Functional Analysis of Porcine Circovirus 2 VP3 Localization Through Truncation Mutagenesis

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Abstract

The Porcine Circovirus 2 (PCV2) VP3 protein induces apoptosis in transformed cells. This apoptotic activity, like that of other VP3 homologs, may be related to subcellular localization. Previous studies of other VP3 homologs indicate that nuclear localization is important apoptosis induction, however, PCV VP3 localizes to the cytosol. In this study, we isolate the nuclear localization sequence and nuclear export sequence through the use of truncation mutants tagged with green fluorescent protein (GFP). Experiments were also performed to assess the pathway used to export the protein from the nucleus. These results provide further evidence that nuclear localization may not be necessary for induction of apoptosis for PCV2 VP3.

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Background

Porcine Circovirus (PCV) is a member of the circoviridae family. The circoviridae family contains other viruses such as Chicken Anemia Virus and Torque-teno virus. Many of these viruses are associated with pathologies in various animals.[1] The first reported case of PCV infection occurred in porcine kidney cells (PK-15) in 1974. The serum and antibody to this particular infectious agent was found in pigs. The isolated virus was introduced into non-infected pigs to observe the pathology of the virus in newly infected pigs, but no pathology was seen. The virus was assumed to be non-pathogenic. In 1991, a new virus with antigenic properties similar to the previously characterized porcine circovirus was discovered. This virus was seen in wasting pigs and led to speculation that new or modified form of the porcine circovirus had emerged. This particular form of PCV was designated the name Porcine Circovirus 2 to distinguish it from Porcine Circovirus 1, the infectious agent found in the PK-15 cells. PCV2 was found to be the causative agent of Postweaning Multisystemic Wasting Syndrome (PWMS).[2]

Postweaning Multisystemic Wasting Syndrome is an illness which afflicts young pigs, especially pigs within the 5 to 12-week age group.[1] The disease is characterized by severe weight loss, respiratory complications, and diarrhea. The severe weight loss is caused by low feed intake and diarrhea. The ailing pigs also show red and purple maculae on their hind legs. These macules, as the pathology progresses, will fuse and the pigs show large discolored areas. These symptoms are fatal for the infected individual.[3] To better understand this particular pathology, the molecular details of the porcine circoviruses have been elucidated through various studies. In particular, attempts have been made to determine the differences between Porcine Circovirus 1 and Porcine Circovirus 2.

The two serotypes of porcine circovirus have some stark differences at the nucleotide and protein level. The two different viruses show approximately at 68-76% homology at the nucleotide

level. The nucleotide homology for the two ORFs, Rep and Capsid, are drastically different. The homology of the Rep gene between the two different serotypes is approximately 83 % at the nucleotide level and 86 % on the amino acid level. On the other hand, the capsid gene shows greater variability. The Cap gene from PCV1 shares a nucleotide homology of 67% and an amino acid homology of 65% with the cap gene from PCV2.[1] The VP3 proteins from the two serotypes share 70 common amino acids. This comprises 34% of the PCV1 VP3 polypeptide sequence or 67% of the PCV2 VP3 polypeptide sequence. This level homology is similar to the homology found at the amino acid level for the other two open reading frames of PCV.

Aside from showing some stark differences at the nucleotide and protein-level, the two viruses also have stark differences in their pathology. PCV1 has not been shown to cause PWMS, whereas the PCV2 is the etiological agent for the pathology.[1] Although not proven experimentally, the differences in the viral proteins may be responsible for the wasting characteristics of PMWS. In the process of determining a molecular explanation for the difference in pathological capabilities, studies have provided a great deal of molecular details regarding PCV.

Porcine Circovirus, like many viruses of the circovirdae family, has a circular negative-sense (template strand) single-stranded DNA genome containing approximately 1.76 kilobases. This genome contains six open reading frames. Of these six reading frames, three different open reading frames are used to produce four proteins: Rep, Rep', Capsid, and VP3.[1,4] The Rep and Rep' protein form the replicase complex in PCV. The capsid protein of this virus is involved in forming the protein envelope for the genome. The icosahedral structure of the virus is derived from a self-assembly of these particles.[1,5] The third open reading frame codes for the VP3 protein and this protein is not involved in either the structural protein cap or the replication machinery. This protein has been implicated in the pathogenecity of Porcine Circovirus 2.[4,6] Due to the pathogenecity of PCV2 more molecular studies have been performed for this particular protein.

Many of the proteins show a significant degree of homology and many of the characteristics of the encoded proteins are common between the two viruses.

The replicase proteins for porcine circovirus are encoded by the Rep gene or ORF1 on the viral genome. The porcine circovirus utilizes the endogenous alternative splicing mechanism to create multiple mRNA transcripts from the same gene. These transcripts are created through the use of alternative splice-site selection. Of the various transcripts created from the Rep Gene, only two are actually translated into protein. The role of the various non-translated mRNA transcripts has yet to be determined.[7] The two proteins, Rep and Rep', are necessary for replication of the genome. This is a unique characteristic amongst Porcine Circovirus. It has been shown that PCV uses a RCR (rolling circle replication) method to replicate its genome and many viruses that utilize this method have only one functional replicase enzyme; the need for two different replicase enzymes is unique to PCV.[8]

Apart from the replication machinery, another crucial protein component of any virus is its protein cap monomer. Currently, a 30 kDa protein has been identified as the monomeric unit of the PCV2 protein cap. The capsid monomer for PCV1 has been identified as a 36 kDa protein. It is not clear whether this difference in mass is a vestige of experimental uncertainty or whether there is an actual difference in the mass and therefore size of the capsid proteins from the two serotypes.[5] The capsid protein is encoded from the Cap gene or ORF2 on the PCV viral genome. This particular capsid protein has been shown to self-assemble and form capsid structures. Although the capsid protein particles were able to self-assemble, the structure of the self-assembled particles was less-ordered than the structure of the purified PCV2 particles. This indicates either the presence of a smaller secondary protein to promote proper orientation of the capsid protein or the interaction of the capsid protein with the genome to create properly ordered particles.[5] Aside from the three

proteins, Rep, Rep', and Cap, PCV also encodes a fourth protein. This particular protein is not involved in cap formation or genome replication and therefore it eluded scientists.

