

Determining the importance of localization of CAV VP3 and TTV VP3 in selective apoptosis of cancer cells

A Major Qualifying Project Submitted to the Faculty of
WORCESTER POLYTECHNIC INSTITUTE

In partial fulfillment of the requirements for the
Degree of Bachelor of Science

By

Jessica Desmond and Fernando Galvez
Biochemistry

April 28, 2016 Approved:

Dr. Destin Heilman, Advisor (Biochemistry)
Department of Chemistry and Biochemistry, WPI

This report represents the work of WPI undergraduate students submitted to the faculty as evidence of completion of a degree requirement. WPI routinely publishes these reports on its website without editorial or peer review.

Abstract

Viral proteins produced by the Chicken Anemia Virus and the Torque Teno Virus have demonstrated the ability to selectively induce apoptosis in cancer cells independent of the p53 tumor suppressor pathway. Each protein has specific localization; CAV VP3 localizes heavily to the nucleus in cancer cells while TTV VP3 localizes to the cytoplasm. The extent of the importance of the localization of these proteins in respect to their ability to induce apoptosis in cancer cells is still unknown. Cell viability assays and localization experiments were utilized to better understand the killing capacity of these proteins under conditions opposite to the natural wild-type localization. The mutant construct of CAV VP3 has mutations that alter amino acids 86-88 and 116-118 so the protein trans-locates to the cytoplasm as opposed to the nucleus which is seen in the wild-type. The CAV VP3 mutant construct was shown to trans-locate to the cytoplasm instead of the nucleus and was unable to induce apoptosis from the cytoplasm. TTV VP3 will be pushed to the nucleus instead of the cytoplasm by fusing GFP-tagged TTV VP3 with an additional SV40 NLS. Efforts to obtain this construct are ongoing.

Contents

Abstract.....	i
Introduction	1
Chicken Anemia Virus (CAV)	2
Torque Teno Virus.....	3
Project Overview.....	4
Materials and Methods.....	6
Construct Design.....	6
TTV Construct Restriction Digest and Ligation.....	6
Transformation of Chemically Competent E. coli Cells.....	8
Tissue Culture Cell Passage	8
Transfection of H1299 Non-Small Human Lung Carcinoma Cells.....	9
Fluorescent microscopy	9
Cell Viability Assay	10
Results.....	12
Discussion.....	15
Figures.....	18
References	24

Introduction

Cancer is a global disease that has been studied extensively and attributed to many genetic and environmental causes. While there are treatments that are used to fight cancers and reduce tumor size, no cure has been discovered¹. Through research on tumor biology, the general progression of a healthy cell to a cancerous cell is fairly understood and occurs when damaged cells proliferate uncontrollably. To ensure processes are functioning properly, there are specific checkpoints throughout the cell cycle, which, when triggered, may induce apoptosis to avoid uncontrolled growth. Though there are many checkpoints in the cell cycle, a specific and well known checkpoint utilizes the p53 tumor suppressor protein. If the cell detects defects at this stage, it will express this protein which results in cell cycle arrest in the G2 phase².

This p53 tumor suppressor can initiate DNA repair or activate an alternative apoptotic pathway when broken DNA is detected to promote the formation of the Apoptosome which initiates a caspase-guided apoptosis³. Mutations in p53 are linked to almost every form of cancer and are involved in about 50% of all tumors^{4, 5}. Missense mutations are the most common in the p53 transcription factor encoded by the TP53 gene. These mutations allow a full protein to be formed but with either altered or obliterated functionality. For example, a mutation in TP53 occurs in almost all cases of ovarian cancer⁴. According to an accepted model called the “Vogelgram”, a p53 mutation is an initial step in the formation of a tumor, but subsequent mutations in oncogenes and other tumor suppressor proteins are required for the formation of a tumor⁶.

Current methods of treatment for patients with cancer include chemotherapy, hormone therapy and surgery to remove tumors. The chemicals used in chemotherapy degrade the DNA in tumor cells, causing a response of functional p53 to induce apoptosis. However, in the case of a

p53-mutated tumor, chemotherapy is ineffective because the mutated p53 cannot repair the DNA and subsequently cannot signal for programmed cell death⁷. As a result, researchers have begun to explore different avenues in order to develop alternative therapies that do not rely on a p53-mediated pathway. It is well documented that certain classes of viral proteins have the ability to induce apoptosis through a p53-independent pathway. In fact, certain *Circoviridae* proteins have been found to selectively induce apoptosis in cancer cells⁸. An excellent example of this phenomenon is the third viral protein produced by the Chicken Anemia Virus (CAV).

Chicken Anemia Virus (CAV)

The Chicken Anemia Virus was first identified by Yuasa et al. in 1979 and has been described as a non-enveloped virus that remains active in spite of temperature or chemical changes in the environment⁹. The virus presents itself in the first three weeks of a chicken's life and is contracted through vertical transmission or contact exposure just before a chick hatches. Older chickens can contract the disease and may exhibit transient symptoms but without fatal consequences. The effects of the virus on chickens can include anemia, subcutaneous hemorrhaging, and compromised immunity to bacterial diseases. The genome of CAV is approximately 2.3 Kbp of single stranded, circular DNA¹⁰. When infecting a host cell, the virus introduces its single-stranded DNA into the cell, which then enters the nucleus and uses the host cell polymerase resulting in a double stranded DNA. This double stranded DNA is transcribed by the cell to form mRNA encoding VP1 (viral protein 1), VP2, and VP3 (Apoptin). CAV VP3 has a unique ability to induce apoptosis in malignant human cells without the use of the p53 pathway¹¹. In approximately 50% of human tumors, p53 protein is absent or dysfunctional which is why CAV VP3 may be used as an alternate cancer therapy¹². However, further research on the CAV VP3 apoptotic mechanism and characteristics is necessary to develop a therapeutic.

