Assessment of the Similarities Between Porcine Circovirus 2 VP3 and Chicken Anemia Virus Apoptin

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Abstract

Porcine Circovirus Type 2 (PCV2) is a circular, single stranded DNA virus. A protein located in its third open reading frame, VP3, is of extreme interest as a cancer treatment because of its ability to induce apoptosis in cancerous cells. PCV2 is commonly compared to the more understood Chicken Anemia Virus (CAV) because of its protein apoptin, which also induces apoptosis in cancerous cells. To determine if PCV2 uses the same CRM1 export pathway as CAV, PCV2 transfected H1299 cells were treated Leptomycin B, an inhibitor of this pathway. An increase in nuclear accumulation provided evidence that PCV2 VP3 may utilize the CRM1 pathway, but it is not the only method at work. To further test the similarities between the two virus proteins, they were cotransfected to observe relocalization patterns. No patterns of relocalization were observed, suggesting the two proteins must differentiate enough from one another to not allow multimerization. The conjunction of the results illustrate that although the two viruses may carry out a similar function, they may utilize completely different mechanisms.

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Introduction

Cancer

Cancer. This one word describes a host of diseases that kill 7.6 million people per year globally[35]. Cancer is responsible for more deaths than some of the most frequently heard of diseases in the world such as AIDS and malaria. Unfortunately, this already high death number is predicted to increase to 80% by the year 2030. Inadequate care in the many poverty-stricken countries around the globe would be responsible for this increase[35]. Therefore, the race to find an efficient, yet cost effective cure for a wide spectrum of cancer types is in progress.

To successfully invent a novel cure for cancer, the mechanisms of the diseases it causes must be analyzed and comprehended. In the simplest terms, cancer encompasses over 100 types of diseases that possess abnormal and indefinitely dividing cells[2,12]. These 100 varieties of cancer are further broken down into the major categories of carcinoma, sarcoma, leukemia, lymphoma and central nervous system[12].

Despite the numerous different types of cancer, their foundations are all relatively similar. All cancer begins in a cells' DNA, thus it is important to differentiate between a normal and a cancerous cell. A normal cell grows and divides as it is needed in the body. Once a cell has reached mature growth, it divides to produce two new cells through the processes of fission, mitosis or meiosis. In these processes, the goal is conserved; DNA must be apportioned to new daughter cells. The growth pathways taken by a cell are coded within the cell's DNA.

Equally as important as cell growth and reproduction, normal cells also undergo apoptosis, or cell death. Similar to the growth and division pathways of a cell, apoptosis is managed by the cells DNA[12]. Apoptosis is actually the default process for all cells; the only instance in which a cell does not undergo apoptosis is when it receives survival signals[23]. Normal cells stop dividing and undergo apoptosis after 50-70 generations[2]. Apoptosis can also be triggered by events such as stress or infection[6]. The entire process of apoptosis is directed and controlled by genes in a cascade of events. Death receptors and mitochondria play the most significant roles in this process. Death receptors are

analogous to docking areas on the cell surface. They are able to receive signals from the body that indicate whether a cell should undergo apoptosis or not. For example, death receptors can receive signals from the tumor necrosis factor alpha (TNFa), which produces immune activation and can stimulate apoptosis. Once the appropriate signals have been received, the mitochondrion is the organelle within the cell that dictates whether or not apoptosis is going to occur. It contains enzymes that both initiate and halt the process. Cytochrome C is one of the major enzymes released from the mitochondria that trigger apoptosis. Cyotchrome C activates enzymes known as caspases, which initiate the cellular events of apoptosis. Specifically, caspases further stimulate other enzymes that cleave DNA, transcription factors, protein kinases, etc[11]. Caspases are further classified into two categories: initiator and effector caspases. Initiator caspases are considered to be upstream and activate effector caspases, which are considered to be downstream[11]. Morphologically, as the DNA and other inner cellular components are destroyed, the cell begins shrinking, which can be seen most noticeably in the nucleus and cytoplasm under a microscope[6]. Following these morphological changes, the apoptotic cell is phagocytosed[24]. Because DNA regulates both the division and apoptotic processes of the cell, they can be disrupted through mutations commonly found in DNA. As is further discussed below, these mutations that may alter the division and apoptotic pathways, are often responsible for causing cancer[22].

Cancer often forms due to the loss or overexpression of signals sent to the cells in the body. Activator and suppressor genes are important for a cell's ability to receive and respond to these signals appropriately. Activator genes stimulate the cell to grow while suppressor genes induce the cell to stop growing[2]. The functionality of these genes help to dictate whether or not a cell will become cancerous. One extremely critical suppressor gene is p53. In primary cells, this gene induces apoptosis when DNA is damaged or the cell is under stress[32]. In these situations the amount of p53 increases and can solicit one of three responses: growth arrest, DNA repair or apoptosis[3]. However, cancerous cells contain mutated DNA that affects their division and growth processes as well as their ability to appropriately respond to external signaling[12]. These mutations are accumulated over time and are found in different genes in a specific group of cells. There are a number of different genes that can be mutated and result in cancer. Mutations can be

found in oncogenes, which normally promote cell growth, tumor suppressor genes, which normally suppress growth or induce apoptosis, and DNA repair genes, which normally correct naturally occurring DNA mistakes. In most cases, all of these mutations are seen in conjunction with one another to cause cancer. p53 is one of the most commonly mutated suppressor genes[19] . When p53 is stimulated, apoptosis is promoted. In many cancers, components of the p53 pathway are altered, for example more than 50% of human cancers show loss of p53 activity[24]. With the loss of p53 activity, apoptosis is no longer initiated through that pathway. Bcl-2 is a commonly mutated proto-oncogene or activator within cancerous cells. It is responsible for the disruption of apoptosis, thus prevention of cell death in most cells. Within cancerous cells it is frequently overexpressed and may explain cancer cell resistance by evading apoptosis[8]. Within the bcl-2 family there are a number of both antiapoptotic and proapoptic proteins. Most importantly is the antiapoptotic bcl-xL and proapoptotic BAD and BAX. These proteins interact with one another to regulate apoptosis. In cancerous cells, Bcl-2 is often overexpressed. Its overexpression sometimes works in concert with upregulation of VEGF, vascular endothelial growth factor, which is a family of growth factor proteins produced by tumors. They function by attaching to VEGF receptors on blood vessels, which causes new blood vessels to be made. These blood vessels surround tumors and provide them with a copious amount of blood to survive and grow. An additional method cancerous cells utilize to evade apoptosis is the inactivation of BAX[23]. With the loss of either BAX or BAD, or both, cancer cells become completely resistant to induced apoptosis[23]. As a result of these mutations, cancerous cells divide uncontrollably and do not respond to growth inhibiting signals or the actions of activator and suppressor genes[12]. The alterations in genes like bcl-2 and p53 are some of the root causes of normal cell transformation into immortal cancer cells[12].