The protein encoded by the third open reading frame was not identified and reported upon till 2005.[4] This 104 amino acid polypeptide was later discovered to be responsible for inducing apoptosis in infected cells and cause the wasting pathology in infected pigs.[6] A study by Liu et. al. showed the activation of the caspase-8 pathway. Caspase-8 activates caspase-3, which in turns activates an exonuclease caspase activated DNase (CAD) by cleaving inhibitor of CAD (ICAD). This allows CAD to enter the nucleus and start degrading the DNA content of the cell.[4] This degradation of DNA is a classic sign of apoptotic cells. The exact mechanism of VP3-induced apoptosis, especially the activation of Caspase-8, has yet to be fully revealed, but certain parts of the mechanism are known. Some of the interactions involved in the apoptosis mechanism have been discovered. One of these interactions involves PCV2 VP3 and pPirh2.

pPirh2 (Porcine p53-induced ring H2) is an E3 ubiquitin ligase and ubiquitylates p53. The poly-ubiquitylation of p53 signals its degradation through the proteosome and thus lowers its concentration in the cell keeping it at low and healthy steady-state. An increase in p53 concentration within the cell initiates apoptosis within the cell. p53 is involved in initiating apoptotic mechanisms within the cell through a transcription factor.[9] The chemical trigger for p53 mediated apoptotic induction is the phosphorylation of a key serine residue at position 146. The phosphorylation of this residue in p53 causes the activation of a transcriptional factor p53AIP1 (p53 regulated Apoptin Inducing Protein 1). This particular transcriptional factor initiates apoptosis through the dissipation of mitochondrial $\Delta\Psi_m$ (the mitochondrial membrane potential). This dissipation of the membrane potential is a sign of induced apoptosis.[10]

In vivo, PCV2 VP3 binds the pPirh2 and prevents its binding to p53. PCV2 VP3 inhibits binding of p53 to pPirh2 through competitive binding. It has been shown that the p53 binding motif

on pPirh2 is bound by PCV2 VP3 in the presence of VP3. This motif comprises of amino acids 120 to 137 on the pPirh2 protein and it shows 100% identity to the hPirh2 and mPirh2.[9] This indicates that VP3 possess the ability to induce apoptosis via the p53-mediated pathway in both humans and mice.

In addition to p53-modulation through pPirh2, PCV2 VP3 has also been shown to modulate p53 activity through interactions with two kinases: JNK and p38 MAPK. JNK and p38 MAPK are both at the bottom of the MAPK (Mitogen-Activated Protein Kinase) signal cascade pathway and rather than phosphorylating other MAPKs these kinases phosphorylate other molecules such as p53 and other transcription factors. Wei et. al. in a recent publication showed that these two pathways are activated during PCV2 viral replication and are necessary for efficient replication of the genome and formation of new viral progeny. This study went further on to show the effect of JNK and p38 MAPK pathway inhibitors on VP3-induced apotosis. Wei et. al. were able to show a decrease in Caspase-3 activity, which is involved in initiating apoptosis through the action of CAD (caspase activated DNase).[4,11] Wei et. al. propose the change in JNK and p38 MAPK pathway affects the phosphorylation JNK and p38. The phosphorylation of JNK causes the dissociation of p53 from JNK. JNK, aside from phosphorylating, also promote ubiquitylation of molecules, in particular p53. The dissociation of p53 from JNK prevents the ubiquitylation of p53 and thus prevents the proteosomal degradation of p53. The phosphorylation of p38 plays a role in the transcription of two genes which are involved in the p53-mediated apoptotic pathway.[11] From these two studies, it seems that PCV2 VP3-induced apoptosis is p53-dependent. This is in stark difference with Apoptin, which induces apoptosis through a novel p53 independent pathway.[12]

Apoptin is one of the proteins encoded by Chicken Anemia Virus. Chicken Anemia Virus (CAV) is the sole member of the gyroviridaie genera, which is part of circoviridae family. The virus shares many features with PCV2 and encodes a protein which is homologous to PCV2 VP3. CAV VP3,

Apoptin, has been shown to induce apoptosis in cortical thymocytes and erythroblastoid cells.[12] In addition, Apoptin is able to induce apoptosis in transformed cells, but does not induce apoptosis in primary cell-lines, even after multiple replication cycles. This unique ability of Apoptin to distinguish transformed cells from primary cells may be related to the difference in localization pattern which is observed amongst primary and transformed cells. Apoptin, in primary cells, shows a cytosolic localization, whereas in transformed cells Apoptin shows localization to the nucleus.[12] Although Apoptin shows a differential localization in transformed and primary cells and shows differential apoptotic ability, the localization is not responsible for the difference in apoptotic ability. Heilman et. al. determined the shuttling action of Apoptin to be the process responsible for the apoptotic ability of Apoptin. In the study, Heilman et. al. propose the shuttling activity of Apoptin promotes the movement of APC (anaphase promoting complex) components from the cytosol to the nucleus and induce apoptosis.[12] Due to the homology of PCV2 VP3 to Apoptin, this study focuses on the localization of PCV2a VP3 to show functional nuclear export, nuclear localization, and shuttling. The study goes further to determine whether PCV2a VP3 utilizes the exportin CRM1 to negotiate nuclear export through the nuclear pore complex.

CRM1 (Chromosome Region Maintenance) protein belongs to the Karyopherin superfamly of protein. These proteins are involved in transport across the nuclear membrane through the Nuclear Pore Complex. The Karyopherin superfamily is divided into two large groups: exportins and importins. Exportins, as the name implies, are involved in moving cargo through the nuclear pore complex from the nucleus to the cytosol. CRM1 is one of the canonical exportin and many different proteins utilize CRM1-mediated export to transverse the nuclear membrane. The CRM1-mediated export pathway requires several components to form the export complex. This complex, aside from CRM1, consists of RAN-GTP, the cargo, and the nuclear export sequence located on the cargo.[13]

The nuclear export sequence is a sequence of peptide located on the protein, which associates with CRM1 and acts as the linkage between CRM1 and the cargo protein. This nuclear export sequence must possess certain characteristic to promote high affinity binding of the cargo protein with CRM1. Most CRM1 nuclear export sequences are leucine-rich sequences and interact with CRM1.[13] The mechanics of the interaction are not fully understood and very little structural information is available. Therefore very little is known about the spatial arrangement of the nuclear export in relation to the cargo protein and CRM1.

LMB (Leptomycin B), an inhibitor of CRM1-mediated nuclear export, was used to show the dependence of the PCV2a VP3 on CRM1 for export. LMB is a metabolite produced by *Streptomycetes*. A study by Kudo et. al. showed the direct binding of LMB to CRM1 and showed knock-out of CRM1-mediated export activity in Hela cell extracts.[14]

Materials and Methods

Synthesis of Insert

PCR, or Polymerase Chain Reaction, was employed to amplify the Nuclear Export Sequence and Nuclear Localization Sequence of the PCV2A VP3. The primers were designed by the previous MQP team to frame the two separate regions of the gene. The primers were also designed with an EcoRI restriction site at the 5' end, and a BamHI restriction site in the 3' end. The recipe for each PCR tube included the following: 1μL template DNA, 2μL Taq Buffer, 1μL (20x) dNTPs, 1μL Taq Polymerase, 13μL ddH_2O, 1μL (5 pMol/μL) forward primer, and 1μL (5 pMol/μL) reverse primer.