An important attribute to note in CAV VP3 is that it maintains a nuclear steady-state localization in cancer cells but remains largely in the cytoplasm in normal cells¹³. It has been hypothesized that the nucleocytoplasmic shuttling of CAV VP3 to the nucleus is crucial for the protein to selectively destroy abnormal, transformed cell lines. When cancer cells are transfected with CAV VP3 then fused with normal cells transfected with CAV VP3, the VP3 in the normal cells trans-locates to the nucleus as it does in the cancer cells. However, when the normal cells are not fused, CAV VP3 is localized to the cytoplasm. The localization of the protein occurs in the early stages of the transformation of a normal cell into a cancerous cell⁸. Based on the proposed mechanism, it is understood that after CAV VP3 localizes to the nucleus, the protein interacts with APC/C (anaphase-promoting complex/cyclosome) subunit 1 in p53-null cells. The interaction between CAV VP3 and APC/C subunit 1 causes G2/M cell cycle arrest and induces apoptosis¹⁴.

Being a member of the Circoviridae family, the Chicken Anemia Virus shares a large homology with the Torque Teno Virus (TTV). TTV VP3, a homologue to CAV VP3, is also capable of inducing apoptosis regardless of a p53 mediated pathway, but demonstrates a subcellular localization completely opposite to that of CAV VP3. Due to the homology of both third viral proteins, the known characteristics of CAV VP3 may elucidate potential means for an apoptotic mechanism in the Torque Teno Virus.

Torque Teno Virus

The Torque Teno Virus was first discovered in a Japanese patient known as “TT” who contracted what was thought to be post-transfusion hepatitis. Research on the serum from the patient showed no homology to hepatitis sequences and therefore was characterized as a Transfusion-Transmitted virus. Though TTV is typically asymptomatic, it is thought to be

associated with autoimmune, liver, respiratory tract, and hematopoietic diseases¹⁵. Through further research and clinical trials, it was determined that TTV is prevalent among various genotypes, especially in Asian populations¹⁶. Regardless of how common the Torque Teno Virus is, when it was first identified, there was no record of any related viral DNA sequence¹⁷. Since then, TTV has been classified as a *Circoviridae* virus assigned to the floating genus *Anellovirus*.

As with CAV, the third ORF of the TTV genome produces a 105 amino acid long protein, TTV VP3, that has been shown to induce apoptosis selectively in transformed cells^{18, 19}. Unlike CAV VP3, the TTV VP3 steady-state localization is primarily in the cytoplasm in cancer cells while it is cytoplasmic in normal cells^{19, 20}. Although the mechanism for CAV VP3 is mostly understood, the homologous characteristics to TTV VP3 are being used to help identify if TTV VP3 forms a multimer similar to CAV VP3²¹. In regards to the feasibility of drug development to mimic the functions of the proteins, it is necessary to understand the mechanisms by which they induce apoptosis and if subcellular localization to specific compartments is integral in the function of the protein.

Project Overview

The extent of the importance of re-localization of CAV VP3 and TTV VP3 is still unknown. Throughout this project, cell viability assays and localization experiments will be utilized to better understand the killing capacity of these proteins when shuttled to subcellular compartments opposite of their wild-type; CAV VP3 was shuttled to the cytoplasm instead of the nucleus and TTV VP3 was shuttled to the nucleus as opposed to the cytoplasm. The NLS (nuclear localization signal) of CAV VP3 contains mutations that alter the amino acid sequence so the protein relocates to the cytoplasm in cancerous cells as opposed to the wild-type location

in the nucleus. For TTV VP3, this will be accomplished by fusing GFP-tagged TTV VP3 with an additional SV40 NLS.

Materials and Methods

Construct Design

The GFP-Ap-pmNLS mutant previously synthesised by the Heilman group was used in the localization and cell viability assays⁸. This construct contained point mutations that mutated the NLS amino acids 86-88 from KKR to AAA and amino acids 116-118 from KRR to AAA (Figure 1).

Primers were designed for PCR resulting in a TTV VP3 sequence with an attached SV40 NLS flanked by restriction sites for BamHI and EcoRI. The forward primer was 5' GCGCGCG AATTCTATGAT 3' which contained an EcoRI cut site and an overhang complementary to the 5' end of TTV VP3. The reverse primer was 5' GCGCGCGGATCCCTACACCTTCCGC TTCTTCTTCGGGCAGGTCTGCGTCTTCGG 3' which added the SV40 NLS sequence and contained a BamHI cut site and an overhang for the 3' end of TTV VP3. Using Gotaq master mix polymerase, a PCR protocol was run with an initial step at 95°C for 30 seconds followed by 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 65°C for 30 seconds then a final two minutes and thirty seconds at 68°C and stored at -20°C.

TTV Construct Restriction Digest and Ligation

A GFP plasmid was digested using 3 µl of plasmid, 1 µl of EcoRI, 1 µl of BamHI, 2 µl of Buffer E, and 13 µl of nuclease free water. This was incubated in a 37°C water bath for two hours and run alongside the insert digest on a 0.9% agarose gel at 90V and purified using the Promega Wizard SV Gel and PCR Clean-up System.

For all Promega Wizard SV Gel and PCR Clean-up System steps, the nuclease free water used to elute was heated to 90 °C and allowed to incubate on the column for one minute at 50 °C instead of room-temperature water and a room-temperature incubation.

The TTV-SV40 NLS PCR product was then subject to a double-restriction digest using 16 μ l of the primary PCR product, 1 μ l of EcoRI, 1 μ l of BamHI, and 2 μ l of Buffer E for a total volume of 20 μ l. The mixture was then incubated in a 37°C water bath for two hours to ensure sufficient restriction. The digest was then co-purified with an excised gel band of restricted GFP plasmid using the Promega Wizard SV Gel and PCR Clean-up System.

A GFP plasmid was simultaneously digested using 3 μ l of plasmid, 1 μ l of EcoRI, 1 μ l of BamHI, 2 μ l of Buffer E, and the volume adjusted to 20 μ l using nuclease free water. This was incubated in a 37°C water bath for two hours and run alongside the insert digest on a 0.9% agarose gel at 90V and gel using the Promega Wizard SV Gel and PCR Clean-up System.

For the ligation of the co-purified insert and plasmid, 16 μ l of co-purified insert and plasmid was used, 2 μ l of the 10X ligase buffer, and 2 μ l of ligase to a total volume of 20 μ l. This was then incubated overnight at 4°C and 7 μ l were used for transformation of chemically competent JM109 *E. coli* (Figure 2).