Though alterations in genes are the causative agent of cancer, not all detrimental changes to genes are inherited[2]. There are a number of alternate causes of cancer and related risk factors that play an equally important role in mutating the genes of normal cells. Environmental factors are some of the most documented cancer causative agents. Carcinogens are a group of chemicals that are known to readily cause cancers including formaldehyde and benzene[2,4,12]. Acrylamide and some artificial sweeteners in foods have

also been linked to causing cancer. Bacteria and viruses, which are collectively referred to as infectious agents, can be indicators of cancer. Some common viruses such as Hepatitis C, Hepatitis B and Epstein Barr virus and the bacteria H. pylori have been associated with the formation and progression of some cancers[4]. Other more obvious causes of cancer stem from radiation that individuals receive from routine X-rays, CT scans, excess UV exposure from the sun and factors as simple as weight, physical activity and diet[2,4].

Understanding the basics of cancerous cells, as well as where they originate, allows scientists to produce targeted cancer treatments. Cancer treatments target the cellular changes and signals within cells that allow cancer to develop. Cancer treatments are grouped based on what part of the cell they act upon. One such group targets and inhibits enzymes. Enzymes are a crucial group to target because of their extensive roles in controlling various processes throughout the body. Enzymes are the protein catalysts used to drastically increase the rate of almost all of the biochemical processes that occur in the body. Without enzymes most reactions would take years to complete. They play roles in the processes of digestion, metabolism, homeostasis, etc.[9] Most commonly, cancer therapies look to block particular enzymes that aid in cancer cell growth signaling; these are referred to as enzyme inhibitors. By blocking these enzymes, cancer cells cannot receive the signals necessary for them to grow and divide uncontrollably. There are a number of enzyme inhibitors that are usually identified by the enzymes they act to block. Some include tyrosine kinase inhibitors, growth factor inhibitors and signal transduction inhibitors[5].

Angiogenesis inhibitors are another group of drugs used to kill cancer cells. Angiogenesis is defined as “the process of making new blood vessels”, thus, angiogenesis inhibitors aid in stopping this process[5]. This is important in treating cancer because all tumors require an adequate blood supply to grow and divide. When their blood supply ceases, the tumors can no longer grow. Specifically, many of these drugs target the vascular endothelial growth factor (VEGF) that was described above. It is a family of growth factor proteins produced by tumors that function by attaching to VEGF receptors on blood vessels. This causes new blood vessels to be made and surround tumors supplying them with the blood they need for continued growth[5].

Apoptosis-inducing drugs are currently an important area of study in discovering a cure to cancer. Some therapies are able to target proteins within cancer cells causing them to undergo apoptosis. The common treatments of chemotherapy and radiation fall within this category. Unfortunately, these types of treatment have a difficult time distinguishing between cancerous and normal cells, causing extensive cell death within a cancer patient. More recently, target therapies have been studied that can differentiate between the two cell types and only cause apoptosis within cancer cells[5]. Cyclin-dependent kinases (cdks), epidermal growth factor receptors (EGF) and Livin/ML-AIP are common targets of these therapies. CDKs regulate cell cycle progression and RNA transcription. In cancer patients these are often overactive, and by inhibiting them, the cell cycle stops and apoptosis is induced. Inhibition of Cdk4 and 6 arrests the G1 phase of the cell cycle, Cdk 2 and 1inhibition effects the S and G2 phases as well as induces apoptosis and transcriptional Cdk 9 inhibition causes apoptosis[33]. Similarly, receptors in the EGF receptor family, especially EGFr and ErbB2, are important for cancerous cells because they can bind to either EGF or transforming growth factor-alpha. When EGF receptors bind these, there is a complete signal transduction pathway that allows for cell proliferation. In transformed cells, overexpression of EGFr, increased concentration of ligands or decreased receptor turnover can lead to increased cell proliferation[16]. With the use of monoclonal antibodies that bind and block EGF receptors, this signal transduction pathway and consequent cell growth can be inhibited in cancer cells [27]. Livin/ML-IAP is a part of a protein family known as inhibitors of apoptosis proteins (IAP). This family encodes negative regulatory proteins that do not allow a cell to undergo apoptosis[21]. Specifically, Livin appears to be upregulated in a number of cancers and may play a crucial role in tumor cell resistance. Therapies are now looking to down regulate this protein to increase apoptosis, reduce tumor growth and stimulate tumors to be more sensitive to chemotherapy[21]. As has been demonstrated, apoptosis-inducing therapies are and have the potential to be strong candidates for curing cancer. Especially those that are capable of targeting only cancerous cells.

With the apparent evidence that cancer therapies that induce apoptosis exclusively in cancer cells could be extremely successful, much research has been done in this area. There are a number of viruses found in species other than humans that show the

capabilities of killing only cancerous cells. The two viruses focused on in the research and experiments of this paper are the chicken anemia virus (CAV) and porcine circovirus 2 (PCV2). Both viruses induce apoptosis exclusively in cancerous cells and are of an extreme interest in discovering a novel cancer treatment. Chicken anemia virus is documented and understood more extensively than porcine circovirus. Because of their similar functionalities, CAV has and is being used to try and better deduce the mechanisms behind PCV2.

Chicken Anemia Virus (CAV)

Chicken Anemia Virus (CAV), discovered in 1979 by Yuasa, causes disease in young chicks. It has been circulating in the United States for at least 25 years[31]. Characteristically, the disease causes severe anemia, subcutaneous hemorrhage, lymphoid atrophy and decreased resistance to bacterial diseases. The virus specifically targets the erythroid and lymphoid progenitor cells[1]. The virion itself is a small, circular, nonenveloped, single stranded DNA virus. It is a polyhedron containing 20 plane faces, also known as icosahedral with a 2.3 kilobase genome consisting three overlapping reading frames[17,31]. These reading frames code for the three viral proteins VP1, VP2 and VP3. VP1 has been identified as the capsid protein. It includes a relatively basic N terminal region of 50 amino acids that is thought to interact with the packaged DNA. The C terminal region is more complex and is associated with rolling circle replication (RCR) of DNA. Thus, the C terminal region is thought to have both structural and functional roles making it the only structural protein in the virus. VP2 is a dual specificity protein phosphatase that may interact with VP1 in assembly and stabilization processes. VP3 is a nonstructural protein that is highly rich in proline. VP3 is now also referred to as apoptin because of its association with the apoptotic process in transformed cells. Apoptin depletes both thymocytes and erthyroblastoid cells in chickens with CAV by inducing apoptosis[17]. Due to apoptin's ability to induce apoptosis in cancerous cells, it is the main protein of interest in this study.

Apoptin's ability to induce apoptosis in transformed cells has made it a strong candidate for cancer therapy. Its unique structure and combination of signaling sequences allows for it to function as a natural cancer cell killer. Apoptin has been

proven to cause apoptosis in only transformed cells. Primary cells are left unharmed, which makes it an appealing possible treatment over current chemotherapies. Additionally, apoptin can cause apoptosis in cells that lack p53, which as previously described is an important tumor suppressor that aids in cancer progression when it is mutated. Most current treatments try to reactivate the p53 pathway, but apoptin can kill cells independent of this factor.