The following temperature protocol was utilized in an attempt to optimize yield. The initial denaturing step was at 95°C for 30 seconds, followed by 30 cycles of a three-part amplification cycle comprised of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute. There was a final extension period at 72°C for 5 minutes.

Cloning into pGEM T-Vector

Before the insert could be cloned into GFP, it first had to be cloned into Promega T-Vector for sequencing. The PCR product was purified through gel electrophoresis in a 0.9% agarose gel with 2μL ethidium bromide run in Tris-acetate-EDTA (TAE) Buffer diluted to 1x from a 50x stock (50x 2M tris, 1M acetate 0.05M EDTA). 20μL of the samples were loaded with 5μL 5x loading buffer (30% Glycerol, 0.25% bromophenol blue, 69.75% ddH_2O). The gel was run between 80-100 volts until the bromophenol blue had advanced more than half the way down the gel.

The appropriate bands were then excised with a razorblade and purified using a GENECLEAN® kit from MP Biomedicals. The gel slices were weighed and added into a microfuge

tube with 3 volumes of NaI (0.1g = 100 μL). The tubes were then incubated at 55°C to melt the gel. 5μL of glassmilk was added and mixed in for 5 minutes to bind the DNA. The glassmilk was then pelleted by centrifugation for 5 seconds, and the supernatant aspirated off. The pellet was then washed by resuspension in three times in 700 μL New Wash buffer (pre-made solution of NaCl, Tris and EDTA diluted in 155 mL ddH_2O and 155 mL 200 Proof ethanol). The pellet was then dried and resuspended in 20μL TE with DNAse-free RNAse to remove the DNA from the glass fines. The pellet was centrifuged and the supernatant was transferred to a new tube.

The PCR product was then ligated to the T-Vector by combining the following in a microfuge tube and leaving it overnight at 4°C: 5μL 2x Ligation Buffer, 1μL pGEM-T Easy Vector, 3μL PCR product, 1μL T4 DNA Ligase (3 units of activity/μL). 5μL of the ligation product was then added to 50μL of chemically competent DH5α *E. Coli*. This sat for 20 minutes before being heat shocked at 42°C for 45 seconds and then cooled on ice for 2 minutes. 400μL of LB recovery media was added to this and allowed to recover at 37°C for one hour. 150μL of transformation was then plated on Ampicillin LB agar plates with 20μL X-Gal and 40μL of Isopropyl-ß-D-thiogalactopyranodise (IPTG) and left overnight at 37°C.

In addition to conferring ampicillin resistance, the T-vector disrupts the lacZ gene of the *E.Coli* resulting in the bacteria being unable to produce ß-galactosidase. One of the products of ß-galactosidase metabolizing X-gal is 5,5'-dibromo-4,4'-dichloro-indigo, a compound which gives the colony a blue appearance. Since these blue colonies did not contain the plasmid insert, only the white colonies were selected for the next step. These white colonies were picked off the plate and incubated overnight at 37°C in 3mL of LB media with 100μg/mL ampicillin.

The following Miniprep method was used to extract the DNA. The culture was centrifuged at 12,000g for 30 seconds and the medium was aspirated off. The resulting pellet was resuspended in

100μL ice-cold solution I (50mM glucose, 25mM TRIS Cl, 10mM EDTA). The cells were then lysed by the addition of 200μL of freshly prepared Solution II (0.1mL 10N NaOH, 0.5mL 10% SDS, H_2O up to 5mL). The lysis was halted by the addition of 150μL ice-cold Solution III, (60mL 5M potassium acetate, 11.5mL glacial acetic acid, 28.5mL H_2O). The resulting liquid was centrifuged 5 minutes at 12,000g and the supernatant transferred to a fresh tube. The DNA was then be precipitated by the addition of 2 volumes 200 proof ethanol. This was again centrifuged at 12,000g for 5 minutes and the supernatant aspirated off. The nucleic acids were then washed three times with 1mL of ice-cold 70% ethanol. Following the wash, the constructs were then re-dissolved in 50μL TE containing DNase-free RNase.

The resulting DNA was checked for the proper plasmid and insert through a restriction digest. Because of the BamHI and EcoRI restriction sites built into the construct, the insert was easily cut out and analyzed. 3μL of DNA was combined with 1μL (10 activity units) BamHI, 1μL (12 activity units) EcoRI, 2μL Promega Buffer E, 1μL RNase A, 2μL (1:10) BSA and 10μL ddH_2O for final volume of 20μL. This was incubated in 37°C water bath for three hours. The resulting product was analyzed through gel electrophoresis as described above with the resulting bands checked for size against a 2-log ladder.

The original culture from the samples that tested positive for our insert DNA was then used to inoculate 100μL of LB media and was left shaking at 37°C for 18 hours. The Promega Midiprep kit was then used to extract the DNA from this culture. The cells were pelleted by centrifugation at 10,000g for 10 minutes at 4°C. The supernatant was removed and the cells resuspended in 3mL Cell Resuspension Solution (50mM Tris-HCl, 10mM EDTA, 100μg/mL RNase A). The cells were then broken down by the addition of 3mL of cell lysis solution (0.2M NaOH 1% SDS). The digestion was then halted by adding 3mL of neutralization solution (1.32M Potassium Acetate). The resulting mixture was then centrifuged at 14,000g for 15 minutes at 4°C to separate the cellular debris from

the nucleic acids. The supernatant was then transferred to a Midicolumn along with 10mL of DNA purification resin. The Midicolumn was then attached to a vacuum manifold and negative pressure applied. After the liquid had passed through the column, 15mL of Column Wash Buffer (80mM potassium acetate, 8.3mM Tris-HCl, 40mM EDTA) was added; this washing was repeated three times. When all of the liquid had passed through the column, the filter was cut off of the column with a razorblade. The column was centrifuged in a microfuge tube to remove any residual Column Wash Buffer. The dried column was then loaded into a new microfuge tube with 300μL 70°C ddH_20 on top of the column. This was then centrifuged at 10,000g for 20 seconds, the warm water passed through the column taking the nucleic acids from the column into the microfuge tube. This was then analyzed by restriction digest and gel electrophoresis as described above. A sample of this DNA was then prepped and sent away for sequencing.

Cloning into eGFP Vectors

The T-Vector constructs and the eGFP vector were restriction digested and analyzed using an agarose gel. The insert band from the gel electrophoresis was then excised and Genecleaned through the above methods. The insert was then ligated to the eGFP vector with the slightly altered recipe of; 2μL Ligation Buffer, 2μL cut eGFP vector, 6μL insert DNA, 1μL Ligase. This was then transformed similarly and plated on LB agar plates with kanamycin instead of the above-mentioned plates with ampicillin. The eGFP vector is not a blue-white screen, but instead just a positive or negative test, so the transformation was plated without X-gal and IPTG. Any colonies that appeared were picked, Miniprepped, and Midiprepped according to the above procedures.