For a positive control, GFP-tagged PCV1 plasmid was digested with the same method as the GFP plasmid above, but not gel-purified. After incubating in a 37°C water bath for two hours, the digested plasmid was purified using the Promega Wizard SV Gel and PCR Clean-up System and eluted in 30 μ l of nuclease free water. From this, 16 μ l were added to 2 μ l of ligase and 2 μ l of 10X ligase buffer. The mixture was incubated at 4°C overnight then 7 μ l of the ligation was used to transform competent JM109 *E. Coli*.

A negative control was also developed using a GFP plasmid lacking an insert. This was done by performing the same restriction digest mentioned above. However, the 20 μ l of restricted plasmid were then run on a 0.9% agarose gel at 90V and the 3.5 Kb GFP band was excised and gel purified using the Promega Wizard SV Gel and PCR Clean-up System and

eluted in 30µl of nuclease free water. From this elution, 16 µl was added to 2µl of ligase, 2µl of 10X ligase buffer and incubated at 4°C overnight incubation, 7 µl was and used to transform competent JM109 *E. coli*.

Transformation of Chemically Competent *E. coli* Cells

A tube stored at -80°C containing 50 µl of competent JM109 *E. coli* was thawed on ice and 6 µl of DNA construct were added to the tube and gently flicked 3-5 times to mix. Then, the tube was incubated on ice for 15-20 minutes to allow DNA complexes to form then heat-shocked in a water bath at 42°C for exactly 60 seconds. The tube was returned to ice for 2 minutes. Following this, 450 µl of warm LB media were added to the tube. The sample was incubated for one hour at 37°C on a rotor. Lastly, 150 µL of the sample were plated on warm LB agar containing 1X kanamycin.

Tissue Culture Cell Passage (T75 flask)

In a sterile hood, the pre-existing media was aspirated from the H1299 non-small human lung carcinoma cells. A wash of 5 mL of PBS solution removed any remaining media then 1 mL of trypsin protease solution was added to the flask and gently swirled. The trypsin was almost immediately aspirated from the flask and the cells were viewed under a light microscope. After ensuring the cells had detached, 5 mL of D10 media were added to the flask. The media was forcefully pipetted up and down over the surface of the flask multiple times to ensure a homogenous mixture. Of this mixture, 5 ml were transferred to a clean T75 flask containing and an additional 5 mL of D10 media.

Transfection of H1299 Non-Small Human Lung Carcinoma Cells *(For Duplicate Wells)*

H1299 non-small human lung carcinoma cells were passed into a 6-well round bottom plate between 40% and 60% confluency a day before transfection. Using a Nano-drop, the DNA of the $\Delta\Delta$ NLS mutant, wild-type CAV VP3 and eGFP was diluted to 1 μg of DNA in 200 μl of EC buffer using the Qiagen Effectene Transfection kit. Enhancer (6.4 μl) was added to the Eppendorf tube, vortexed, and incubated at room temperature for 2-5 minutes. Following the incubation, 20 μl of Effectene reagent was added and gently flicked to mix, then incubated at room temperature for 10-15 minutes to allow complexes to fully form. Meanwhile, the growth media was aspirated from the 6-well round bottom plate, washed once with 1 mL 1X PBS, and 1.5 mL of fresh D10 media were added to the wells. Once the incubation was complete, 1.2 mL of D10 media were added to each tube and then ~ 710 μl of the solution was added dropwise to each well. The plate was swirled and returned to 37°C incubator for 2 days depending. For fluorescent microscopy experiments, the wells contained sterile glass microscope cover slides prior to the cells being added in order for the cells to mount.

Fluorescent microscopy

The 6 well round bottom plate was removed from the incubator and the media was aspirated exposing the glass coverslips. The slides were washed with 1X PBS and covered with 1 mL of 4% paraformaldehyde in PBS. The plate was then transferred to a nutator for 15 minutes. Following the incubation, the paraformaldehyde was aspirated off and the cells were washed again with 1mL of 1X PBS and 2 mL of 70% ethanol were added to each well. The coverslips were gently removed from the wells and mounted on glass slides using 15 μl of mounting media containing dapI. The cover slides were then pressed to expunge any excess mounting media then

sealed with clear nail polish and viewed under a confocal microscope to assess localization of CAV VP3.

Cell Viability Assay

Beginning 24 hours after transfecting ~70% confluent cells, the plates were viewed under a fluorescent microscope to determine adequate transfection efficiency (~50-60%) and similar transfection across all samples. When the confluency of the cells became 90% or higher, the first round of G418 Geneticin treatment was carried out. The culture media was aspirated and the cells were washed with 1 mL of 1X PBS. After swirling the plates to gently shear the dead cells from the flask, the PBS was aspirated then treated with 0.05X trypsin diluted in D10 media and allowed to sit for 20 seconds. The trypsin was then aspirated off and the cells were resuspended in 2 mL of media containing a concentration of Geneticin at 800 $\mu\text{g}/\text{mL}$. The media was pipetted onto the walls of the wells carefully to reduce the amount of stress placed on the cells. Then, 825 μl were transferred to another 6-well round bottom flask and 1.175mL of G418 laced media, the same as before, were added to raise the volume to 2 mL. Two days later, the same selection process was performed; however, the concentration of G418 laced media was increased to 1200 $\mu\text{g}/\text{mL}$. Two days later, the remaining media was then aspirated off the cells and washed with 1 mL of 1X PBS. Following the wash, the PBS was aspirated and the cells were fixed with 4% paraformaldehyde prepared in 1X PBS. The plates were placed on a nutator and the cells allowed to fix for 15 minutes. After incubating the cells on the nutator, 0.75 mL of 0.1% (w/v) crystal violet was prepared in 10% ethanol and then added to the cells, incubating them for an additional 20 minutes. The cells were then washed with distilled water until there was no trace of a violet tinge. The wells were then allowed to dry and photos were taken to evaluate the remaining viable cells. The staining agent for the photo evaluation was solubilized by 2% (w/v) sodium dodecyl

sulfate (SDS). Utilizing a Genesys 20 Visible Spectrophotometer set to 590 nm, the wells optical densities (OD) were measured. Cell viability was calculated through the equation: (Optical Density of Experimental/Optical Density of controls) x100%.