In order to understand the mechanisms apoptin uses to induce apoptosis in tumor cells many studies were and are being done. One such study was to observe whether or not apoptin interacts with the caspases that are responsible for apoptosis in normal cells. To do this, viral inhibitors that are known to block specific caspases were used to block the caspases in cancer cells that lacked p53. These cells were then exposed to apoptin to see if the lack of certain caspases altered the efficiency of apoptin-induced apoptosis. It was found that apoptin requires activation of effector, or the downstream caspases in order to induce rapid apoptosis. Rapid apoptosis was inhibited by p35, which is a viral inhibitor of downstream caspases. Although the rapid response was inhibited, it was found that caspase inhibition does not prevent all aspects of apoptin-induced apoptosis. Importantly, it was discovered that caspase 3 is a crucial component of apoptin's pathway. In a human breast cancer cell line that lacked caspase 3, apoptin did not cause the rapid cell death that was seen in cancer cell lines that contained caspase 3. Additionally, Bcl-2, which ceases apoptosis in normal cells, does not inhibit apoptin-induced apoptosis. Knowing that caspases are important but not completely necessary for apoptin activity in cancer cells, the big question that remains is how does apoptin selectively target just cancer cells[11].

Many theories have been proposed as to how apoptin selectively targets cancerous cells, but one theory has gained more prevalence over the others: localization. Many studies show a difference in apoptin localization between tumor and primary cells. Normal cells show heavy cytoplasmic localization of apoptin while tumor cells show strong nuclear localization. It is believed that apoptin's antitumor proapoptic activity is directly correlated to its localization patterns. Shown by a number of studies, transformed cells contain significantly more apoptin localized in the nucleus than primary cells. The key nuclear targeting determinant is in the C terminus of the protein. Amino

acids 74-121 of the C terminus are tumor cell specific for nuclear targeting whereas the amino acids 1-73 of the N terminus confer cytoplasmic localization. The important component found within the C terminus of apoptin that allows for nuclear localization is the presence of a nuclear localization sequence (NLS) that is functional in only cancerous cells[30]. One mutagenesis study delved further into the mechanistic action of nuclear localization sequences to better understand their role. Through this study it was found that apoptin contains not one, but two NLS. To determine whether or not the two NLS work in concert or are independent of one another, each NLS was fused to a GFP construct and introduced into a cancerous cell. What was discovered is that both NLS are located in the C terminus and are interdependent on one another. Thus, for sufficient nuclear targeting, they need to work in concert, indicating they are bipartite nuclear localization signals. NLS 1 is located from amino acid 82-88 while NLS 2 is found from amino acid 111-121. In addition to the two NLS, it was revealed that apoptin contains two domains that induce apoptosis separately from each other. Although independent, both domains heavily rely on nuclear localization for their ability to kill. In an attempt to prove that localization is the key to inducing apoptosis, a NLS was fused to the naturally cytoplasmic localized N terminus. This caused the N terminus to relocalize to the nucleus. Interestingly, there was an increase in apoptosis by the N terminus, demonstrating that localization indeed must be significant. To further test this localization theory, the apoptin was altered to form a NLS-Apoptin construct. This construct contained the apoptin gene fused to N terminally to a NLS to cause nuclear localization in primary cells, where they are usually cytoplasmic. However, when in the nucleus of nontransformed cells, apoptosis was not induced. This experiment provided evidence that there must be additional factors that allow for the induction of apoptosis in only cancer cells. Perhaps tumor cells modify the apoptin differently, which triggers apoptosis or the cellular decision machinery could vary between primary and transformed cells as well[10].

The structure of apoptin allows for the mechanisms behind its function to be better understood. Knowing that apoptin contains two nuclear localization sequences explains its relocalization to the nucleus. However, it does not explain why it is relocalized exclusively in transformed cells. A study at Monash University revealed that

there is more to apoptin than the cytoplasmic N terminus and NLS containing C terminus. It was found that apoptin utilizes the CRM1 nuclear export signal (NES) to accumulate within the nucleus of cancerous cells. In addition to the CRM1 NES, apoptin was also found to contain a leucine rich sequence (LRS) from amino acid 33-46. This component allows for the further accumulation of apoptin in the nucleus of cancer cells by acting as a nuclear retention sequence. With the results of this study, it was concluded that apoptin functions to selectively target and kill cancer cells through a combination of factors. Its bipartite type nuclear targeting signal found in NLS1 and NLS 2 of the C terminus allow for apoptin to be relocalized to the nucleus. The CRM1 NES allows for the apoptin to accumulate within the nucleus. The LRS aids in this accumulation, which leads to the induction of apoptosis within the cancer cell. Additionally, it has been suggested that the multimerization of apoptin plays a significant role in inducing apoptosis in cancerous cells as well[29].

Porcine Circovirus (PCV)

The apparent knowledge that has been gained about Chicken Anemia Virus and its proapoptic protein apoptin, has allowed it to be used as model for other similar viruses. One such virus is porcine circovirus (PCV). PCV is a part of the circoviridae family that contains 3 genera of viral pathogens effecting plants, birds and swine[7]. Viruses in this family contain icosahedral protein capsids with genomes approximately 1750-2319 nucleotides in length. These viruses are further categorized into two more specific groups based on differences in capsid morphology and genomic organization. The Gyrovirus genus is comprised of the chicken anemia virus while the circovirus genus contains beak and feather disease virus, nanoviridae of plants and porcine circovirus[26].

Porcine circovrisues (PCV) are the smallest of the animal viruses. Structurally they are circular, non-enveloped, single stranded DNA viruses that replicate autonomously in mammalian cells[28]. They infect pigs and wild boars, as their names suggests. With regards to pathogenicity, there are two types of porcine circovirus. Porcine circovirus type 1 (PCV1) infects swine, but does not cause disease. Porcine circovirus type 2 (PCV2) however, is the causative agent of postweaning multisystem wasting syndrome (PMWS). PMWS is characterized by growth retardation, loss of weight and death in

weaned piglets. PCV2 is further divided into two genotypes: A and B. PCV2B is much more virulent than PCV2A commonly causing death in the swine it infects[15].

The structure of PCV is important for its virulence. Its genome is 1767-1768 nucleotides long and codes for eleven open reading frames (ORF). Unlike chicken anemia virus, porcine circovirus is less understood. Of the eleven ORFs, protein expression is only known for three of them. The three known ORFs are used to produce four major proteins: Rep, Rep', Capsid and VP3[20]. ORF1 is responsible for the Replicase (Rep) protein and its splicing variant Rep'. These proteins are needed for viral replication. ORF2 encodes the Capsid (Cap) protein, which is the only structural and most immunogenic protein of PCV2. ORF3 encodes for VP3, which is a non structural protein associated with viral replication and pathogenesis[15].

Although PCV1 and PCV2 have the same basic genomic structure, their distinct difference in pathogenicity proves they must vary. Overall, they have a DNA sequence homology of 68-76%, with their ORF1 being the most similar. ORF1 between the two types has 83% nucleotide homology and 86% amino acid homology. ORF2 is more variable between the types with 67% nucleotide homology and 65% amino acid homology[15]. Based on these percentages, there is 83% nucleotide homology and 86% amino acid homology between the two serotypes' Rep gene and 67% nucleotide homology and 65% amino acid homology for the Cap gene. ORF3, which encodes VP3, have 70 common amino acids between PCV1 and PCV2[13]. Thus, a difference in the remaining amino acids in ORF3 is what separates PCV1 and PCV2 at the pathogenic level[14].