Transfection into H1299

After both the Nuclear Export Sequence and Nuclear Localization Sequence from PCV2 VP3 were successfully ligated into eGFP and Midiprepped, the next step was to transfect human cells with the constructs. H1299 cells, an immortalized line of non-small cell lung carcinoma cells were

transfected. These cells do not express p53, an important tumor suppressor, which partially accounts for their prolific nature. Three six-well plates were prepared, two of them being identical sets of ANES, ANLS and Control, the other being WT and control. To perform the transfection 0.8μg DNA was diluted into a final volume of 200μL. Then 6.4μL of enhancer and 20μL effectene reagent were added and allowed to sit for 10 minutes. The media was removed from the cells, they were washed with PBS, and 1.6mL of fresh media was added to each well. 1.2mL of media (DMEM with 10% FBS and PSF) was added to the DNA complex and was added drop wise to the relevant wells. The dishes were swirled and returned to the incubator.

Leptomycin B and Microscopy

After the cells had been growing for a day, half of them were spiked with 10μL (540 μg/mL) Leptomycin B from LC Laboratories. This was added as a single drop from a micro syringe to the center of the appropriate cover slips in the six-well plates. After three hours, these were mounted to slides with 20μL of Mounting Media (50% glycerol, 100mM Tris pH 7.5, 2% DABCO, DAPI). These slides were then examined with florescence microscopy at 400x with an oil immersion lens.

Results

Determination of NES and NLS location

The location of the PCV2a VP3 NES and NLS was determined by aligning the primary sequences of Apoptin and PCV2a VP3 (Figure 1). The canonical nuclear export sequence (NES) for Apoptin is located from residue 37 to residue 46. The homologous region of PCV2a VP3 spans from residue 40 to residue 47 in the polypeptide chain and residue 118 to residue 141 on the VP3 gene (Figure 1). Although there is no canonical nuclear localization sequence (NLS), the putative NLS for Apoptin extends from amino acid 70 to 121. The homologous region for PCV2 VP3 comprises of a region from amino acid 65 to the C-terminus of the protein. Although this seems like a very large fragment, the lack of homology from residue 96 to 104 between PCV2a VP3 and Apoptin suggests the presence of a bipartite NLS. Due to the small size of the NES fragment, PCR primers sitting at the 5'-end and at the 3'-end of the gene fragment could not be used to isolate the fragment. Although the primers can amplify the small amplicon, the resulting gene fragment would be very difficult to isolate and purify. To avoid the post-amplification issues, a primer set was created to amplify a larger amplicon (Figure 2). The isolated amplicon extended from the start of the gene to nucleotide 192. This much larger amplicon would be easier to purify and ligate. The amplicon codes for the first 64 amino acids. This particular fragment of VP3 consists of the NES and the polypeptide sequence up to the start of the predicted nuclear localization sequence. A different set of primers was used to amplify the possibly bipartite NLS. The forward primer was designed to start amplification at nucleotide 193, whereas the reverse primer for this particular set was designed to bind the 3' end of the gene (Figure 2). The amplicon generated from this particular set of primers consists of the entire predicted nuclear localization sequence.

PCV2a and PCV2b show a large degree of homology at the nucleotide level

To determine the level of homology between the PCV2a and PCV2b VP3 genes, the two genes were aligned. The alignment showed a great degree of homology between the two genes with a 98% identity at the nucleotide level. The differences occur at nucleotide 121, 207, 283, 291, 304, 310 (Figure 3). This six amino acid variation translates to a difference of three amino acids between the two isoforms (Figure 4).

Functionality of the Nuclear Localization and Export Sequences

The GFP fusion truncation mutants, with the Nuclear Export and Localization Sequences of the PCV2a VP3, when expressed in H1299 non-small cell lung carcinoma cells, suggested significant results. In the absence of Leptomycin B, as seen in figure 5, the NES and NLS appeared to have a significant level of functionality. Figure 5A, the GFP tagged NES was largely diffused throughout each of the three cells depicted, with the exception of a darker circle in each of the three cells. Upon examination of figure 5b, the same set of cells with DAPI stained chromatin, the areas of brighter blue appeared to correlate with the areas of lesser green from the previous image. This shows that the GFP was cytosolic. This was confirmed by figure 5c, a combination of the previous two images, a finding that suggests the presence of a functional Nuclear Export Sequence in PCV2a VP3.

A similar result was found in the analysis of figures 5d – 5f. These figures show H1299s transfected with the NLS GFP-fusion construct. Figure 5d shows a certain level of GFP diffusion throughout the cell, with an area of significantly greater intensity. This area of greater intensity aligns with the area of greater DAPI concentration in figure 5e, which is confirmed in figure 5f. This demonstrates a significantly increased level of GFP within the nucleus, suggesting a functional Nuclear Localization Sequence.

Nuclear Export is CRM1 Dependant

The same GFP-tagged mutants in H1299 cells with the addition of Leptomycin B suggested further results. Leptomycin B impairs the functionality of the CRM1-mediated export, a canonical export pathway. By applying Leptomycin B to H1299 cells transfected with the same mutants as in figure 5, we see a different set of results in figure 6. While figures 6d – 6f, the NLS mutants, show similar results to the corresponding cells in figure 5d – 5f, the NES mutants suggest different data. Figures 5a – 5c demonstrate a functional NES by the presence of a greater concentration of GFP outside the nucleus. However the area of greatest GFP concentration in figure 6a is in the same area of the cell that contains the greatest concentration of DAPI in figure 6b. This demonstrates that the GFP-fusion is still inside the nucleus, rather than in the cytosol as expected. This loss of function of the NES can be explained by (is due to) the presence of LMB, suggesting that nuclear export is CRM1 dependant.

Presence of punctuate structures

The full-length PCV2a VP3 showed punctate structures in the absence of LMB in transformed H1299 cells (Figure 7a). These punctate structures appear as points of intense GFP concentration not located in the nucleus as shown by the DAPI-stained chromatin (Figure 7b) and the overlayed image of the GFP location and the DAPI stain location (Figure 7c). Aside from the punctate structures, PCV2a VP3 shows a cytosolic localization in the H1299 cells (Figure 7c). This is in agreement with results of previous studies with GFP-fusions of wild-type PCV2a VP3.

PCV2a VP3 shows functional shuttling activity

In transformed H1299 cells, PCV2a VP3 showed functional shuttling activity. The GFP-fusion protein showed localization to one distinct area within the cell (Figure 8a) in the presence of LMB. This particular localization of GFP correlated with the location of the nucleus as indicated by a DAPI stain (Figure 8b) and showed a nuclear localization of the GFP (Figure 8c). The LMB was used

to inhibit the CRM1-dependent export of the wild-type protein from the nucleus and to see whether the native nuclear localization sequence would localize to the nucleus. It has been shown that PCV2a VP3 utilizes CRM1 to transverse the membrane (Figure 6). The nuclear accumulation of GFP shows an active transport of the GFP into the nucleus.