Results

Currently, most medical treatments for patients with cancer are dependent on a functional p53 tumor suppressor protein to induce apoptosis. Considering about 50% of malignant cancers have dysfunctional p53, there has been a push to research novel methods of cancer treatment which rely on different mechanisms⁵. Potential candidates for p53 independent anti-cancer therapy are viruses that have been discovered which induce apoptosis selectively in cancer cells⁸. Of these viruses, the Chicken Anemia Virus protein 3 (CAV VP3) is the most characterized and its homologue, the Torque Teno Virus protein 3 (TTV VP3), is currently being studied. In cancer cells, CAV VP3 and TTV VP3 have unique localizations and understanding the relevance of this localization on the apoptotic ability of the proteins is an important step in determining the feasibility of mimicking their function for cancer drug development. CAV VP3 resides predominantly in the nucleus in cancer cells as opposed to the cytoplasm in normal cells. However, the TTV VP3 steady-state localization is primarily cytoplasmic in both cancerous and primary cells¹⁸. Thus, it has become an area of interest to determine if a change in subcellular localization is necessary for TTV VP3 or CAV VP induced apoptosis.

CAV VP3

In order to investigate how the localization of CAV VP3 affects protein function, a mutant construct was developed to functionally knock out the NLS of the protein. The mutant was tagged with GFP for visualization of localization and compared to WT-CAV VP3. The GFP-Δp-mNLS CAV VP3 mutant construct contained point mutations in the NLS to drive the steady-state equilibrium toward the cytoplasm (Figure 1). These point mutations included altered amino acids 86-88 (KKR) and 116-118 (KRR) to alanine residues. In an effort to determine if the changes to the NLS were effective, the plasmid containing the point mutations was transfected into H1299 non-small human lung carcinoma cells. In comparison to the GFP-WT-CAV VP3

control, the GFP-Ap-pmNLS showed extreme cytoplasmic localization (Figure 3). In order to quantify the dispersion of protein around the cell, GFP fluorescence of de-convoluted confocal microscope images were measured using ImageJ. The mutant CAV VP3 transfected cells had a nuclear to cytoplasmic ratio of 1:10 which is opposite to the GFP-WT-CAV VP3 that had a ratio of 10:1 (Figure 4). The localization assay results conclusively demonstrate that the GFP-Ap-pmNLS mutant causes CAV VP3 to localize to the cytoplasm rather than the nucleus. Once it was understood that the mutant protein localized to the cytosol, the killing capacity of the GFP-Ap-pmNLS needed to be determined in comparison to WT-CAV in a cell viability assay.

In order to determine cell viability of our construct, H1299 cells were transfected with mutant, GFP, or Flag-WT-CAV VP3 plasmid and allowed to grow. The cells were fixed to a 6-well plate and stained with crystal violet after four days of G418 treatment. All constructs were observed with specific controls using pure D10 media and non-transfected H1299 cells. Transfected cells contained a resistance to G418 and survived selection while all non-transfected cells died. The G418-treated non-transfected cells showed no viable cells present after the fourth day of selection while the non-G418-treated non-transfected cells showed normal cell growth. After visually observing the differences between the Flag-WT-CAV VP3 and GFP-Ap-pmNLS, it was determined that there was a significant difference between their apoptotic functionality. The GFP-Ap-pmNLS and the GFP control qualitatively showed a similar amount of viable cells after staining (Figure 5).

To attempt to quantify the difference in killing capacity, the crystal violet stripped from the GFP-transfected cells that were subject to G418 treatment were measured using a spectrophotometer at 590 nm, obtained an absorbance of 9.216 O.D. compared the WT-CAV VP3-transfected cells which read a 0.3065 O.D. and the mutant at 19.01 O.D. While it is

apparent that the wild type terminated the vast majority of cells within the wells, the mutant appeared to have a comparable killing capacity to that of GFP, which is known to leave all transfected cells unharmed.

TTV-VP3

It was hypothesized that adding a stronger NLS to TTV VP3 would shift the steady-state equilibrium to the nucleus rather than the cytoplasm. The construct design contained GFP-TTV VP3 with an attached SV40 NLS on the C-terminus of TTV VP3 (Figure 2).

Figure 7 shows the TTV VP3-SV40 NLS PCR product on an agarose gel. This product was then subjected to a restriction digest using EcoRI and BamHI in order to create homologous sticky ends of the PCR product and the GFP plasmid to allow ligation. Separately, a GFP plasmid was also digested with EcoRI and BamHI then purified from an agarose gel (Figure 6). To increase the concentration for the subsequent ligation, the restricted GFP and PCR product were co-purified through a column. The presence of both restriction products prior to the ligation reaction were confirmed using gel electrophoresis (Figure 7). Despite the presence of both insert and plasmid, the ligation failed regardless of the insert to plasmid ratio. Efforts to obtain this construct are still ongoing.

Discussion

Proteins produced by the Chicken Anemia Virus and the Torque Teno Virus may provide insight to develop new p53 independent drugs for cancer patients. These viral proteins are being studied to potentially develop new cancer therapies that mimic their function. Considering approximately 50% of cancer treatments rely on a functional p53 tumor suppressor to induce apoptosis in transformed cells, treatments ranging from chemotherapy and radiation are not effective in tumors with mutated p53. It is well understood that the Chicken Anemia Virus VP3 exists in the cytoplasm in normal cells but localizes to the nucleus in cancer cells. Heilman et al has shown that CAV VP3 most likely interacts with the APC/C subunit 1⁸. Upon binding to the APC/C subunit, CAV VP3 induces G2/M cell cycle arrest. While it has been documented that the APC/C- CAV VP3 interaction occurs in the nucleus, it has also been found that the APC/C subcomplex homologues also reside predominantly in the cytoplasm but transiently shuttle to the nucleus. Due to the localization of APC/C subunit homologues in both compartments of the cell, it was important to analyze whether nuclear localization is necessary for CAV VP3 binding of the APC/C complex or if CAV VP3 can interact with cytoplasmic APC/C subunits to achieve the same apoptotic result.