Just as PCV1 and PCV2 vary from one another, the variance in virulence between PCV2A and PCV2B suggests there must be differentiations between them as well. The differences between isotypes A and B is much less than that between PCV1 and PCV2. PCV2A and PCV2B have 98% homology at the nucleotide level. There are only six nucleotides that are not the same between the two genotypes. These are located at positions 121, 207, 282, 291, 304 and 319 in ORF3. As a result of the different nucleotides at these positions, PCV2A and PCV2B have alternate amino acids. Three changes in amino acids occur at positions 41, 102 and 104 at the C terminal end. The most important amino acid change is at position 41, which sits in the dead center of a

nuclear export signal sequence[13]. As was previously mentioned, these NES sequences are necessary to transfer proteins out of the nucleus and into the cytoplasm. The alterations between PCV2A and PCV2B found in ORF3, are what confer the difference in virulence between the two[13].

As has been demonstrated through the variable differences in ORF3 between PCV1 and PCV2 as well as between PCV2A and PCV2B, ORF3 appears to play a crucial role in pathogenicity. As was stated, the product of this ORF is VP3. Interestingly, PCV2 VP3 is very similar to the VP3 or apoptin protein produced by chicken anemia virus. Just as apoptin targets and induces apoptosis in cancer cells, PCV2 VP3 does the like[25]. Both apoptin and PCV2 VP3 are encoded in the third ORF and have similar localization patterns. Additionally, there is high homology between apoptin and PCV2 VP3 nuclear export sequence (NES) sites and nuclear localization sequences (NLS). Again, as was seen with apoptin, the NES acts to transport protein from the nucleus to the cytoplasm in normal cells. The NLS, located in the C terminus, as it was for apoptin, induces apoptosis through the activation of caspases 3 and 8. Caspase 8 is an initiator caspase, which acts to stimulate the effector caspase 3. Because caspase 3 is a death protease it catalyzes the cleavage of many cellular proteins leading to apoptosis[20].

Although it is understood that PCV2 VP3 is able to target and kill cancerous cells, many of the mechanisms behind it are still unknown. Based on previous studies, some completed in this laboratory, experiments were done in an attempt to decipher the apoptotic capabilities of PCV2 VP3 more extensively. VP3 provides an equally significant candidate for cancer treatment because of its selectivity and efficiency in killing cancer cells. Additionally, because of its similarity to CAV apoptin, there is a strong foundation of potential answers for how VP3 works.

In this study two hypotheses were tested. The first of which was whether or not apoptin and PCV2 VP3 use the same CRM1 export pathway by utilizing the CRM1 export inhibitor Leptomycin B. Secondly, was to test if apoptin was able to relocalize normally cytoplasmic PCV2 VP3 into the nucleus. This was observed in a previous experiment and was to be tested further.

Materials and Methods

Transformation of Chemically Competent E. coli

Stock dsRed apoptin, PCV2A GFP, PCV2B GFP and GFP ΔΔ NLS were used to transform JM109 Competent *E. coli* cells (50 uL). The *E. coli* cells were thawed on ice and mixed with 1uL of DNA from the stock constructs. The tubes were flicked to ensure mixing and were incubated on ice for 15 minutes to allow complexes to form. After the incubation period, the cells were heat shocked in a 42 degree Celsius water bath for 60 seconds and immediately returned to ice for the following two minutes. Next, warmed LB media was added to each *E. coli* tube. The *E. coli* was then placed on the rotator at 37 degrees Celsius for one hour for recovery. To ensure only transformed cells were being selected for, 50 uL of the transformed *E. coli* was placed in a autoclaved flask with 100mL of LB media and 100uL of kanamycin antibiotic. The flasks were placed in the shaking incubator overnight at 37 degrees Celsius.

Large Scale Plasmid DNA Preparation- Midiprep

Following the growth of the transformed *E. coli* cells overnight, the plasmid DNA was purified using the PureYield Plasmid Midiprep kit (Promega) published protocol. To verify the size of the DNA constructs, the purified DNA was run through a gel electrophoresis restriction digest.

Gel Electrophoresis Restriction Digest

The restriction digests of the purified DNA were done using 1uL of EcoRI, 1uL of BamHI, 2uL of Buffer E, 3uL of DNA and 13uL of ddH20. This mix was placed in eppendorf tubes and incubated in a 37 degree Celsius water bath for 2 hours. Following the restriction, agarose gel electrophoresis was performed using a 1% gel.

DNA Quantification

The purified DNA from the midiprep was quantified and sequenced by measuring its absorbance at 260nm on the UV-Vis Spectrophotometer. A 100-fold dilution of the DNA sample was placed into a mixture with a total volume of 300uL. Once the absorbencies were read, approximate concentrations were calculated by diluting the DNA to 0.2ug/uL. These samples were submitted and sequenced by Macrogen USA.

Transfection in H1299 Cells

H1299 lung carcinoma cells were used to express the DNA constructs dsRed apoptin, PCV2A GFP, PCV2B GFP and GFP ΔΔ NLS. To eliminate the issue of contamination, these cells were grown and transfected in a sterile environment. They were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum and 1% PSF antibiotic formulation of penicillin, streptomycin and fungizone. The cells were kept at 37 degrees Celsius in 5% CO2 air. The cells utilized in this study were grown to 70% confluency and passed into two 6 well plates containing sterilized square microscope cover slips. The H1299 cells were then transfected using an Effectene Transfection Kit (Qiagen) after 24 hours of incubation. The first 6 well plate was transfected in duplicates with PCV2A GFP, PCV2 GFP and GFP ΔΔ NLS. The second 6 well plate was cotransfected in duplicates with PCV2A GFP/dsRed Apoptin, PCV2B GFP/dsRed Apoptin and exclusively dsRed apoptin. Twenty-four hours post transfection, the cells in the 6 well plate containing PCV2A GFP, PCV2B GFP and GFP ΔΔ NLS were treated with 20 uL of leptomycin B in one of the two wells for each construct. The cells were incubated at 37 degrees Celsius for 3 hours to allow the leptomycin B to take effect. Next, the media was aspirated from both 6 well plates and the cells were washed with 1X PBS. The cells were then fixed using 4% paraformaldehyde diluted in PBS for 15 minutes. Following the fixing period, the cells were mounted on slides with mounting media (2.5% DABCO, 50% glycerol,1x PBS; pH 8.6). Once mounted, the slides were imaged by confocal microscopy.

Note that in the repeat experiment, the 6 well plate containing PCV2A GFP, PCV2B GFP and GFP ΔΔ NLS was not treated with leptomycin B, but rather were incubated for 48 hours post transfection before being fixed, mounted and imaged.

Image Analysis and Quantification

The program Image J was utilized to quantify the confocal images taken. The rectangular tool was used to produce running average histograms. The rectangle was placed over a cell so that it contained an area of both cytoplasm and nucleus. To produce surface plots of the images, the images first had to be converted to 8-bit color. Then the surface plot tool was selected from the analyze menu.