Discussion

In this study we showed the presence of functional nuclear export and localization sequences in PCV2a VP3 as well as the presence of shuttling activity. In addition, we also showed the export sequence to be CRM1 dependant. Lastly we observed the presence of punctate formations of the wild-type protein within the cytosol of transformed cells.

As a preliminary attempt to determine the presence of functional localization sequences, the PCV2a VP3 gene was aligned with apoptin *in silico*. This alignment showed significant homology between the nuclear export sequences of apoptin and PCV2a VP3. The level of homology between the nuclear localization sequences is not as strong as the export sequences. Previous studies have shown that Apoptin possesses both a functional nuclear export and nuclear localization sequence. From the high level of homology present within the nuclear export sequences, it is likely that PCV2a VP3 possesses a functional NES. Due to the lack of homology, the same conclusions cannot be drawn regarding the NLS. To conclusively show the lack or presence of a functional nuclear localization sequence, studies were performed by expressing truncated NLS-GFP mutants.

Of the two isoforms, studies show PCV2b to be more highly pathogenic with a faster onset and more potent symptoms. Although the molecular basis for this difference in pathology has not been determined, it is likely the result of the few differences at the amino acid level, between the two VP3 isoforms. As a part of this study, the two VP3 isoforms were aligned to determine any differences in primary structure. The three amino acid differences between the two isoforms exist at residues 41, 102, and 104. All three of these differences are located within the predicted NES and NLS. At residue 41, PCV2a VP3 and PCV2b VP3 have amino acids Glycine and Serine, respectively. These two amino acids are not considered functional substitutes of each other and therefore may affect the function of the NES. At residue 102, PCV2a VP3 contains a Phenylalanine, whereas PCV2b

VP3 contains a Leucine. Finally, at residue 104, PCV2a VP3 contains a Lysine, whereas PCV2b VP3 contains a Glutamine. The two substitutions at residues 102 and 104, in certain situations, can be considered functional substitutes of each other. These three differences within the localization sequences may attribute to the pathological differences between the two isoforms.

Through the transient transfection of H1299 cells with the GFP-fusion constructs, we show the presence of a functional nuclear export sequence and more importantly a functional nuclear localization sequence. The presence of a functional nuclear export sequence was expected due to the cytosolic localization of the wild-type protein in transformed cells. The presence of a functional nuclear localization sequence, however, was unknown due to VP3 previously only being observed in the cytosol. By separating the nuclear export sequence from the nuclear localization sequence, we were able to study the effect of the nuclear localization sequence on the protein. This uncoupling is particularly important in the case where the nuclear export sequence outcompetes the nuclear localization sequence and therefore masks the effects of the localization sequence. The GFP-fused nuclear localization sequence increases the concentration of GFP in the nucleus of the cell. This increased concentration in one particular locale within the cell suggests an active transport mechanism. In particular, the localization of GFP into the nucleus from the cytosol can only be achieved in the presence of a functional nuclear localization sequence.

It is known that PCV2a VP3 possesses a functional NES, but very little is known about the molecular mechanisms involved in the export. One of the canonical export pathways relies on the karyopherin CRM1 to negotiate export through the Nuclear Pore Complex. We show that a CRM1 dependant pathway is used by PCV2a VP3 to exit the nucleus. This finding suggests that PCV2a VP3 NES functions in a manner very similar to the Apoptin NES, in agreement with the results shown by our alignment.

The presence of a functional nuclear export and nuclear localization sequence alludes to shuttling activity across the nuclear membrane. The nuclear localization of GFP in wild-type VP3 protein in the presence of LMB suggests an active shuttling activity. It has already been shown that the nuclear export sequence is functional in wild-type VP3. As mentioned above, a functional nuclear localization sequence has not previously been observed in wild-type VP3. In order to shuttle between the nucleus and the cytosol, VP3 must have both an active export and an active localization sequence. The presence of an active NLS was shown by this study. The presence of shuttling activity is strongly suggest PCV2a VP3 utilizes a pathway similar to Apotpin to induce apoptosis in cells. One major difference between the two proteins is the localization pattern in transformed cells. PCV2a VP3 shows a cytosolic localization whereas Apoptin shows a nuclear localization in transformed cells. This suggests that PCV2a VP3 utilizes a target other than the APC to induce apoptosis. The presence of a shuttling activity cannot be determined from the truncation mutants due to the lack of the full peptide within the GFP construct, especially in the case of the nuclear localization construct.

An unexpected observation in the H1299 cells transfected with the wild-type VP3 GFP-fusion, without the presence of LMB was the presence of punctate structures in the cytosol of cells. It is not clear whether these particular structures are evidence of multimerization activity. It is worth noting that Apoptin has been shown to form multimers in both the cytosol and nucleus. The signficance of these Apoptin multimers is not known, but it is linked to the NES. Our study suggests that in PCV2a VP3, these punctate structures are not linked to the NES. This suggests that these structures may not be multimers of PCV2a VP3. The alternative possibilities include non-specific aggregation of the protein or the binding of VP3 to one of the non-nuclear organelles. It is not possible to discern between these possibilities through the techniques used in this study. Further molecular studies including yeast 2-hybrid assays to show multimerization of PCV2a VP3 should be

conducted. Some other molecular methods to determine the components of these punctate structures include co-immuno precipitation or western blots.

Through this study, we have shown functional export and localization sequences, CRM1 dependant export, and the presence of puncate structures in the cytosol of transformed cells. Further studies need to be performed in order to determine whether the B isoform of PCV2 has the same characteristics as the A isoform despite subtle differences in protein composition. In addition to these, future studies need to determine the apoptotic capabilities of PCV2 VP3 in both transformed and primary cells.

