of EIF5A hypusination in viral protein synthesis remain to be fully explored for many viruses, including CVB3. CVB3’s polyprotein contains 8 di-prolines, likely making it require EIF5A-hypusine for efficient translation. Due to cholesterol not fully rescuing CVB3 binding to GC7 treated cells, we hypothesized that CVB3 requires EIF5A-hypusine for the efficient translation of its polyprotein.

Results

Inhibition of Hypusination Limits CVB3 Replication

We previously found CVB3 attachment was sensitive to hypusination inhibition through GC7 treatment, and viral attachment was rescued with exogenous cholesterol addition. To explore if EIF5A-hypusine is involved in other stages of CVB3 infection, we first treated Huh7 cells with increasing doses of GC7 plus 500 µM aminoguanidine, to inhibit diamine oxidases in the serum, for 16h\(^{365}\). Cells were then infected with CVB3 at an MOI of 0.1 for 24h. Cell supernatant was collected and used for viral titer
enumeration via plaque assay. Viral titers significantly decreased in a dose-dependent manner and by ~2.5 log for 500 µM GC7 (Fig. 32A). We previously showed that the doses tested are non-toxic to Huh7 cells (Fig. 21E). To determine the kinetics of CVB3 in cells treated with GC7, untreated and treated cells were infected with CVB3 and supernatant was collected at different hpi (Fig. 32B). Viral titers were significantly reduced in GC7-treated cells 24 hpi and later. CVB3 titers remained depressed throughout infection. To determine what stage of CVB3 infection is sensitive to hypusination inhibition, cells were treated with GC7 at various times before and after infection (Fig. 32C). Cells treated with GC7 16 and 6 hours before infection significantly decreased viral titers by ~2 log. Cells treated 2 hours before infection, at infection, and 2 hpi exhibited a significant decrease of titers by ~1-0.5 log. This suggests that while inhibiting hypusination before infection significantly impacts viral titers, potentially through cholesterol's requirement for eIF5A-hypusine and thus CVB3 binding\textsuperscript{20}, inhibiting hypusination just before or after infection significantly impacts CVB3 later during its replication cycle.
CVB3 Translation is not Affected by GC7 Treatment

Due to the presence of multiple di-proline motifs within CVB3’s polyprotein, we hypothesized that eIF5A-hypusine was required for CVB3 polyprotein translation. To test if the initial translation of CVB3 required eIF5A-hypusine, Huh7 cells were treated with 500 µM GC7 and, as a control, 400 µM ribavirin 30 minutes before transfection with the CVB3 replicon plasmid encoding Renilla luciferase or a Renilla control plasmid (SiCheck) (Fig. 33A). Transfected cells treated with GC7 alone exhibited no significant difference in Renilla activity compared to the Renilla control plasmid. However, due to the replicon’s ability to self-replicate and potentially produce more Renilla luciferase, we treated cells with both GC7 and guanidine HCl (GuHCl), a CVB3 RNA polymerase inhibitor, 30 minutes before transfection (Fig. 33A). GC7 and GuHCl had less replicon...
Renilla luciferase activity compared to replicon cells treated with GuHCl alone; however, this was not significant. To determine if eIF5A-hypusine is required for CVB3 translation throughout infection, we performed live-cell imaging of cells infected at an MOI of 1 with CVB3 that expresses GFP (CVB3-GFP)\textsuperscript{334} and treated with GC7 and ribavirin at the time of infection (Fig. 33B). There was significantly lower GFP fluorescence in cells treated with GC7 compared to untreated cells (Fig. 33C), which was also observed in cells treated with ribavirin, as expected. To control for the number of genomes present during infection, cells were infected with CVB3-GFP and relative viral genomes were enumerated at several time points during infection via RT-qPCR (Fig. 33D). There were significantly fewer viral genomes in GC7 treated cells at 10 hpi compared to untreated cells, potentially leading to less GFP production. We next calculated the mean GFP fluorescence divided by the number of relative CVB3-GFP genomes to determine if there was a difference in the amount of GFP produced per genome (Fig. 33E). We observed no significant difference between GC7 and untreated cells. These data together suggest that eIF5A-hypusine is not required for efficient CVB3 genome translation.
Identification of GC7 Resistant CVB3 Mutants