Results

Porcine Circovirus 2 (PCV2) and Chicken Anemia Virus (CAV) have been shown to have many similar structural characteristics. Both viruses contain a protein in their third open reading frame, apoptin in CAV and VP3 in PCV2, that induces apoptosis in cancer cells. Both apoptin and PCV2 VP3 are encoded in the third ORF and have similar localization patterns. Additionally, there is high homology between apoptin and PCV2 VP3 nuclear export sequence (NES) sites and nuclear localization sequences (NLS). The NLS, located in the C terminus, as it was for apoptin, induces apoptosis. Because much more is known about the structure and function relationships in CAV, PCV2 is often compared to it in an attempt to identify whether or not the mechanisms behind PCV2 behave similarly to that of CAV.

With respect to PCV, there are two forms delineated PCV1 and PCV2. These two forms vary distinctly in virulence. PCV2 causes death in swine through Post Weaning Multisystemic Wasting Disease, while PCV1 appears to be nonpathogenic. PCV2 is further categorized into the isoforms PCV2A and PCV2B. Again, there is a difference in virulence between the two types; PCV2B is much more pathogenic. The differentiation between the two isoforms lies in a change of six nucleotides. These are located at positions 121, 207, 282, 291, 304 and 319 in ORF3. Alternate amino acids are

encoded as a result of these nucleotides changes. Three changes in amino acids occur at positions 41 (Glycine to Serine), 102 (Phenylalanine to Leucine), and 104 (Lysine to Glutamine). The most important amino acid change is at position 41, which sits in the dead center of a nuclear export signal sequence (NES). Through research done on CAV, it is known that its ability to induce apoptosis is greatly aided by its NES. Thus, it has been suggested that the modification of the amino acid at position 41 between PCV2A and PCV2B is what allows for the difference in virulence.

In order to distinguish the mechanisms and functions behind PCV2, foundations for research are often based on how CAV is known to work. Therefore, in this study, two different experiments were done utilizing information on CAV's apoptin. First, PCV2A and B GFP and GFP ΔΔ NLS constructs were transfected in duplicates into an H1299 line of cells. Half of the cells were then exposed to Leptomycin B (LMB), a CRM1 export inhibitor. In the case of apoptin and cancerous cells, the CRM1 export pathway is used for nuclear accumulation. Therefore, it was tested to see if PCV2 VP3 uses the same CRM1 export pathway.

CRM1 Export

To determine if PCV2 VP3 utilizes the same CRM1 export pathway as CAV apoptin, H1299 cells transfected with GFP tagged PCV2A, PCV2B and GFP ΔΔ NLS were exposed to the CRM1 inhibitor Leptomycin B. Cells that were not treated with Leptomycin B acted as a control. It was expected that if PCV2 VP3 uses the same CRM1 export pathway as CAV apoptin, the addition of Leptomycin B would drastically increase the amount of virus accumulated within the nucleus. Figure 3 illustrates the surface plots of treated and untreated cells. Figure 3 Panel A displays that PCV2A shows a distinct difference in intensity between the nucleus and cytoplasm. The nucleus appears to have less of a signal than the cytoplasm demonstrating that PCV2A is cytoplasmic. However, with Leptomycin B treatment, the intensity becomes more evenly dispersed between the cytoplasm and nucleus. This suggests that more of the virus is being kept within the nucleus due to the inhibition of its export. Similar results can be seen for PCV2B shown in Figure3 Panel B. Again, PCV2 is more heavily localized in the cytoplasm, but when

treated with Leptomycin B, a stronger signal in the nucleus can be observed. As a control, GFP ΔΔ NLS acted to demonstrate what a strong response to Leptomycin B would look like. GFP ΔΔ NLS is known to utilize the CRM1 export pathway, thus with the addition of Leptomycin B a drastic change in localization was anticipated. Figure 3 Panel C show just this. GFP ΔΔ NLS shows a distinct depression at the location of the nucleus demonstrating its high cytoplasmic localization. With the addition of Leptomycin B, that depression quickly becomes a peak because of the inhibition of export from the nucleus.

To quantify the results from the imaging, histograms were produced to display the GFP expression in the H1299 cells. Figure 1 is divided with a red line to delineate between nucleus and cytoplasm GFP intensity. As can be seen, these histograms further support the results of the surface plots. PCV2A is heavily cytoplasmic, however once PCV2A is treated with Leptomycin B the location of higher intensity shifts to the nucleus. Similarly, PCV2B shows the most intensity in the cytoplasm. When treated with Leptomycin B, the intensity of the cytoplasm decreases and the intensity within the nucleus increases. However, the shift in intensity is not as extreme as was seen with PCV2A. GFP ΔΔ NLS shows the most distinct change in intensity with the addition of Leptomycin B. Alone, GFP ΔΔ NLS is heavily cytoplasmic, but when treated with Leptomycin B, it becomes heavily nuclear.

Further analysis of the GFP imaging data was done through quantifying the histograms by calculating the cytoplasmic localization/nuclear localization fraction. The bar graph shown in Figure 2 illustrates these calculations for each treated and non-treated cell type. The graph shows that the addition of Leptomycin B to all the cell types causes an increase in accumulation in the nucleus. The most distinct change can be seen with the control, GFP ΔΔ NLS, as was expected. However, both PCV2A and PCV2B appear to show a clear change from heavily cytoplasmic to less cytoplasmic when treated with the CRM1 inhibitor Leptomycin B. These results suggest that PCV2A and PCV2B are at least partially utilizing the CRM1 export pathway to induce apoptosis in cancerous cells and suggests another mechanism may be contributing as well.

Apoptin Relocalization of PCV2

To further test the extent of similarity between PCV2 VP3 and CAV apoptin, PCV2A and B GFP constructs were cotransfected with dsR apoptin into H1299 cells. In a previous study, it appeared that apoptin was able to relocalize normally cytoplasmic PCV2 VP3 into the nucleus. Thus, this experiment was repeated to identify whether or not this finding was accurate. If found to be accurate it would suggest that PCV2 VP3 and CAV apoptin are similar enough to multimerize with each other. The results of the cotransfection can be seen in the confocal images in Figure 4. To distinguish between the presence of PCV2 VP3 and apoptin, each was bound to a different fluorescent tag. Apoptin is bound to a dsRed tag whereas PCV2 VP3 is bound a GFP tag. Therefore, through confocal imaging, both apoptin and PCV2 VP3 could be visualized. Figure 4 Panel A shows the PCV2A/dsR construct. The first panel is the PCV2A GFP construct, followed by the apoptin dsR construct, followed by a merged image to show the localization of both constructs together. As can be seen, PCV2A and apoptin remain in their own compartments; PCV2 in the cytoplasm and apoptin in the nucleus. The same results are seen for PCV2B/dsR experiment seen in Figure 4 Panel B. Both proteins remain separate and in their expected locations suggesting that the relocalization results seen in the previous study may have been a chance result.