Figures

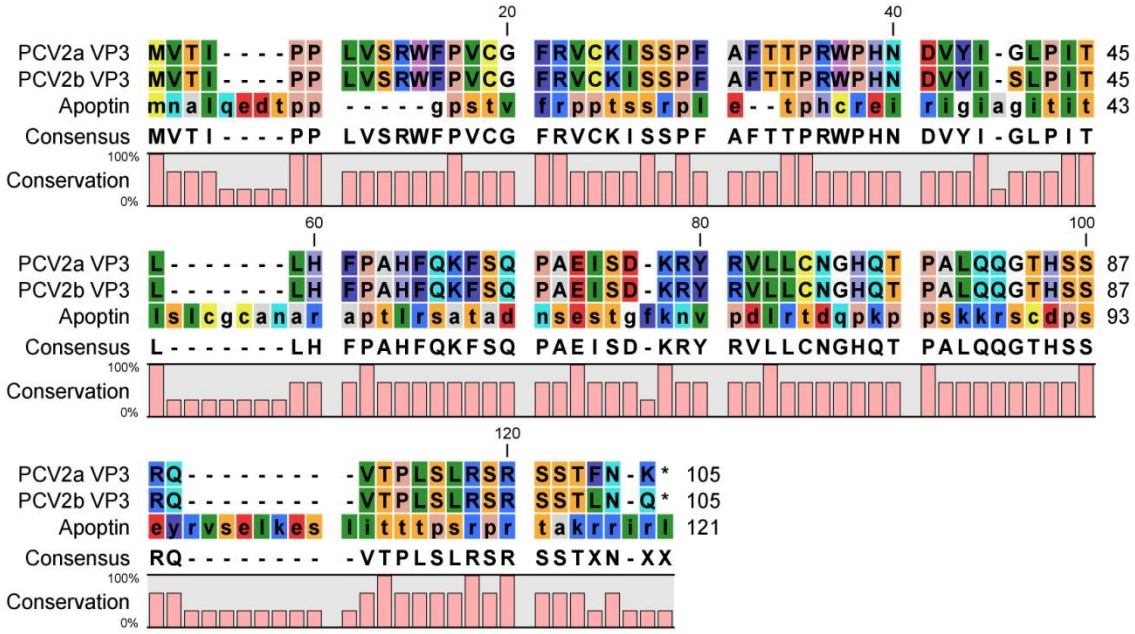

Figure 1: Amino Acid Alignment between VP3 from PCV2A and PCV2B as well as Apoptin from Chicken Anemia Virus

aNES Forward	5’ CGA ATT CAA TGG TAA CCA TCC CAC CAC TT 3’
aNES Reverse	5’ GCG GAT CCT CAG TCA GAA ATT TCC GC 3’
aNLS Forward	5’ GCG AAT TCA AAA CGT TAC AGG GTG CT 3’
aNLS Reverse	5’ GCG GAT CCA ATT ACT TAT TGA ATG TG 3’

Figure 2: Primers utilized in framing the NES and NLS from the PCV2A VP3

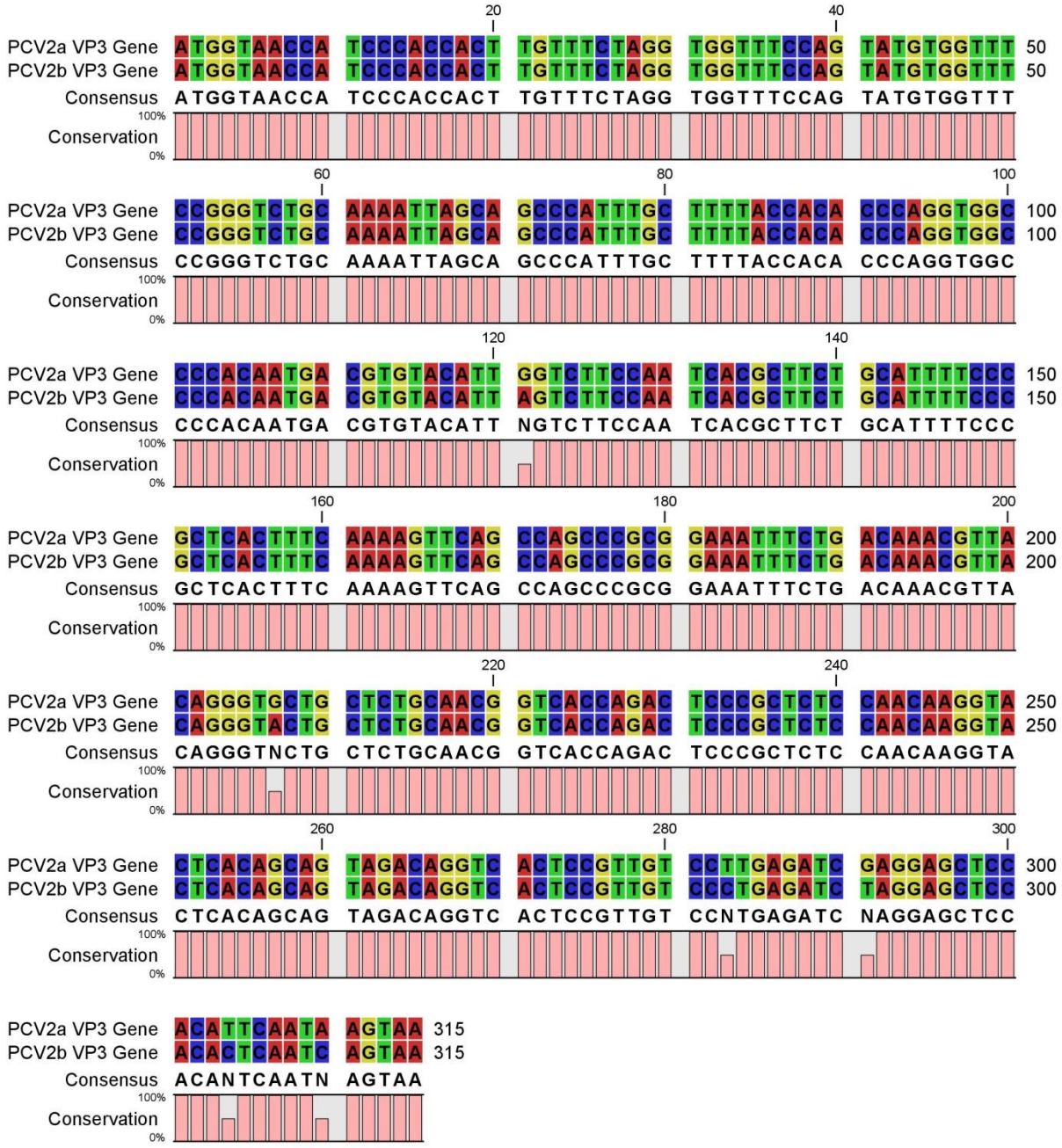

Figure 3: Nucleotide Alignment between PCV2a and PCV2b VP3 gene, illustrating a great deal of homology between the two isoforms of the VP3 gene