To determine how inhibiting hypusination limits CVB3 infection due to GC7’s limited impact on CVB3 translation, we used the previously described polyamine depletion resistant CVB3 protease mutants $2A^{Q29R3C^{Q52K}}$ (2A3C)$^{15}$ and $2A^{S35G}$ (S35G)$^{17}$ to probe if there is overlapping resistance. The 2A3C mutant was found by passaging...
CVB3 in DFMO treated cells and the S35G mutant was found in cells treated with DENSpm which promotes polyamine catabolism through the enzyme SAT1. We infected GC7 treated cells with WT and mutant viruses and measured viral titers to see if they were resistant to hypusination inhibition (Fig. 3A). We saw no significant difference in viral titers between WT and the mutant viruses. We next sequentially passaged CVB3 a total of 23 times in Huh7 cells treated with GC7. After passage, we subjected the viral population to increasing doses of GC7 to determine if CVB3 could gain resistance. Virus from the 23rd passage was resistant to GC7 treatment compared to virus passaged in untreated cells, as titers were higher with GC7 treatment when infected with virus passaged in GC7 (Fig. 3B). Interestingly, the passaged virus was not resistant to DFMO treatment, suggesting a different mechanism of resistance compared to the previously described mutants (Fig. 3C). Upon sequencing the passaged virus, several synonymous and non-synonymous mutations were present compared to untreated passaged virus; however, only two non-synonymous mutations were present in two of the three passaged viruses (Fig. 3D). One mutation was in the 2A protease (Q85L), which is near the cleft of the protease active site, and the other was in 3A (H57Y). The 3A^{H57Y} was previously described as being able to overcome PI4Kβ inhibition and no longer forms replication organelles upon PI4Kβ inhibition^{284}. We confirmed that CVB3 containing both mutations (2A3A) were resistant to PI4Kβ inhibition with GW5074 (Fig. 3E). Both single and double mutants were found to be significantly resistant to hypusination inhibition to varying degrees (Fig. 3F) and were found to have similar growth kinetics to WT virus (Fig. 3G).
Figure 34. Identification of GC7 Resistant CVB3.

**Panel A:**
- Y-axis: Normalized PFU/mL
- X-axis: GC7 (μM)
- Legend: WT, 2A3C, S35G

**Panel B:**
- Y-axis: Normalized PFU/mL
- X-axis: GC7 (μM)
- Legend: WT, GC7p23

**Panel C:**
- Y-axis: Normalized PFU/mL
- X-axis: DFMO (mM)
- Legend: WT, GC7p23

**Panel D:**
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**Panel E:**
- Y-axis: log₁₀ Titer (PFU/mL)
- X-axis: GW5074 (μM)
-Legend: WT, 2A3A

**Panel F:**
- Y-axis: Normalized PFU/mL
- X-axis: GC7 (μM)
- Legend: WT, 2A Q85L, 3A H57Y, 2A3A

**Panel G:**
- Y-axis: log₁₀ Titer (PFU/mL)
- X-axis: Time (h)
- Legend: WT, 2A Q85L, 3A H57Y, 2A3A
Since CVB3 translation was not affected by GC7 treatment, and the emergence of the $3A^{H57Y}$ mutant, we performed immunofluorescence microscopy to determine if there was a change in ROs. Cells were treated with 500 µM GC7 and infected with CVB3 at an MOI of 5 for 6h. dsRNA (green) was then stained to determine if there was a change in ROs. Cells were treated with 500 µM GC7 and infected with CVB3 at an MOI of 5 for 6h. dsRNA (green) was then stained to determine if there was a change in ROs.

**Figure 34. Identification of GC7 Resistant CVB3.** (A) Viral titers of WT, 2A3C, and S35G CVB3 normalized to no treatment. Huh7 cells were treated with the indicated doses of GC7 and 500 µM aminoguanidine 16h before infection with virus at an MOI of 0.1. (B) Viral titers of passaged virus normalized to untreated Huh7 cells. Cells were treated with the indicated doses of GC7 and 500 µM aminoguanidine 16h before infection with virus at MOI 0.1. (C) Viral titers of passaged virus normalized to untreated Huh7 cells. Cells were treated with indicated doses of DFMO 96h before infection with virus at MOI 0.1. (D) Table of nucleotide changes in CVB3 passaged 23 times in GC7 treated cells relative to CVB3 passaged 23 times in untreated cells. Done in biological triplicates. † mutation occurred in 2 of 3 triplicates. (E) Viral titers of WT CVB3 and $2A^{Q85L}3A^{H57Y}$ in Huh7 cells treated with GW5074 at the time of infection. (F) Viral titers of CVB3 normalized to untreated Huh7s. Cells were treated with indicated doses of GC7 and 500µM aminoguanidine at the time of infection. (G) Viral titers of infected Huh7 supernatant collected at indicated times post infection. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ by the Student’s $t$ test.