It was hypothesized that the point mutations made to the NLS of CAV VP3 in the GFP-Ap-pmNLS mutant would disrupt the ability of the protein to bind to the necessary factors that would otherwise transport the protein to the nucleus. In order for a protein to localize in the nucleus, the NLS of the protein must bind to a nuclear transport receptor (importin) and RanGTPase. The importin/RanGTPase/CAV VP3 complex is transported through a nuclear pore on the nuclear membrane. Since it has been shown that importin will bind to various NLS sequences and a consensus sequence has yet to be identified, it is hypothesized that importin

relies on a secondary or tertiary structure which exposes the necessary amino acids for binding²²⁻²⁴. In this experiment, it was theorized that the mutations of leucine and arginine residues 86-88 and 116-118 to alanine residues have either induced a conformational change or directly altered importins binding site on CAV VP3. De-convoluting laser confocal microscopy images of GFP-WT CAV VP3 and GFP-Ap-pmNLS mutant transfected cells allowed analyses of the effect of mutations in the NLS on the localization of the protein. The images of the WT-CAV VP3 localization corroborated previous data that indicated extreme subcellular steady state localization in the nucleus. Contrary to this, the mutant construct indicated steady-state levels to be cytoplasmic as previously shown by the Heilman group⁸. These results indicate that the interaction with importin was disrupted as hypothesized.

After determining that the changes to the NLS were successful, the killing capacity of the GFP-Ap-pmNLS mutant was analyzed. When observing the stained 6-well plates in the cell viability assay, it was clear that the Flag-WT-CAV VP3 successfully induced apoptosis in all transfected cells. It was also apparent that the GFP control and GFP-Ap-pmNLS mutant had similar amounts of staining on the plate. Considering GFP was used as a control indicating that all transfected cells retained the ability to proliferate freely and the wells of both GFP and GFP-Ap-pmNLS were qualitatively the same, it is apparent that the mutant CAV VP3 lacks an ability to induce apoptosis. Since the cytosolic CAV VP3 was comparable to GFP, it can be concluded that localization is integral to the apoptotic function of CAV VP3.

The epitope tag was used on the Flag-WT CAV VP3 construct because it is known that the addition of a GFP chromophore to WT-CAV VP3 renders it nonfunctional, but the use of the Flag tag allows the WT-CAV VP3 construct to maintain its killing capacity. A fluorescent chromophore may have the same effect on the GFP-Ap-pmNLS which could be an alternate

explanation of the results obtained in the cell viability assay. Diminished apoptotic capacity between the Flag and GFP-tagged protein could be attributed to the size of the GFP chromophore (238 amino acids) in comparison to the 8 amino acid epitope tag^{25, 26}. The inability of the GFP-Ap-pmNLS construct to kill the cancer cells could either be due to the re-localization to the cytoplasm or the addition of the chromophore. Further evaluation using the cell viability assay should be done using an epitope-tagged Ap-pmNLS mutant to determine whether the GFP or the change in localization is causing the protein to be non-functional in the cytoplasm.

The quantifiable data obtained in the localization assay could not be normalized because the transfection efficiency of the Flag-WT-CAV VP3 could not be visually determined. However, when the crystal violet was stripped from the plates and analyzed for relative concentration at 590 nm, the raw data showed an extreme difference between the Flag-WT-CAV VP3, the GFP control and GFP-Ap-pmNLS construct. Qualitatively, this extreme difference, even without normalizing the data, supports the conclusion that the cytoplasmic GFP-Ap-pmNLS construct does not induce apoptosis.

In regards to TTV VP3, the attempts to obtain the GFP-TTV VP3-SV40 NLS construct are still ongoing. Additional construct designs could involve the addition of another strong NLS or mutations in the NES of TTV VP3 to alter the protein localization in cancer cells. Subsequently, a cell viability assay should be conducted to determine the killing capacity of TTV VP3 when localized to the nucleus instead of the cytoplasm.

Figures

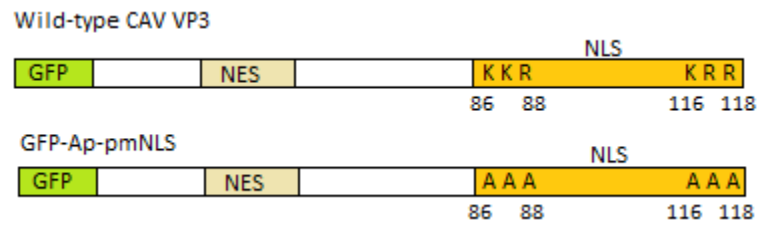


Figure 1: The GFP-Ap-pmNLS CAV VP3 mutant construct previously synthesized in the Heilman lab. Point mutations were made in the gene to cause triple alanine amino acid changes in the protein. The wild type CAV VP3 protein has a KKR sequence at amino acid residues 86-88 and a KRR sequence at amino acid residues 116-118⁸.

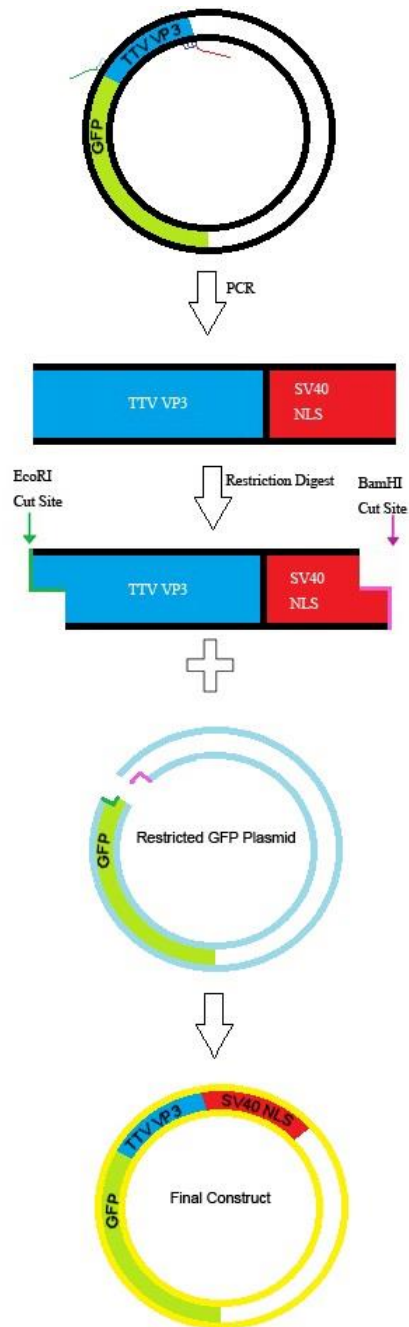


Figure 2: Synthesis design to obtain a construct with GFP-tagged TTV VP3 with an attached SV40 NLS. Primers containing EcoRI and BamHI cut sites and an SV40 NLS were used in PCR to attach to the ends of the TTV VP3 sequence. This product was then digested with EcoRI and BamHI and ligated into a GFP vector previously restricted with the same enzymes.