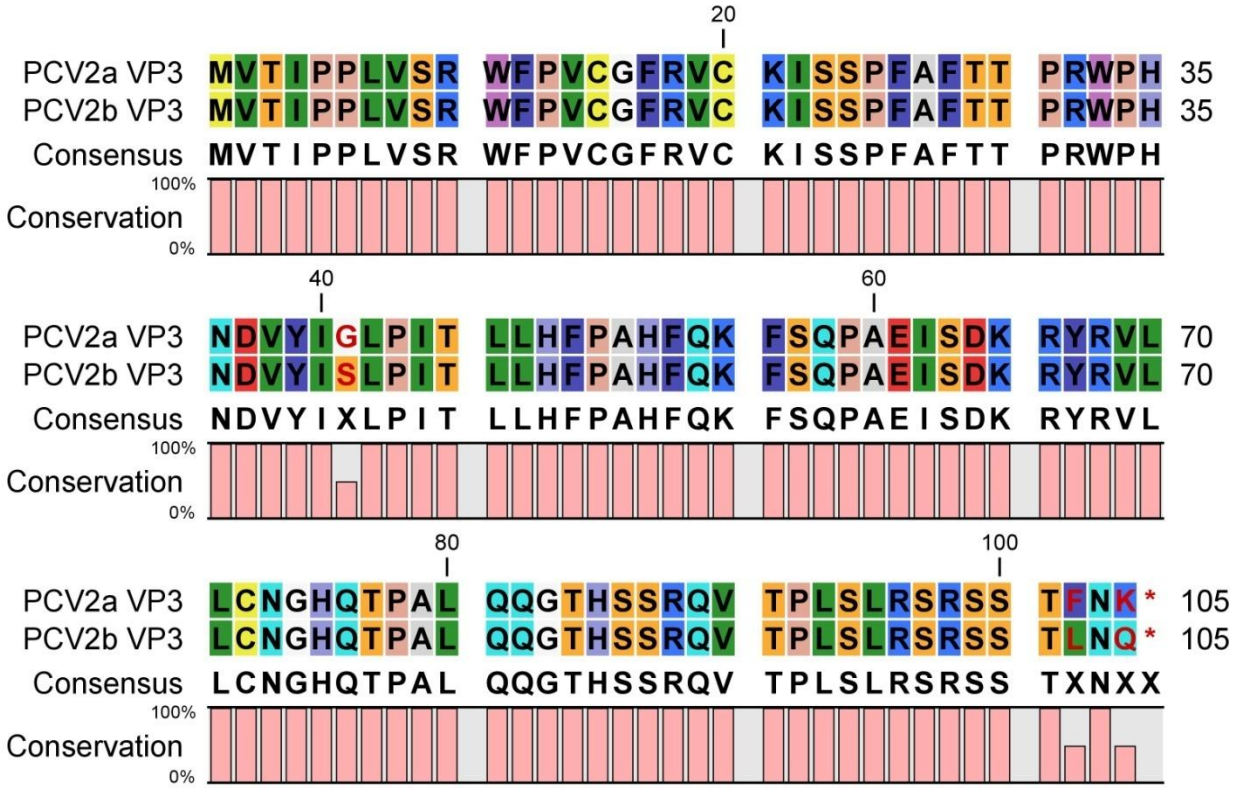

Figure 4: Amino Acid alignment of PCV2a VP3 and PCV2b VP3. The non-conserved amino acids are highlighted in red.

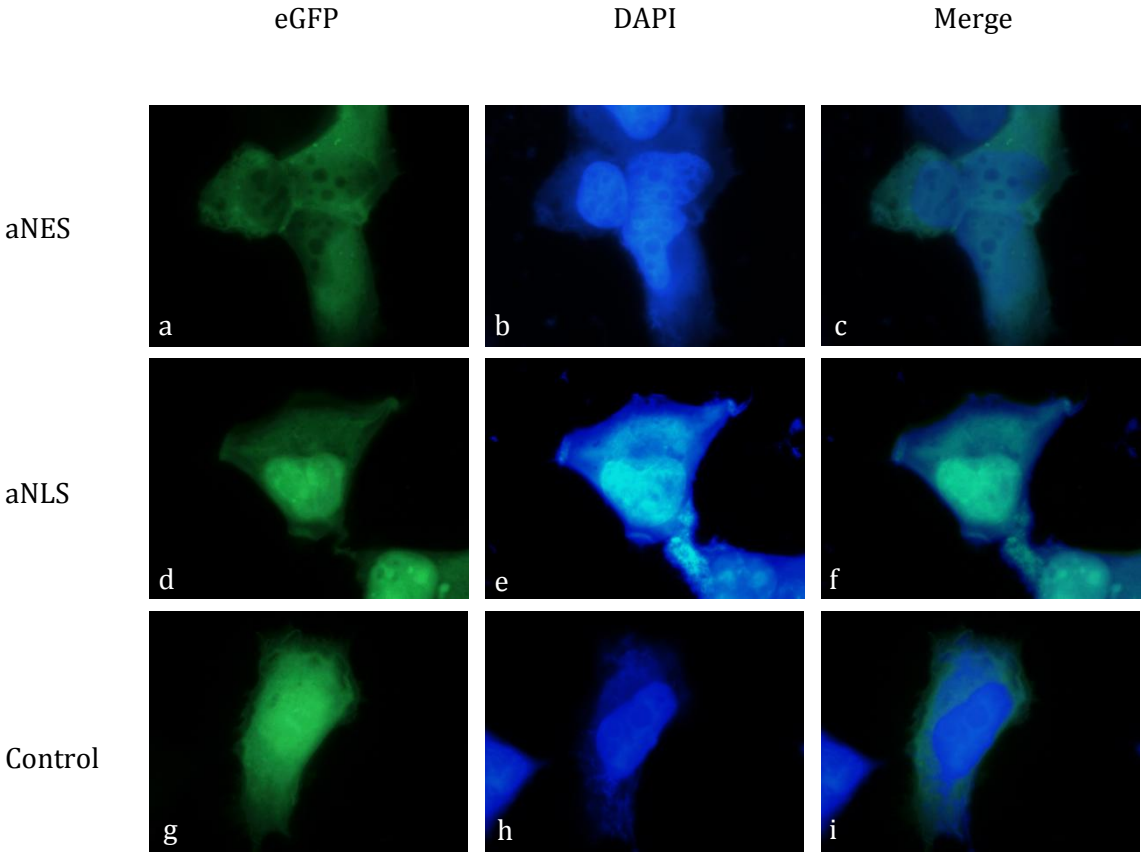

Figure 5: aNES and aNLS eGFP constructs along with an eGFP control transfection expressed in H1299 cells without Leptomycin B. (A-C) H1299 cells containing the GFP-fused NES under: a GFP filter (A), a DAPI filter (B), and merged images (C). (D-F) H1299 cell containing the GFP-fused NLS under: a GFP filter (A), a DAPI filter (B), and merged images (C). (G-I) H1299 cell containing a GFP control under: a GFP filter (A), a DAPI (B), and merged images (C).