**GC7 Alters CVB3 Replication Organelles**

![Immunofluorescence imaging of cells infected with CVB3 at an MOI of 5 for 6h. Cells were treated with 500 µM GC7 and aminoguanidine at the time of infection. Nuclei were stained with DAPI (blue) and dsRNA (green) was stained with K2 antibody.](image)

Since CVB3 translation was not affected by GC7 treatment, and the emergence of the $3A^{H57Y}$ mutant, we performed immunofluorescence microscopy to determine if there was a change in ROs. Cells were treated with 500 µM GC7 and infected with CVB3 at an MOI of 5 for 6h. dsRNA (green) was then stained to determine if there was a change in ROs.

**Figure 35. Immunofluorescence of CVB3 Infection.** Immunofluorescence imaging of cells infected with CVB3 at an MOI of 5 for 6h. Cells were treated with 500 µM GC7 and aminoguanidine at the time of infection. Nuclei were stained with DAPI (blue) and dsRNA (green) was stained with K2 antibody.
a phenotypic change in ROs (Fig. 35). We observed less dsRNA staining in cells treated with GC7 and the stained area was more confined. To better determine any change in ROs, transmission electron microscopy (TEM) was performed on GC7 and untreated cells infected with CVB3 at an MOI of 5 for 6h (Fig. 36). ROs were widely dispersed in untreated cells and seemed to be the dominating structure of the infected cells. However, in GC7 treated cells, there were fewer cells infected and the ROs were smaller and less dispersed.
Figure 36. Replication Organelles of CVB3 Infected Cells. TEM of Huh7 cells infected with CVB3 at an MOI of 5 for 6h. Cells were treated with 500 µM GC7 and aminoguanidine at the time of infection. Black arrows point to ROs.
TEM was also performed with cells infected with the 2A3A mutant under the same conditions above (Fig. 37). The ROs of untreated and GC7 treated were phenotypically similar. The ROs were less developed compared to those of WT infected cells and there seemed to be less membrane alterations within 2A3C infected cells regardless of treatment.

**eIF5A-hypusine is Required for Efficient CVB3 Replication Through Cholesterol**

The phosphatidylinositol kinase PI4Kβ is crucial for creating the rich phosphatidylinositol-4-phosphate environment needed by CVB3 to create replication organelles. PI4Kβ also has two di-proline motifs present, making it potentially sensitive to hypusination inhibition and causing CVB3 to mutate its 3A protein. However, upon GC7 treatment, there was no change in PI4Kβ protein levels (Fig. 38A). Since we observed no significant change in CVB3 translation though we find changes in replication organelles, we considered our previous work showing that polyamines facilitate cholesterol synthesis, which could in turn impact ROs. To determine if these
mutations arose due to GC7 impacting intracellular cholesterol, we treated cells with U18666A 4h before infection with WT CVB3 or 2A3A. Supernatant was then collected 24hpi and titers were enumerated via plaque assay (Fig. 38B). We found significantly less WT CVB3 at all doses tested of U18666A compared to 2A3A. To determine if GC7 treatment differently affected genome accumulation between WT and 2A3A, cells were treated with GC7 at the time of infection and genomes were measured at 0, 2, 4, and 8hpi (Fig. 38C). Treatment with GC7 significantly decreased WT CVB3 genomes by 8hpi while the 2A3A genomes were the same.