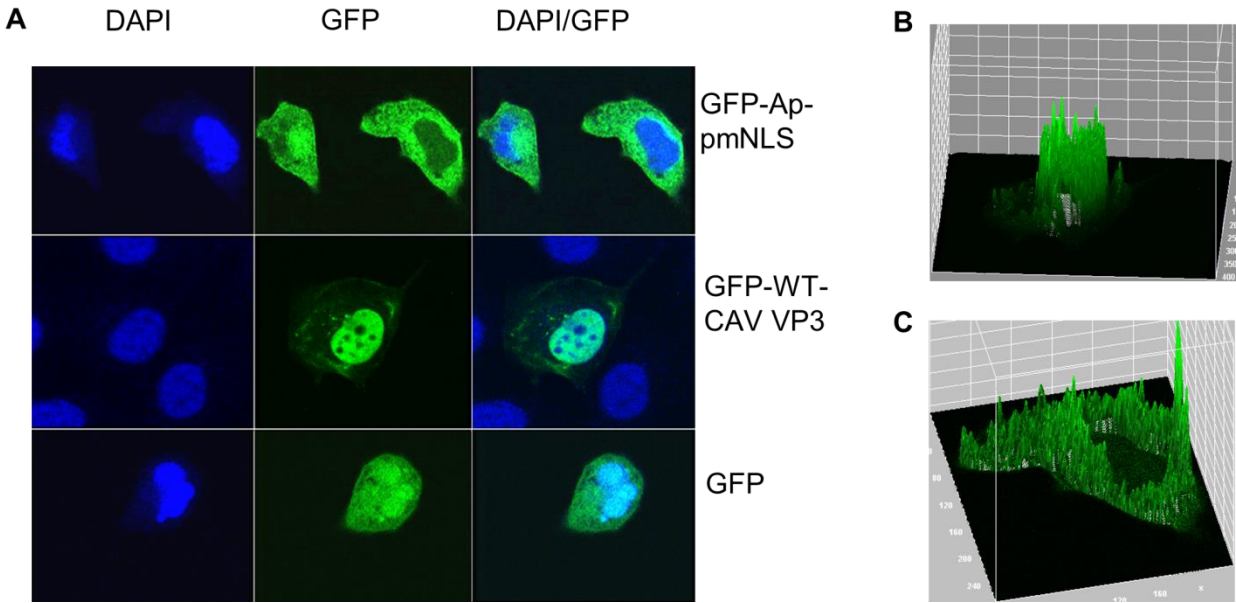


Figure 3: Subcellular localization of GFP-Ap-pmNLS CAV-VP3, GFP-WT-CAV VP3, and GFP. H1299 cells were transfected with GFP-Ap-pmNLS mutant, GFP, or GFP-tagged WT-CAV VP3 in 6-well plates containing glass microscope cover slips. After two days, the cover slips were carefully removed from the wells and mounted to microscope slides using mounting media and DAPI. The cover slips were secured to the slides and viewed under a confocal microscope. A.) Confocal microscope photographs of transfected cells. DAPI staining represents the nucleus of cells. GFP represents expression of the transfected gene. The overlay of both in the GFP-Ap-pmNLS mutant shows very little GFP in within the DAPI staining. This means that the protein is cytoplasmic. The GFP shows ubiquitous GFP expression as a positive control. GFP-WT-CAV VP3 is almost entirely nuclear. B.) This is the GFP ratio throughout different areas of a GFP-Ap-pmNLS transfected cell. The hole in the middle corresponds to the nucleus which expressed very little protein. C.) The GFP expression in the GFP-WT-CAV VP3 is almost completely nuclear as shown by the large spike in the middle. These images were derived using ImageJ technology.

Nuclear to Cytoplasmic Ratio

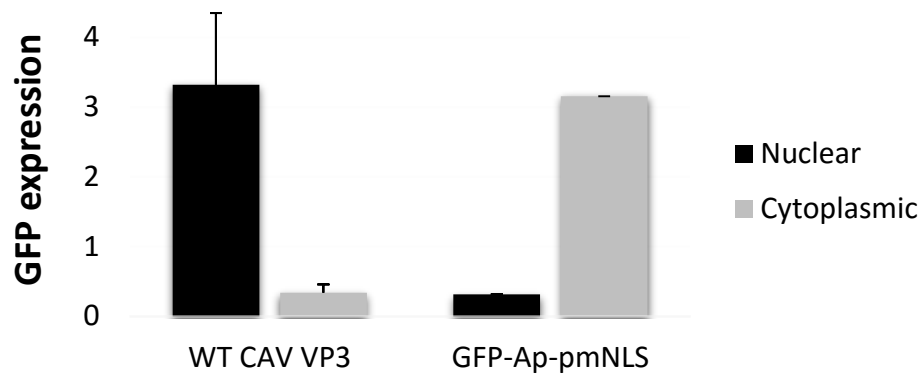


Figure 4: A graph of GFP expression in the nucleus versus the cytoplasm with respect to the GFP control set to one. The GFP-WT CAV VP3 shows a nuclear to cytoplasmic expression ratio of 10:1 indicating that the majority of the protein resides in the nucleus. In contrast, the GFP-Ap-pmNLS had a nuclear to cytoplasmic ratio of 1:10. This supports the hypothesis that the point mutations made in the NLS the GFP-Ap-pmNLS construct causes the protein to re-localize to the cytoplasm. No standard deviation was determined for the GFP-Ap-pmNLS mutant as only one cell was observed.