Discussion

The discovery of novel treatments for cancer is a necessity in a world that loses 7.6 million people per year to cancerous diseases. Through studies on viruses, like Chicken Anemia Virus and Porcine Circovirus, an innovative treatment is possible in the immediate future. The proteins found within Chicken Anemia Virus and Porcine Circovirus are strong candidates for cancer treatment because of their apoptosis inducing properties in cancerous cells. CAV is classified and understood much better on both structural and functional levels. Because of this, knowledge of the structure-function relationships and their corresponding mechanisms of CAV are known. The distinct similarities between CAV and PCV allow for a generalized foundation of how PCV may

function. The rationale that PCV may function in the same manner as CAV was the basis of this study.

Both the Chicken Anemia Virus and Porcine Circovirus 2 contain a protein of interest in the third opening reading frame, apoptin in CAV and VP3 in PCV2. These two proteins are potential candidates for cancer therapy because of their strong association and induction of the apoptotic process in cancerous cells while leaving primary cells unharmed. Due to their similarities, this study was completed to analyze whether or not the two proteins function in an analogous mechanistic fashion. First, knowing that CAV apoptin utilizes the CRM1 export pathway, it was hypothesized that PCV2A and PCV2B may use this pathway as well. To test this hypothesis, H1299 cells were transfected with both PCV isoforms A and B and exposed to Leptomycin B. Leptomycin B inhibits the CRM1 export pathway, causing accumulation of proteins in the nucleus. If PCV utilizes the CRM1 export pathway, a distinct difference in nuclear protein accumulation would be seen between cells not treated with Leptomycin B and cells treated with Leptomycin B.

The CRM1 export analysis of PCV2A and PCV2B provides evidence that Leptomycin B inhibition of CRM1 export increases the accumulation of PCV2 VP3 in the nucleus. The surface plots, histograms and localization graph indicate that both PCV2A and PCV2B responded to the Leptomycin B treatment. Although the response was not as strong as that of the control, there was a distinct decrease in cytoplasmic localization and increase in nuclear localization. The degree of PCV2 VP3 repsonse may be a result of protein escaping to use an alternate pathway or the proteins ability to bind efficiently to the CRM1 receptors. These results suggest that PCV2A and PCV2B utilize the CRM1 export pathway to a certain degree, but may not bind to CRM1 receptors as tightly as CAV apoptin. It can be speculated that PCV2 VP3 must be using additional factors in order to accumulate and remain within the nucleus of cancerous cells. Additionally, there was a difference in response level between the two PCV2 isoforms A and B. PCV2B appeared to have a stronger response to the Leptomycin B treatment, which could be related to the virulence difference between the isoforms. PCV2B is the more pathogenic form of PCV2, and may explain why it had a greater response to the Leptomycin B treatment. From previous research on apoptin, it is known that the NES is

extremely important in the CRM1 export process. Therefore, future studies could analyze the effects of altering or removing the NES site in PCV2. This could provide additional evidence regarding the function and importance of CRM1 export in PCV2. These results suggest that although apoptin and VP3 both selectively induce apoptosis in cancerous cells, they are more than likely utilizing different mechanistic processes to accumulate within the nucleus and carry out their function.

To further investigate the similarities between CAV and PCV2, a relocalization analysis was completed to determine whether or not apoptin could relocalize PCV2 VP3 to the nucleus. As is shown by the confocal images, relocalization was not observed; apoptin localized in a punctate fashion in the nucleus, while VP3 remained in the cytoplasm. These results provide evidence that apoptin and VP3 are not adequately similar to interact strongly, cross-multimerize, and cause relocalization. The differences between apoptin and VP3, that did not allow them to cross-mulitmerize, may be located in their nuclear export sequences. These sequences are essential for multimerization for each of the proteins, but differences in their amino acid sequences could provide an explanation. Comparison of amino acid alignments of the NES found in both apoptin and VP3 could reveal their exact homology. At the places the two sequences differ, future studies may look into mutating the sequences to make them as homologous as possible. If the NES of apoptin and VP3 are virtually identical, this cotransfection experiment could be repeated in an attempt to observe colocalization.

Using CAV as a foundation for investigation of its function has certainly provided ample options as to how PCV2 may work, but through these experiments it has been demonstrated that there are distinct differences between the two virus proteins. Therefore, although the use of CAV has proven to be helpful, it should not be used as a definitive model for PCV2. Functionally, CAV and PCV2 VP3 proteins are quite similar, but the crux of their importance in being potential cancer treatments, lay in the mechanisms behind the function. The results of this study provide evidence that PCV2 VP3 is not exclusively using the CRM1 export pathway for nuclear accumulation, nor is PCV2 VP3 similar enough to apoptin for cross-localization. These results illustrate very rudimentary ideas that PCV2 and CAV may be functioning completely differently, thus, as time progresses, the use of CAV as a model for PCV2 may become less relevant. If

the mechanistic pathways of PCV2 and CAV prove to be different, it suggests provocative findings that these two viruses diverged to execute a common function. Previous studies that emphasized the importance of relocalization of CAV apoptin from the cytoplasm to the nucleus to cause apoptosis may be invalidated. It is possible that relocalization is not necessary to induce such a response. Based on this, future studies should aim to identify where, why and how these two viruses diverged from one another or if they are truly homologues hidden behind artifacts.

Figures

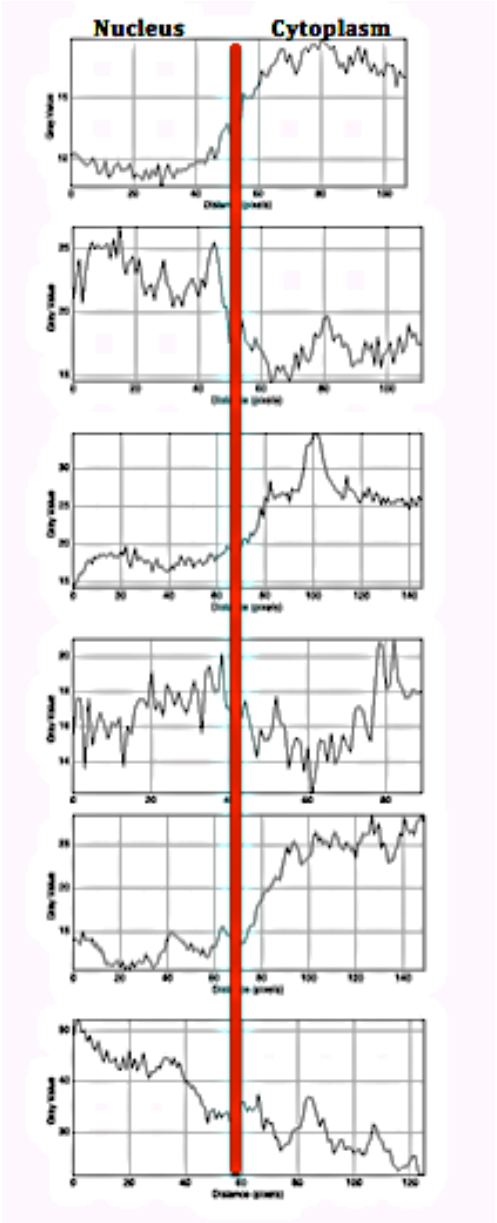


Figure 1: GFP expression histogram in H1299 cells

Fluorescent images were quantified using ImageJ. The histograms illustrate the pixel density for each cell by measuring from the nucleus to the cell wall. The red line delineates the intensity in the nucleus (right) versus the cytoplasm (left). The cells not treated with Leptomycin B are heavily cytoplasmic, while those treated with Leptomycin B become noticeably more nuclear, suggesting partial use of the CRM1 export pathway.