![Figure 38](image)

**Figure 38. Resistance to eIF5A-Hypusine Confers Resistance to Cholesterol Inhibition and Rescues Viral Genome Replication.** (A) Huh7 cells were treated with increasing doses of GC7 and cellular PI4Kβ and hypusine levels were measured by western blot. (B) Cells were treated with increasing doses of U18666A 4h before infection to block cholesterol transport and subsequently infected with WT or 2A3A mutant virus. Supernatant was collected at 24hpi for plaque assay. (C, D) Huh7 cells were treated with 500 µM GC7 and infected with WT (C) or 2A3A (D) CVB3. Viral genomes were measured by qPCR at the times indicated. *p < 0.05, **p < 0.01, and ****p < 0.0001 by the Student’s t test. Data from three independent experiments.
Diverse RNA Viruses are Sensitive to Hypusination Inhibition

To test if CVB3’s requirement for eIF5A-hypusine was unique, we tested GC7’s ability to block viral titers with several different viruses (Fig. 39). The related picornavirus human rhinovirus 16 (HRV16) had a significant decrease in viral titers by ~1 log (Fig. 39A). The bunyavirus La Crosse virus (LACV) also had a significant reduction in viral titers (Fig. 39B) along with the alphavirus chikungunya virus (CHIKV) (Fig. 39C). Curiously, Vaccinia virus (VACV), a DNA virus, was not significantly impacted by hypusination inhibition under these conditions (Fig. 39D). These data suggest that hypusination likely plays a role in the replication of diverse viruses, potentially at multiple or different points in their replication cycles.

![Figure 39. RNA Viruses are Sensitive to GC7 Treatment.](image)

Discussion

We found that CVB3 requires hypusinated eIF5A for a productive infection and the formation of ROs. Blocking hypusination with the specific inhibitor GC7 significantly decreased viral titers even when cells were treated shortly after infection. Due to the importance of eIF5A-hypusine in translation of di-prolines, we investigated its role in the
initial translation of CVB3 and the translation of CVB3 throughout infection. Interestingly, we saw no difference in the amount of GFP reporter produced per genome in cells treated with or without GC7. To further investigate how eIF5A-hypusine affects CVB3 infection, we passaged CVB3 in cells treated with GC7 to ascertain if CVB3 could become resistant and which proteins were involved. Two mutations were found in two of the three viruses passaged in GC7 at $2A^{Q85L}$ and $3A^{H57Y}$. Based on these viral protein’s role in polyprotein processing and RO formation, we investigated if GC7 inhibited RO formation. Using transmission electron microscopy and immunofluorescence, we found that CVB3 ROs were smaller in cells treated with GC7 compared to untreated cells. Furthermore, 2A3A double mutant was resistant to the cholesterol transport inhibitor U18666A. These data together show cholesterol’s requirement for eIF5A-hypusine affects CVB3’s ability to form replication complexes and that eIF5A-hypusine is minimally involved in CVB3 translation. We also found that other positive strand RNA viruses were sensitive to GC7 treatment to varying degrees. However, VACV did not require eIF5A-hypusine under these conditions.

Both LACV and ZIKA were sensitive to hypusination inhibition. This could be a result of directly requiring eIF5A-hypusine for their translation or an indirect effect of reducing hypusinated eIF5A. Both flaviviruses (ZIKA) and bunyaviruses (LACV) require cholesterol for the formation of replication complexes, and the data shown here gives evidence of a potential indirect role of eIF5A-hypusine on these viral infections. However, more work is needed to fully determine the role eIF5A-hypusine during flavivirus and bunyavirus infection. VACV, on the other hand, is a large DNA virus (200+ genes) that packages polyamines to stabilize its genome and promote transcription,
similar to both flavi- and bunyaviruses\textsuperscript{174,179,368}. However, hypusination inhibition did not decrease viral titers of VACV. Unlike the other viruses tested, VACV does not appear to require cholesterol for replication. Treatment with U18666A fails to decrease VACV titers and disruption of lipid rafts only affected entry\textsuperscript{369,370}. This cholesterol independence could explain why GC7 failed to reduce titers. It is also possible that some of VACV’s proteins allow it to circumvent hypusination inhibition.

Due to cholesterol being the main cellular factor affected by polyamine depletion, we hypothesized that CVB3 replication mutants derived from passaging in DFMO or DENSpm treated cells should be resistant to hypusination inhibition. However, this was not the case. The 2A3C and S35G mutants had comparable sensitivity to GC7 treatment compared to WT CVB3. This points to polyamines potentially playing a direct role in viral protease activity; however, polyamine depletion may also be impacting other cellular processes that ultimately puts selective pressure on the virus for these mutations. Either way, more investigation is needed to determine the full extent of polyamines’ influence on CVB3 infection.