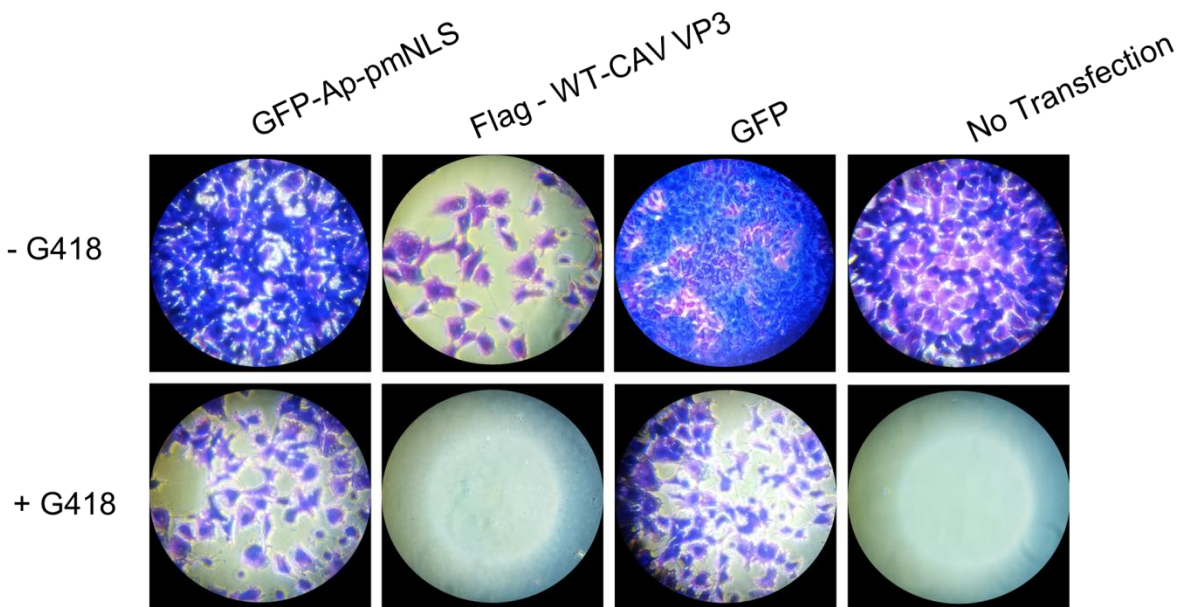


Figure 5: Cell viability assay. G418 drug selection was performed over a 4 day period to determine the percentage of viable cells present. The plasmid used to transfect H1299 non-small human lung carcinoma cells contained a resistance to G418 resulting in only transfected cells surviving both days of drug selection. **Top:** Transfected cells were not subjected to the G418 drug selection. All samples contained many surviving cells with the exception of the Flag-WT-CAV VP3 which inherently kills cancer cells. The GFP-Ap-pmNLS construct allowed for similar growth as the GFP transfected cells. **Bottom:** Transfected cells were subjected to G418 selection. Only transfected cells survived this selection. Flag-WT-CAV VP3 wells were almost completely empty as all successfully transfected cells underwent apoptosis and all non-transfected cells died in selection. Non-transfected cells in this case were all terminated due to the lack of G418 resistance. The survival of GFP-Ap-pmNLS transfected cells was very similar to the GFP transfected survival.

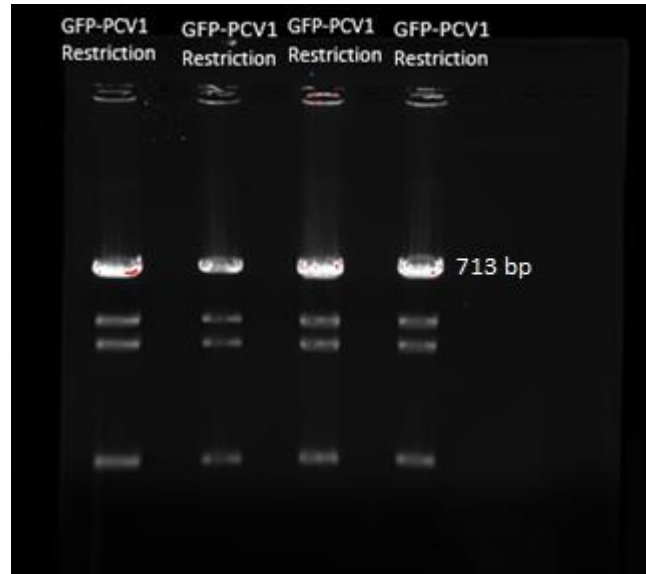


Figure 6: EcoRI and BamHI digested PCV1 VP3 GFP plasmid. The 713 bp band was excised and purified for ligation with the TTV VP3 - SV40 NLS insert. This band contains the GFP sequence without the PCV1 VP3 sequence.

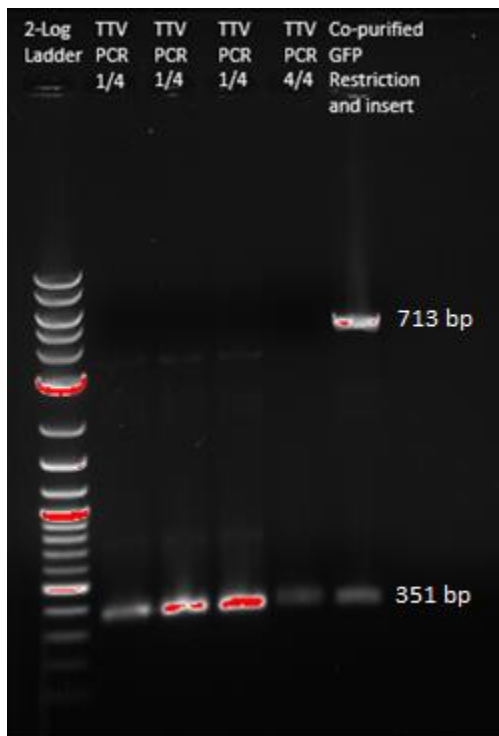


Figure 7: TTV PCR Products are shown in lanes 2-5. The PCR protocol resulted in a 351 bp sequence containing TTV VP3 and an SV40 NLS flanked by EcoRI and BamHI cut sites. Lane 6 shows the presence of both the restricted GFP plasmid and the restricted TTV VP3-SV40 insert after co-purification through a column.