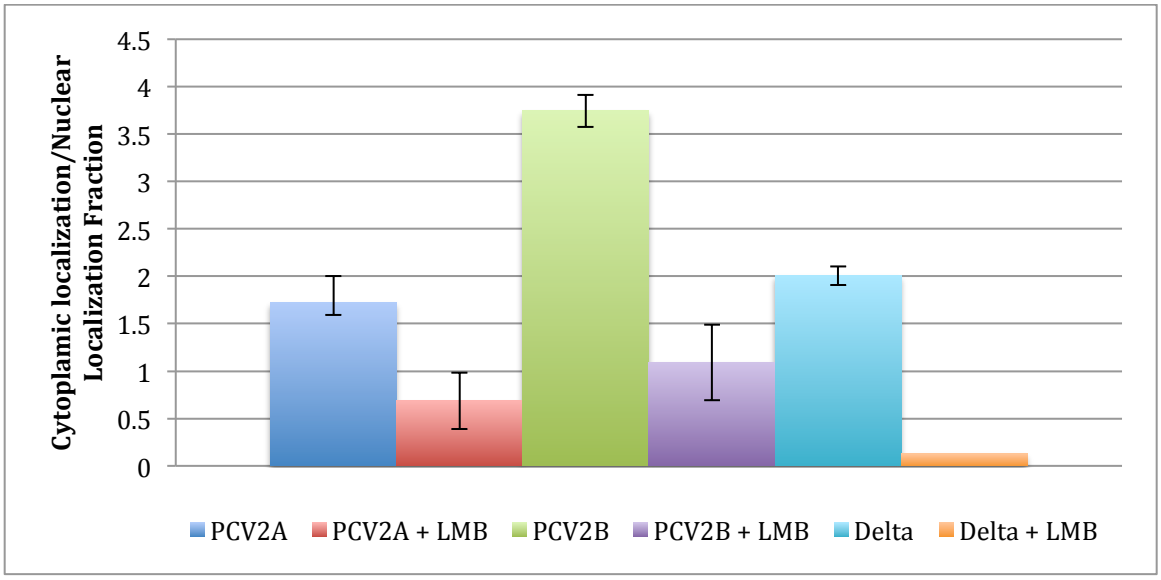
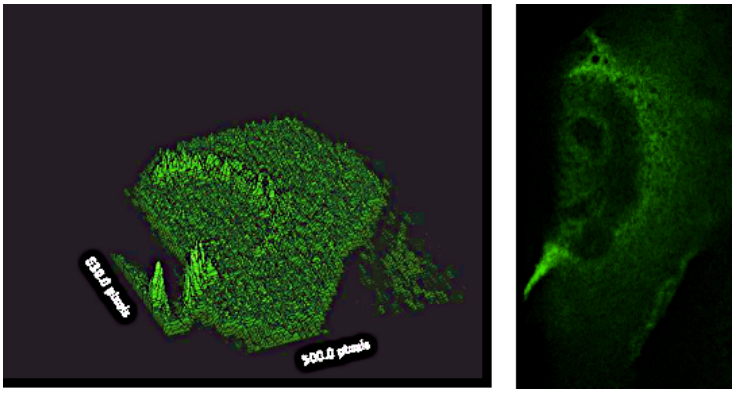


Figure 2: Localization analysis of PCV2 constructs

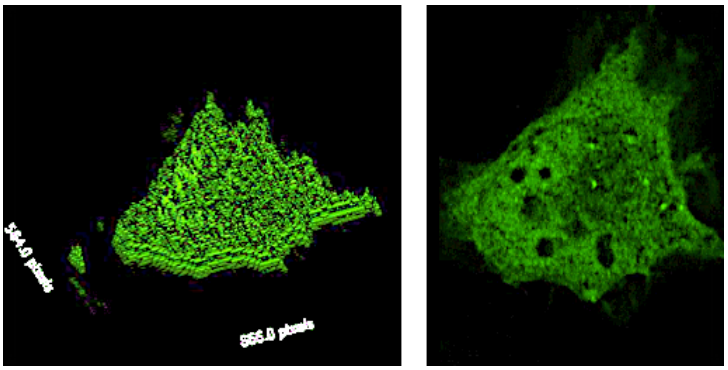
Utilizing measurements in ImageJ, the cytoplasmic/nuclear localization fraction was calculated to quantify the confocal images of the PCV2 constructs. To calculate this fraction, the mean green intensity in the nucleus and cytoplasm of three cells for each type of construct was calculated. The graph reinforces PCV2's use of a CRM1 export pathway. Those cells not exposed to Leptomycin B show a stronger cytoplasmic localization, whereas those treated with Leptomycin B are more heavily nuclear.

A.

PCV2A

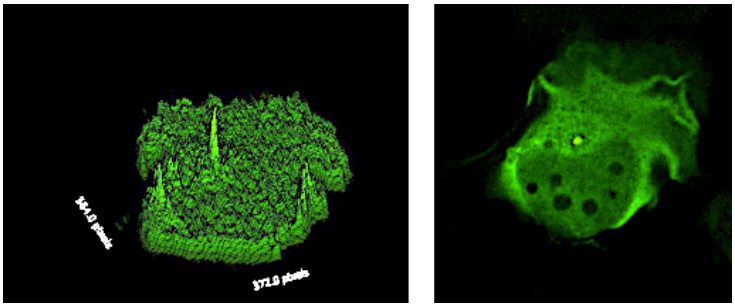


PCV2A LMB

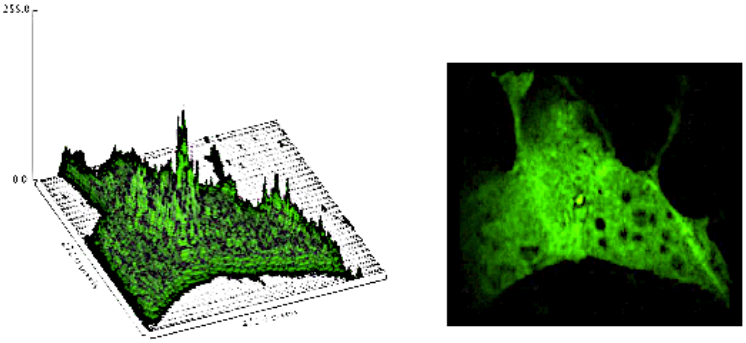


B.

PCV2B

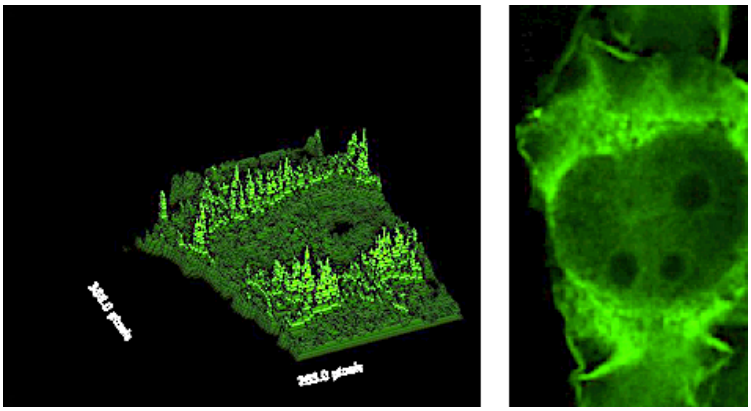


PCV2B LMB



C.