CVB3’s 2A protease is a chymotrypsin-related endopeptidase and has several important roles during infection\textsuperscript{371}. It is responsible for cleaving the polyprotein into two sections, separating the structural proteins from the non-structural proteins\textsuperscript{372}. It is most well-known for its ability to cleave eIF4G, abrogating cap-dependent translation\textsuperscript{313}. 2A was also found to upregulate lipid synthesis genes through the MEK/ERK pathway through a yet unknown mechanism\textsuperscript{314}. The 2A\textsuperscript{Q85L} mutant recovered from passaging CVB3 in GC7 has yet to be described. However, based off NMR data of CVB4’s 2A protease, Q85 is in a dityrosine flap region on one side of the substrate-binding cleft and
is considered a gatekeeper which helps control the size of the cleft\textsuperscript{373,374}. CVB4’s (strain JVB/Benschoten/New York/51) 2A protein has a 90.6\% amino acid identity match to CVB3’s (Nancy) 2A. Due to the position of Q85L, it is possible that this mutation alters 2A’s substrate specificity to aid in the lack of eIF5A-hypsine present.

The 2A\textsuperscript{Q85L} and 3A\textsuperscript{H57Y} mutants either individually or together were not fully resistant to GC7 treatment, with \(~50\%\) viral titers at 500 \(\mu\text{M}\) GC7 compared to untreated cells. This lack of complete resistance could be due to multiple reasons. eIF5A-hypsine is crucial for alleviating ribosomal pausing at poly-proline motifs\textsuperscript{21}. CVB3 (Nancy) contains 8 di-proline motifs along with a proline-rich region within 3A that is required for its function\textsuperscript{375}. Proline’s unique, rigid cyclic structure is important for protein folding including setting borders for \(\alpha\)-helices and \(\beta\)-sheets\textsuperscript{376}. Due to proline’s importance, CVB3 may be unable to mutate them without serious deleterious effects to polyprotein folding. It is still possible that CVB3 requires eIF5A-hypsine for its translation, thus resulting in only partial resistance to GC7. However, the techniques used to measure viral translation suggest that CVB3 does not seem to require eIF5A-hypsine for efficient translation and that eIF5A-hypsine plays an indirect role.

Blocking eIF5A hypusination may also affect other host-cell proteins that are important for CVB3 replication that the virus cannot overcome through a mutation in its genome. This makes targeting the hypusination pathway a potentially viable strategy for limiting CVB3 infection since it is unable to fully overcome its inhibition. However, more work is needed to determine its efficacy and if there are any deleterious side effects.

The ROs CVB3 forms in cells lacking eIF5A-hypsine are visually distinct compared to those of untreated cells. The electron microscopy and
immunofluorescence reveal that the ROs appear smaller and are less spread out in cells treated with GC7. Upon measuring viral titers throughout infection, up to 48 hpi, we found that CVB3 never reaches the same titer as untreated conditions, and its peak viral production is stunted (Fig. 32C). Since there was no difference in reporter GFP production per CVB3 genome, a lack of viral protein translation does not explain reduced ROs. We previously showed that eIF5A-hypusine is vital in maintaining cellular cholesterol, and blocking hypusination results in a significant decrease in intracellular cholesterol levels\textsuperscript{20}. Previous studies have shown the importance of cholesterol and lipid droplets in the formation of enterovirus ROs\textsuperscript{10,12,317}. The viral protein 3A is responsible for shuttling cholesterol to the ROs by recruiting PI4Kβ. Blocking the function of PI4Kβ results in the \textsuperscript{3A\textsuperscript{H57Y}} mutation and the virus no longer forming ROs when there is a lack of PI4P\textsuperscript{284,330}. \textsuperscript{3A\textsuperscript{H57Y}} is also resistant to OSBP inhibition through GW5074, thus not requiring cholesterol from the ER\textsuperscript{329,377}. Additionally, cholesterol regulates enterovirus polyprotein processing, potentially through the 2A protease\textsuperscript{300}. The emergence of both the \textsuperscript{2A\textsuperscript{Q85L}} and \textsuperscript{3A\textsuperscript{H57Y}} mutants in GC7 treated cells point to a lack of cholesterol within the cell which prevents CVB3 from forming ROs and limit polyprotein processing. Treatment of cells with the cholesterol transport inhibitor U18666A resulted in significantly higher viral titers with the 2A3A mutant compared to WT, suggesting that these mutations arose due to limited cholesterol present within the cell. Furthermore, treatment with GC7 had no effect on PI4Kβ (UniProt #Q9UBF8) protein levels, even though it contains 2 di-proline motifs. There is the possibility that OSBP translation is impacted by hypusination inhibition since it contains 3 di-proline motifs (UniProt #P22059). Together, these data underscore the importance of
cholesterol in CVB3 replication and suggest CVB3’s ROs requirement for cholesterol synthesis drives CVB3’s reliance on eIF5A-hypusine.
CHAPTER 7
IMPLICATIONS