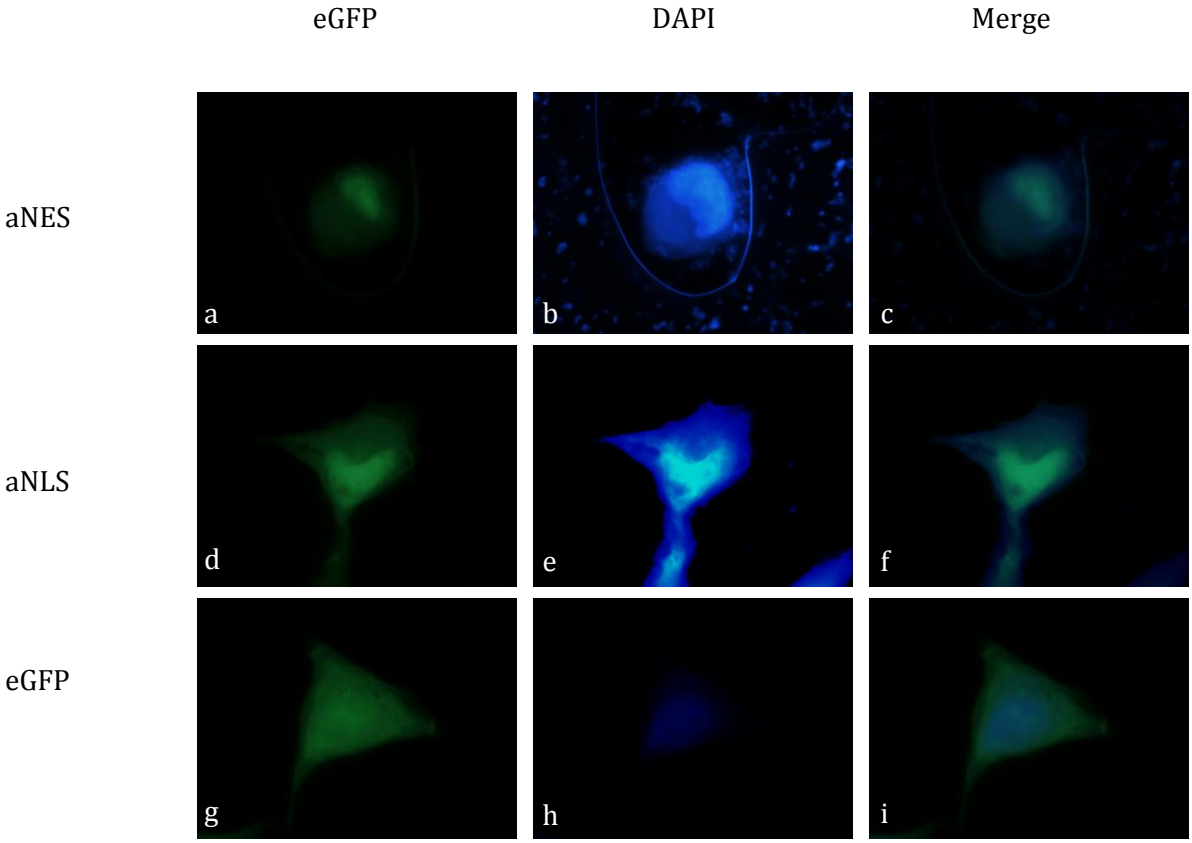

Figure 6: aNES and aNLS eGFP constructs along with an eGFP control transfection expressed in H1299 cells with Leptomycin B, a CRM1 Inhibitor. (A-C) H1299 cells containing the GFP-fused NES under: a GFP filter (A), a DAPI filter (B), and merged images (C). (D-F) H1299 cell containing the GFP-fused NLS under: a GFP filter (A), a DAPI filter (B), and merged images (C). (G-I) H1299 cell containing a GFP control under: a GFP filter (A), a DAPI (B), and merged images (C).

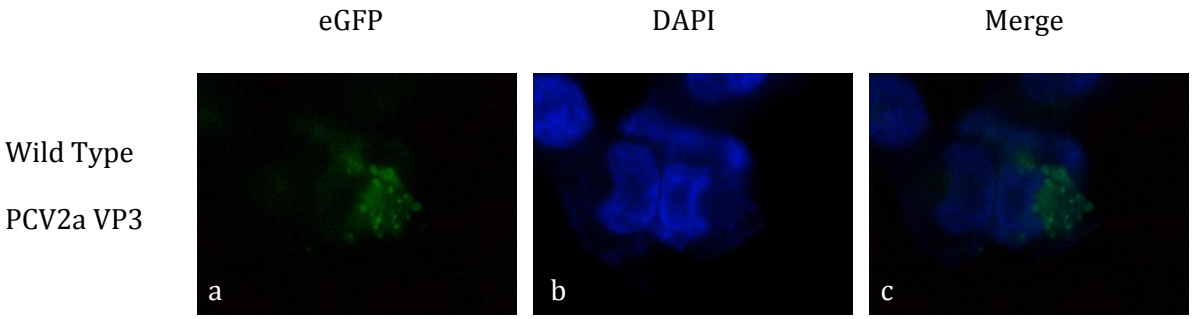

Figure 7: PCV2a VP3 Wild Type tagged with eGFP, transfected into H1299, without Leptomycin B. (A-C) H1299 cells containing the GFP-fused Wild-Type PCV2a VP3 under: a GFP filter (A), a DAPI filter (B), and merged images (C).

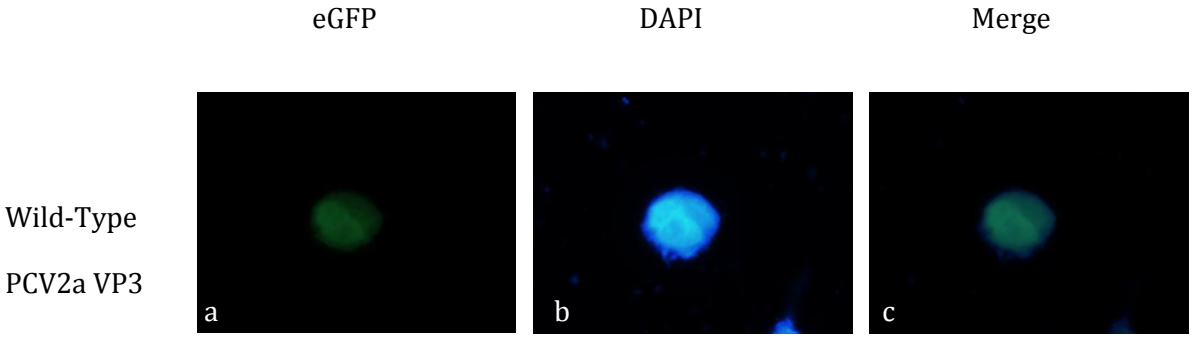

Figure 8: PCV2a VP3 Wild Type tagged with eGFP, transfected into H1299, with Leptomycin B, a CRM1 Inhibitor. (A-C) H1299 cells containing the GFP-fused Wild-Type PCV2a VP3 under: a GFP filter (A), a DAPI filter (B), and merged images (C).

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