GFP ΔΔ NLS



GFP ΔΔ NLS LMB

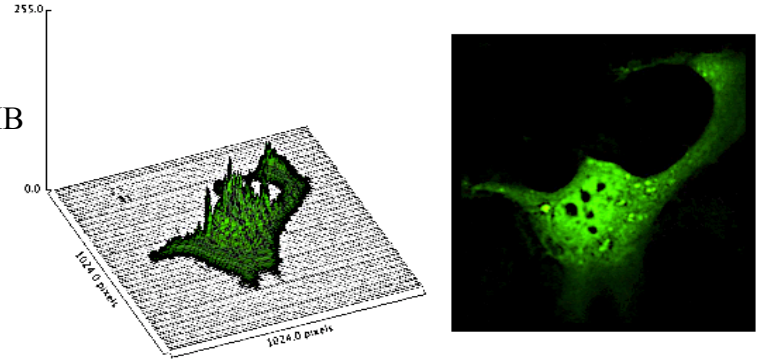
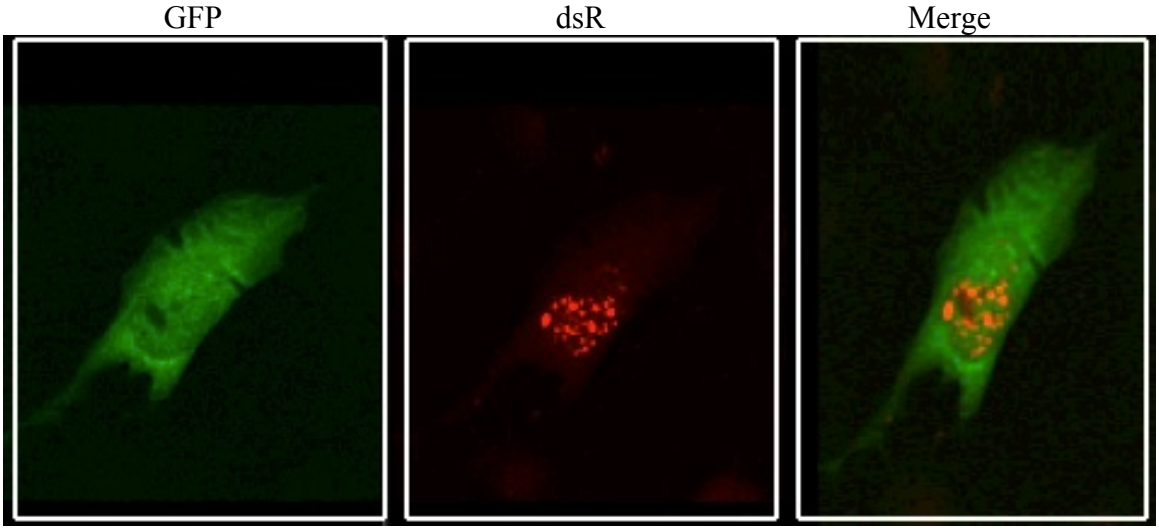


Figure 3: Surface plots of PCV2 constructs

The confocal images were graphed topographically using ImageJ to better illustrate GPF intensity in the cells. Panel A shows the surface plots for PCV2A and PCV2A exposed to Leptomycin B (PCV2A LMB). PCV2A is heavily cytoplasmic, whereas exposure to Leptomycin B increases its nuclear localization. Panel B illustrates the surface plots for PCV2B and PCV2B treated with Leptomycin B. Similar results to PCV2A are seen; PCV2B is cytoplasmic while PCV2B LMB is more nuclear. Lastly, Panel C provides the surface plot for GFP ΔΔ NLS and GFP ΔΔ NLS LMB. Here, the strongest response is seen with GFP ΔΔ NLS being distinctly cytoplasmic and GFP ΔΔ NLS LMB becoming strongly nuclear.

A.



B.

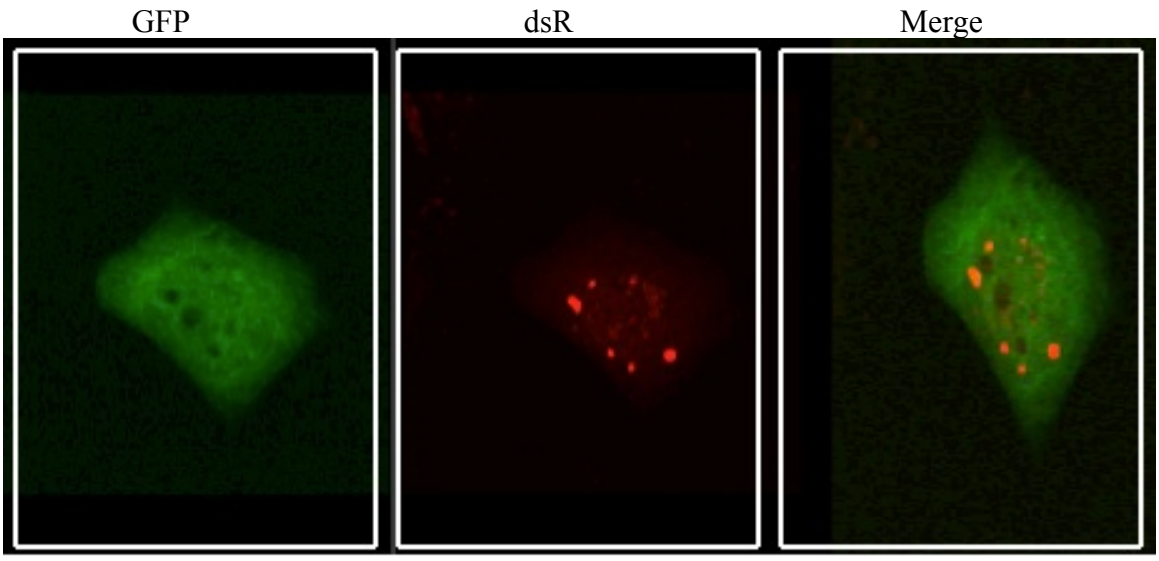


Figure 4: Confocal images of PCV2 VP3/apoptin localization

H1299 cells were contransfected with PCV2 and apoptin. The PCV2 constructs were fused to a GFP tag, while the apoptin construct was fused to a dsRed tag. Through confocal imaging, both constructs could be visualized. Panel A shows the confocal images for the H1299 cells contransfected with PCV2A GFP and Apoptin dsRed. PCV2A remains heavily in the cytoplasm and apoptin is punctate in the nucleus. Panel B illustrates cells contransfected with PCV2B GFP and Apoptin dsRed. Again, PCV2B is localized in the cytoplasm whereas Apoptin is localized in the nucleus. No relocalization of either construct is seen.

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