Figure 40. Hypusination’s Role in Cholesterol Synthesis Impacts Multiple Stages of CVB3 Infection. Spermidine is used and modified by DHPS and DOHH to hypusinate eIF5A. eIF5A-hypusine then aids in the translation of the global sterol transcription factor SREBP2 which is then translocated to the nucleus to up-regulate cholesterol synthesis genes when cholesterol is low. Cholesterol produced within the ER is then moved to the plasma membrane where it helps with lipid raft formation. This lipid raft formation is required by CVB3 when it binds to CAR so that it can undergo endocytosis. Once in the cell, CVB3 releases its genome where it undergoes translation, forming its polyprotein. The polyprotein then undergoes a series of cleavage events, releasing the viral proteins. These viral proteins then go on to alter the cell to make it more favorable for viral replication. One of these alterations is the formation of ROs where 3A binds to ACBD3, recruiting PI4Kβ, and increasing the concentration of PI4P on the RO membrane. This allows OSBP to exchange PI4P for cholesterol which is important for RO membrane formation and is associated with polyprotein processing. The loss of cholesterol through hypusination inhibition prevents CVB3 from forming ROs and forces it to mutate its 2C and 3A proteins to combat this loss of cholesterol.
Polyamines are vital for multiple cellular and viral processes. By first investigating polyamines’ effect on cellular transcription, we found that one underlying pathway that was most affected by polyamine depletion was cellular cholesterol homeostasis. While the mTORC1 pathway had a slightly lower decrease in its enrichment score compared to cholesterol homeostasis, investigation of it was pursued since it is a broad pathway that is involved in diverse signaling cascades related to cell growth and nutrient abundance. Meanwhile, we found that the decrease in cholesterol homeostasis gene transcription was primarily due to a decrease in hypusinated eIF5A, resulting in a decrease in translation of SREBP2. Reduced SREBP2 translation resulted in reduced cellular cholesterol which ultimately inhibited CVB3 binding and RO formation. The CVB3 2A\textsuperscript{Q85L}3A\textsuperscript{H57Y} mutant derived from passaging CVB3 in GC7 overcame hypusination inhibition and had markedly different ROs compared to WT virus. These results are summarized in Figure 40.

There are broad implications to human health with the discovery of polyamines’ influence on cholesterol homeostasis and how it impacts the cardiotropic CVB3. Many studies have shown that high circulating cholesterol can lead to cardiovascular disease and increased fat on the heart\textsuperscript{378}. With cholesterol being important for multiple stages of CVB3 infection, it is possible that increased blood cholesterol and fat deposits on the heart could enhance infection and lead to an increased risk in the development of myocarditis and DCM. A study in 1990 showed that CVB3 infection of mice hearts led to a significant increase in cholesterol and lipid accumulation\textsuperscript{379}. We also saw an increase in cholesterol gene transcription in CVB3 infected cells\textsuperscript{20}. Furthermore, in an autoimmune myocarditis model, rats treated with cholesterol lowering agents had
reduced heart-to-body weight size, inflammation, and expression of apoptosis related proteins\textsuperscript{380}. This provides evidence that cholesterol enhances myocarditis and that CVB3 is able to increase cholesterol within cardiac cells leading to increased apoptosis and immune cell infiltration. Rats treated with DFMO had reduced serum cholesterol levels\textsuperscript{18}, potentially through polyamines’ role in hypusination and SREBP2 translation described here. This makes treatment of CVB3 infected patients with DFMO a potentially viable solution to directly reduce CVB3 infection and serum cholesterol to prevent myocarditis. Treatment with GC7 could also be a viable strategy; however, it is not FDA approved and may have unforeseen side effects due to its role in proliferation and immune cell function\textsuperscript{123,141}. CVB3 may also develop resistance to these treatments through the course of infection, making it harder to clear from the body.