References

- [1] American Cancer Society. (2015) What causes cancer, American Cancer Society.
- [2] Abrahamson, J., Lee, J. M., and Bernstein, A. (1995) Regulation of p53-mediated apoptosis and cell cycle arrest by Steel factor, *Molecular and cellular biology* 15, 6953-6960.
- [3] Haupt, S., Berger, M., Goldberg, Z., and Haupt, Y. (2003) Apoptosis-the p53 network, *Journal of cell science* 116, 4077-4085.
- [4] Rivlin, N., Brosh, R., Oren, M., and Rotter, V. (2011) Mutations in the p53 Tumor Suppressor Gene: Important Milestones at the Various Steps of Tumorigenesis, *Genes & Cancer* 2, 466-474.
- [5] Wang, Z., and Sun, Y. (2010) Targeting p53 for Novel Anticancer Therapy, *Translational Oncology* 3, 1-12.
- [6] Thiagalingam, S. (2006) A cascade of modules of a network defines cancer progression, *Cancer Res* 66, 7379-7385.
- [7] Breen, L., Heenan, M., Amberger-Murphy, V., and Clynes, M. (2007) Investigation of the role of p53 in chemotherapy resistance of lung cancer cell lines, *Anticancer research* 27, 1361-1364.
- [8] Heilman, D. W., Teodoro, J. G., and Green, M. R. (2006) Apoptin nucleocytoplasmic shuttling is required for cell type-specific localization, apoptosis, and recruitment of the anaphase-promoting complex/cyclosome to PML bodies, *Journal of virology* 80, 7535-7545.
- [9] Yuasa, N., Taniguchi, T., and Yoshida, I. (1979) Isolation and Some Characteristics of an Agent Inducing Anemia in Chicks, *Avian Diseases* 23, 366-385.
- [10] Rosenberger, J. K., and Cloud, S. S. (1998) Chicken anemia virus, *Poultry science* 77, 1190-1192.
- [11] Noteborn, M. H. M. (2004) Chicken anemia virus induced apoptosis: underlying molecular mechanisms, *Veterinary Microbiology* 98, 89-94.
- [12] Zhuang, S.-M., Shvarts, A., van Ormondt, H., Jochemsen, A. G., van der Eb, A. J., and Noteborn, M. H. (1995) Apoptin, a protein derived from chicken anemia virus, induces p53-independent apoptosis in human osteosarcoma cells, *Cancer research* 55, 486-489.
- [13] Danen-van Oorschot, A. A., Zhang, Y.-H., Leliveld, S. R., Rohn, J. L., Seelen, M. C., Bolk, M. W., van Zon, A., Erkeland, S. J., Abrahams, J.-P., and Mumberg, D. (2003) Importance of nuclear localization of apoptin for tumor-specific induction of apoptosis, *Journal of Biological Chemistry* 278, 27729-27736.
- [14] Teodoro, J. G., Heilman, D. W., Parker, A. E., and Green, M. R. (2004) The viral protein Apoptin associates with the anaphase-promoting complex to induce G2/M arrest and apoptosis in the absence of p53, *Genes & Development* 18, 1952-1957.
- [15] Hino, S., and Miyata, H. (2007) Torque teno virus (TTV): current status, *Reviews in Medical Virology* 17, 45-57.
- [16] Nishizawa, T., Okamoto, H., Konishi, K., Yoshizawa, H., Miyakawa, Y., and Mayumi, M. (1997) A Novel DNA Virus (TTV) Associated with Elevated Transaminase Levels in Posttransfusion Hepatitis of Unknown Etiology, *Biochemical and Biophysical Research Communications* 241, 92-97.
- [17] Mushahwar, I. K., Erker, J. C., Muerhoff, A. S., Leary, T. P., Simons, J. N., Birkenmeyer, L. G., Chalmers, M. L., Pilot-Matias, T. J., and Dexai, S. M. (1999) Molecular and biophysical characterization of TT virus: evidence for a new virus family infecting humans, *Proceedings of the National Academy of Sciences of the United States of America* 96, 3177-3182.
- [18] McCauley, S. (2011) Determining Localization Domains and Apoptotic Ability of the Human Torque Teno Virus VP3 Protein, WORCESTER POLYTECHNIC INSTITUTE.
- [19] Mueller, B., Maerz, A., Doberstein, K., Finsterbusch, T., and Mankertz, A. (2008) Gene expression of the human Torque Teno Virus isolate P/1C1, *Virology* 381, 36-45.

- [20] McCauley, S., Stone, N.P.,. (2011) Determining Localization Domains and Apoptotic Ability of the Human Torque Teno Virus VP3 Protein, In *Chemistry and Biochemistry*, WPI.
- [21] Leliveld, S. R., Zhang, Y.-H., Rohn, J. L., Noteborn, M. H., and Abrahams, J. P. (2003) Apoptin induces tumor-specific apoptosis as a globular multimer, *Journal of Biological Chemistry* 278, 9042-9051.
- [22] Cooper, G. M. (2000) The Nuclear Envelope and Traffic between the Nucleus and Cytoplasm.
- [23] Chook, Y. M., and Süel, K. E. (2011) Nuclear import by Karyopherin- β s: recognition and inhibition, *Biochimica et biophysica acta* 1813, 1593-1606.
- [24] Kosugi, S., Hasebe, M., Matsumura, N., Takashima, H., Miyamoto-Sato, E., Tomita, M., and Yanagawa, H. (2009) Six classes of nuclear localization signals specific to different binding grooves of importin α , *Journal of Biological Chemistry* 284, 478-485.
- [25] Li, X., Zhang, G., Ngo, N., Zhao, X., Kain, S. R., and Huang, C.-C. (1997) Deletions of the *Aequorea victoria* green fluorescent protein define the minimal domain required for fluorescence, *Journal of Biological Chemistry* 272, 28545-28549.
- [26] Einhauer, A., and Jungbauer, A. (2001) The FLAG™ peptide, a versatile fusion tag for the purification of recombinant proteins, *Journal of Biochemical and Biophysical Methods* 49, 455-465.