While polyamines seem to support cholesterol homeostasis, no evidence exists that people with high polyamine diets have increased circulating cholesterol or an increased risk in contracting a viral infection. However, enhanced polyamine metabolism has been linked to cancers and dysfunctional regulatory T cell ratios in HIV infected cells\textsuperscript{381,382}. In the case of CVB3, there is no current evidence that it enhances polyamine metabolism during infection and that addition of excess polyamines increases CVB3’s replication \textit{in vitro}. If and how polyamine metabolism may change in response to CVB3 infection \textit{in vivo} or in an organoid model remains unknown.

Persistent CVB3 infection has been implicated in the development of DCM. Roughly 50\% of hearts with DCM are positive for CVB3 viral RNA and CVB3 genomes can be detected months after the initial infection\textsuperscript{5}. In another study, 37\% of patients with DCM and 32\% of patients with chronic coronary disease had detectable levels of
enterovirus\textsuperscript{383}. However, negative strand enterovirus RNA and VP1 was only detected in 2 of the 70 individuals that were positive for infection. CVB3 from these persistent populations often have significant alterations within their genomes, specifically deletions in their 5’ UTR\textsuperscript{384}. These data suggest that persistent CVB3 is deficient in replication, potentially allowing the virus to remain undetected by immune cells but still cause damage in infected cells. Unfortunately, there is no current \textit{in vitro} model to study CVB3 persistence in heart cells. Nevertheless, CVB3 can form a persistent infection in the PANC-1 pancreatic cell line. We found several interesting mutations in CVB3’s capsid proteins and in its polymerase, causing higher fidelity\textsuperscript{198}. We also found that persistently infected cells had decreased polyamines and ODC1 translation. Whether CVB3 caused this decrease in polyamine levels or if the cells compensated to combat infection is unknown. However, it is possible that the decrease in polyamines could cause CVB3 to have a higher fidelity polymerase due to polyamines’ role in polymerase function shown in other viruses\textsuperscript{227}. The lack of polyamines and potentially other cellular components, like cholesterol, could potentially lead to the 5’ UTR deletions seen in persistent CVB3. This could help control viral replication and may lead to an equilibrium between polyamines present and the number of CVB3 virions produced in persistently infected cells. Treatment of the persistently infected PANC-1 cells with DFMO cleared CVB3 infection. This is most likely due to the polyamine levels becoming too low for CVB3 to replicate. This provides further evidence that polyamine depletion remains a viable strategy to limit CVB3 infection and potentially clear a persistent infection in the heart. Being able to clear a persistent infection could decrease the development of DCM and thus limit the number of heart transplants needed.
Cholesterol’s dependence on eIF5A-hypusine may also affect other aspects of cell signaling and CVB3 infection. Lipid rafts are important for viral sensing and signal transduction during an IFN response\textsuperscript{385}. MAVS, one of the intracellular proteins responsible for recognizing CVB3, oligomerizes at sites with high cholesterol content on the mitochondrial membrane\textsuperscript{386}. With cholesterol synthesis reliant on eIF5A-hypusine, blocking hypusination could potentially be beneficial for CVB3 by preventing MAVS signal transduction. However, CVB3’s 3C already cleaves MAVS and blocking hypusination did not increase any aspect of CVB3 replication \textit{in vitro}. Depletion of cholesterol with statins also limits a cell’s ability to respond to IFN α/β. IFNAR expression is down and is no longer endocytosed in these statin treated cells\textsuperscript{387}. Treatment with a hypusination inhibitor could result in a similar phenotype and prevent cells from entering an antiviral state, making it easier for subsequent CVB3 infections. Besides cholesterol’s role in lipid rafts, it is also used as a precursor molecule for 25-hydroxycholesterol. This molecule elicits an inflammatory response and is upregulated during an IFN response\textsuperscript{388}. 25-hydroxycholesterol blocks the fusion of enveloped viruses with the plasma or endosomal membranes and also limits enterovirus replication by preventing PI4P/cholesterol shuttling\textsuperscript{276,389}. 25-hydroxycholesterol can also bind to SCAP, preventing SREBP2 maturation and thus further production of cholesterol\textsuperscript{244}. The inhibition of hypusination would limit the production of 25-hydroxycholesterol due to a lack of intracellular cholesterol. This would be potentially beneficial for CVB3; however, as shown here and in other works, CVB3 is able to overcome a lack of cholesterol within ROs through mutation of 3A. Enteroviruses are also not impacted as
much by 25-hydroxycholesterol compared to enveloped viruses, so the lack of 25-
hydroxycholesterol would most likely not significantly increase CVB3 infection.

Cholesterol is a vital metabolite for cells, but it is also linked to inflammation. During acute infections and in autoimmune disorders, the levels of high-density lipoprotein (HDL) are decreased\textsuperscript{390}. HDL is thought to be anti-inflammatory by protecting low-density lipoprotein (LDL) from being taken up by artery wall cells and being oxidized\textsuperscript{391}. If LDL is high or HDL is low, LDL can be taken up by cells where it can be modified and presented externally as a ligand for macrophage pattern recognition receptors, directly triggering an inflammatory response\textsuperscript{390}. Modified LDL can also be engulfed by macrophages, enhancing toll-like receptor signaling. This process is the mechanism behind the chronic disease atherosclerosis, where cholesterol is deposited on arteries and triggers a macrophage inflammatory response. Other diseases, such as lupus and rheumatoid arthritis, are more severe if there are defects in the cholesterol efflux pathway (HDL production)\textsuperscript{392,393}. Treatment of patients with these diseases often include nonsteroidal or steroid anti-inflammatory drugs as well as other immune-modulatory drugs. It is possible that treatment with DFMO or other drugs targeting the polyamine pathway could help alleviate symptoms by decreasing cholesterol and thus, macrophage activation. However, it is unknown how DFMO treatment affects LDL and HDL levels \textit{in vivo} and if polyamines’ role in hypusination is responsible for the decrease in serum cholesterol seen in animal models.

Cells require a variety of metabolites for proper function and viruses are dependent on these metabolites to infect cells. Previous studies had suggested a link between the two metabolites polyamines and cholesterol, which are vital for both
cellular function and CVB3 infection. The goal of this dissertation was to elucidate how polyamines were altering intracellular cholesterol and how this underlying mechanism impacted CVB3 infection. We found that polyamines’ role in translation, through the hypusination of eIF5A, was required for the proper translation of SREBP2. Without hypusinated eIF5A, SREBP2 translation did not occur and resulted in significantly reduced intracellular cholesterol. The impact that the polyamine-cholesterol axis had on CVB3 infection was two-fold. The loss of cholesterol through polyamine depletion or hypusination inhibition resulted in reduced CVB3 binding that could be rescued with the addition of cholesterol. Furthermore, hypusination inhibition altered CVB3’s ability to form replication organelles. This forced CVB3 to mutate its 3A protein so that it no longer required cholesterol to gain partial resistance to hypusination inhibition. This novel relationship between polyamines and cholesterol has broad implications for the treatment of human diseases ranging from viral infections to autoimmune disorders and cancer. The use of DFMO or GC7 could prevent CVB3 from establishing a persistent infection and using them in conjunction with other antivirals would decrease the possibility of CVB3 becoming resistant to treatment. Polyamine lowering drugs could also be used to lessen symptoms from certain autoimmune disorders. Nevertheless, further exploration is needed to determine how the polyamine-cholesterol axis impacts human health.


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VITA

The author, Mason Richard Firpo, was born in Pleasanton, CA, on April 5\textsuperscript{th}, 1996, to Melinda and Anthony Firpo. He attended California Polytechnic State University, San Luis Obispo, where he graduated Cum Laude with a Bachelor of Science in Biological Sciences and a Minor in Microbiology in 2018. During his undergraduate studies, Mason worked in the laboratory of Dr. Sandra Clement studying mRNA binding proteins in macrophages during an inflammatory response.

In 2019, Mason matriculated into the Loyola University Chicago Interdisciplinary Program in Biomedical Sciences. He joined the department of Microbiology and Immunology and the laboratory of Dr. Bryan Mounce. In Dr. Bryan Mounce’s laboratory, he studied how polyamines influenced viral infection with support from the T32 Immunology Training Grant awarded to Dr. Katherine Knight and a pre-doctoral fellowship awarded from The American Heart Association.

After completion of his graduate studies, Mason will continue to pursue his interest in scientific research and begin a postdoctoral position at The University of Chicago in the laboratory of Dr. Glenn Randall. He will investigate hepatitis C virus replication and the role of lipids during infection.